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Research Article

Microscopy and Molecular Detection of Haemoparasites in Birds in Ibadan, Nigeria

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ABSTRACT

Introduction: Avian haemoparasites pose significant threats to poultry health and productivity. While microscopy is traditionally used for parasite identification, molecular methods, such as polymerase chain reaction (PCR), offer enhanced sensitivity and specificity. The present study aimed to determine the prevalence of haemoparasites in poultry species in Ibadan, Nigeria, and to compare the diagnostic performance of microscopy and nested PCR for detection and identification.

Materials and methods: Blood samples were obtained from 390 healthy birds, including commercial layers chickens (153), turkeys (75), broiler chickens (69), local chickens (60), pigeons (30), and ducks (3), randomly selected from 10 farms in Ibadan, Nigeria. Thin blood smears were Giemsa-stained and examined microscopically. Genomic DNA was extracted and subjected to nested PCR targeting the mitochondrial cytochrome b gene of haemosporidian parasites. Positive amplicons were sequenced and phylogenetically analyzed.

Results: The haemoparasites were microscopically detected in 44.6% (174/390) of samples, including *Plasmodium* (*P.*) spp. (18.2%), *Haemoproteus* spp. (15.9%), *Leucocytozoon* spp. (5.6%), *Babesia* spp. (2.8%), and microfilariae (2.1%). The PCR detected infections in 53.3% (208/390), confirming *P. gallinaceum* (12.3%), *Haemoproteus* spp. (19%), *Leucocytozoon* spp. (9%), *Babesia* spp. (3.3%), and additional unidentified haemosporidian lineages (8.7%). The PCR demonstrated significantly greater sensitivity than microscopic analysis. Infections were more prevalent among females (60.3%), adult birds (55.2%), and during the rainy season (54%). Sequencing confirmed the presence of *P. gallinaceum* as the most prevalent pathogen (97.87-97.94%). Phylogenetic analysis supported the molecular identification and revealed evolutionary relationships among detected lineages.

Conclusion: The present study confirmed a high prevalence of haemoparasites in poultry in Ibadan, Nigeria, and underscored the superior sensitivity of PCR over microscopy for detection. The integration of molecular and morphological approaches enhanced diagnostic accuracy and provided deeper insights into parasite diversity and epidemiology.

1. Introduction

Agriculture remains the backbone of most sub-Saharan African economies, contributing an average of about 25% of the Gross Domestic Product (GDP)[1]. Poultry birds are effective at converting feed into usable protein in meat and eggs[2]. The industry currently provides a significant number of jobs and a low-cost source of animal protein for the continent's massive and expanding population[3]. It has been estimated that 14.718 billion chickens are raised worldwide. Of this population, 11.038 billion live in developing nations such as Nigeria, Ghana, Kenya, and Tanzania[4]. Poultry production is affected by several diseases, including parasitic diseases, which are a primary

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cause of reduced production worldwide[5].

Haemoparasites are harmful to their hosts and can result in high fatalities[6]. Haemoparasitic infections have been linked to environmental factors, including altitude, temperature, and precipitation. In the last few decades, an increase in the incidence of some infectious diseases, such as avian malaria and other haemosporidian diseases have been reported in wild populations, which can significantly impact avian populations[7]. Haemoparasites can infect different bird clades and are widespread globally[7]. In birds and other vertebrates, blood parasites can be extracellular, including *Trypanosoma* species and microfilariae of filariid nematodes[8].

The intracellular blood parasites are extremely varied and belong to a number of taxa within the phylum Apicomplexa, including the orders Haemosporida (common genera include *Plasmodium, Haemoproteus*, and *Leucocytozoon*), Piroplasmida (*Babesia, Theileria*, and *Sauroplasma*), and Eucoccidiorida, which includes the two suborders Adeleorina (*Hepatozoon*, and *Hemogregarina*) and Eimeriorina (*Lankesterella, Schellackia*, and *Lainsonia*)[8]. Haemosporidian parasites and their hosts, such as chickens and some mammals, are commonly used as ecological and evolutionary models, and the parasites are mostly transmitted through the faeco-oral route[7-9]. The prevalence of haemosporidian parasites in Iran has been reported to range from 20% to 60%[9]. Common clinical signs of haemaparasitism in birds include dehydration, pale combs and wattles indicating anaemia, and somnolence[6]. Relatively basic evolutionary groupings, including *Struthioniformes* and *Pelecaniformes*, have the lowest parasite diversity (less than three species).

