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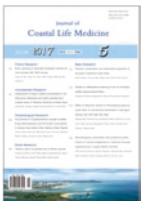

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Occurrence of *Cryptosporidium* oocysts in edible frogs (*Rana* species) sold for human consumption in Hanwa frog market, Zaria, Kaduna State, Nigeria

Grace Sabo Nok Kia*, Blessing Iveren Ukuma, Mathias Bello Odoba, Jerome Unubi Okpanachi

Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria

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ABSTRACT

Objective: To determine the occurrence of *Cryptosporidium* oocysts in edible frogs (*Rana* spp.) sold at the Hanwa frog market, Zaria, Kaduna State, Nigeria.
Methods: A total of 117 frogs (*Rana* spp.) were randomly collected based on consent and availability at the market. The intestinal contents from the frogs were examined by staining flotation and sedimentation smears with modified Ziehl-Neelsen staining technique and microscopy of the oocysts.
Results: Overall, 35.9% of frogs sampled from the Hanwa frog market were positive for *Cryptosporidium* oocysts. There were more *Cryptosporidium* oocysts detected by sedimentation test (28.2%) than flotation test (23.9%). There was no significant statistical association between sex of frogs and oocyst detection ($\chi^2 = 0.5349$, $P > 0.05$). Also, there was no correlation between the weights of frogs and the sizes of the *Cryptosporidium* oocysts detected ($r = 0.0109$, $P > 0.05$). Nevertheless, female frogs (40%) and frogs within the weight range 170–219 g were more infected with *Cryptosporidium* (66.7%). Oocysts size range 6.10–7.00 μm had the highest frequency of 10 (23.8%). By size 28.2% of the oocysts detected suggested infection with *Cryptosporidium parvum* and *Cryptosporidium maleagridis*.
Conclusions: This study has established that edible frogs (*Rana* spp.) sold at the Hanwa frog market for human consumption were infected with *Cryptosporidium* spp. which constitutes a valid public health risk especially for immunocompromised individuals.

1. Introduction

Cryptosporidiosis is a highly transmissible protozoan disease that affects all species of animals including both immunocompromised and healthy humans[1]. It has a high morbidity in susceptible populations. Due to the ubiquitous nature of the infective oocyst, the disease has a worldwide epidemiology[2]. This study was designed to determine the occurrence of *Cryptosporidium* oocysts in edible frogs (*Rana* spp.) sold at the Hanwa frog market, Zaria, Kaduna State, Nigeria, for human consumption. This was achieved by isolation of oocysts from frog samples by flotation and sedimentation techniques, microscopic identification and measurement of isolated oocysts by examination of smeared slides stained by modified ZiehlNeelson method. Since *Cryptosporidium* can be transmitted by ingestion of infected food animals and poorly treated water and by direct contact[3], it is possible for infection to occur through ingestion of under cooked frogs and through poor personal hygiene after handling and processing frogs that may be infected.

Although the epidemiology of *Cryptosporidium* includes a variety of mammals, birds, reptiles, fish and amphibians[3,4], there is paucity of information as regard occurrence of *Cryptosporidium* in aquatic animals in Nigeria. Only recently, the occurrence of *Cryptosporidium* in fish from natural and artificial water in Northern Nigeria was reported[4].

Frogs from wild waters are a unique delicacy in some African countries[5]. In Burkina Faso, the frogs are properly cleaned and eviscerated then fried before consumption; while in Nigeria, they are frequently sundried or smoked and seldom fried prior to consumption. This may pose as health risk for transmission of cryptosporidiosis from infected frogs to humans. In Nigeria, frog meat is fast gaining popularity with frog meat markets springing up

*Corresponding author: Dr. Grace Sabo Nok Kia, Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.
Tel: +2349056362828
E-mail: gracegracekia@gmail.com

All experimental procedures involving animals were conducted in accordance to Humane Anaesthesia and Pain Management in Amphibian Protocol and approved by Ahmadu Bello University Ethics Committee.

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nationwide[5].

Cryptosporidium was among agents implicated in cases of diarrhoea in Human Immunodeficiency Virus (HIV)/Acquired Immune deficiency Syndrome (HIV/AIDS) patients in Kano, Northern Nigeria[6]. Owing to the number of HIV/AIDS patients who commonly suffer from cryptosporidial enteritis and cough, the epidemiology and subsequent policies geared towards the control of cryptosporidiosis in animals and man is very important[7].

Presence of *Cryptosporidium* oocysts in frogs may by implication also reveal the *Cryptosporidium* status of water bodies from various sources where the frogs were caught, aside the risk it poses to other animals and humans.

2. Materials and methods

2.1. Study area

Hanwa frog market, Zaria, Kaduna State, Nigeria is located at latitude 11°04' N and longitude 7°42' E. Edible frogs (*Rana* spp.) used for this study were sourced from different areas in Kaduna and neighbouring states and brought to Hanwa frog market which serves as collection, slaughter, processing and distribution point.

