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Molecular Detection of Avian Haemosporidian Parasites in Village Chickens (*Gallus gallus Domesticus*) in Gombe State, Nigeria

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Abstract

The present study aimed to determine the prevalence and molecularly identify the species of haemosporidian parasites in village chickens in Gombe State, Nigeria, using microscopy and nested Polymerase Chain Reaction (PCR) techniques. Whole blood samples were collected from 1600 village chickens from eight local government areas of Gombe State. Thin blood films and buffy coat smear from each blood samples were first screened for the presence of intracellular or extracellular parasites using microscopy; this revealed prevalence of 20.4%, while DNA extracted from 220 selected whole blood samples used for PCR amplification, revealed prevalence rate of 76.8%; however, 63 (28.6%) samples were positive for *Plasmodium*, 45 (20.5%) for *Haemoproteus* while 33 (15.0%) samples were positive *Leucocytozoon*. 23 (10.5%) had mixed *Plasmodium* + *Haemoproteus* infection while *Plasmodium* + *Haemoproteus* + *Leucocytozoon* was detected in 5 (2.3%) of the positive samples. Sequencing of positive amplicons revealed *Plasmodium* species, *Leucocytozoon schoutedeni* and *Haemoproteus columbae* in a single and co-infection. Phylogenetic trees were constructed to determine the closely related species. This present study represents the first attempt to molecularly detect avian haemosporidian parasites in the village chickens in Gombe State, Nigeria. In conclusion, haemosporidian parasites are prevalent in Gombe State and nested PCR was more sensitive in haemoparasite detection than microscopy. Further research should be conducted to determine the prevalence of haemosporidian parasites in other poultry species in Nigeria to further evaluate the epidemiology of the diseases for effective control and prevention.

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Introduction

Haemosporidian parasites (Sporozoa: Haemosporida) are obligate heteroxenous parasitic protists that parasitize amphibians, reptiles, birds, and mammals and are spread by dipteran insects (Insecta: Diptera) as vectors [1]. Changes in the environment and the growing human population increase the chances of poultry coming into contact with wild birds, which may facilitate disease transmission among avian species [2]. To overcome the limitations of microscopic morphological detection of haemoparasites, molecular diagnosis, genotyping, and amplification of unique haemoparasite gene fragments have been used in recent years [3]. Molecular methods that use PCR-based techniques to amplify specific regions have enhanced parasite species genome recognition and have proven to be more sensitive in diagnosing haemoparasitic infection when parasitaemia is low [4,5]. Just a few studies have compared microscopic techniques for detecting haemoparasites with molecular techniques [6-8]. Notably, both PCR-based and microscopy-based methods have drawbacks when it comes to diagnosing haemoparasites in avian blood [9,10].

More than 200 haemosporidian species have been identified, with taxonomists classifying them into the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, and *Fallisia*. However, one of the most challenging aspects of researching the diversity and distribution of avian haemosporidian parasites is correct species identification [11]. *Plasmodium* and *Haemoproteus* species, the haemosporidian parasites that cause avian malaria, have been found in blood smears almost uniformly among domesticated and wild birds [12,13]. However, a nested-PCR assay that targets the parasites' cytochrome b gene is effective, allowing for the screening and typing of *Leucocytozoon* species alongside *Haemoproteus* and *Plasmodium* species in avian blood samples. This new approach appears to be highly repeatable and effective, making it an important tool for studying *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* species in birds at the same time. The use of a molecular approach allows for a better understanding of the diversity of avian haemoparasitosis, particularly in cases where the microscopy method can restrict parasite diagnosis at low parasitaemia levels. The prevalence of avian haemosporidian parasites in domesticated birds appears to have increased in developing countries, especially in endemic areas, including Africa, and this may be due to climate change [14-16]. The phylogenetic affinities of avian haemosporidian parasites must therefore be investigated, especially in geographical areas such as the Guinea Savannah region of northeastern Nigeria, which includes Gombe State.

The current study aims to detect the existence of the *cyt-b* gene from the *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* genera in village chickens (*Gallus gallus domesticus*)

in Gombe State, Nigeria. This is also the first time these avian haemosporidian parasites have been detected molecularly in village chickens in Northern Nigeria.

Materials and methods

Study area

This study was carried out in Gombe State, Northeastern Nigeria. The state is located between latitude 9° 30' and 12° 3' N and longitude 8° 45' and 11° 45' E [17], it has a mean annual rainfall of 818.5mm, with a mean maximum temperature of 37°C and a mean minimum temperature of 12°C. The total poultry population in Gombe State is approximately 508, 305 comprising 462,000 backyard poultry and 46,305 exotic poultry [18].