Traditional microscopic analysis of stained blood smears and molecular methods utilizing polymerase chain reaction (PCR) assays have been used to diagnose blood parasites. Following Giemsa staining, the different developmental phases of the hemoparasites could be identified under a microscope in tissue or blood smears[10]. The present study aimed to identify haemoparasites, ascertain their prevalence in birds, and evaluate the diagnostic efficacy of conventional microscopy and PCR in identifying haemoparasites in birds in Ibadan, Nigeria.

2. Materials and Methods

2.1. Ethical approval

Approval for the use of animals, as prescribed by international and institutional guidelines, was obtained from the University of Ibadan Animal Care and Use Research Committee (UI ACUREC) under approval number UI ACUREC NO: 034-0423/18.

2.2. Study location

The study was conducted in Ibadan, Oyo state, Nigeria, located between latitude 7˚ 3 and 9˚ 12 North of the equator and longitudes 2˚ 47 and 4˚ 23' East of the Meridian with a total land mass of 27,249 square kilometers ranging from an altitude of 500 m in the South to 1,219 m in the North. The climate is tropical, characterized by distinct wet and dry seasons influenced by the intertropical convergence zone. The dry season typically spans from November to March, and is marked by low humidity and minimal rainfall. From December to February, the dry and dusty Harmattan wind from the Sahara Desert is prevalent. The wet season is bimodal, running from April to October. The wet season features two peaks of heavy rainfall. The first rainfall peak occurs from April through July, a brief period of reduced rainfall, known locally as the August Break, often occurs in mid-August, and a second rainfall peak follows from September to October. The average annual rainfall ranges from 1,194 mm in the North to 1,278 mm in the South. The mean temperature is 27°C, with daily sunshine hours ranging from 24 hours in August to 7 hours in February.

2.3. Sample collection

A total of 390 apparently healthy birds were randomly selected for the present study. Health status was determined by physical examination prior to sample collection, and no birds exhibited overt clinical signs of disease at the time of sampling. Birds were sampled from 10 farms (growing different breeds) across different locations (39 birds were sampled in each farm) in Ibadan, Nigeria, between January and August 2023. The sampled population comprised commercial layers chickens (153), broiler chickens (69), local chickens (60), turkeys (75), ducks (3), and pigeons (30) in Ibadan, Nigeria. The sample consisted of a mixed population of male (126) and female (264) birds, young (189) and adult (201), with an average weight of 4.3 kg. Approximately 2 mL of blood sample collected from the jugular vein of each bird was divided into two aliquots stored in an EDTA-coated tube for microscopy and DNA analysis as described by Nourani et al.[9]. Samples were immediately transported in ice packs to the Veterinary Clinical Pathology Laboratory at the University of Ibadan, Ibadan, Oyo State, Nigeria, for further analysis.

2.4. Microscopic examination

Thin blood smears were prepared from the blood samples on clean glass slides, allowed to air dry, fixed with absolute methanol (100%, Brentag NV, Deerlijk, Belgium), and stained with Giemsa stain (Brentag NV, Deerlijk, Belgium). The slides were then screened for the presence of blood parasites using a CX21 FS1 light microscope (Olympus®, Japan) at different magnifications (40x, 100x, 400x, and 1000x) as described by Nourani et al.[9].

2.5. Molecular detection

Subsequently, whole blood samples from the 390 birds, collected in EDTA tubes, were used for genomic DNA extraction with the PrimePrep Genomic DNA Isolation Kit (GENETBIO Inc., Daejeon, South Korea) according to the manufacturer's instructions. The DNA extraction process used 50-100 µl of blood samples. The quality of the extracted DNA was checked by a spectrophotometer (DeNovix Inc.®, USA).

