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Molecular Epidemiology of *Cryptosporidium* Species, in Human Immunodeficiency Virus Patients Attending Selected Hospitals in Nasarawa State, Nigeria

Madukwe H. A, Gyar, S. D and Ekeleme I. K

Department of Microbiology, Faculty of Natural and Applied Science, Nasarawa StateUniversity, Keffi, P.M.B 1021 Keffi, Nasarawa State, Nigeria

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Corresponding Author: **Gyar SD**
E-Mail: **silasgyar@nsuk.edu.ng**

ABSTRACT

Cryptosporidium infection is transmitted through the faecal oral route, mostly through the consumption of contaminated water. The current study was aimed at detecting Cryptosporidium species in Human Immunodeficiency Virus patients attending selected Hospitals in Nasarawa State, Nigeria. A total of one hundred and ten stool samples were collected from HIV patients attending selected hospitals in study area. Faecal samples were analyzed using formal diethylacetateusing the cheesbrough vortex and polymerase chain reaction technique to detect the presence of Cryptosporidium species. The prevalence of Cryptosporidium species among HIV patients attending selected Hospitals in Nasarawa state was 29 (26.4%) Cryptosporidium parvum (25.5%) and Cryptosporidium hominis (11.8%). Prevalence in relation to health care centers was highest in OLHA (24.0%) and lowest in DASH (17.1%). In relation to gender, the prevalence of Cryptosporidium parvum was higher in males (35.7%) than females (19.1%) and Cryptosporidium hominis was higher in males (11.9%) than in females (11.8%). The Prevalence in relation occupation showed that Cryptosporidium parvumwas highest among the business class (40.0%) and lowest among students (14.8%) while Cryptosporidium hominis had high prevalence among civil servants (18.8%), but low among others (17.14%). The prevalence in relation to age shows that Cryptosporidium parvumhad the highest prevalence at age 31-45 years (38.0%) and lowest at age 16-30 years (12.0%). While Cryptosporidium hominis had the highest prevalence at ages of 0-15 years (22.0%) and the lowest at age 16-30 years (7.5%). There was no significant difference between the different age groups at P > 0.05. The prevalence in relation to education shows Cryptosporidium parvumhad the highest prevalence among the uneducated (37.5%) and lowest among primary education (20%), while Cryptosporidium hominis, the highest prevalence was among the uneducated (18.8%) and lowest was among primary education (17.4%). The prevalence in relation to livestock farming shows Cryptosporidium parvum had a prevalence of 58.0% amongst livestock farmers and 10.0% among non-livestock farmers. Cryptosporidium hominis had 25.0% among livestock farmers and 7.0% among non-livestock farmer. It is interesting to note the high prevalence rate of cryptosporidium infection in the study area. This study establishes that Cryptosporidiasis is a coinfection amongst HIV infection individuals thereby further predisposing such patients to more health hazards. Cryptosporidiasis is therefore a contaminant disease.

Keywords: *Cryptosporidium, HIV patients, health care centers.*

1. Introduction

Cryptosporidiosis is an intestinal disease caused by *Cryptosporidium*, a protozoan parasite belonging to the phylum Apicomplexa. It is a significant cause of diarrheal illness worldwide and is transmitted primarily through the fecal-oral route, often via contaminated water or food. While the infection is typically self-limiting in healthy individuals, it can lead to severe, chronic, and life-threatening complications in immunocompromised patients, particularly those living with HIV/AIDS.

The disease was first identified in laboratory mice in 1912 and later linked to human infections in 1976. Initially, it was believed that only one *Cryptosporidium* species was responsible for human disease; however, the advent of molecular diagnostic tools has revealed the existence of multiple pathogenic species. Among these, *Cryptosporidium hominis*, which is primarily found in humans, and *Cryptosporidium parvum*, which can infect both humans and cattle, are the most prevalent.