Target population

Rural and urban areas within eight (8) out of the eleven (11) local government areas of the study were visited for sample collection viz: Gombe, Akko, Funakaye, Kwami, Dukku, Yamaltu-Deba, Kaltungo and Balanga Local Government Areas.

Sample size determination for microscopy detection

A cross-sectional study design adopting non-probability convenience sampling techniques was used with emphasis on areas with large populations of village chickens. Blood samples were collected from 1600 (200 chickens/LGA) apparently healthy village chickens.

Sample size determination for molecular detection

The sample size was calculated using the prevalence rate of avian haemosporidian parasites in Maiduguri, Borno State, Nigeria which was found to be 17.0% as reported by Lawal JR et al. [19]. A standard epidemiological formula (Fisher's formula for cross-sectional descriptive study) using the equation given by Thrusfield [20] for simple random sampling in research was used to calculate the sample size as follows;

$$N = \frac{Z^2 Pq}{d^2}$$

Where N = Sample size

Z = standard normal distribution at 95% confidence level = 1.96

Therefore Z2 = 1.962 = 3.8416

P = prevalence rate of 17% = 0.60

q = 1 - P, therefore q = 1 - 0.17 = 0.83

d = maximum value of probability (allowable error taken as 5%) = 5/100 = 0.05

Therefore d^2 = 0.052 = 0.0025

$$N = \frac{3.8416 \times 0.17 \times 0.83}{0.0025}$$

$$N = \frac{0.54204976}{0.0025}$$

Therefore, N = 216.819904 ≈ 216.82 ≈ 217

From this calculation, 217 samples were arrived at, but to be on the safest side of sample size selection, a total of 220 village chickens blood samples were selected for the molecular detection of avian haemoparasites in the present study at the biotech laboratory in NVRI-Vom Plateau State.

Microscopy examination for avian haemosporidian parasites in village chickens from Gombe State, Nigeria

Thin blood and buffy coat smears were made from each blood sample on two different clean dry slides and were left for few minutes to air dry and then labeled appropriately. The slides were then fixed with methanol for five (5) minutes, allowed to air dry, packaged and then transported to the Department of Veterinary Parasitology and Entomology Research Laboratory, University of Maiduguri for staining with diluted 10% Giemsa

stain 7.4 according to the standard procedures described by Zajac and Conboy [21]. The slides were later viewed at low magnification (×40) and at high magnification (×100) using the light Olympus® (Japan) microscope under oil immersion for the presence of intracellular or extracellular blood parasites and their gametocytes as previously described by Valkiûnas [22], Valkiûnas et al. [10].

Samples selection for molecular detections of avian haemosporidian parasites in village chickens from Gombe State, Nigeria

Thin blood smears were made from two hundred and twenty (220) blood samples, which were initially examined and screened for the presence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species in village chicken in Gombe State, Nigeria using microscopy method at Department of Veterinary Parasitology and Entomology Laboratory, University of Maiduguri, Borno State, Nigeria. The 2ml of frozen (at – 80°C) whole blood samples in Ethylene Diamine Tetraacetic Acid (EDTA) tubes were well labeled and transported in cold chain to the Biotechnology Research Laboratory, National Veterinary Research Institute, Vom–Plateau State, Nigeria for molecular detections of the parasites using the nested PCR techniques. However, all kits, reagents and primers required for the PCR were ordered from Inqaba Biotec Laboratories, South Africa (info@inqababiotec.ng). All molecular procedures were carried out according to the standard recommendations and protocols of the manufacturers.

Genomic DNA extraction from blood samples

Deoxyribonucleic Acid (DNA) was extracted from frozen whole blood using the commercial DNA extraction kit (Quick-DNATM Miniprep Plus Blood and Tissue kit; Catalog Nos. D4068 & D4069; Zymo-SpinTM Technology, USA) according to the manufacturers' protocol.

Test for the presence and quality of extracted DNA

Ten extracted DNA was randomly selected, the presence and quality of the extracted DNA was assessed by electrophoresis of 2µl of the extract in 2% agarose containing ethidium bromide, and visualized under UV light. All protocol of the procedures involved in the quality assessment of the DNA template were carried out according to the standard procedures described by Cosgrove et al. [23].