Amplification of the 480 bp fragment of mitochondrial cytochrome b (cytb) DNA was carried out using a nested PCR approach for two isolates, T2 (sample from an adult

female commercial layer chicken) and T4 (young male broiler chicken). The primers used in the first round were the primer set HaemNF1 and HaemNR3, followed by HaemF, HaemR2, HaemFL, and HaemR2L[9] in the second round, as indicated in Table 1. The final PCR reaction volume was 25 µL, consisting of 12.5 µL of AMPLIQON Red PCR Master Mix (AMPLIQON, Denmark), 1-2 µL of genomic DNA template (50-100 ng/µl), 0.6 µl of each primer (10 µM stock), and nuclease-free water to reach the final volume. Ultrapure water and a previously confirmed positive PCR product were included as negative and positive controls, respectively. This reaction setup was consistent with established nested PCR protocols for avian haemosporidians[9,12]. The resulting positive amplicons were purified and sequenced using the primers HaemF and HaemR2 (for *Haemoproteus* and *Plasmodium*) and HaemFL and HaemR2L (for *Leucocytozoon*) by BIONEER Inc.® (Seoul, South Korea) as indicated in Table1.

Table 1. Primers used for nested PCR amplification of the mitochondrial cytochrome b gene in avian haemoparasites

PCR round	Primer name	Primer sequence (5' → 3')	Target parasite(s)	Reference of primer
First	HaemNF1	CATATATTAAGAGAAITATGGAG	General haemosporidians (*Plasmodium, Haemoproteus, Leucocytozoon*)	Nourani et al.[9]
	HaemNR3	ATAGAAAGATAAGAAATACCATTC	General haemosporidians (*Plasmodium, Haemoproteus, Leucocytozoon*)	Nourani et al.[9]
Second	HaemF	ATGGTGTTTTAGATACTTACAT	*Plasmodium* and *Haemoproteus*	Nourani et al.[9]
	HaemR2	GCATTATCTGGATGTGATAATGGT	*Plasmodium* and *Haemoproteus*	Nourani et al.[9]
	HeamFL	ATGGTGTTTTCGATATTTACAT	*Leucocytozoon*	Nourani et al.[9]
	HaemR2L	CATCCAATCCATAATAAAGCAT	*Leucocytozoon*	Nourani et al.[9]

Following the manufacturer's instructions, the fragments were sequenced using the Nimagen Brilliant Dye™ Terminator Cycle Sequencing Kit V3 (BRD3-100/1000, Zymo Research, Irvine, CA, USA). The ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053, Zymo Research, Irvine, CA, USA) was used to purify the labeled products. The clean-up kit used a silica-membrane purification system designed to remove unincorporated dyes, salts, and other reaction components, ensuring cleaner templates and more reliable Sanger sequencing results. The purified products were then loaded onto an Applied Biosystems ABI 3500XL genetic analyzer equipped with a 50 cm array and POP7 polymer (Thermo Fisher Scientific, Cat. No. 4406016, Zymo Research, Irvine, CA, USA) for sequence data collection.

Phylogenetic reconstruction was performed to elucidate the evolutionary relationships of the detected haemoparasites. Nucleotide sequences obtained from PCR-positive samples were aligned with reference sequences retrieved from the GenBank database. Evolutionary history was inferred using the Neighbour-Joining method[13]. The robustness of the tree topology was assessed using bootstrap analysis with 1,000 replicates; bootstrap values ≥70% were considered indicative of significant clustering[14]. Evolutionary distances were calculated using the Jukes-Cantor model[15] and expressed as the number of base substitutions per site. The final dataset comprised 10 nucleotide sequences and 570 aligned positions. All codon positions (first, second, and third) and non-coding regions were included, and ambiguous alignment positions were removed using the pairwise deletion option. All phylogenetic analyses were conducted using MEGA11 software according to Tamura et al.[16].