2.2. Sample collection

Live edible frogs (*Rana* spp.) were collected from eight locations based on availability between the months of February and April 2016. Some of the frogs were brought in from neighbouring states (Katsina, Zamfara and Kano) and within Kaduna (Sabon Gari, Tudun Wada, Lokoro) to the frog market at Hanwa Zaria. The frogs were collected in clean sacks then transported to the Veterinary Public Health and Preventive Medicine Parasitology laboratory, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, where they were processed.

2.3. Sample processing

The frogs were sedated with chloroform and their weights were measured in grams using a weighing balance. The sexes of frogs were determined by physical examination ante mortem and dissection post mortem. The abdominal walls of the frogs were dissected aseptically using a dissecting kit to isolate the intestines. The intestinal contents were then milked out and put into sample bottles containing normal saline (0.85% NaCl in distilled water) for analysis by flotation and sedimentation techniques. All experimental procedures involving animals were conducted in accordance to Humane Anaesthesia and Pain Management in Amphibian Protocol[8] and approved by Ahmadu Bello University Ethics Committee.

2.4. Flotation and sedimentation techniques

The intestinal contents were homogenized in normal saline using a glass rod and filtered using gauze and a plastic funnel, the filtrate was centrifuged at 2000 r/min for 5 min, the centrifuged samples were decanted and the flotation media added up to the brim of the sample bottle until a convex meniscus was formed. Glass slides were gently placed on top of the flotation medium and allowed to stay for 30 min. After 30 min the glass slides were gently taken off and the flotation smear, the flotation medium was discarded and the sediment at the bottom of the sample bottle was smeared at another corner of the glass slide, thus each glass slide had two smears: one for flotation and the other for sedimentation. The slides were allowed to air dry for 24 h at room temperature.

2.5. Staining technique

Modified Ziehl-Neelsen staining technique was carried out on the smeared slides. The slides were fixed with methanol for 2–3 min, then stained with carbol-fuschin without heating for 15 min and washed with water. The slides were thereafter decolorized using 5% acid alcohol for 10–15 s, and then washed with water. The slides were counter-stained with 0.3% methylene blue for 30 s, then washed with water and allowed to air-dry[9,10]. The stained slides were viewed using a light microscope at ×40 objective lens.

2.6. Description of Cryptosporidium oocyst

Cryptosporidium oocysts appeared red on a blue background. The degree and proportion of staining varied between oocysts. The internal structures took up various degrees of staining (from amorphous to eccentric). *Cryptosporidium* oocysts appeared as spherical discs with diameters ranging from 4–6 μm[9].

2.7. Micrometry of the oocysts

Measurement of the oocysts from the positive sample was estimated by using a calibrated eyepiece. *Cryptosporidium* oocyst size range was 4–6 μm[10]. A calibrated eye piece was inserted into the micrometer, a stage micrometer graticule was focused to align with the calibration on the eye piece, and the calibration on the eyepiece was then determined[11]. The positive slide was placed on the stage of the microscope and focused while viewing from the eye piece. The oocyst was moved towards the calibration by adjusting the knob to achieve alignment of the oocyst and the calibration, and measurement was taken from one end of the oocyst to the other end. This measurement was done in the Helminthology Laboratory of the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria.

2.8. Statistical analysis

Using Graghpad prism version 5, Pearson's correlation (r) and Chi square (χ^2) statistics were calculated to determine association between independent and dependent variables. P-values less than 0.05 were considered statistically significant.

2.9. Limitation

Sensitivity and specificity of the flotation and sedimentation tests could not be ascertained as we were unable to conduct an ELISA test, which is the gold standard on the samples. This was due to limited funds.

3. Results

Cryptosporidium oocysts were found in 42 (35.9%) of the 117 frogs sampled. Of these 117 frogs sampled 72 were males (61.5%) and 45 were females (38.5%).

The samples were evaluated for statistical relationship between the sex of the frogs and the *Cryptosporidium* occurrence rate. Among the 42 frogs which had *Cryptosporidium* oocysts, 18 (40.0%) of females and 24 (33.3%) of the males were positive, thus giving a higher female to male ratio of *Cryptosporidium* occurrence. This relationship was not statistically significant (χ^2 = 0.5349, *df* = 1, $P > 0.05$). The occurrence of *Cryptosporidium* oocysts was also evaluated based on the source, location, and distribution (Table 1) which revealed the highest occurrence of *Cryptosporidium* in frogs sourced from Zamfara State (46.7%) followed closely by Sabon Gari, Zaria, Kaduna State (45%).

Table 1
Locational distribution of frogs (*Rana* spp.) infected by *Cryptosporidium* oocysts from the Hanwa frog market, Zaria, Kaduna State, Nigeria [*n* (%)].