The parasite's life cycle is completed within a single host. Upon ingestion, *Cryptosporidium* oocysts—infectious at doses as low as 10–30—attach to the epithelial cells of the small intestine. These oocysts are intracellular but extracytoplasmic, allowing them to evade host immune responses and persist even under adverse environmental conditions. Notably, they are highly resistant to standard water treatment methods, including chlorine disinfection. After replication, the parasite sheds large numbers of oocysts in the host's feces, facilitating further transmission.

Clinical symptoms typically develop between 2 to 10 days post-exposure, with an average onset around day 7.

Common symptoms include watery diarrhea, abdominal cramps, nausea, and low-grade fever. In most cases, symptoms resolve within two weeks; however, in immunocompromised individuals, especially HIV/AIDS patients, the infection can persist and cause life-threatening complications. Asymptomatic carriers also pose a risk of transmission, as they can continue to shed oocysts even after clinical recovery.

In Nigeria, cryptosporidiosis remains an under-researched but significant public health issue. Northern regions of the country, in particular, report high incidences among children and HIV-positive individuals. An estimated 2.3 million children have died due to diarrhea-related diseases, including cryptosporidiosis, underscoring the urgent need for intervention. The World Health Organization has raised concerns over the disproportionate impact of the disease on vulnerable populations such as women and children.

This study aims to address the gap in epidemiological data by focusing on the molecular detection and identification of *Cryptosporidium* species among HIV-positive patients in selected health centers in Nasarawa State, Nigeria. Through advanced molecular techniques, this research seeks to improve understanding of the distribution and species diversity of *Cryptosporidium* in immunocompromised populations and support the development of targeted public health interventions.

2. Mateials and Methods

Study area

Individuals attending HIV clinics for HIV/AIDS-related cases at selected health centers in Nasarawa State namely: Model Hospital Akwanga (MHA) and Our Lady of Apostles Hospital (OLAH), Dalhatu Araf Specialist Hospital (DASH), and Federal Medical center, Keffi (FMCK).

2.1 Questionnaire Administration

An interviewer, administered a questionnaire to each participant to obtain written demographic and clinical data. The questionnaire contains information on their bio-data, educational history, occupation, source of drinking water and involvement in livestock farming

2.2 Research Design

A cross sectional and descriptive study was used for this study.

2.3 Consent Form

A confidential consent form was prepared heightening the purpose of the research, type of specimen required and assurance of their confidentiality, without coercion was given.

2.4 Ethical Approval

Ethical Approval was obtained from the ethics committee of the Ministry of Health, Lafia; Nasarawa State following the submission and defense of the research protocol where patients' right to consent and confidentiality was assured

2.5 Sample Size Determination

The sample size formula for this study is: $N= (Z^2pq)/e^2$

The appropriate sample size formula chosen above is that designed for non-comparative single proportion study [8].

The calculation of the sample size

N=Desired sample size in a population

Z=Standard deviation set at 1.96, corresponding to the 95% confidence interval.

P= Prevalence of the target population estimated to have a particular characteristic. In this case *Cryptosporidium*

Q= 1-p complimentary probability to P

e= the degree of precision or absolute error set at 5 % (0. 5)

In this Study P was taken as 7% representing the prevalence rate of *Cryptosporidium* species [9].

Mathematically, sample size

N= (1.96) ^2 x prevalence x (1-prevalence)/(0 05^2)

0.31462704/0.0025= 100

Minimum sample size=100

10% attrition=10

Sample size=110

2.6 Sample Collection

Fresh stool samples were collected from 3 categories of participants as follows: loose samples, whether they were on anti-retroviral therapy or not; watery stool, whether they were on anti-retroviral or not; any stool texture from immunocompromised participants whose CD4 count of 200 cells/mm'3 and below 100,000 copies/'3 and above .A total of 110 fresh stool samples that met the criteria were collected into a sterile, wide open ,dry, leak-proof container with 35 samples from DASH; 35 from MHA ;25 stool samples from OLAH; and 15 from FMCK. Samples collected from these canter were transferred to Omega Int'l Medical Laboratory Ltd Akwanga, Nasarawa State for analysis

2.7 Sample Processing

Macroscopic examination of fecal samples was performed prior to microscopy. Observations included the consistency of the sample and the presence or absence of mucus and blood. Approximately 1 gram of feces was emulsified in 4 mL of 10% formalin (formol water) using a sterile rod, and the mixture was placed into a screw-capped bottle. An additional 3–4 mL of 10% formol water was added to the suspension, which was then thoroughly mixed by shaking.