2.6. Statistical analysis

All data were managed and analyzed using IBM SPSS Statistics for Windows Version 21.0 (IBM corp., Armonk, NY, USA). Descriptive statistics were employed to summarize the prevalence of haemoparasite infections, expressed as percentages with 95% confidence intervals (CI), stratified by host species, sex, age group, and season. The diagnostic performance of microscopy and nested PCR was compared using Cohen's kappa (k) statistic to assess agreement beyond chance. The sensitivity of microscopy was calculated using PCR as the reference standard.

Associations between categorical variables such as sex, age (young compared to adult), season (dry compared to rainy), and haemoparasite infection status (positive compared to negative) were assessed using the Chi-square test of independence. Odd ratios (ORs) with 95% CIs were calculated to quantify the strength of association. A p-value less than 5% ($p < 0.05$) was considered statistically significant for all inferential tests.

3. Results

Haemoparasites were identified in 174 birds (44.6%), 53 commercial layers (34%), 27 broiler chickens (46%), 40 local chickens (67%), 33 turkeys (44%), 2 ducks (67%) and 19 pigeons (63%) samples via microscopic analysis, while PCR test detected parasites in 208 of the 390 blood samples (53.3%). The agreement between microscopy and PCR was moderate and significant (Cohen's $k = 0.42$, $p < 0.05$). The PCR demonstrated significantly higher sensitivity ($\chi2 = 15.8$, $p < 0.05$) and identified 34 additional positive cases that were negative on microscopic examination.

Microscopic examination revealed the presence of *Haemoproteus* spp. (15.9%), *Plasmodium* spp. (18.2%), *Leucocytozoon* spp. (5.6%), *Babesia* spp. (2.8%) and microfilariae (2.1%). Nested PCR and subsequent sequencing confirmed *Haemoproteus* spp. Infections (19%), *Plasmodium gallinaceum (P. gallinaceum*; 12.3%), *Leucocytozoon* spp. (9%), and additional unidentified haemosporidian lineages (8.7%). Representative gel electrophoresis images of PCR products for *Plasmodium* at 600 bp and 400 bp, and *Haemoproteus* at 400 bp are shown in Figure 1 and Figure 2, respectively.