Sources of positive control and negative controls

For positive controls, reference standard of avian haemosporidian parasites from inqaba biotechnology laboratory were used for the positive control and incorporated into each PCR run, while the negative control used was sterile Type-1^{+} purified water instead of a sample containing DNA template. The positive controls were used in running the PCR of the rest of the samples. All samples were kept frozen at -80 0C (Sanyo Japan) until further molecular analysis.

PCR amplification

All kits, reagents and primers required for the PCR technique were sourced from Inqaba Biotec Laboratories, South Africa. A nested PCR assay was employed to amplify a 500bp fragment of the mitochondrial cytochrome b gene (*cyt-b*) of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* using published primers [4]. The primary amplification was carried out using the primer pairs HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3'), which is a general primer for the amplification of *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. For the secondary amplification, primer pairs specific for *Plasmodium* and *Haemoproteus* HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') [24], and *Leucocytozoon* HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3') [4], were used. Images were captured using a digital camera and computer software (GeneSnap TM, Bio-Rad Laboratories). For differentiation of *Haemoproteus* and *Plasmodium* species, an enzymatic digestion was performed in accordance with methodology previously described by Kistler et al. [25]. Bands compatible with *Haemoproteus* and *Plasmodium* species were seen as shown in Kistler et al [25].

Gel purification and sequencing

Positive amplicons of 500bp were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturers' protocol. Plasmid extraction was also conducted using the GeneAll® Hybrid-QTM Plasmid RapidPrep Kit (GeneAll, Korea) according to manufacturer's protocol. The eluted plasmid DNA containing the *Plasmodium*, *Haemoproteus* and *Leucocytozoon cyt-b* gene fragment was stored at -20°C and later sequenced using the Nimagen, BrilliantDye™ Terminator cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions . Sequencing was carried out at Inqaba biotec laboratories in South Africa following standard procedures.

Analysis of the *Plasmodium*, *Haemoproteus* and *Leucocytozoon cyt-b* gene

The nucleotide sequences were subjected to phylogenetic analysis using MEGA5 [26]. All positions containing gaps and missing data were eliminated. Phylogenetic affinities were determined by constructing a Neighbour-Joining (NJ) tree [27] employing the p-distance model. The heuristic search was obtained automatically by applying Neighbor-Join and then selecting the topology with superior log likelihood value. Cyt-b gene sequences of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* from other avian species curated by NCBI GenBank were incorporated into the phylogenetic analysis. The strength of the phylogenetic trees analysis were tested using 1000 (NJ) bootstrap replicates [28].

Results

Out of 1600 blood samples examined, 326 (20.4%) were found to be infected with avian haemosporidian parasites. the microscopy prevalence of *Plasmodium* (163/1600) and *Haemoproteus* (93/1600) species were 10.2% and 5.8% respectively, that of mixed infection of *Plasmodium* + *Haemoproteus* (24/1600) was 1.5% and that of *Leucocytozoon* species (5/1600) was 0.3%. Out of the 220 DNA samples extracted from whole blood and tested, 169/220 (76.8%) were positive for avian haemosporidian parasites by nested PCR. Presence of the haemosporidian parasites was determined by PCR amplification of the cytochrome b gene. However, out of the positive samples, 63/220 (28.6%) samples were positive for *Plasmodium* spp., *Haemoproteus* spp. 45/220 (20.5%), 33/220 (15.0%) were positive for *Leucocytozoon* and 23/220 (10.5%) for mixed *Plasmodium* + *Haemoproteus* infection. However, 5/220 (2.3%) amplicons showed mixed infection with *Plasmodium* + *Haemoproteus* + *Leucocytozoon* as shown in Table 1.

Table 1: Molecular Detection and Prevalence of avian haemosporidian parasites in Village chickens (Gallus gallus domesticus) in Gombe State, Nigeria.

Diagnostic Technique	Number (%) of chicken infected with Avian haemosporidian parasites					Overall Prevalence (%)
	Plasmodium	*Haemoproteus*	*Leucocytozoon*	*Plasmodium + Haemoproteus*	*Plasmodium + Haemoproteus + Leucocytozoon*	
Microscopy	163 (10.2)	93 (5.8)	24 (1.5)	5 (0.3)	0 (0.0%)	326 (20.4%)
95% CI (LL – UL)	8.8–11.8	4.8–7.1	1.0–2.2	0.1–0.7	0.0–0.2	18.5–22.4
Nested PCR	63 (28.6)	45 (20.5)	33 (15.0)	23 (10.5)	5 (2.3)	169 (76.8%)
95% CI (LL – UL)	23.1-34.9	15.7–26.3	10.9–20.3	7.1–15.2	1.0–5.2	70.8–81.9

Presence of the DNA of *Plasmodium* and/or *Haemoproteus* or *Leucocytozoon* species in infected blood was determined by PCR amplification of the cytochrome b gene as shown in Gel electrophoresis in Figure 1 and 2 showing the amplified fragment of cytochrome-b gene of these haemoparasites.

