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Molecular Characterization of Hookworm Detected among Peasant Farmers and their Haemoglobin Profile in selected Local Government Areas of Kaduna State, Nigeria.

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ABSTRACT

This study aimed at determining the prevalence of hookworm infection among peasant farmers in selected Local Government Areas (LGAs) of Kaduna State, Nigeria. A cross-sectional, descriptive study was carried out between November 2014 and October 2015. One thousand two hundred eligible peasant farmers were enrolled in the study. One LGA was selected from each of the three Senatorial Zones of Kaduna State by simple random sampling method. Samples were analysed for the presence of hookworm ova using direct wet mount and formal-ether concentration technique as well as determine the haemoglobin profile and characterize the hookworm species. The haemoglobin profile of the peasant farmers in relation to hookworm infection was determined using Mission Haemoglometer Stirps Test Kits. The stool samples that were highly positive microscopically for hookworm ova were selected for molecular analysis, 5 from each LGA, making a total of 15 samples. The stool samples were subjected to DNA extraction, DNA concentration/Purity estimation, PCR, Agarose gel electrophoresis, Sequencing, BLAST and Phylogenetic analysis of the isolates were carried out. The results obtained showed the overall prevalence of hookworm infection in the study population to be 18.7%. In respect to LGA, the prevalence of hookworm infection was 24.0% in Jema'a, 17.3% in Chikun and 14.8% in Zaria LGA. The prevalence of hookworm infection was found to be significantly associated in the studied LGAs (P< 0.002). The distribution of hookworm infection in relation to Haemoglobin (Hb) level revealed that, the farmers with the Hb/PCV level of 6-10/18-30% had the highest prevalence of 43.0% (128/298) while farmers with Hb/PCV of 16-20/48-60% had the lowest prevalence of 3.7% (1/27) and a statistically significant difference of P=0.000. The electrophoresis of 15 amplicons obtained from 15 positive stool samples from the three selected LGAs showed a corresponding base pair band of 310bp which corresponds with the base pair of the *N. americanus* positive control. The sequencing and basic local alignment search tool (BLAST) revealed that Sequence of Sample No. 5 from Zaria LGA had 95% identity with that of *N. americanus*, sequence of Sample No. 10 from Chikun LGA had 99% identity with that of *N. americanus* and finally sequence of Sample No.15 from Jema'a LGA also gave a similar correlation of 97% as that of *N. americanus.* The result of the species-specific identification of the two human hookworm species (i.e. *N. americanus* and *A. duodenale*) obtained shows that *N. americanus* was predominant in the study area (Kaduna State, Nigeria) during the study. The results of this study indicated an overall prevalence rate of hookworm infection of 18.7% among peasant farmers in Kaduna State. This indicates a potential risk of severe anaemia among individuals particularly the vulnerable groups such as pregnant women and children of school age who participate in farming. In conclusion, this study revealed the transmission of human hookworm among peasant farmers in Kaduna State to be at an alarming rate. Thus, major prevention and control measures should be adopted to avoid further spread of the infection; and more so, there is a need for prompt treatment of the infected persons as well as creating a law that will prohibit indiscriminate defeacation on farmlands.

Introduction

Hookworms are nematodes belonging to the family Ancylostomatidae, super-family Strongyloides. Human hookworm infection is a soil-transmitted infection caused by Necator americanus and Ancylostoma duodenale. It is the leading cause of anaemia and protein malnutrition, afflicting an estimated 740 million people in the developing nations of the tropics (Cheesebrough, 2005; CDC, 2013).

Hookworm infection is spread by faecal contamination of the soil; infection occurs when infective third-stage filariform larvae (L_3) penetrate the hands, feet, arms or legs, especially when a person walks bare-footed (Paniker and Jayaram, 2007). While farmers are the most vulnerable group to acquire this infection, signs of advanced severe infection include anaemia and protein deficiency, including emaciation, cardiac failure and abdominal distension (Ayoya et al., 2006; Drisdelle, 2006). The infection has been noted to be more common in families who are involved in agricultural pursuits (Damen et al., 2007). The resultant effect of hookworm infection includes severe anaemia leading to high morbidity and mortality and consequently causing low productivity and food insecurity in Nigeria.

