UJMR, Vol. 10 No. 1, June, 2025, pp. 141 - 154 *E-ISSN: 2814 – 1822; P-ISSN: 2616 – 0668*

https://doi.org/10.47430/ujmr/25101.015

Received: 16th February, 2025 **Accepted:** 12th May, 2025

Molecular Detection of Benzimidazole Resistance Genes and Associated Risk Factors in Hookworms from HIV-positive and HIV-negative Patients in Plateau State, Nigeria

[1]H.Y. Azi, [2]H.I. Inabo, [2]O.S. Olonitola and [2]H.G. Bishop

[1]Department of Microbiology, Faculty of Natural Sciences, University of Jos, Plateau, Nigeria

[2]Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University Zaria, Kaduna, Nigeria

Corresponding Author: hezekiahazi@gmail.com

Abstract

An individual may be predisposed to infection with hookworms through various activities that bring them into contact with filariform larvae in soil. Parasite control programs that rely on mass drug administration (MDA) over prolonged periods using the same antihelmintic drugs would exert selection pressure on hookworm populations and favour the development of drug resistance. This cross-sectional study was conducted to detect benzimidazole resistance genes and assess risk factors of hookworm infection among HIV-positive and HIV-negative patients in Plateau State, Nigeria. A total of 526 stool samples were collected and analysed for hookworm eggs using wet mount and formol-ether concentration techniques. Specimens confirmed to be hookworm-positive by microscopy were further subjected to molecular analyses on Real-Time qPCR. A well-structured questionnaire was administered to each participant to obtain information on risk factors. The result showed an overall prevalence of 6.46%; with 3.62% and 10.36% for HIV-positive and HIV-negative patients, respectively. Subjects who arrived at their farms before sunrise recorded higher hookworm prevalence (9.63%) compared to those who arrived at their farms after sunrise (2.30%). Also, subjects who conduct farm activities barefoot had a higher hookworm prevalence (7.99%), while those who wear footwear had a lower prevalence (3.07%). Again, subjects who had never been dewormed had a higher hookworm prevalence (20.93%); while those who were dewormed less than 6 months ago had a lower prevalence (1.85%). A statistically significant association was found to exist between hookworm infection and these risk factors at P ≤ 0.05. Out of the 34 samples positive by microscopy for hookworms, 16 were confirmed to be Necator americanus on qPCR. However, 5 out of the 16 samples were from HIV-positive patients, while 11 were from HIV-negative patients. Single-nucleotide polymorphisms (SNPs) were detected in 2 samples on codons 167 and 200. The occurrence of these SNPs was found only among HIV-negative patients. In conclusion, the detection of SNPs suggests that mutant genes associated with benzimidazole resistance are circulating in hookworms in Plateau State, Nigeria.

Key words: Benzimidazole resistance, Hookworms, HIV patients, Single-nucleotide polymorphism.

INTRODUCTION

Hookworm infections are associated with the highest global burden of disease, resulting in an estimated 500 million infections and a loss of 3.5 million disability-adjusted life-years (WHO, 2023). Hookworm is a blood-feeding intestinal worm, and the mature larvae ingest the blood, rupture the erythrocytes, and degrade the hemoglobin by attaching to the gut wall, which results in iron deficiency anemia (Zeleke *et al.*, 2021). Hookworm-associated clinical symptoms, such as abdominal pain, diarrhea, and protein malnutrition, commonly occur; however, the principal clinical manifestation of hookworm disease is Iron Deficiency Anemia (IDA) as a result of intestinal blood loss (Abuzeid *et al.*, 2020). The most damaging effects of hookworm infections include impaired physical, intellectual, and cognitive development of children, increased mortality in pregnant women and their infants, and reduced work capacity of adolescents and adults (Zeleke *et al.*, 2021).

In regions where sanitation is poor, human faeces may contaminate the soil, allowing hookworm eggs to hatch and develop into infective larvae. Walking barefoot on contaminated soil increases the risk of acquiring the infection. Furthermore, hookworm prevalence is often higher in rural agricultural areas, where individuals have more frequent contact with soil and limited access to healthcare (Brooker *et al.*, 2004). Strategies for control of hookworms and other Soil-transmitted helminths (STHs) rely on periodic deworming of groups at greatest risk of morbidity. The World Health Organization (WHO) currently recommends annual or twice-yearly mass drug administration (MDA) of antihelmintics, including mebendazole or albendazole, to school-age children (WHO 2023). The current benzimidazoles administered in large-scale deworming programs, albendazole (ALB) and mebendazole (MEB), are both given as a single oral dose of 400 mg and 500 mg, respectively (Walker *et al.*, 2021). At the population level, these two drugs exhibit very high therapeutic efficacy (measured as the percent reduction in arithmetic mean egg counts following drug administration in a population, known as the egg reduction rate, ERR) against A. lumbricoides but poor efficacy against *T. trichiura*. For hookworm infections, ALB is significantly more potent than MEB (Levecke *et al.*, 2014; Moser *et al.*, 2017).

Recent evidence challenges the long-term impact of current, age-targeted control strategies on hookworm morbidity, because large populations of untreated adults bolster community-wide transmission, and adoption of mass-drug administration has been recommended to support sustained reduction of morbidity (Colella *et al.*, 2021). Additionally, a single dose of either of these drugs shows suboptimal efficacy against hookworms, and treatment with these drugs is the primary hookworm control strategy recommended by the WHO, as there is currently no vaccine available (WHO, 2022). A major concern is that MDA, when used over prolonged periods with the same antihelminthics, would exert selection pressure on hookworm parasite populations and favour the development of resistance (Rashwan *et al.*, 2016). Some studies have suggested an emergence of drug resistance by reporting low cure rates and faecal egg count reductions (Flohr *et al.*, 2007; Albonico and Savioli, 2017).

Resistance to benzimidazoles, characterized by single nucleotide polymorphisms (SNPs), cause amino acid substitution in the ß-tubulin isotype 1 gene at codons 167 (from TTC, TTT/phenylalanine to TAC, TAT/tyrosine) as well as codon 200 (from TTC/ phenylalanine to TAC/tyrosine) (Diawara *et al.*, 2013; Zuccherato *et al.*, 2018). Furthermore, a glutamate-to-alanine change at codon 198 (GAG, GAA/glutamic acid to GCG, GCA/alanine) has recently been associated with benzimidazole (BZ) resistance (Mottier and Prichard 2008; Zuccherato *et al.*, 2018). This study was therefore conducted with the aim of detecting benzimidazole resistance genes and associated risk factors in hookworms from HIV-positive and HIV-negative patients in Plateau State, Nigeria

MATERIALS AND METHODS

Study Area

This cross-sectional study was conducted in Plateau State, North Central Nigeria. Plateau State is located in the Middle Belt of Nigeria. The State is named after the picturesque Jos Plateaus, predominantly a mountainous area in the northern part of the state, characterized by attractive rock formations (Agabi *et al.*, 2023). It is the twelfth-largest state in Nigeria, and is roughly located in the center of the country. With an area of 26,899 km^2, the state has an estimated population of about three (3) million people at the 2006 census. It is located at latitude 80^{0}24'N and 80^{0}32' and 100^{0}38'E. Bare rocks are scattered across the grasslands which cover the plateau. The altitude ranges from around 1,200 meters (about 4,000 feet) to a peak of 1,829 meters above sea level in the Shere Hills range (Ajayi *et al.*, 2016).