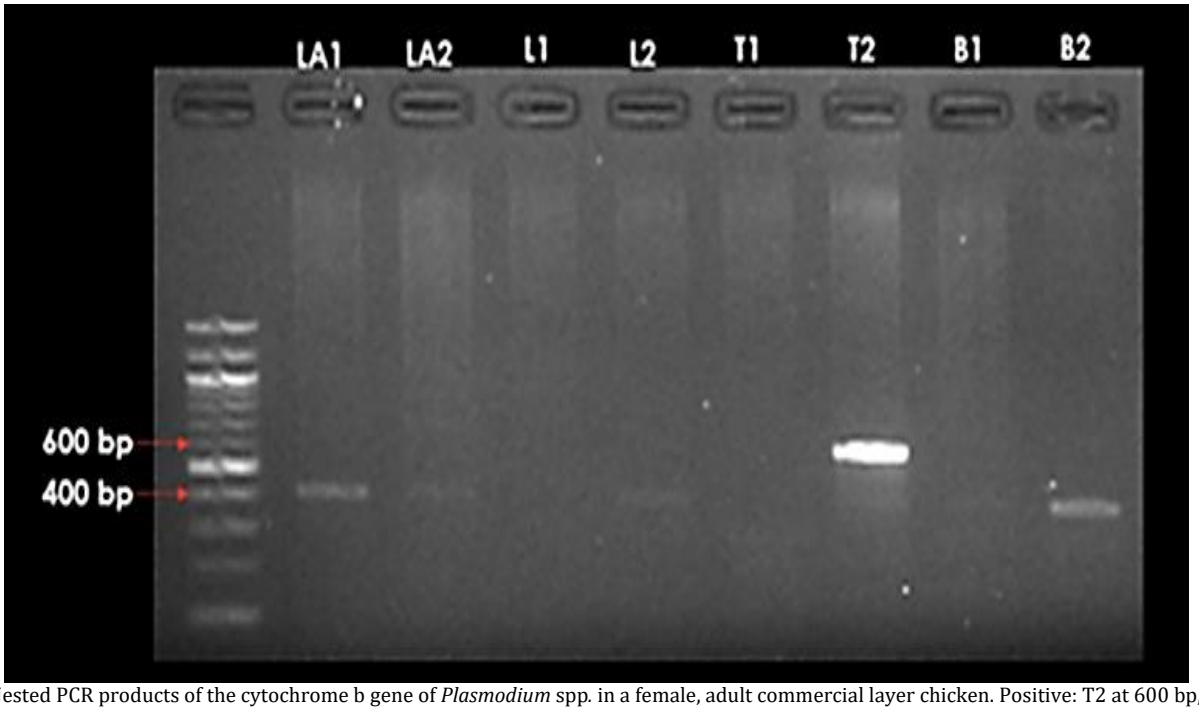

Figure 1. Nested PCR products of the cytochrome b gene of *Plasmodium* spp. in a female, adult commercial layer chicken. Positive: T2 at 600 bp, Lanes LA1 and B2 at 400 bp for *Plasmodium spp*. Negative: Lanes L1, L2, T1, and B1.

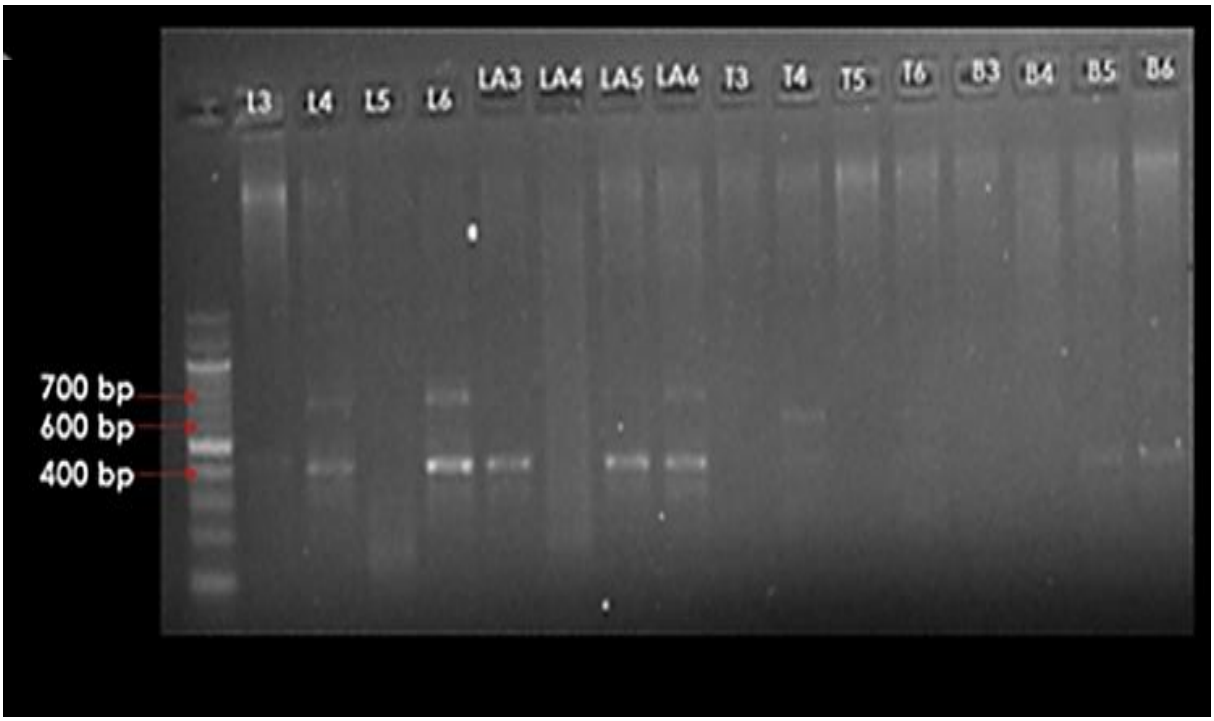

Figure 2. *Haemoproteus* spp. amplified fragment of the cytochrome b gene in a male young broiler chicken at 400 bp. Positive: Lanes L4, L6, LA3, LA5, and LA6. Negative: Lanes L3, L5, T3, T5, T6, B3, B4, B5, and B6.