Results and Discussion

Out of the 1,200 stool samples examined, the overall prevalence of hookworm was 18.7% (224/1200). In respect to LGA, The highest prevalence of Hookworm

infection was in Jema'a LGA of the Southern Senatorial Zone 96 (24.0%), followed by Chikun LGA of the Central Senatorial Zone 69 (17.3%) and Zaria LGA of Northern Senatorial Zone had the least 59 (14.8%) with a statistical significant difference (P=0.002) (**Table1**). The differences may be due to the topography of the location, the climatic condition, type of soil and the tradition as well as the agricultural cultural practices of the people and their environmental sanitary attributes. It was also noted that farmers in Zaria LGA have the privileged of using pipe-borne water and boreholes as their source of drinking water while most of the farmers in Jema'a and Chikun uses mostly water from the rivers and wells which are more exposed to all kinds of pollution or faecal contamination. They also had a lower number of toilet facilities and many of them undergo backyard farming barefooted (Brooker et al, 2004; Thomas, 2009). Hence, these could be the reasons for their differences in prevalence rate.

The prevalence of hookworm infection among farmers in relation to their Haemoglobin (Hb) profiles were also obtained. Those having Hb level 6- 10g/dl (pcv18-30%) had the highest prevalence of 43.0%, while those with Hb 16-20g/dl (PCV 48-60%) had the least prevalence of 3.7% with a statistically significant difference of P=0.000 (**Table 2**). The results obtained from this study revealed that majority of the farmers did not have their haemoglobin level fallen within the expected normal values as previously reported by Hoffbrand et al., (2011) who stated the normal adult red cell values as 11.5 to 17.5g/dl (36 to 52%). The result of this study revealed that hookworm infection has a serious effect on the haemoglobin level of the farmers infected, which can lead to severe anaemia. This is also attributed to the fact that Hookworm has the ability to suck a large quantity of blood meal from their host per day. for example WHO, 1991 estimated blood loss in the hookworm-infected individual as 0.20 mL per worm per day (range 0.14 – 0.26 mL) for A. duodenale and 0.04ml per worm per day (range 0.02 – 0.07 mL) for *N. americanus.*

It was observed that the concentration and purity of the human hookworm DNA extracted from the positive stool samples selected were pure except for the few samples that suggested the presence of little impurities such as samples number 8 and 13 that had a purity of 2.02 and 2.01 respectively (**Table 3**) as recognized by (Clark and Kimberley, 2001). This could be due to the fact that highly positive stool samples were selected from each LGA and there was no contamination of proteins or other concomitant impurities.

The electrophoresis of 15 amplicons to detect the target gene (Cytochrome oxidase 1 gene) revealed a corresponding base pair band of 310bp which corresponds with the base pair of the N. americanus positive control in all the three LGAs (**Figure 1**). The resultant sequences obtained from the three representative amplicons of positive samples after the Basic Local Alignment Search Tool (BLAST) showed identity similarity of N. americanus as obtained from Togo by Hu *et al*., (2003) with 99%, 97% and 95% identity in Jema'a LGA, Chikun LGA and Zaria LGA respectively (**Table 4**).

The species-specific identification of the two human hookworm species (i.e. *N. americanus* and *A. duodenale*) obtained in this study revealed that N. americanus is predominant in the study area (Kaduna State Nigeria). However, we strongly believe that there could be the presence of a mixed infection of *A. duodenale* missed out during the selection of samples for molecular study. In addition, the migration of people from Europe and other endemic areas of N. americanus during Slave trade, Missionary era and even up till today to Sahara and Sub- Saharan Africa could have contributed to the transmission of the *N. americanus* to Kaduna State, Nigeria and other West African countries such as Togo. Furthermore, considering the fact that climatic condition plays a vital role in the growth, replication, and transmission of hookworm and that *N. americanus* is a worldwide parasite which strives better in the warm region of China, Sahara, and Sub- Saharan Africa, it is possible that after being transferred to these regions became highly proliferative due to conducive environmental factors suitable for them, hence dominate the presence of *A. duodenale.*

The Phylogenetic analysis generated, gave a cladogram (**Figure 3**), this revealed that the *N. americanus* obtained from the study area are all from

the same genetic origin, even though that of Jema'a LGA and Chikun LGA tends to be more closely related than that of Zaria LGA. There is also a close genetic relationship between the isolates (*N. americanus*) from this study area (Kaduna, Nigeria) and those of Togo and China. It is also obvious that there is a close genetic relationship between the hookworm isolates (*N. americanus*) from Nigeria and that of Togo, this could be due to the fact that Nigeria has a closed border with Togo hence easy migration by infected individuals from Togo to Nigeria and verse versa, on the other hand, the close genetic relationship between the hookworm isolates (*N. americanus*) from Nigeria and that of China could also be due to movement or migration of Chinese engineers who came to Nigeria and constructed railway lines particularly in the study area (Kaduna State).