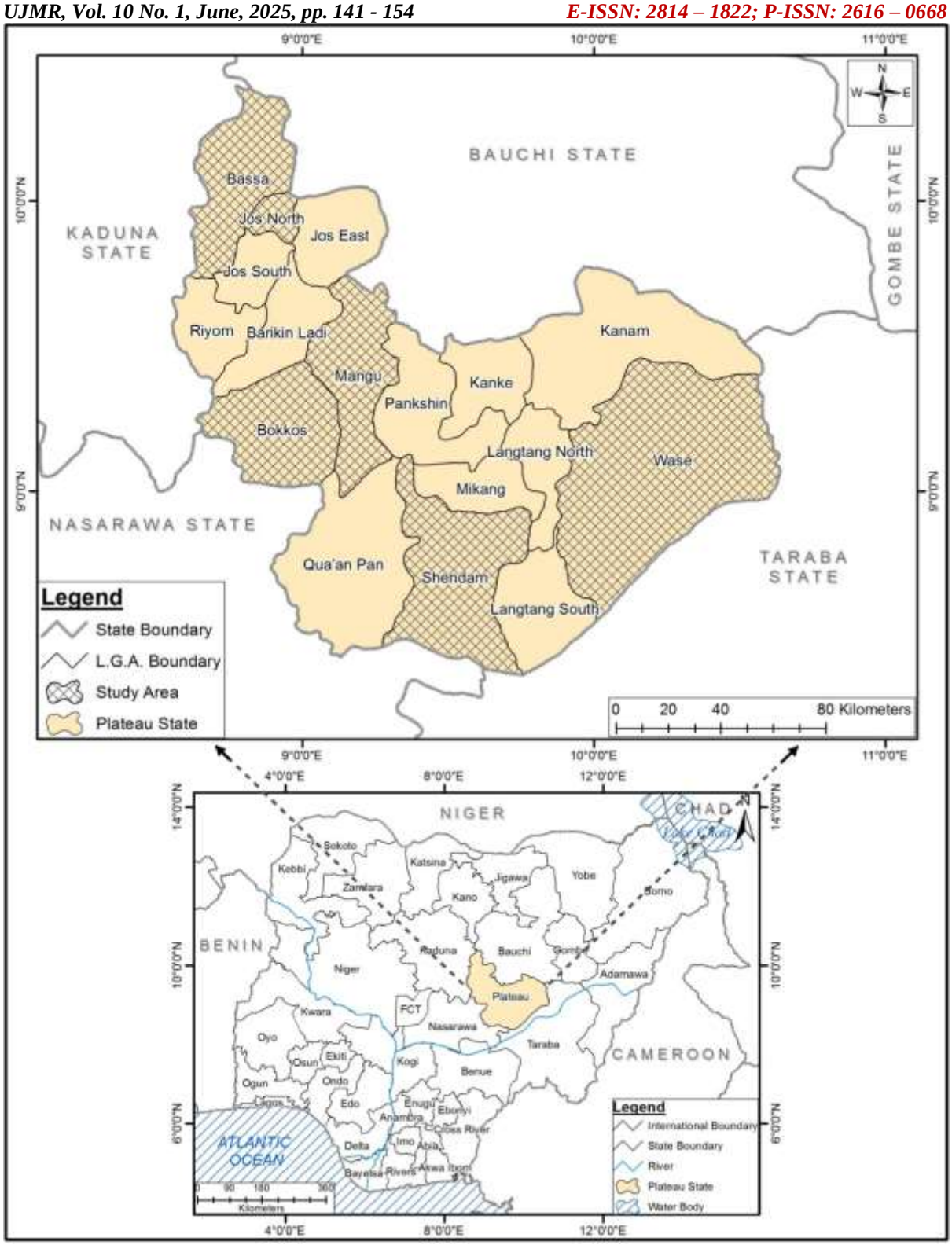

Figure 1: Map of Plateau State, Nigeria showing the Study Area

Study Population

The study population consisted of HIV-positive patients who are undergoing antiretroviral therapy (ART), ART-naïve patients, as well as HIV-negative patients in the selected study areas across the State. All the recruited subjects gave consent to participate in the study by signing a written informed consent form

Inclusion Criteria

The study subjects included any HIV-positive and non-HIV-positive adolescents (10-17 years) and adults (18 years and above) in all the study locations.

Ethical Approval

Ethical approval was obtained from the Research and Ethics Committee of Plateau State Specialist Hospital, Jos, Nigeria (PSSH/ADM/ETH.CO/2018/044). After reading an information sheet, all consenting subjects were requested to sign an informed consent form, which was later retrieved. For adolescent subjects, consent was obtained from their parents/guardians. Permission was obtained from the Plateau State Ministry of Health to enable sample collection from General Hospitals in the respective study locations (HMB/ADM/423/II/21).

Data Collection

Information about the recruited subjects was collected through structured questionnaires. Data on risk factors, such as walking barefoot on soil and touching the soil with bare hands, as well as sanitary behaviors, including hand-washing, were also collected. Additionally, information on the use and duration of exposure to antiretroviral therapy and antihelmintic treatment was collected using the questionnaire.

Collection of Samples and Laboratory Analyses

Exactly 5g stool samples was collected from each of the study subjects. Clean, grease-free, leak-proof sample bottles were provided and the samples were received from the subjects on the day of collection. All specimens collected were divided into two portions. One portion was subjected to standard parasitological analyses. The other portion, if positive, was stored at -80^{0}C and subjected to molecular analyses at Plateau State Human Virology Research Centre (PLASVIREC) Jos Nigeria.

Parasitological Analysis

Each stool sample was subjected to direct microscopic examination for the presence of hookworm eggs (Akue *et al.*, 2011). Thereafter, the samples were subjected to Formol-ether sedimentation technique (Cheesbrough, 2010).