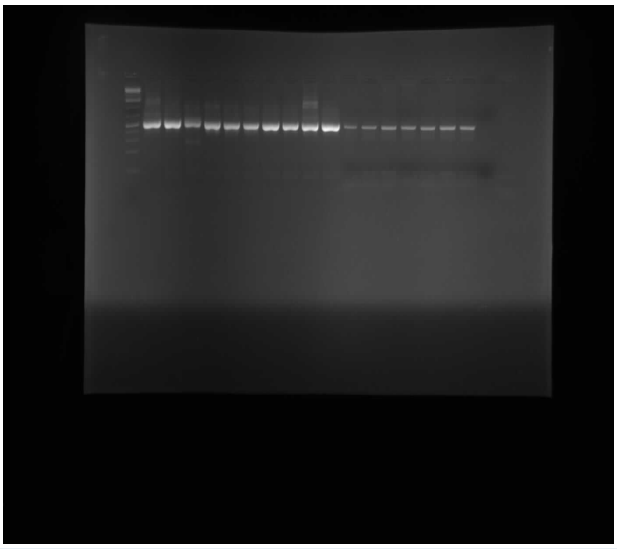

Figure 1: MGel electrophoresis showing the amplified fragment of cytochrome-b gene of *Plasmodium/Haemoproteus* (lanes 1-16 were positive of the parasites) from village chickens (Gallus gallus domesticus) in Gombe State, Nigeria.

Key: M: 100bp size marker; P: Positive Control; N: Negative Control; Amplicons are in the numbered lanes.

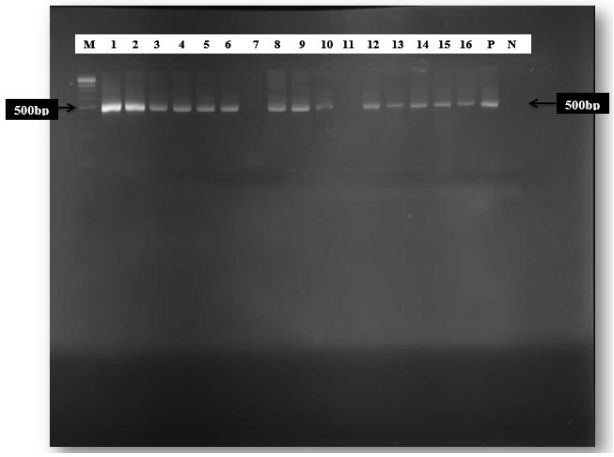

Figure 2: Gel electrophoresis showing the amplified fragment of cytochrome-b gene of *Leucocytozoon* (lanes 1-6), (lanes 8-10) and (lanes 12-16) were positive; but lanes 7 and 11 were negative of the parasites from village chickens (*Gallus gallus domesticus*) in Gombe State, Nigeria.

Key: M: 100bp size marker; P: Positive Control, N: Negative Control; Amplicons are in the numbered lanes.

Avian haemosporidian parasites species composition and co-infection in village chickens (*gallus gallus domesticus*) in Gombe State, Nigeria

Sequencing of 20 positive amplicons from the PCR amplification revealed 13 species of haemosporidian parasites, comprising of 4 species of *Plasmodium*, 8 *Leucocytozoon* species and 1 *Haemoproteus* species namely, *Plasmodium* species, *Leucocytozoon schoutedeni*, and *Haemoproteus columbae* in the present study.

Composition and co-infection of *Plasmodium* species in village chickens (*Gallus gallus domesticus*) in Gombe State, Nigeria

Sequencing of the PCR amplicons revealed the occurrence of four different species of *Plasmodium* in village chickens in Gombe State. Both single and mixed infection of *Plasmodium* species and other species of haemosporidian parasites was detected in the infected village chickens. Double and triple infections of *Plasmodium* species + *Haemoproteus columbae* as well as *Plasmodium* species + *Haemoproteus columbae* + *Leucocytozoon schoutedeni* were recorded by the molecular technique in the present study.