The result of this work agrees with the work of many researchers who observed that *N. americanus* is endemic in many Tropical and Subtropical regions of the world, including parts of Africa, India, China, South-east Asia, the South-west Pacific Islands, South and Central America, the Caribbean Island and Southern USA. Liu *et al*., 1999; Behnke *et al*., 2000; Gandhi *et al*., 2001).

Table 1: Prevalence of Hookworm Infection in Three Local Government Areas of Kaduna State (Using Stool Microscopy).

Local Govt. Area	Number Examined	Number Positive (%)	P-Value
Jama'a	400	96 (24.0)	0.002
Chikun	400	69 (17.3)	
Zaria	400	59 (14.8)	
Total	**1,200**	**224 (18.7)**	

Table2: Prevalence of Hookworm Infection among Peasant Farmers in Relation Haemoglobin Level g/dl, Pack Cell Volume (PCV%) in Kaduna State, Nigeria.

Haemglobin level g/dl /PCV (%)	Number Examined	Number Positive (%)	P-Value
0-5 (0-15)	4	1(25.0)	
6-10(18-30)	298	128(43.0)	0.000
11-15(33-45)	87`	94(10.8)	
16-20(48-60)	27	1(3.7)	

Normal Adult red cell value: Haemoglobin is 11.5 - 17.5g/dl and PCV is 36 – 52% (Hoffbrand et al., 2011).

Table 3: Concentration and Purity of DNA Extracted from Human Hookworm

Sample No.	Absorbance at 260 nm (A_{260})	Absorbance at 280 nm (A_{280})	A_{260}/ A_{280} Ratio (Purity of DNA)	Concentration of DNA Extract (µg/ml)
1	1.250	0.640	1.95	62.50
2	1.150	0.620	1.85	57.50
3	0.945	0.502	1.88	47.25
4	1.055	0.555	1.90	52.75
5	1.048	0.580	1.81	52.40
6	0.975	0.543	1.80	48.75
7	0.965	0.488	1.98	48.25
8	0.990	0.450	2.02	49.50
9	1.023	0.568	1.80	51.15
10	0.875	0.485	1.80	43.75
11	1.003	0.530	1.90	50.15
12	0.875	0.485	1.80	43.75
13	1.325	0.660	2.01	66.25
14	1.295	0.648	2.00	64.75
15	1.003	0.510	1.96	50.15