Molecular Analysis

Genomic DNA Extraction

Genomic DNA was extracted from the hookworm eggs using the Viasure DNA/RNA extraction Kit according to the manufacturer's protocol. Two hundred microliters (200µl) of the sample was transferred into a 2ml collection tube, after which 200µl of lysis buffer, 20µl of carrier RNA, and 20µl of proteinase K were added and vortexed vigorously. The mixture was incubated for 10 minutes at 65 °C and then for 10 minutes at 95 °C, while shaking continuously. Thereafter, 260 µL of binding buffer was added and mixed by vortexing, and then incubated at room temperature for 5 minutes. The lysate was then transferred into a Mini Spin Column without touching the membrane and centrifuged for 1 minute at 11,000 rpm. The filtrate and the RTA collection tube were discarded. Thereafter, the Mini Spin Column was transferred into a new RTA collection tube, and 600 µL of Wash Buffer I was added. The mixture was then centrifuged for 1 minute at 11,000 rpm. Again, the filtrate and the RTA collection tube were discarded, and the Mini Spin column was transferred into a new RTA collection tube, where 700 µL wash Buffer II was added. This was centrifuged for 1 minute at 11,000 rpm, and the filtrate was discarded. The Mini Spin column was then returned to the used RTA collection tube. This washing step was repeated, after which the mixture was centrifuged for 5 minutes at 11,000 rpm, and the RTA collection tube was discarded. The Mini Spin column was placed into a 1.5 mL collection tube, and 200 µL of Elution Buffer (pre-heated to 65 °C) was added. The mixture was then incubated for 1 minute at room temperature, followed by centrifugation for 1 minute at 11,000 rpm. The Mini Spin column was discarded. Finally, the 1.5 mL collection tube was closed, and the extracted DNA was stored at -20 °C until further use.

Hookworms ß-tubulin isotype 1 gene amplification using PCR

To confirm the presence of hookworms in the samples, Real-time quantitative PCR (qPCR) was performed to amplify a specific fragment of the ß-tubulin isotype 1 gene, including codon positions 167, 198, and 200. The forward and reverse primers, each containing one of the three codons, were supplied by Cambridge University, London (Table 1). All PCR amplifications were performed in a 20µl reaction containing 10µl master mix (Luna Universal – Biolabs); 0.5µl of each of the forward and reverse primers (1µl); 4µl template DNA and 5µl nuclease-free water. Negative control (no template) was also included for quality control. The PCR amplifications were carried out according to the following thermocycling conditions: 95 °C for 60 seconds, followed by 45 cycles at 95 °C for 15 seconds, and a final extension at 60 °C for 20 minutes. The PCR program was run on the Bio-Rad CFX96 Real-Time PCR, and the SYBR Green channel was set as the detection channel.

Detection of ß-tubulin Mutations (SNPs) using PCR

To confirm the presence of Single Nucleotide Polymorphisms on the ß-tubulin gene that is associated with benzimidazole resistance in hookworms, qPCR was performed.

Specific fragments of the ß-tubulin isotype-1 gene, containing codons 167, 198, and 200, were amplified using primers synthesized to carry the SNPs for each of the codons. The SNP primers were supplied by Cambridge University, London (Table 1). All PCR amplifications were performed in a 20µl reaction containing 10µl master mix (Luna Universal – Biolabs); 0.5µl of each of the forward and reverse primers (1µl); 4µl template DNA, and 5µl nuclease-free water. Negative control (no template) was also included for quality control. The PCR amplifications were carried out according to the following program: 95 °C for 60 seconds, followed by 45 cycles at 95 °C for 15 seconds, and a final extension at 60 °C for 20 minutes. The PCR program was run on the Bio-Rad CFX96 Real-Time PCR, and the SYBR Green channel was set as the detection channel.

In real-time qPCR, the Ct value (threshold cycle) represents the amplification cycle at which the fluorescent signal exceeds the background noise. It is the intersection between an amplification curve and a threshold line that gives a relative measure of the concentration of the target (DNA) in the PCR reaction. The Ct value is inversely proportional to the amount of target DNA in the sample. Hence, a lower Ct value (typically < 30-35) indicates a higher amount of target DNA in the sample, which is generally considered positive. A higher Ct value (typically > 35-40) indicates a lower amount of target DNA in the sample, which may be considered negative or inconclusive. A Ct value of above 40 or higher is considered negative, as it indicates that the target DNA is either absent or in very low amounts.

Statistical analysis

All data from this study were analyzed using IBM SPSS Statistics Version 21 software. Relationships between categorical variables were assessed using the Chi-square test and a *p*-value of 0.05 (some 0.01) was considered statistically significant.

Table 1: List of primers and sequences for Hookworm (*N. americanus*) identification and SNP detection on the ß-Tubulin Isotype-1 Gene

Codon	Primer sequences (5'-3')	Temperature (^{0}C)	Expected size (bp)	Reference
167	Forward: AAGAAGCTGAAGGATGTGACTG	60	227	Rashwan *et al.*, 2016
	Reverse: GAAGCGAAGACAGGTAGTAACAC			
	SNP Fwd: CATGTCCTCGTATTCCGTTG		265	
	SNP Rev: CAACGGAATACGAGGACATG			
198	Forward: AAGAAGCTGAAGGATGTGACTG	60	214	
	Reverse: GAAGCGAAGACAGGTAGTAACAC			
	SNP Fwd: AGATGCGACCTTCTGTATTGATAATG		231	
	SNP Rev: CATTATCAATACAGAAGGTCGCATCT			
200	Forward: AAGAAGCTGAAGGATGTGACTG	60	208	
	Reverse: GAAGCGAAGACAGGTAGTAACAC			
	SNP Fwd: AGATGAGACCTACTGTATTGATAATG		215	
	SNP Rev: CATTATCAATACAGTAGGTCTCATCT			

RESULTS

An overall prevalence of 6.46%; with 3.62% and 10.36% for HIV-positive and HIV-negative patients respectively, was obtained. Some risk factors associated with hookworm infections among HIV-positive and HIV-negative subjects are presented in Table 2. Subjects whose residence were close to dump sites recorded a lower occurrence of hookworm infections (4.45%) as compared to those whose residences were not close to a dump site, with a higher occurrence of 8.24%. Analysis of the results based on walking on bare soil shows that subjects who rarely do so had a high prevalence (7.98%), while those who do not walk barefoot had a lower prevalence of hookworm infection (3.45%). The results analysis according to hand-contact with soil showed that subjects who often have hand-contact with soil recorded a high prevalence of hookworm infection (8.65%), followed by those who rarely have hand-contact with soil (4.58%). Subjects who do not have hand-contact with soil recorded the lowest prevalence of 1.20%.

Analysis of the results with respect to washing hands after contact with soil indicates that subjects who do not wash their hands have a higher prevalence of hookworm infection (18.03%), while a low prevalence is observed among those who often wash their hands after contact with the soil (4.38%). The results of statistical analysis show that a significant association exists between hookworm infection and the frequency of washing hands after contact with soil ($P \leq 0.01$).

Based on the proximity of residence to underdeveloped bushy land, a higher prevalence was observed among subjects whose residence was not close to an underdeveloped bushy land (9.09%), while a lower prevalence was observed among subjects who reside close to an underdeveloped bushy land (4.73%). The result of statistical analysis reveals a significant association between hookworm infection and proximity of residence to underdeveloped, bushy land at P ≤ 0.05. Regarding engagement in outdoor activities from morning to noon, a high prevalence of hookworm infection was recorded among those who participate in outdoor activities between morning and noon (7.98%), while subjects who do not engage in outdoor activities during this time had a lower prevalence of 5.43%. In terms of the Time of farm visitation, as reported by the study subjects, the lowest prevalence of hookworm infection was recorded among subjects who visit their farms after sunrise (2.30%), while those who visit their farms before sunrise recorded the highest prevalence of 9.63%. The results of statistical analysis show that a significant association exists between hookworm infection and the time of farm visitation by the study subjects (P ≤ 0.01). Regarding the use of footwear during planting/harvesting, the subjects with the highest prevalence of hookworm infection were those who did not wear footwear (7.99%), while those who wore footwear had a lower prevalence of 3.07%. The result also shows that there is a statistically significant association between hookworm infection and use of foot wears during planting/harvesting at P≤0.05.