A significantly higher prevalence of haemoparasites was observed in male birds across all the bird species (54.76%) compared to females (39.77%; $p < 0.05$; Table 2). By age, adults had a significantly higher prevalence of haemoparasites (55.2%) than young birds (44.8%; $p < 0.05$; Table 3). Haemoparasite prevalence was significantly higher in all the birds during the rainy season (54%) compared to the dry season (46%; Table 4).

Table 2. Prevalence of haemoparasite infection in birds stratified by sex in Ibadan, Nigeria, 2023

Sex	Samples (number)	Positive (number)	Prevalence rate
Male	126	69	54.76%[a]
Female	264	105	39.77%[b]

[a,b] Mean different superscript letters in a column indicate significant differences ($p<0.05$).

Table 3. Prevalence of haemoparasite infections in birds stratified by age in Ibadan, Nigeria, 2023

Age	Samples (number)	Positive (number)	Prevalence rate
Young	189	78	41.27%[b]
Adults	201	96	47.76%[a]

[a,b] Mean different superscript letters in a column indicate significant differences ($p<0.05$).

Table 4. Prevalence of haemoparasite infection stratified by season of sample collection in Ibadan, Nigeria, 2023

Seasons	Samples (number)	Positive (number)	Prevalence rate
Dry	200	80	40.00%[b]
Rainy	190	94	49.47%[a]

[a,b] Different superscript letters in a column indicate significant differences ($p<0.05$).

Blast analysis of cytb sequences from two isolates (T2 and T4) indicated high nucleotide identity (97.87% and 97.94%, respectively) with *P. gallinaceum* reference strains KY653786.1 and HM179147.1 (Table 5). Protein BLAST of the corresponding sequences confirmed identity percentages of 93.67% and 92.21% with *P. gallinaceum* cytb protein entries (Table 6). The phylogenetic tree constructed using the Neighbour-Joining method demonstrated that the detected sequences clustered closely with *P. gallinaceum* lineages, supported by bootstrap values ≥ 85% (Figure 3).

Table 5. BLAST-nucleotides analysis of cytochrome b sequences from *Plasmodium gallinaceum* isolated from chickens in Ibadan, Nigeria

Sample ID	Organism	Sequence length (bp)	Identity	Accession number of BLAST hit	E-value	Alignment score	Highest query coverage
T2	*Plasmodium gallinaceum*	474	97.87%	KY653786.1	0.0	≥ 200	98%
T4	*Plasmodium gallinaceum*	481	97.94%	HM179147.1	0.0	≥ 200	90%

T2: Sample 2 from an adult female commercial layer, T4: Sample 4 from a young male broiler chicken. E-value: Expected value, which indicates statistical significance of the sequence match. E-value of 0.0 indicated an extremely significant result, meaning the sequences were almost certainly from *P. gallinaceum*.

Table 6. BLAST-protein analysis of cytochrome b sequences from *Plasmodium gallinaceum* isolated from chickens in Ibadan, Nigeria

Sample ID	Organism	Sequence length (bp)	Identity	Accession number of BLAST hit	E-value	Alignment score	Highest query coverage
T2	*Plasmodium gallinaceum*	158	93.67%	AOG15972.1	5e-100	≥ 200	100 %
T4	*Plasmodium gallinaceum*	159	92.21%	ABQ65778.1	6e-94	≥ 200	96 %

T2: Sample 2 from an adult female commercial layer, T4: Sample 4 from a young male broiler chicken. E-value: Expected value, indicates statistical significance of the sequence match. E-values of 5e-100 and 6e-94 indicated a strongly significant result, meaning that the translated protein sequences were homologous to *P. gallinaceum* cytochrome b.