The emulsified feces were filtered through a sieve, and the resulting suspension was collected in a sterile beaker. This suspension was then transferred into centrifuge tubes made of durable glass, copolymer, or polypropylene. Next, 3–4 mL of diethyl ether was added to each tube. The contents were mixed vigorously for 15 seconds using a vortex mixer (Stuart SA7 Vortex Mixer, Cole-Parmer Ltd).

The samples were then centrifuged immediately at 3000 rpm for 1 minute. After centrifugation, the layer of fecal debris adhering to the tube walls was loosened using a plastic pipette stem or stick. The tubes were then inverted to discard the ether layer. The remaining components—fecal debris, formol water, and sediment—were retained for microscopic examination [10].

Microscopic Examination of the Slides

Microscopic examination was conducted using a LEICA microscope (Leica Microsystems GmbH, Wetzlar, Germany), which was calibrated using an ocular graticule to aid in differentiating *Cryptosporidium* oocysts from other coccidian oocysts. Prepared slides were initially examined under low-power magnification to detect the presence of oocysts. Identification was confirmed under oil immersion objective.

Oocysts that appeared as small, round to oval, pink to red-stained bodies measuring 4–6 µm in diameter, or as structures showing a single deeply stained red dot, were considered positive for *Cryptosporidium* spp. [10].

2.7.1 Staining Procedure

A smear was prepared from the sediment obtained using the formol-ether concentration technique. The smear was air-dried and subsequently fixed with methanol for 2–3 minutes. Staining was performed using unheated carbol fuchsin, which was applied to the smear for 15 minutes. After staining, the slide was gently rinsed with clean water.

Decolorization was carried out using 1% acid-alcohol for 10–15 seconds, followed by another rinse with water. The slide was then counterstained with 0.05% methylene blue for 30 seconds and washed again with water. After staining, the slide was placed on a draining rack to air dry completely.

Once dry, each slide was examined under a microscope. Initial screening was conducted using low-power magnification, followed by detailed examination using an oil immersion objective lens to confirm the presence and identification of *Cryptosporidium* oocysts [11].

2.7.2 DNA extraction

DNA extraction was carried out using the TEL-DNA extraction kit as instructed by the manufacturer TELSA-DX consult Australia. 250-300 mg of each stool sample was properly mixed and added into the beads. The beads were secured horizontally on a vortex adapter for 1.5 -2.0 ml, then vortex at maximum speed for 10 minutes at 15000g (14800 rpm) for 1 min and the supernatant was transferred to clean 2 ml microcentrifuge tube avoiding the beads.200µl of wash buffer A was added and mixed thoroughly by vortex ; the mixture was pipetted into the spin column already placed in a 2ml collection tube, centrifuged for 1 min at 8000 rpm. The flow-through was then discarded completely. With the collection tube reused for the next step. The spin column was placed into the collection tube and 200µl of wash buffer B was added. This was centrifuged for 1 min at 8000rpm , discarding the flow-through. After the spin column was transferred to new 2 ml microcentrifuge tube . DNA eluted by adding by adding 50µl of the elution buffer to the centre of the spin column membrane. It was then incubated at room temperature for 1 min and centrifuged for 1 min at 8,000 rpm. DNA was then ready for TEL-Crypto multiplex PCR