Table 2: Risk Factors of Hookworm Infection among HIV-positive and HIV-negative based on risk factors

Risk Factor	Response	HIV-PositiveNo. Examined	No. Positive (%)	HIV-NegativeNo. Examined	No. Positive (%)	Total Examined	Total Positive (%)	P-value
Residence close to dumpsite	Yes	142	5 (3.52)	105	6 (5.71)	247	11 (4.45)	
	No	162	6 (3.70)	117	17 (14.53)	279	23 (8.24)	0.078
Walking on soil barefooted	Often	151	5 (3.31)	100	11 (11.00)	251	16 (6.37)	
	Rarely	101	5 (4.95)	87	10 (11.49)	188	15 (7.98)	
	No	52	1 (1.92)	35	2 (5.71)	87	3 (3.45)	0.363
Hand-contact with soil	Often	171	8 (4.68)	141	19 (13.48)	312	27 (8.65)	
	Rarely	62	2 (3.33)	69	4 (5.80)	131	6 (4.58)	
	No	71	1 (1.41)	12	0 (0.00)	83	1 (1.20)	0.437
Washing hands after contact with soil	Often	187	4 (2.14)	110	9 (8.18)	297	13 (4.38)	
	Rarely	93	3 (3.23)	75	7 (9.33)	168	10 (5.95)	
	No	24	4 (16.67)	37	7 (18.92)	61	11 (18.03)	0.001**
Bushy land close to residence	Yes	107	3 (2.80)	210	12 (5.71)	317	15 (4.73)	
	No	197	8 (4.06)	12	11 (84.62)	209	19 (9.09)	0.047*
Outdoor activity (morning-noon)	Yes	101	4 (3.96)	112	13 (11.61)	213	17 (7.98)	
	No	203	7 (3.45)	110	10 (9.09)	313	17 (5.43)	0.243
Time of farm visitation	Before sunrise	107	9 (8.41)	194	20 (10.31)	301	29 (9.63)	
	After sunrise	182	2 (1.10)	15	2 (13.33)	197	4 (2.30)	
	None	15	0 (0.00)	13	1 (7.69)	28	1 (3.57)	0.003**
Use of footwear during farming	Yes	114	2 (1.75)	49	3 (6.12)	163	5 (3.07)	
	No	190	9 (4.74)	173	20 (11.56)	363	29 (7.99)	0.034*
Total	-	304	11 (3.62)	222	23 (10.36)	526	34 (6.46)	-

***Statistically significant association exists at p ≤ 0.05, **Statistically significant association exists at p ≤ 0.01**

Key: No=Number; %=Percentage

The occurrence of hookworm infections in relation to the medical history of the study subjects was investigated, and the results are presented in Table 3. Subjects who confirmed having a history of a rash on their foot after working on a farm barefoot had a lower prevalence of 1.97%, while those who never had a rash on their foot after working on a farm barefoot recorded a higher prevalence of 8.29%. Statistical analysis reveals a significant association between hookworm infection and a history of having a rash on the feet of the study subjects after working barefoot on a farm (P ≤ 0.01). With respect to the medical history of previously testing for hookworm infection, a high prevalence was recorded among subjects who confirmed having previously tested (7.81%), while subjects who had not tested previously had a lower prevalence of 5.97%. With regards to the history of last deworming, a high prevalence of hookworm infection was recorded among subjects who have not dewormed previously (20.93%) followed by those who dewormed more than 12 months ago (5.76%) while a low prevalence was recorded among subjects who dewormed about 6 months ago (1.85%). Statistical analysis indicates a significant association between hookworm infection and a history of deworming among the study subjects at P ≤ 0.01.

Table 3: Prevalence of Hookworm Infection among HIV-positive and HIV-negative Patients in Relation to Medical History

Medical History	Response	HIV-Positive No. Examined	No. Positive (%)	HIV-Negative No. Examined	No. Positive (%)	Total Examined	Total Positive (%)	P-value
Rash on foot after working on farm barefooted	Yes	3	0 (0.00)	149	3 (2.01)	152	3 (1.97)	
	No	301	11 (3.65)	73	20 (27.40)	374	31 (8.29)	0.008**
Previously tested for hookworm	Yes	83	4 (4.82)	58	7 (12.07)	141	11 (7.81)	
	No	221	7 (3.17)	164	16 (9.76)	385	23 (5.97)	0.45
Last deworming (months)	≤ 6	23	0 (0.00)	31	1 (3.23)	54	1 (1.85)	
	<12	59	1 (1.69)	23	3 (13.04)	82	4 (4.88)	
	>12	196	5 (2.55)	151	15 (9.93)	347	20 (5.76)	
	No	26	5 (19.23)	17	4 (23.53)	43	9 (20.93)	0.001**
Total	-	304	11 (3.62)	222	23 (10.36)	526	34 (6.46)	-

***Statistically significant association exists at p ≤ 0.05**Statistically significant association exists at p ≤ 0.01**
Key: No=Number; %=Percentage

Real Time qPCR Results Interpretation
A standard qPCR amplification curve resembles a sigmoidal amplification pattern with three distinct phases: baseline phase, exponential phase, and plateau phase. The detection of amplification through the Sybr Green channel with a threshold cycle (Ct) value of ≤35 was considered to be a positive sample (Figure 2). Amplifications with Ct values >35 were considered negative samples (Figures 3 and 4).

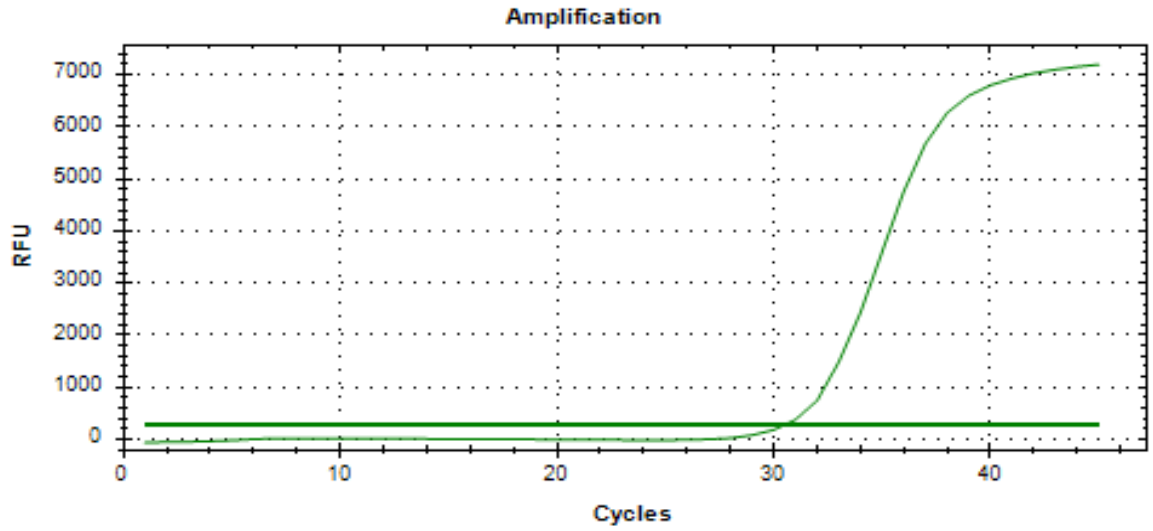

Figure 2: Positive Hookworm (*N. americaus*) ß-Tubulin Isotype-1 gene Amplification Keys: RFU: Relative fluorescence units; Ct value < 35 indicates a positive result