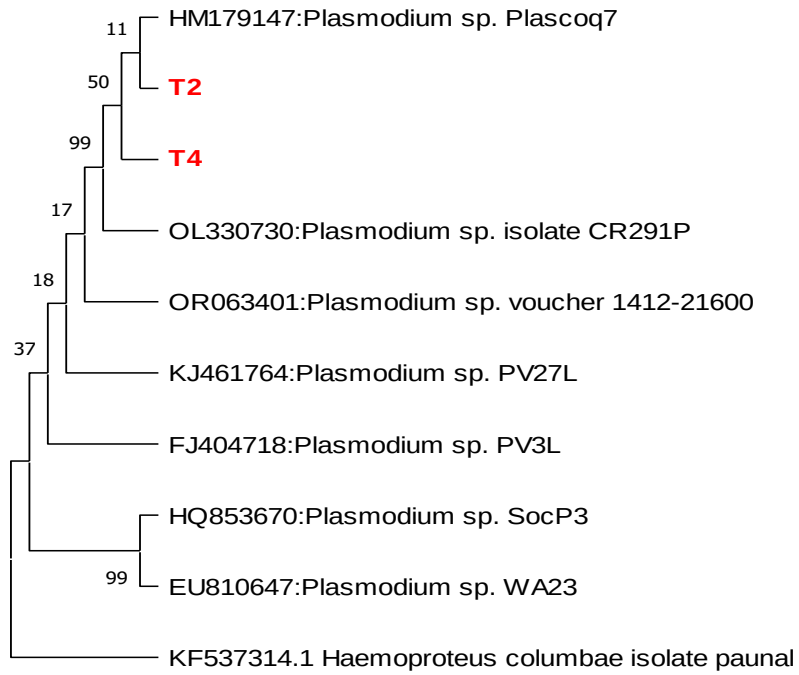

Figure 3. Phylogenetic tree of avian haemosporidian parasites based on mitochondrial cytochrome b gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Evolutionary relationships among the taxa in the samples are shown in red.

4. Discussion

Numerous studies have examined the prevalence, detection, and identification of haemoparasites. In a study conducted in Northwest Italy, blood samples were typically obtained from free-living birds to assess the shape of haemoparasites in blood cells, grade parasitaemia using light microscopy, and identify several lineages[17]. Blood parasite distribution depends on mosquito feeding habits, host-parasite and vector-parasite compatibility, and climate; temperature, relative humidity, and rainfall are indicators of the quantity and activity of their insect vectors[10].

The present study used microscopic techniques, considered by most haemoparasitologists to be the gold standard for detecting blood parasites[6], and PCR for accurate diagnosis. The present study emphasized the superiority of PCR over microscopy in detecting avian haemosporidians, revealing a higher prevalence of infections using molecular techniques. This finding was consistent with that of Lawal et al.[18], who reported that nested PCR demonstrated higher sensitivity for analyzing haemoparasite prevalence than microscopic analysis. The study by Ciloglu et al.[19] indicated the effectiveness and high sensitivity of multiplex PCR in identifying single and mixed infections, which aligns with the present findings.

During the present study, parasites including *Haemoproteus* spp., *Plasmodium* spp., *Leucocytozoon* spp., Microfilaria, and *Babesia* spp. were detected, which differed from that of Ogbaje et al.[20] who encountered only three genera of haemoparasites, including *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* using the microscopic method in Benue state, Nigeria. Additionally, Lawal et al.[6] reported two genera of avian haemoparasites (Plasmodium and *Haemoproteus* spp.) in domesticated and wild birds in Maiduguri, Northeastern Nigeria, using microscopy. Using nested PCR, the current study found a greater overall frequency of avian hemoplasmidian parasites in poultry birds than microscopic analysis in Ibadan, Nigeria. Thus, the present study confirmed that nested PCR is more sensitive than microscopic analysis and can detect very low levels of infection, consistent with the findings of Lawal et al.[21].

Weather conditions are a crucial factor in haemoparasite infections in poultry, which are more prevalent during warmer than colder periods, most likely due to increased arthropod vector activity[22]. In the present study, the prevalence of haemoparasite infection was higher in the rainy season, consistent with the findings of Lapointe et al.[23] in Hawaii, United States, who indicated that differences in seasonal or annual precipitation might affect transmission patterns across the landscape. The seasonal variation is consistent with the study by Lawal et al.[18] in Gombe state, Nigeria, which emphasized the role of arthropod vectors and environmental factors in influencing the prevalence of avian haemosporidians.