2.7.3 Amplification

Amplification for *Cryptosporidium parvum*and *Cryptosporidium hominis*subgenotypingA 60-kDa glycoprotein (GP60) gene fragment was amplified by nested PCR as previously described [12]. TEL-Master mix, primers/probe mix, and positive and negative controls which was stored at -20°C, .Repeated freezing and thawing of positive control and contamination was avoided. QMaster mix was prepared by mixing 12.6µl (one well), multiplied by 110 to give a total of 1380 µl. The primer (*Cryptosporidium* gp60 for *Cryptosporidium parvum*and hsp90 for *Cryptosporidium hominis*and a probe mix containing SYBR green labeled fluorescent dye of FAM for *Cryptosporidium parvum*and HEX for *Cryptosporidium hominis,* was prepared by mixing 2.4 µl (one well), multiplied by 110 to give a total of 246µl. Both 1380µl of master mix and 264 µl of primers/probe mix was mixed in one tube as a reagent mix resulting to 1650µl.

The master mix reagent was also prepared for positive and negative controls. Aliquots of 15µl of the mixed master mix reagents were then distributed into each well of an appropriate 32-well reaction tube. Including the controls, after that 5µl of the extracted gDNA or non template control (NTC) was added making a total of 20µl reaction set up on each well. This was then thoroughly vortex with the reagent mix and briefly centrifuged. Reaction tubes were then covered with appropriate optical lids and placed appropriately in a thermocycling machine (MYGO, Pro; an opened real time PCR Real time detection system, fully installed with its software already loaded in computer system, calibrated and maintained according to manufacturer instructions and recommendations. incorporated with fluorescent detection). The activation (Holding time of 1 min) at a temperature of 95^0c was achieved. The amplification cycle has 2 steps (The annealing, extension and acquisition). First 95^0c for 10 secs and 60^0c for 30 secs, with the number of cycles of the 2 steps amplification of 45 cycles.After amplification, the software amplification on the computer system of the MYGO Pro thermocycling machine was carefully followed and imputations were made on the settings for different dyes. The menu for targets contains the dye (Target 1 –*Cryptosporidium parvum* fluorescence is FAM and target 2 *Cryptosporidium hominis* fluorescence is HEX). The manufacturers instructions on the imputed software was carefully followed. A quality control curve was generated showing , no amplification for NTC, no amplification for negative controls, no CT values and no detection occurred (meaning no contamination or trouble shooting occurred during the analysis), but positive samples were amplified as shown in appendix E. A curve was also generated from the system showing the Relative Fluorescence Unit (RFU) and different amplification of the samples emissioins after acquiring fluorescence dye as either positive or negative with their CT values.

2.8 Data Analysis

Statistical analysis was carried out using R software amplication version 3.6.1. Chi-square was used to test for significance.

3. Results and Discussion

Figure 1 shows the prevalence of *Cryptosporidiumparvum* and *Cryptosporidium*hominis amongst HIV patients in relation to Hospitals.The overall prevalence of *Cryptosporidium parvum* was (25.5%) and *Cryptosporidium hominis* (11.8%). In the prevalence of *Cryptosporidium parvum* in relation to selected hospitals was found to be highest in OLHA (24.0%) and lowest in DASH Lafia (17.1%). For *Cryptosporidium hominis*, the highestprevalence was in FMCK (16.7%) and lowest was in GHA (2.86%) as shown in Figure 1. It is statistical insignificant relation to hospitalsatp=0.05. This prevalence is lower, when compared with other studies reported in Gboko, Benue state by Ishar*et al*. [13], in Wammako, Sokoto State by ShinkafiandMuhammed [7], and in Osun state by Adesiji*et al*. [14], with prevalence rates of 40.7%, 62.5% and 52.7% respectively. These high rates reported by these studies may be attributed to contamination of drinking water, aquatic environment, recreational water, and heavy livestock farming.