Source of frog	Total No. of frogs sampled (%)	No. of frogs positive (source specific rate)	Proportional positivity rate (%)
Tudun Wada, Zaria	20 (17.1)	8 (40.0)	19.0
Sabon Gari, Zaria	20 (17.1)	9 (45.0)	21.4
Lokoro, Zaria	20 (17.1)	3 (15.0)	7.1
Katsina	15 (12.8)	5 (33.3)	11.9
Zamfara	15 (12.8)	7 (46.7)	16.7
Kano	10 (8.5)	4 (40.0)	9.5
Kaduna	10 (8.5)	3 (30.0)	7.1
University farm, ABU	7 (5.9)	3 (42.9)	7.1
Total	117 (100.0)	42 (35.9)	100.0

The samples were also evaluated for relationship in weight and the occurrence of *Cryptosporidium* (Table 2). Findings revealed the highest occurrence (66.7%) was among the weight range 170–219 g, while the weight specific rate showed that 50.0% of frogs within the weight range of 70–119 g were found with *Cryptosporidium* oocysts.

On evaluation of the detection of *Cryptosporidium* oocysts using the two techniques, *viz*. flotation and sedimentation tests. Higher oocyst detection was obtained using the sedimentation (28.2%) than the flotation (23.9%) technique.

The diameter of the oocysts varied from 3.49 µm to 13.96 µm, with the highest frequency of 10 occurring for the oocyst size range 6.10–7.00 µm followed by the frequency of nine occurring for the oocyst size range 10.10–11.00 µm. There was no statistical significance between the weights of frogs and the size of oocyst ($P > 0.05$).

4. Discussion

The occurrence of *Cryptosporidium* oocysts in edible frog (35.9%) in this study may indicate that the water bodies may have been contaminated with human and/or animal faecal material. The water bodies from which the *Cryptosporidium* infected frogs were caught are by implication unsafe for human consumption and unfit for irrigation farming. This is because cryptosporidia are generally resistant to the common water treatments such as chlorination and aeration. There is also the risk of infection by ingestion of fruits and vegetables contaminated with irrigation water rich in infective oocysts. This finding agrees with Green *et al*.[11] who reported a case of *Cryptosporidium* oocysts in a 2-year-old female South African clawed frog (*Xenopus laevis*). This finding also agrees with Crawshaw and Mehren[12] who reported naturally acquired *Cryptosporidium* infection in a Bell's horned frog (*Ceratophrys ornate*), and Magi *et al*.[13] who reported a case of *Cryptosporidium* infection in an American toad (*Bufo americanus*). The frogs sampled in this study were also gotten from natural water bodies. This implies high levels

Table 2
Locational distribution of frogs (*Rana* spp.) infected with *Cryptosporidium* oocysts using flotation and sedimentation tests from frogs sold at the Hanwa frog market, Zaria, Kaduna State, Nigeria.

Source	Total No. sampled	Flotation test positives (SSR)	Sedimentation test positives (SSR)	Positives by both Flot. and Sed.	PPR for both Flot. and Sed.
Tudun Wada, Zaria	20	7 (35.0)	5 (25.0)	8 (40.0)	19.0
Sabon Gari, Zaria	20	5 (25.0)	8 (40.0)	9 (45.0)	21.4
Lokoro, Zaria	20	3 (15.0)	1 (5.0)	3 (15.0)	7.1
Katsina	15	5 (33.0)	4 (26.0)	5 (33.0)	11.9
Zamfara	15	4 (26.0)	6 (40.0)	7 (46.0)	16.7
Kano	10	2 (20.0)	4 (40.0)	4 (40.0)	9.5
Kaduna	10	0 (0.0)	3 (30.0)	3 (30.0)	7.1
Uiversity farm, ABU	7	2 (28.0)	2 (28.6)	3 (42.0)	7.1
Total	117	28 (23.9)	33 (28.2)	42 (35.9)	100.0

SSR: Source specific rate; PPR: Proportional positivity rate; Sed: Sedimentation test; Flot: Flotation test.

of contamination with cryptosporidia oocysts.

Cryptosporidium is resistant to chlorine and other water disinfectants[14]. This poses a challenge for the water management system in the study area as pipe borne water will contain this organism which is highly resistant to chlorination of water, making the water unsafe for human consumption even after treatment of the water[15].

This study also points out that frog samples sourced from Zamfara State have the highest number of positives (46.7%) followed by Sabon Gari (45%), Zaria, suggesting that there may be greater level of water contamination with human and or animal faecal material in these water sources than other water sources within the study area. Most houses in these areas have no toilets and people defaecate in the open fields, while animals are found roaming around with no supervision. Water runoff during the rains may have transported *Cryptosporidium* among dirt and debris into the water bodies which serve as water sources for cooking, drinking and other household chores in these various areas. Sedimentation technique resulted in a higher detection of *Cryptosporidium* oocysts as compared to flotation which agrees with previous reports[15].

Oocysts size range of 6.10–7.00 μm had the highest frequency of 10 (23.8%), 28.2% of the oocyst sizesuggested infection with *Cryptosporidium parvum* and *Cryptosporidium maleagridis*[9]. Frog meat should be thoroughly cooked before consumption. Proper hygiene and sanitation should be ensured when processing frogs for human consumption.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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