The current findings demonstrated that female birds had a higher specific prevalence rate of haemoparasites than male birds. This finding was consistent with those of Rukambile et al.[24], who reported high infection rates in female local chickens and pigeons in Tanzania, a trend linked to higher levels of progesterone and prolactin. This finding contrast with the findings of Lawal et al.[18], who reported a higher prevalence of the parasite in male chickens. Scaglione et al.[17], in a study conducted in Northwest Italy, reported no difference in the prevalence of bird diseases across age groups, which contrasted with the present results, as haemoparasitism was more prevalent in adult birds. In this study, the higher parasite intensity observed in adult birds appeared to be driven by selective mortality against the most severely parasitized adults. The higher parasitism in younger birds observed in the present study aligned with the immunity hypothesis demonstrated by Sol et al.[22] in feral pigeons. Sol et al.[22] indicated that elevated parasite intensity in juveniles was due to an underdeveloped immune response, a deficit that is typically resolved as birds mature and acquire protective immunity.

Previous studies in Northern Nigeria[21] and Eastern Madagascar[25] have demonstrated that microscopic analysis was less sensitive than PCR methods, which aligned with the current findings. The sensitivity of microscopic analysis is notably influenced by the quality of blood smears and the investigator's proficiency. Eliminating these uncertainties requires achieving test parameters that match the performance of optimal PCR and ensuring the superior quality of blood smear preparations[25]. The limitations of microscopic analysis underscore its low sensitivity in detecting low-level infections and the challenge of determining species composition during simultaneous infections. Despite these limitations, microscopic analysis retains advantages, such as its low cost, wide availability, and ability to determine infection identity and intensity. These advantages of microscopic analysis align with the findings of Valkiūnas et al.[26] regarding its use in routine screening and the identification of blood parasites. Several studies have demonstrated that PCR amplification and sequencing of the cytb gene have helped identify distinct lineages and have produced a sizable database of avian haemosporidian parasites for sequence comparison[26].

5. Conclusion

The present study indicated a high prevalence of haemoparasites (53.3%) in poultry in Ibadan, Nigeria, using the nested PCR and microscopic methods, with prevalence of *Haemoproteus* spp., *P. gallinaceum*, and *Leucocytozoon* spp. Nested PCR demonstrated greater sensitivity than microscopic analysis, making it essential for detecting low-level and mixed infections. Epidemiological patterns revealed higher infection rates among female and adult birds during the rainy season, linking transmission to host factors and vector ecology. Molecular characterization confirmed *that P. gallinaceum* was highly genetically identical to previously recognized strains. While microscopic analysis remains valuable for species identification and parasitaemia estimation, its diagnostic limitations underscore the need to integrate molecular tools into routine surveillance. Future studies should adopt an integrated One Health approach that combines vector ecology, molecular epidemiology, host-pathogen

interactions, and climate-linked surveillance to develop sustainable, field-applicable diagnostic and control strategies for haemoparasites in birds.

Declarations

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Authors' contributions

The concept of the study was developed by Theophilus Aghogho Jarikre and Diano Oumar Mamadou Diop. The literature search and the original draft were conducted by Diano Oumar Mamadou Diop and Abdulrauf Adekunle Usman, who also collected the samples and analyzed the data. The manuscript was reviewed by Theophilus Aghogho Jarikre and Oluwole Oyetunde Oni. Microscopic analysis and molecular diagnosis were performed by Diano Oumar Mamadou Diop and Abdulrauf Adekunle Usman. Theophilus Aghogho Jarikre and Oluwole Oyetunde Oni supervised the study. All authors have read and confirmed the final edition of the manuscript.

Availability of data and materials

Data supporting the present study are included within the article and supporting materials.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors declared that this original study article has not been published or submitted elsewhere. The manuscript underwent plagiarism screening using standard software. The authors did not use AI tools during any phase of the study or in preparing this manuscript.

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