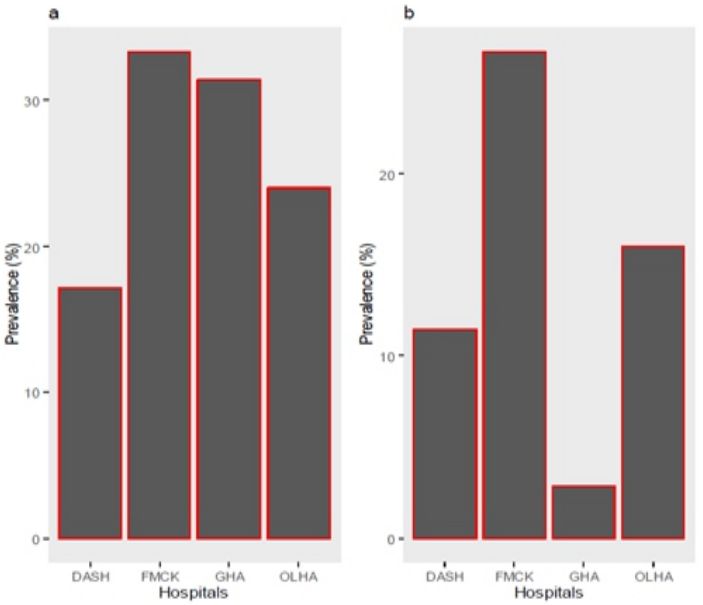

Figure 1: Prevalence of Cryptosporidiumparvum and Cryptosporidium hominis amongst HIV patients in relation to Hospitals (a) represents Cryptosporidiumparvum (b) represents Cryptosporidiumhominis.

The prevalence of *Cryptosporidium* species in relation to gender showed that *Cryptosporidium parvum* was higher in male (35.7%) than females (19.1%). for *Cryptosporidium hominis*the prevalence was higher in male (11.9%) than in females (11.8%) as shown in Figure 2. Statistically, the prevalence of both *Cryptosporidium parvum* and *Cryptosporidium hominis* in relation to gender of those infected with HIV in selected hospitals were insignificant (p=0.05). This is however different from the findings from study done at two general hospitals in Adamawa state by Raphael *et al*. [15] with a prevalence of 43.1% for females and 32.6% for males. This sharp contrast in gender values may be, as a result of exposure to risk factors.

The prevalence of *Cryptosporidium parvum* and *Cryptosporidium hominis* in relation to occupation of HIV patients in selected hospitals is as shown in Figure **3**. The highest prevalence of *Cryptosporidium parvum* was among those doing business (40.0%) and lowest among students (14.8%) while for *Cryptosporidium hominis* the prevalence was high among the civil servants (18.8%), but low among others (17.14%) and it is statistical insignificant in relation to the occupation.

However, the prevalence of *Cryptosporidium* species in relation to age shows *Cryptosporidium parvum* has the highest age 31-45 years (38.0%) and lowest age of 16-30 years(12.0%). *Cryptosporidium hominis* has the highest prevalence between the ages of 0-15 years (22.0%) and the lowest age range 16-30 years(7.5%) as shown in Figure **4**. These results could imply that there are anthroponotic transmissions, of *Cryptosporidium* species amongst children and teenagers, but zoonotic transmissions amongst the adult population. However, this study is at variance with report of Pam*et al*. [16], who reported high prevalence age ranges of 50-59 years (50.0%). This could possibly be as a result of other predisposing factors to diarrhea and poor hygienic practices.

The prevalence of *Cryptosporidium parvum* and *Cryptosporidium hominis* in relation to the level of education among the HIV patients in selected hospitals is as shown in Figure 5.*Cryptosporidium parvum* has the highest among the uneducated (37.5%) and lowest among those with primary education (20%), while for *Cryptosporidium hominis*, the prevalence was also highest among the uneducated (18.8%) and those with primary education was (17.4%), statistical values shows no significant difference (p=0.5). The high values in this two species among the uneducated demonstrate lack of knowledge on personal hygiene, poor understanding on environmental sanitation and drinking of contaminated water.Education is crucial for the epidemiological eradication of cryptosporidiosis as there was no *Cryptosporidium hominis* among those with tertiary education in this study [7,13].