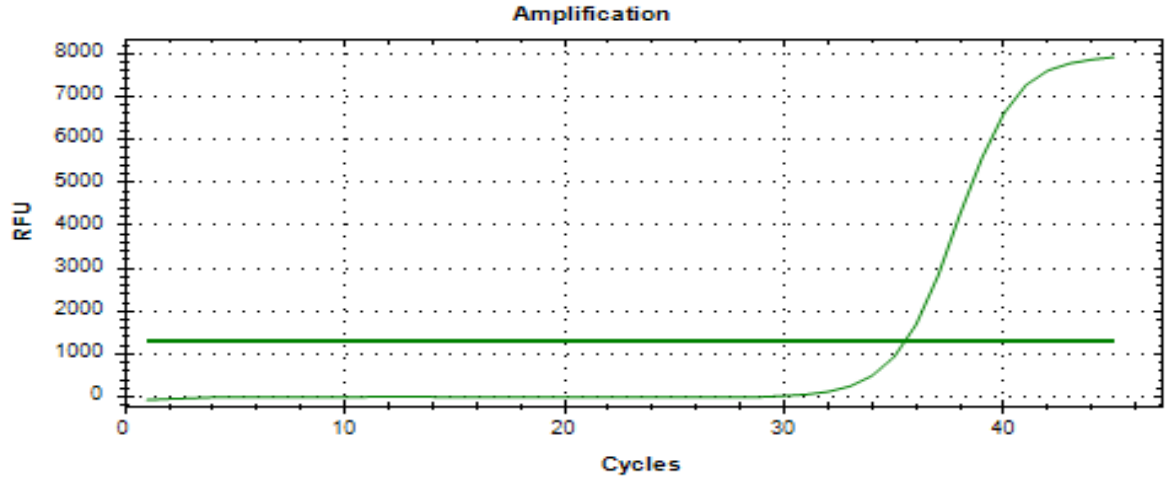

Figure 3: Negative Hookworm ß-Tubulin Isotype-1 gene Amplification
Keys: RFU: Relative fluorescence units; Ct value > 35 indicates a negative result

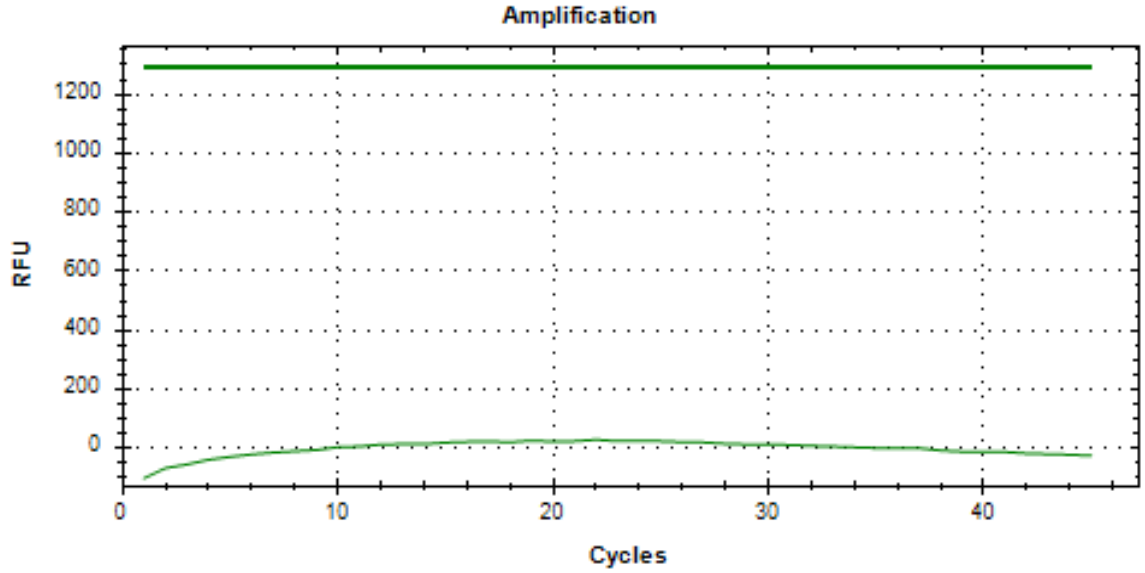

Figure 4: Negative Control for Hookworm ß-Tubulin Isotype-1 gene Amplification
Keys: RFU: Relative fluorescence units; No Ct-value, primers were not used

The results for the identification of hookworms in the samples, obtained through the amplification of the ß-tubulin Isotype 1 gene, are presented in Table 4. Out of the 34 samples that were positive by microscopy for hookworms, 16 were confirmed to *N. americanus* on Real-Time qPCR. Primer sequences specific for the three codons (167, 198, and 200) that are unique to hookworm species were used to amplify the ß-tubulin Isotype-1 gene from the oligonucleotides extracted earlier. All 16 samples had Ct Values <35, which is considered positive. The result further showed that 5 out of the 16 samples confirmed by PCR were from HIV-positive subjects, while 11 were from HIV-negative patients.

The results for the detection of Single Nucleotide Polymorphisms (SNPs) on the ß-tubulin Isotype-1 gene are presented in Table 5. Here, an SNP was detected in 2 samples (No. 27 and No. 36). With respect to the codons, 1 SNP was detected at codon 167, none at codon 198, and 1 SNP at codon 200. In relation to HIV status, no SNP was detected in samples from HIV-positive subjects, while the 2 SNPs were all detected in samples obtained from HIV-negative patients.