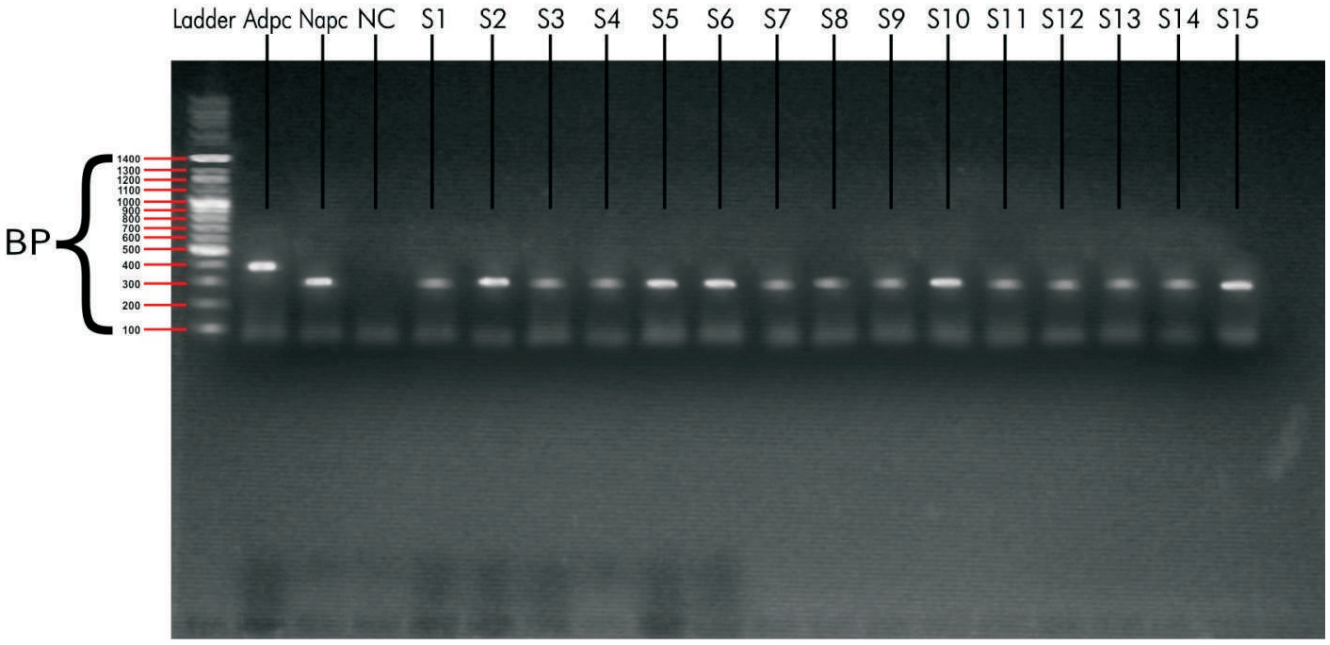

Figure 1: PCR gel Photo of Amplicons of Hookworm isolated from stool samples of peasant Farmers showing the BP of Cytochrome Oxidase 1 Gene (CO1 Gene). BP – Base pair, Adpc – Ancylostoma duodenale Positive control, Napc – Necator americanus positive control, NC – Negative control, S - Sample.

Table 4: Result of Nucleotide Sequences submitted to NCBI Gene Bank (submission ID: 2109788).

Sample No.	LGA	Access No	Max Score	Total Score	Query cover (%)	E-Value	% Identity	Description of organism	Country
5	Zaria	M H 311534	676	676	100	0.0	95	*N. americanus*	Nigeria
10	Chikun	M H 311535	839	839	100	0.0	99	*N. americanus*	Nigeria
15	Jema'a	M H 311533	835	835	99	0.0	97	*N. americanus*	Nigeria

Figure 2: Multiple sequence alignment of hookworm isolates from peasant farmers.