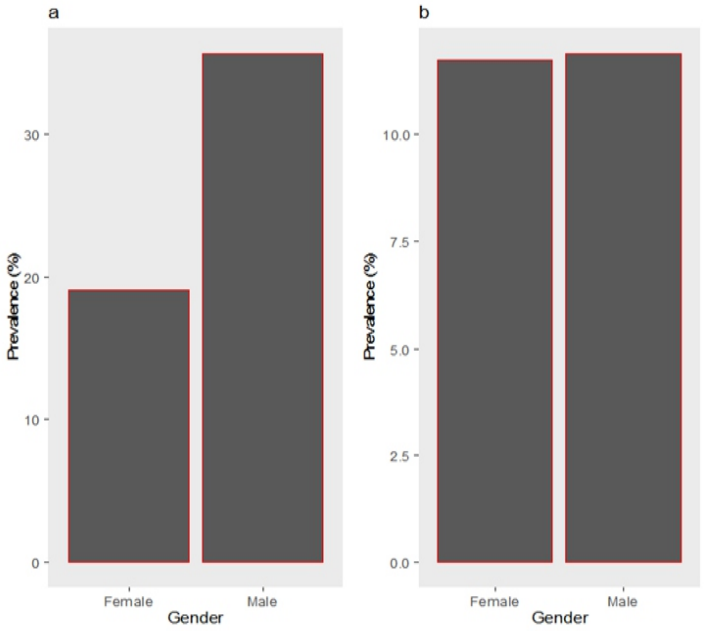

Figure 2: Prevalence of Cryptosporidium parvum and Cryptosporidium hominis amongst individuals infected with HIV in relation to gender. (a) Represents Cryptosporidium parvum

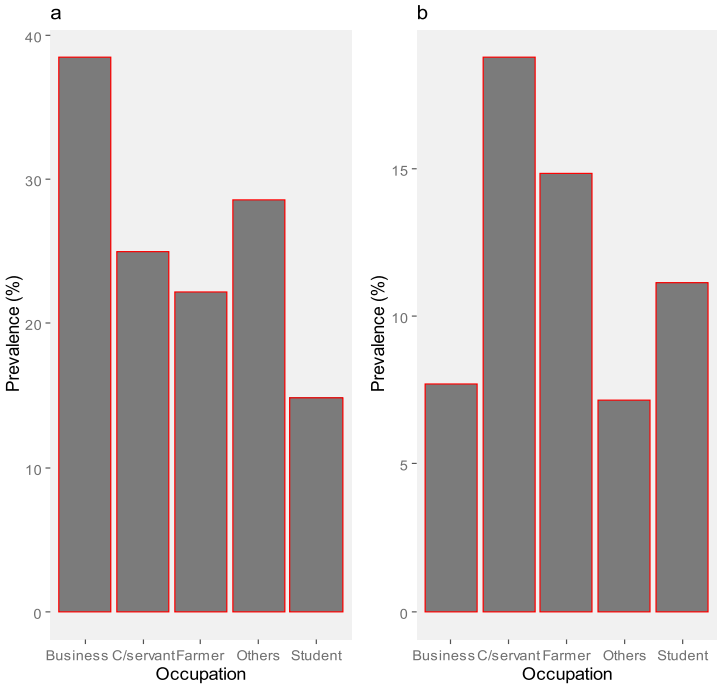

Figure 3: Prevalence of Cryptosporidium parvum and Cryptosporidium hominis amongst individuals infected with HIV in relation to Occupation. (a) Represent Cryptosporidium parvum and (b) represents Cryptosporidium hominis