Table 4: Identification of Hookworms (*N. americanus*) by Amplifying the ß-tubulin Isotype 1 gene among HIV-positive and HIV-negative Patients

Sample ID	HIV Status	Ct value	Hookworm detected
020	+	47.87	-
021	+	38.66	-
022	+	40.86	-
023	+	50.56	-
024	-	30.56	+
025	+	34.24	+
026	+	42.55	-
027	-	30.47	+
028	+	37.28	-
029	-	27.29	+
030	+	39.66	-
031	-	30.25	+
032	+	38.75	-
033	-	30.24	+
034	-	27.15	+
035	+	32.43	+
036	-	31.60	+
037	+	37.64	-
038	-	32.20	+
039	-	31.87	+
040	+	39.92	-
041	+	32.47	+
042	+	33.21	+
043	+	41.12	-
044	-	30.93	+
045	+	39.04	-
046	-	32.26	+
047	+	39.22	-
048	+	32.20	+
049	+	42.44	-
050	+	39.04	-
051	+	40.01	-
052	+	36.09	-
053	+	36.98	-

Key: Ct = Cycle Threshold, + = Positive Result, - = Negative Result, Green = HIV-negative; Hookworm-positive and Red = HIV-positive; Hookworm-positive

Table 5: Detection of Single Nucleotide Polymorphisms (SNPs) on *N. americanus* ß-tubulin Isotype 1 gene among HIV-positive and HIV-negative Patients

Sample No	HIV Status	Ct Value	Results of SNP Detection
020	+	N/A	-
021	+	N/A	-
022	+	N/A	-
023	+	N/A	-
024	-	N/A	-
025	+	N/A	-
026	+	N/A	-
027	-	24.98	+ (codon 167)
028	+	N/A	-
029	-	N/A	-
030	+	N/A	-
031	-	N/A	-
032	+	N/A	-
033	-	N/A	-
034	-	N/A	-
035	+	N/A	-
036	-	30.25	+ (codon 200)
037	+	N/A	-
038	-	N/A	-
039	-	N/A	-
040	+	N/A	-
041	+	N/A	-
042	+	N/A	-
043	+	N/A	-
044	-	N/A	-
045	+	N/A	-
046	-	N/A	-
047	+	N/A	-
048	+	N/A	-
049	+	N/A	-
050	+	N/A	-
051	+	N/A	-
052	+	N/A	-
053	+	N/A	-

Key: Ct = Cycle Threshold, N/A = Not Available, + = Positive Result and - = Negative Result

DISCUSSION

In this research, study subjects who infrequently washed their hands after contact with soil, bushy land around their residence, and did not use footwear during farm work or arrived at their farms before sunrise were significantly more likely to be infected with hookworms. This finding is consistent with the report of Deku *et al.* (2022). Contact with moist soil containing infective filariform larvae of the parasite enhances the chances of their penetration into the skin. This is a common occurrence, particularly among individuals who engage in agricultural activities that involve the use of untreated night soil as fertilizer (Hossain *et al.*, 2016).

Studies have reported a high prevalence of hookworm infections among individuals who conduct farming activities barefoot (Shiferaw and Mengistu, 2015; Adamu and Haruna, 2017). This is particularly serious in areas where untreated human waste is used as manure, as well as in areas prone to open defecation (Sandy *et al.*, 2014). The presence of underdeveloped, bushy land around residential areas can serve as sites for the dumping of refuse and/or open defecation.

Such bushy areas tend to retain soil moisture, creating an ideal environment for hookworm larvae to survive and thrive (Babamale and Ugbomoiko, 2016). Vegetation provides shade, which reduces soil temperature and increases humidity favouring hookworm larval development. Similarly, bushy areas tend to accumulate abundant decaying organic matter, creating microclimates with high humidity that foster hookworm larval development and survival. Dense vegetation also limits sunlight exposure, reducing the natural disinfectant effect of ultraviolet radiation on soil and feces (Hossain and Bhuiyan, 2016).

Furthermore, studies have reported a high prevalence of hookworm infection among those who arrive at their farms before sunrise (Hotez *et al.*, 2004; Loukas *et al.*, 2016). This may be attributed to the fact that early morning hours have high humidity which favours hookworm survival and transmission. Additionally, soil moisture is higher before sunrise, making it easier for hookworm larvae to move through the soil and thereby increasing the likelihood of infection.

Interestingly, this study observed a significantly low degree of hookworm infection among those whose residence is close to a dumpsite. This finding contrasts with the results of Babamale and Ugbomoiko (2016) and Toluwalope et al. (2022), which documented high hookworm infections among individuals residing near dumpsites in a community in Kwara and among pregnant women at the Federal Medical Centre Keffi. This can be attributed to the fact that dumpsites can lead to soil contamination with human feces, organic matter, and other waste, creating an ideal environment for hookworm larvae to survive and proliferate. Furthermore, Nnachi *et al.* (2015) noted that dumpsites can attract moisture, creating a humid environment that promotes the development of hookworm larvae. He further suggests that hookworm eggs and larvae can be present in human faeces, which may be deposited in dumpsites, thereby contaminating the soil and increasing the possibility of infection. Again, Uhuo *et al.* (2011) documented that leachate from dumpsites can contaminate nearby water sources, leading to hookworm infection through contact with the contaminated water.

In this research, the history of having a foot rash after working on a farm barefoot, as well as the history of deworming, was observed to have a significant impact on the occurrence of hookworm infection among the study subjects. A rash on the foot, often referred to as "ground itch" or "cutaneous larva migrans," is a common symptom of hookworm infection (Arora & Arora, 2012). This is because hookworm larvae may be present in contaminated soil and when an individual works barefoot, the larvae can penetrate the skin of the feet. The presence of the larvae in the skin triggers an immune response, resulting in an itchy rash, redness, and swelling. This rash typically appears within 1 to 2 days after exposure and can persist for several days. It is essential to note, however, that not all rashes on the foot are caused by hookworm infection. Other conditions, such as fungal and bacterial infections, allergic reactions, or insect bites, can also cause similar symptoms (Katz, 2018).

Regarding the history of deworming, it is known that deworming eliminates adult hookworms, thereby reducing their number in the intestine and the amount of eggs produced. (Hotez *et al.*, 2004). By reducing the worm burden, deworming decreases the likelihood of contaminating the soil with hookworms as they would have been killed before being passed along with faeces of infected individuals (Bethony *et al.*, 2006).

In this study, hookworms were identified in the stool samples collected from both HIV-positive and HIV-negative patients by amplifying the ß-tubulin isotype-1 gene. The number of positive samples identified by microscopy was greater than that identified by PCR. This finding disagrees with the results of Hii et al. (2018), Basumi et al. (2011), Easton et al. (2016), and Verweij (2014), who reported higher detection rates of hookworms by PCR compared to microscopy. This difference may be attributed to variations in terms of expertise in handling both microscopy and PCR. The variation may also be related to the fact that only *N. americanus* was identified since the primers were specific for it. However, the two human hookworms are indistinguishable from each other by microscopy. Hence, it could be concluded that the remaining microscopy-positive samples were *A. duodenale*.

Single-nucleotide polymorphisms (SNPs) in the ß-tubulin isotype 1 gene, specifically at codons 167, 198, and 200, have been associated with benzimidazole resistance in certain helminths (Furtado *et al.*, 2018). These mutations alter the binding affinity of benzimidazoles, reducing their effectiveness. Detection of these SNPs increases the possibility of intervening to avoid the establishment of a drug-resistant worm population. In this study, two SNPs were recorded at codons 167 and 200 of the hookworm ß-tubulin isotype-1 gene. This finding is similar to that of Schwenkenbecher *et al.* (2007) and Orr *et al.* (2019), who reported SNPs at the same codons (167 and 200) in hookworms collected from school-aged children.