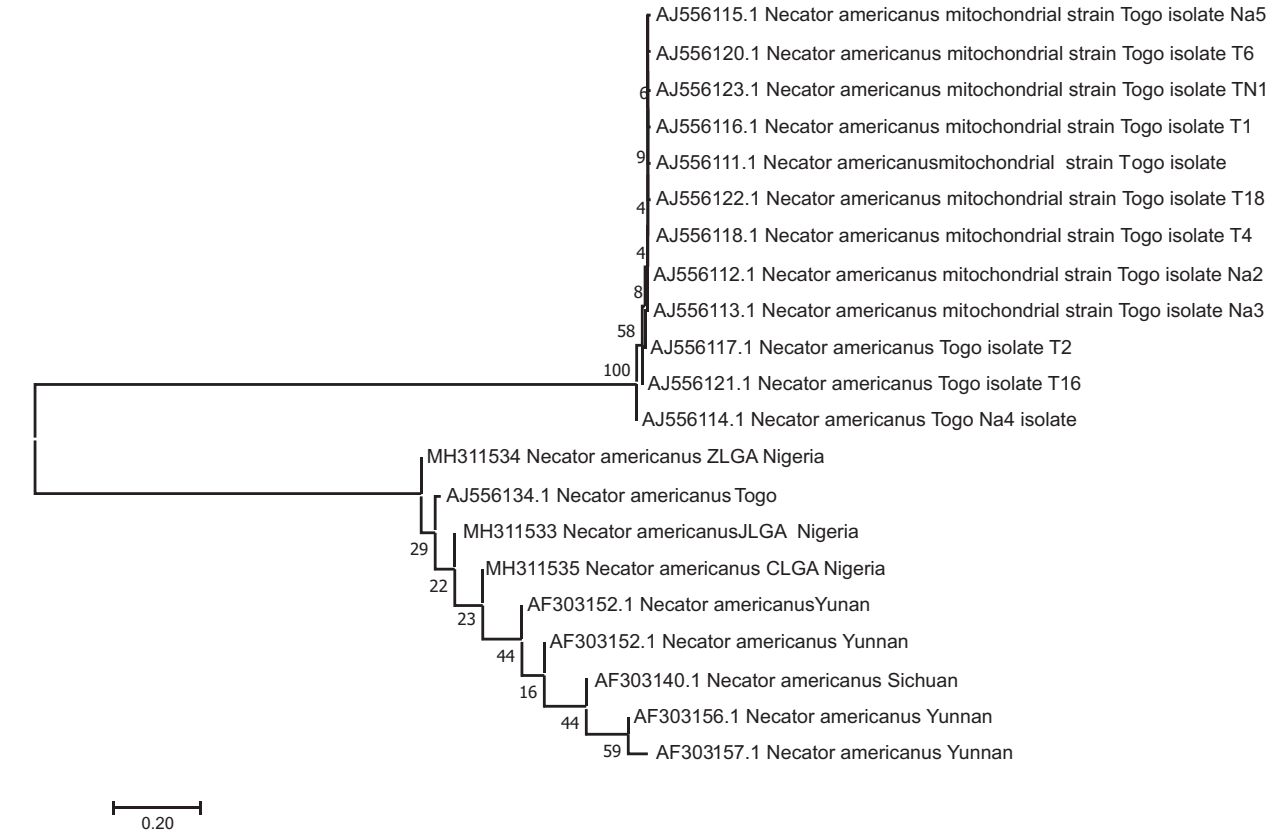

Figure 3: Phylogenetic Tree of The Hookworm Isolates Obtained from Peasant Farmers.

Conclusion

This study reported a prevalence rate (18.7%) of Hookworm infection among peasant farmers in three LGAs in Kaduna State. This indicates a potential danger among individuals particularly the vulnerable groups such as pregnant women and children of school age who participate in farming.

Further studies by molecular detection using the PCR and gel- electrophoresis revealed that *N. americanus* is the main hookworm species common in the study area. However, there is a high possibility of getting some *A. duodenale* or mix infection of *N. americanus* and *A. duodenale* if a higher number of samples are used for molecular detection.

The sequencing and basic alignment search tool (BLAST) further confirmed that hookworm was isolated, and all were *N. americanus* species. Therefore, it can be concluded that *N. americanus* is the major hookworm species common in the study area since the result of the Phylogenetic analysis generated, gave a cladogram identical to that of the gene bank obtained from Togo and China as well as "one clade" (Jema'a LGA, Chikun LGA and Zaria LGA) which confirmed that the *N. americanus* obtained from the study area are all from the same genetic origin.

It is very important to note that farming is an occupation that is practised all over the whole world; there is no nation that can survive without agriculture. Before the discovery of petroleum in Nigeria, agriculture was the number one source of income in the Nigerian economy. Now considering the Nigerian economic recession, the Government is calling on all Nigerians to turn to agriculture which I concur. Finally, from both past and present studies, there is overwhelming evidence that hookworm infection is now a public health problem; there is also good evidence from pilot studies that the problem is amenable to solution.

Recommendations

Based on the findings of this study, the following

recommendations were made:
There should be awareness campaign programmes in respect to the indiscriminate defecation on farmland and the use of untreated human excreta, animal dung or raw sewage should not be used as fertilizer in agriculture unless decomposed to destroy the ova and larvae of hookworm by heat generated or ammonium sulphate should be added to the fresh faeces to a strength of 12% to destroy the hookworm embryo within 24 hours. People should not walk bare-footed especially in known infected areas and the use of personal protective equipment by farmers should be encouraged Pipe-borne water and bore-hole should be made available to the populace to replace rivers and other bad sources of water supply and should be treated.

Mass treatment of infected farmers and the general public is highly recommended, especially in the rural areas where peasant farming is being practised. It is also recommended that if a patient is suffering from severe anaemia ferrous sulphate (200 mg) be administered three times daily at the same time with antihelmintic treatment; this should be continued until haemoglobin values return to normal. Ground-itch, blisters, and wounds were found to be common among farmers in the study area which corresponds to the clinical pathogenic characteristics of *N. americanus.* Hence, blisters and wounds caused by an allergic reaction should be cleaned and properly treated by health personnel.