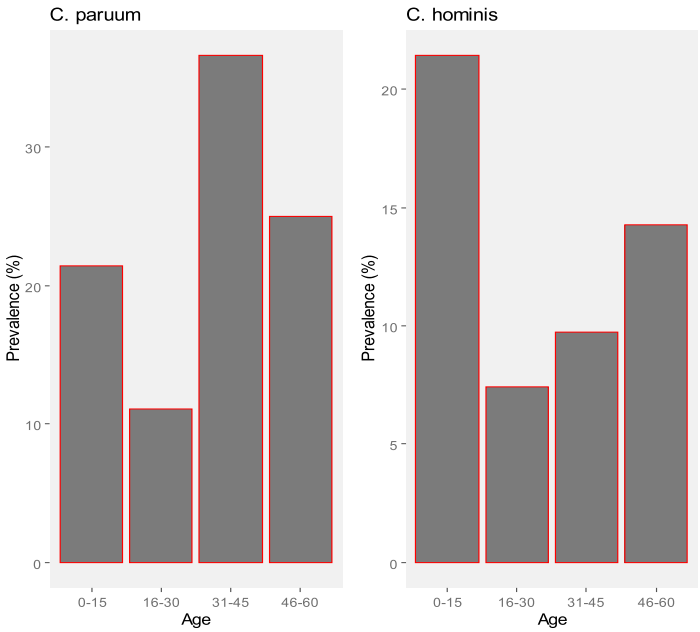

Figure 4: Prevalence of Cryptosporidium parvum and Cryptosporidium homInis amongst individuals infected with HIV in relation to age (a) Represents Cryptosporidium parvum(b) Represents Cryptosporidium hominis

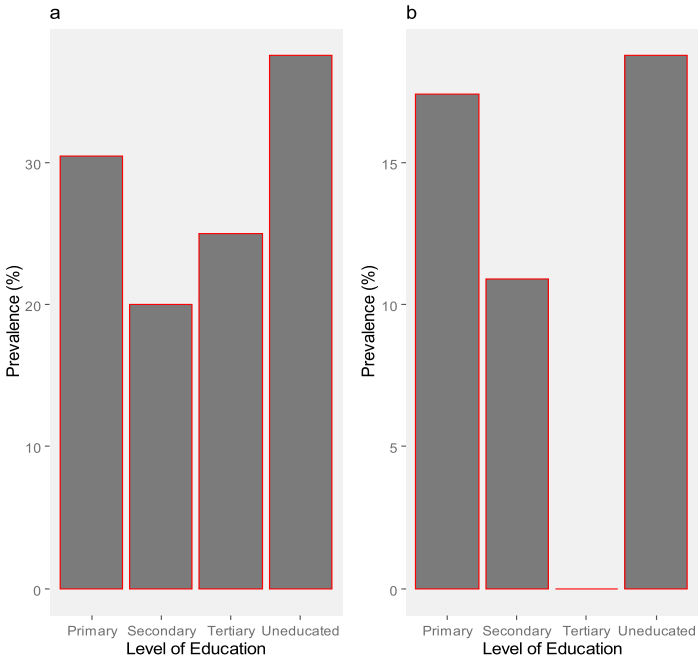

Figure 5: Prevalence of Cryptosporidium parvum and Cryptosporidium hominis amongst individuals infected with HIV in relation to the level of education (a) Represents Cryptosporidium parvum and (b) represesentsCryptosporidium hominis

The Prevalence of *Cryptosporidium parvum* and *Cryptosporidium hominis*in relation to source of drinking water is as shown in Figure 6. The prevalence of *Cryptosporidium parvum*was highest among those that drink sachet water(65.0%) and lowest among those that drink well water (50.0%) and it was statistically significant. While the prevalence of *Cryptosporidium hominis*was highest (65.0%) for those that drink borehole water but insignificant in relation to source of drinking water. This has shown that the two species are present in virtually all the sources of drinking water and that even with sachet water, they can still breed, meaning that modes of purification do not eliminate *cryptosporidium.* Their resistance to conventional water treatments imposes them as endemic pathogens of water origin, hence the need for strict hygienic practices as primary source of control of contamination of drinking water. [13] reported a prevalence of high infection rate which is notably because of source of water which is contaminated with *Cryptosporidium oocyst*. Because of the rigid cell wall of *Cryptosporidium* oocyst, normal purification with chlorine does not remove the parasite. Thisis probably the reason why this study recorded a high prevalence rate as well water due to the reason of large sample size. Well water and borehole arestill a major source of drinking water in developing countries such as is the case with Nigeria where Nasarawa state is the study area where anthroponotic and zoonotic transmission of *Cryptosporidium* oocyst can still occur.