However, the result of the present study differs from that of Zuccherato et al. (2018), who reported SNPs at codons 198 and 200, but not at codon 167. Likewise, this finding disagrees with that of Rashwan *et al.* (2016), who reported an SNP at codon 198 only, but not at codons 167 and 200.

Notably, the detection of SNPs in the ß-tubulin isotype-1 gene of human hookworms in the present study indicates that mutant genes associated with benzimidazole resistance are circulating among hookworms in Plateau State, North Central Nigeria. This carries an unknown potential to impact the effectiveness and sustainability of chemotherapeutic interventions for disease transmission and control.

CONCLUSION

Infrequent handwashing after contact with soil, the presence of underdeveloped, bushy land close to residences, farm visitation before sunrise, and the non-use of footwear while working on farms were found to be significant risk factors that predispose individuals to hookworm infection. Higher infection rates were recorded among subjects who engage in such activities. Medical history of having a rash on the foot after working on the farm barefooted and deworming history were also found to have a significant impact on the occurrence of hookworm infection among the HIV-positive and HIV-negative patients in Plateau State. A higher prevalence of hookworm infection was recorded among subjects who had never been dewormed, signifying the possibility of heavy carriage of adult hookworms in such individuals. Some of the samples that were identified to be hookworms by microscopy were confirmed to be *N. americanus* on real-time qPCR. A total of 34 hookworms were identified by microscopy, out of which 16 were confirmed to be N. americanus, indicating that the remaining 18 were *A. duodenale* or other species. Two (2) SNPs were recorded at codons 167 and 200 of the hookworm ß-tubulin isotype-1 gene. In relation to HIV status, no SNP was detected in samples from HIV-positive subjects, while the 2 SNPs were all detected in samples obtained from HIV-negative patients.

REFERENCES

Abuzeid, A. M., Zhou, X., Huang, Y., & Li, G. (2020). Twenty-five year research progress in hookworm excretory/secretory products. *Parasites & Vectors, 13*, 136. [Crossref]

Adamu, R., & Haruna, M. (2017). The epidemiology of hookworm infection among primary school pupils in Dantube, Dawakin-kudu Local Government Area, Kano State, Nigeria. *UMYU Journal of Microbiology Research*, 118–124. [Crossref]

Agabi, Y. A., Ezeife, C. E., Uneze, S. B., Okojokwu, O. J., Eya, B. S., Nkup, J., Zakari, H., Isaac, M. A., Adoga, M. P., Owuna, J. E., Mawak, J. D., & Anejo-Okopi, J. A. (2023). Prevalence of intestinal helminths among HIV patients accessing health care services at Faith-Alive Foundation, Jos Nigeria. *Microbes and Infectious Diseases, 4*(1), 276–284.

Ajayi, O. T., Makanjuola, O. B., Olayinka, A. T., Olorukooba, A., Olofu, J. E., Nguku, P., & Fawole, O. I. (2023). Predictors of intestinal parasite infection among HIV patients on antiretroviral therapy in Jos, Plateau State, Nigeria: 2016: A cross-sectional survey. *Pan African Medical Journal, 38*(306). [Crossref]

Akue, J. P., Nkoghe, D., Padilla, C., Moussavou, G., Moukana, H., Mbou, A. R., Ollomo, B., & Leroy, E. M. (2011). Epidemiology of concomitant infection due to *Loa loa* and *Mansonella perstans* in Gabon. *PLoS Neglected Tropical Diseases, 5*(10), e1329. [Crossref]

Albonico, M., & Savioli, L. (2017). Hookworm: A neglected resurgent infection. *British Medical Journal, 359*, j4813. [Crossref]

Arora, R. M., & Arora, B. B. (2012). *Medical parasitology* (3rd ed.). CBS Publishers and Distributors.

Babamale, O. A., & Ugbomoiko, U. S. (2016). Hookworm infection: A severe health problem in peri-urban community of North central, Nigeria. *Tropical Biomedicine, 33*(1), 8-13.

Basuni, M., Muhi, J., Othman, N., Verweij, J. J., Ahmad, M., Miswan, N., Rahumatullah, A., Aziz, F. A., Zainudin, N. S., & Noordin, R. (2011). A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. *American Journal of Tropical Medicine and Hygiene, 84*(2), 338–343. [Crossref]

Bethony, J., Brooker, S., Albonico, M., Geiger, S. M., Loukas, A., & Diemert, D. (2006). Soil-transmitted helminth infections: Ascariasis, trichuriasis, and hookworm. *The Lancet, 367*(9521), 1521–1532. [Crossref]

Brooker, S., Bethony, J., & Hotez, P. J. (2004). Human hookworm infection in the 21st

century. *Advances in Parasitology, 58*, 197–288. [Crossref]

Cheesbrough, M. (2010). *District laboratory practice in tropical countries (Part 1)*. Cambridge Low Price Editions.

Clements, A. C., & Alene, K. A. (2022). Global distribution of human hookworm species and differences in their morbidity effects: A systematic review. *The Lancet Microbe, 3*, e72–e79. [Crossref]

Colella, V., Khieu, V., Worsley, A., Senevirathna, D., Muth, S., Huy, R., Odermatt, P., & Traub, R. B. (2021). Risk profiling and efficacy of albendazole against the hookworms *Necator americanus* and *Ancylostoma ceylanicum* in Cambodia to support control programmes in South-East Asia and Western Pacific. *The Lancet Regional Health - Western Pacific, 16*, 100258. [Crossref]

Deku, J. G., Botchway, K. A., Kinanyok, S., Gedzeah, C. K., Duneeh, R. V., & Duedu, K. O. (2022). Intestinal parasitic infection and associated risk factors among HIV-infected patients seeking healthcare in a rural hospital in Ghana. *Journal of Pathogens, 2022*, Article ID 5652637. [Crossref]

Diawara, A., Schwenkenbecher, J. M., Kaplan, R. M., & Prichard, R. K. (2013). Molecular and biological diagnostic tests for monitoring benzimidazole resistance in human soil-transmitted helminths. *American Journal of Tropical Medicine and Hygiene, 88*(6), 1052–1061. [Crossref]

Easton, A. V., Oliveira, R. G., O'Connell, E. M., Kepha, S., Mwandawiro, C. S., Njenga, S. M., Kihara, J. H., Mwatele, C., Odiere, M. R., Brooker, S. J., Webster, J. P., Anderson, R. M., & Nutman, T. B. (2016). Multi-parallel qPCR provides increased sensitivity and diagnostic breadth for gastrointestinal parasites of humans: Field-based inferences on the impact of mass deworming. *Parasites & Vectors, 9*, 38. [Crossref]

Flohr, C., Tuyen, L. N., Lewis, S., Minh, T. T., Campbell, J., Britton, J., Williams, H., Hien, T. T., Farrar, J., & Quinnell, R. J. (2007). Low efficacy of mebendazole against hookworm in Vietnam: Two randomized controlled trials. *American Journal of Tropical Medicine and Hygiene, 76*(4), 732–736. [Crossref]