The Government of Kaduna State should be enforced the law on environmental sanitation and ensure proper disposal of sewage and faecal matter in both rural and urban areas of the state. A similar study should be carried out in the same study area and the number of positive samples used for species specific molecular identification be increased to see if there could be *A. duodenale* or mix infection of *N. americanus* and *A. duodenale.*

Materials and Methods

Study Area

The study was carried out in some selected LGAs of Kaduna state. The state is located between latitude 90° and 140° north of the equator and longitude 70° and 100° east of the Greenwich meridian, it occupies a landmass of about 70,210 square kilometers on the map of Nigeria. The topography is that of an undulating plateau that forms part of the rich tourist attractions in areas like Kufena in Zaria, Kagoro, Kwoi, and Gwantu. According to the National Population Commission (2006) census figure, Kaduna state has a population of over 6 million people. Kaduna State has 23 Local Government Areas and 3 Senatorial Zones (North, Central, and Southern) Senatorial Zones.

The main occupations of the majority of the people in the study area are peasant farming, petty trading, mining and a few white-collar jobs. The state has two distinct seasons (dry and wet or raining seasons). The former takes place from November to March while the latter occurs between April and October.

Study design

A cross-sectional, descriptive study was carried out between November 2014 and October 2015. One thousand two hundred eligible peasant farmers were enrolled in the study. The study was carried out in some selected LGAs of Kaduna State, Nigeria by simple random sampling method.

Study population

The population studied comprised voluntarily consented peasant farmers in Jema'a, Chikun and Zaria Local Government Areas in Southern, Central and Northern zones of Kaduna State respectively, where farming activities are very high involving the vulnerable groups (women and children between ages 10 and 15 years); of the peasant farmers (male and female) considered was 10 years and above. Ethical clearance was obtained from the Kaduna State Ministry of Health. A feasibility study of the selected LGAs was carried out with the co-operation of the district heads; sensitization lecture was given to the people in all the study areas.

Sample Collection and Analysis

Sample Size

Using a reported 36% prevalence of intestinal parasitic contamination of vegetables in Jos, Plateau State,

Nigeria by Damen *et al* (2007), the sample size was calculated using the formula of Israel (1992);

$$n = \frac{Z^2pq}{d^2}$$

Where n = number of samples to be collected

Z = standard normal distribution at 95% confidence limit = 1. 96

P = prevalence rate of infection of previous study = 36% = 0.36

q = 1- p

d = absolute desired precision = 0.05%

The calculated sample size was 354 but was estimated to four hundred (400) stool samples; these were collected from each Senatorial zone of which a total of one thousand two hundred (1,200) stool samples were collected from the three senatorial zones of the state and used for the study.

Inclusion and Exclusion Criteria

Children aged 10 years and above who participate actively or assist their parents in farming were included in the study and all consenting farmers. Children below 10 years of age were excluded from the study, non-consenting parents together with their children as well as all civil servants, business or traders and those who do not depend on farming as their main source of income and livelihood were excluded.

Microscopy

A cross-sectional, descriptive study was carried out between November 2014 and October 2015. A total of 1,200 samples were collected, comprising 400 samples from each Local Government of the three senatorial zones of the state. Specimens were collected using a clean-labeled container and then packed in insulated iceboxes before transported to the microbiology laboratory of Ahmadu Bello University.