However, in terms of *Cryptosporidium* amongst patients with HIV in relation to livestock farmers, *Cryptosporidium parvum* had a prevalence of 58.0% amongst livestock farmers and 10.0% among non-livestock farmers. *Cryptosporidium hominis* had 25.0% among livestock farmers and 7.0% among non-livestock farmers as shown Figure 7. The prevalence of *Cryptosporidium parvum* and *Cryptosporidium hominins* were significantly in associated with livestock farming (p=0.5). This is predictable so, as *Cryptosporidium parvum* is majorly zoonotic. This also predictable as *Cryptosporidium hominis* among livestock farmers can be infected from aquatic habitat, swimming pools and those working at day care centers.

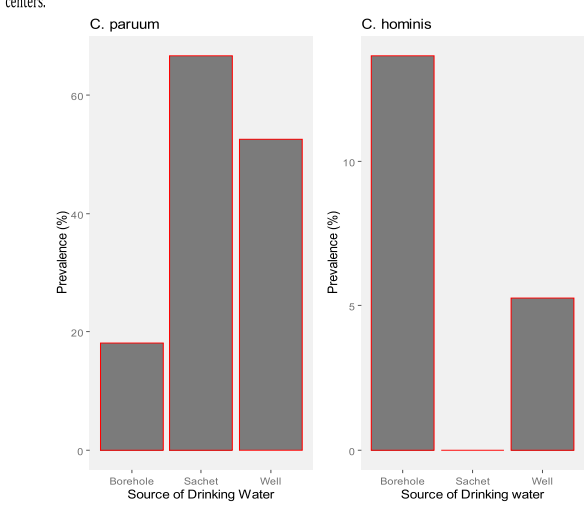

Figure 6: Prevalence of Cryptosporidium parvum and Cryptosporidium hominis amongst individuals infected with HIV in relation to source of drinking water (a) Represents Cryptosporidium parvum (b) Represents Cryptosporidium hominis

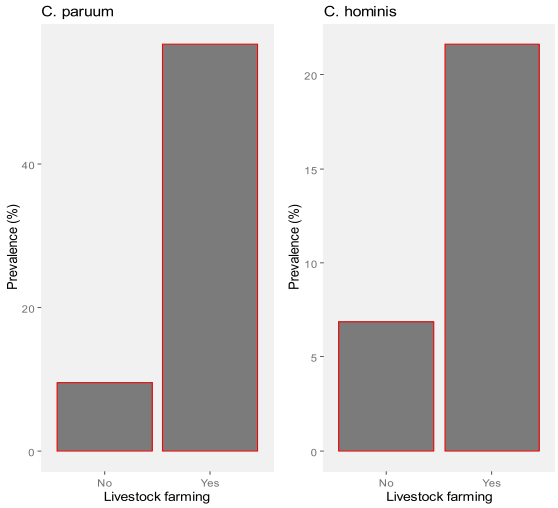

Figure 7: Prevalence of Cryptosporidium parvum and Cryptosporidium hominis amongst individuals infected with HIV in relation to livestock farming. (a) Represents Cryptosporidium parvum(b) Represents Cryptosporidium hominis

4. Conclusion

The overall *Cryptosporidium* parasite prevalences in this study was high.This prevalence should be a source of worry for public health managers in Nasarawa State, because it interprets high population are infected with *Cryptosporidium* and it is a burden in the state. The epidemiology and transmission of this parasite, has shown from this study that the major source of transmission is water, and also livestock, thereby confirming cases of both zoonotic and anthroponotic transmissions covering both male and female genders and higher incidences amongst teenagers and youth populations and the *Cryptosporidium parvum* and *Cryptosporidium hominis*species which are the major species of human host.This study therefore confirms both the prevalence and demography of *Cryptosporidium* among HIV infected individuals in the study population.

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6. Conflict of Interest Statement

The authors declare no conflicts of interest

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