Furtado, L. F., Magalhães, J. G., & Rabelo, E. M. (2018). Standardization and application of a modified RFLP-PCR methodology for analysis of polymorphisms linked to treatment resistance in *Ancylostoma braziliense*. *Parasites & Vectors, 11*, 540. [Crossref]

Hii, S. F., Senevirathna, D., Llewellyn, S., Inpankaew, T., Odermatt, P., Khieu, V., Muth, S., McCarthy, J., & Traub, R. J. (2018). Development and evaluation of a multiplex quantitative real-time polymerase chain reaction for hookworm species in human stool. *American Journal of Tropical Medicine and Hygiene, 99*(5), 1186–1193. [Crossref]

Hossain, M., Alam, M. D. S., Khair, M., Sayeed, M. D. A., & Bhuiyan, M. J. U. (2016). Prevalence and risk factors of *Strongyloides stercoralis* infection in selected tea garden of Sylhet, Bangladesh. *International Journal of Tropical Diseases, 4*, 206. [Crossref]

Hotez, P. J., Brooker, S., Bethony, J. M., Bottazzi, M. E., Loukas, A., & Xiao, S. (2004). Hookworm infection. *New England Journal of Medicine, 351*(8), 799–807. [Crossref]

Hussain, M., & Bhuiyan, M. J. U. (2016). Hookworm infection: A neglected tropical disease of mankind. *Journal of Infection Molecular Biology, 4*(2), 24-43. [Crossref]

Katz, N. (2018). Hookworm infection: A review of the literature. *Journal of Infectious Diseases, 217*(1), 14–25.

Levecke, B., Brooker, S. J., Knopp, S., Steinmann, P., Sousa-Figueiredo, J. C., & Stothard, J. R. (2014). Effect of sampling and diagnostic effort on the assessment of schistosomiasis and soil-transmitted helminthiasis and drug efficacy: A meta-analysis of six drug efficacy trials and one epidemiological survey. *Parasitology, 141*(14), 1826-1840. [Crossref]

Loukas, A., Hotez, P., Diemert, D., Yazdanbakhsh, M., McCarthy, J. S., Correa-Oliveira, R., Croese, J., & Bethony, J. M. (2016). Hookworm infection. *Nature Reviews Disease Primers, 2*, 16088. [Crossref]

Moser, W., Schindler, C., & Keiser, J. (2017). Efficacy of recommended drugs against soil transmitted helminths: Systematic review and network meta-analysis. *British Medical Journal, 358*, j4307. [Crossref]

Mottier, M., & Prichard, R. K. (2008). Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on *Haemonchus*

contortus. *Pharmacogenetics and Genomics, 18*(2), 129–140. [Crossref]

Nnachi, I. A., Odikamnoro, O. O., Nnnachi, A. U., Ani, O. C., Uhuo, C. A., & Egwuatu, C. C. (2015). Epidemiology of hookworm infection and the influence of some epidemiological factors on their prevalence in some farming communities in Ebonyi State, Nigeria. *International Journal of Tropical Disease and Health, 10*(4), 1–10. [Crossref]

Orr, A. R., Quagraine, J. E., Suwondo, P., George, S., Harrison, L. M., Dornas, F. P., Evans, B., Caccone, A., Humphries, D., Wilson, M. D., & Cappello, M. (2019). Genetic markers of benzimidazole resistance among human hookworms (*Necator americanus*) in Kintampo North Municipality, Ghana. *American Journal of Tropical Medicine and Hygiene, 100*(2), 351–356. [Crossref]

Prichard, R. K., Basanez, M. G., Boatin, B. A., McCarthy, J. S., Garcia, H. H., & Yang, G. J. (2012). A research agenda for helminth diseases of humans: Intervention for control and elimination. *PLoS Neglected Tropical Diseases, 6*(4), e1549. [Crossref]

Rashwan, N., Bourguinat, C., Keller, K., Gunawardena, N. K., de Silva, N., & Prichard, R. (2016). Isothermal diagnostic assays for monitoring single nucleotide polymorphisms in *Necator americanus* associated with benzimidazole drug resistance. *PLoS Neglected Tropical Diseases, 10*(12), e0005113. [Crossref]

Sandy, S., Sumarni, S., & Soeyoko, V. (2014). Footwear as a risk factor of hookworm infection in elementary school students. *Universa Medicina, 33*(3), 133–140.

Schwenkenbecher, J. M., Albonico, M., Bickle, Q., & Kaplan, R. M. (2007). Characterization of beta-tubulin genes in hookworms and investigation of resistance-associated mutations using real-time PCR. *Molecular and Biochemical Parasitology, 156*(2), 167–174. [Crossref]

Shiferaw, M. B., & Mengistu, A. D. (2015). Helminthiasis: Hookworm infection remains a public health problem in Dera District, South Gondar, Ethiopia. *PLoS One, 10*(12), e0144588. [Crossref]

Toluwalope, O. F., Idikwu, O. G., & Anzaku, S. A. (2022). Burden of hookworm infestation and association with socio-demographic and clinical factors in a clinical setting in Nigeria. *Science Archives, 3*(3), 168–174. [Crossref]

Uhuo, A. C., Odikamnoro, O. O., & Ani, O. C. (2011). The incidence of intestinal nematodes in primary school children in Ezza North L.G.A, Ebonyi State, Nigeria. *Advances in Applied Science Research, 2*(5), 257–262.

Verweij, J. J. (2014). Application of PCR-based methods for diagnosis of intestinal parasitic infections in the clinical laboratory. *Parasitology, 141*(14), 1863–1872. [Crossref]

Walker, M., Cools, P., Albonico, M., Aine, S. M., Ayana, M., & Dana, D. (2021). Individual responses to a single oral dose of albendazole indicate reduced efficacy against soil-transmitted helminths in an area with high drug pressure. *PLoS Neglected Tropical Diseases, 15*(10), e0009888. [Crossref]

World Health Organization. (2022). *Report of the WHO informal consultation on the use of praziquantel during pregnancy/lactation and albendazole/mebendazole in children under 24 months*. who.int

World Health Organization. (2023). *Soil-transmitted helminth infections* [Fact sheet]. who.int

Zeleke, A. J., Addisu, A., Derso, A., Tegegue, Y., Birhanie, M., Sisay, T., & Aemero, M. (2021). Evaluation of hookworm diagnosis techniques from patients in Debre Elias and Sanja Districts of the Amhara Region Ethiopia. *Journal of Parasitology Research, 2021*, Article ID 6682330. [Crossref]

Zuccherato, L. W., Furtado, L. F., Medieros, C. S., Pinheiro, C. S., & Rabelo, E. M. (2018). PCR-RFLP screening of polymorphisms associated with benzimidazole resistance in *Necator americanus* and *Ascaris lumbricoides* from different geographical regions in Brazil. *PLoS Neglected Tropical Diseases, 12*(9), e0006766. [Crossref]