Microscopy was carried out using Direct Wet Mount and Formal-Ether Concentration Techniques; Using an applicator stick, 1g (pea-size) of the stool was taken from a mixed specimen into 10 mL of normal saline (physiological saline) it was emulsified and sieved through gauze using glass funnel into a pointed end glass centrifuge tube then washed twice by centrifuging at 3000rpm for 5 minutes, the supernatant was discarded and the deposit resuspended and transferred into a screw-cap centrifuge tube then 7 mL of 10% formal saline was added then followed by 3mLof ether, it was covered and was shaken vigorously for 20 seconds, then centrifuged at 3000 rpm for 3 minutes. After centrifuging, the parasite's ova were separated to the bottom of the tube and the faecal – debris was collected in a layer between the ether and formal saline, using the applicator stick , other 3 layers were removed leaving only the sediment at the bottom of the tube which were suspended and a drop of it placed at the centre of a clean grease free slide and covered with cover slip carefully avoiding air bubbles and examined systematically under x10 and x40 objectives respectively (Cheesbrough, 2015). The results were recorded as Scanty 1-3 (+) per preparation, few 4-10 (++), Moderate 11-20 (+++) and Heavy 21-40 (++++) (Cheesbrough, 2010). Remaining Samples were preserved using 95% ethanol and kept in the refrigerator at -4°C for further molecular studies. The Haemoglobin (Hb) analysis was carried out by the use of the Mission- Haemoglometer machine. A lancet was used to prick the finger of the individual after cleaning with a swab and a drop of blood was placed on the sensor path of the Hb strip and within 5 seconds Haemoglobin (Hb) level of the client was read and recorded immediately.

DNA Extraction

The PowerSoil DNA Kit was used according to manufacturer's (Biotechnology Research Center Inqaba, South Africa) instructions, in the DNA Laboratory, Faculty of Veterinary Medicine Ahmadu Bello University Zaria, Nigeria. Genomic DNA was extracted directly from microscopically positive fecal samples. Approximately 0.3 g of fecal sample was added into a Power Bead Tube and incubated at 70°C for 10 minutes with the presence of cell lysis and disruption agent provided by the manufacturer. Subsequently, the fecal sample was subjected to homogenization and lysis procedure for complete cell lysis by mechanical shaking using Vortex Machine (J.P. SELECTA,7001721). The extraction of the DNA was carried out using the cold centrifuge machine (SUNON WEALTH ELEC MACH TGL-16G). Final elution of DNA was performed in 50 mL of elution buffer. The extracted

DNA was stored at –20°C until Polymerase Chain Reaction (PCR) was required. Quantification and purity of DNA were determined using spectrophotometer (Shimadzu 1700) as previously described (Gallagher and Wiley, 2008).

SEMI-NESTED PCR Assay and Sequencing

A two-step semi-nested PCR was used for DNA amplification of hookworm species. For the first amplification, forward primer NC1 (5'-ACG TCT GGT TCA GGG TTC TT-3') and reverse primer NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') was used to amplify approximately 310-basepair and 420-basepair regions of internal transcribed spacer 2 and 28S ribosomal RNA region of *N. americanus* and *Ancylostoma spp.* The PCR was conducted in a 50 mL volume with the final mixture containing 10x PCR buffer, 2.5 Mm dNTPs, 25 mM $MgCl_2$, 10 pmol of each primer, 5 units of Taq polymerase, and 6 mL of DNA template. The sample was heated to 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds (denaturing), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), and a final extension at 72°C for 7minutes. Negative control samples without DNA (DNase/RNase free water) and samples containing N. americanus and *Ancylostoma spp*. genomic DNA (positive control) was included in each PCR run. Subsequently, samples that produced a fragment approximately 310 and/or 420 base pairs in the first PCR were subjected to a second amplification. Amplification was conducted by using forward primer NA (5¢-ATGTGCACGTTATTCACT-3¢) for N. americanus18 and AD1 (5¢-CGA CTT TAG AAC GTTTCG GC-3¢) for Ancylostoma spp and NC2 as a common reverse primer. The secondary amplification reagent concentrations were as described above except that 6 mL of primary PCR product was added instead of DNA. The cycling conditions for the second round of amplification was 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute (denaturing), 55°C for 1 minute (annealing), and 72°C for 1 minute (extension), and a final extension at 72°C for 7 minutes. The integrity of the amplicons was checked using an agarose gel electrophoresis (1%). The amplicons were later sequenced (Sanger sequencer) and the sequences (3) were deposited in the NCBI gene bank with accession number (MH311533-MH311535). A phylogenetic tree was constructed for the sequences using the Neighbour– joining and bootstrap at 2000.

Statistical Analysis

The results obtained were analyzed using the Statistical Package for Social Science (SPSS) Version 22. Pearson chi-square test was used to measure the association between variables. Statistical significance was indicated by a two-tailed test at 95% confidence intervals and $P \leq 0.05$ was considered significant.

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