Composition and co-infection of *Haemoproteus* species in village chickens (*Gallus gallus domesticus*) in Gombe State, Nigeria

Sequencing of the PCR amplicons also revealed *Haemoproteus columbae* as the only *Haemoproteus* species in village chickens in present study area. However, among the amplicons sequenced, both single and mixed infection of *Haemoproteus columbae* with other species of haemosporidian parasites was found. Double infections by *Haemoproteus columbae* + *Leucocytozoon schoutedeni* as well as *Haemoproteus columbae* + *Plasmodium* species were detected in village chickens in Gombe State. Triple infection by *Haemoproteus columbae* + *Leucocytozoon schoutedeni* + *Plasmodium* species were also recorded by the molecular technique in the present study.

Composition and co-infection of *Leucocytozoon* species in village chickens (*Gallus gallus domesticus*) in Gombe State, Nigeria

Sequencing of positive amplicons revealed eight genetically related *Leucocytozoon* species which comprises of the *Leucocytozoon schoutedeni* that were detected in village chickens in Gombe State in the present study. Both single and mixed infection of *Leucocytozoon schoutedeni* and other haemosporidian parasites were detected in village chickens. Mixed infections involving double-species infection by *Leucocytozoon schoutedeni* + *Haemoproteus columbae*, as well as *Leucocytozoon schoutedeni* + *Plasmodium* species were detected in village chickens in Gombe State. Triple-species involving *Leucocytozoon schout-*

edeni + *Haemoproteus columbae* + *Plasmodium* species were also detected in village chickens in Gombe State in the present study.

Phylogenetic analyses

A total of 425 nucleotides residues of the *Plasmodium*, *Haemoproteus* and *Leucocytozoon cyt-b* gene were used for the construction of Neighbour-Joining (NJ) phylogenetic trees using sequences obtained from this present study and those curated at GenBank. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, [27]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Figure 3). All positions containing gaps and missing data were eliminated.

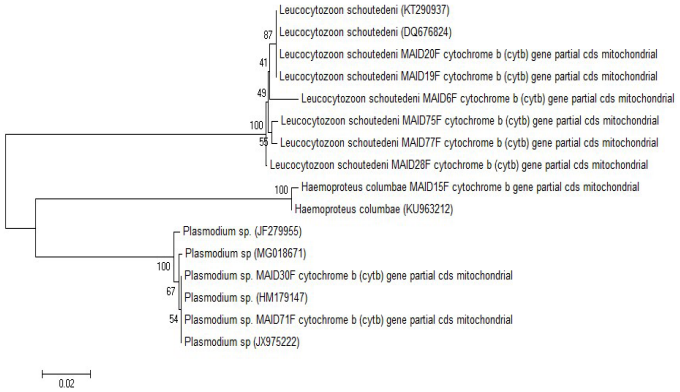

Figure 3: Neighbour-joining phylogenetic tree (1000 bootstrap replicates) of *Plasmodium* species, *Haemoproteus columbae* and *Leucocytozoon schoutedeni* based on nucleotide residues of the cytochrome b gene computed using the p-distance model (Evolutionary relationships of taxa). The *Plasmodium* species (MAID30F and MAID71F), *Haemoproteus columbae* (MAID15F) and *Leucocytozoon schoutedeni* (MAID20F, MAID19F, MAID28F, MAID6F, MAID75F and MAID77F) cytochrome b (*cytb*) gene partial cds mitichondrial isolated from *Gallus gallus domesticus* in this study are indicated with NCBI GenBank accession numbers.

Discussion

This present study represents the first attempt to molecularly detect avian haemosporidian parasites namely *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in the village chickens in Gombe State, Nigeria. Although genus of these haemosporidia have been previously detected from village chickens and other domesticated poultry species in Nigeria based on morphological identification of the organisms in stained blood films [19,29-32][this study has reconfirmed their presence using molecular techniques. The present study represents the first attempt to amplify the parasite's cytochrome b gene for the determination of the molecular prevalence in village chickens in Nigeria. The present use of molecular techniques also provides evidence and confirmed the occurrence of these avian haemosporidian parasites namely *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in single and mixed infection among village chickens in Gombe State, Nigeria.

In this study, the overall prevalence of avian haemosporidian parasites in village chickens using nested PCR is 76.8%. It was higher than 20.4% recorded by microscopy in the study area. Thus, confirming the fact that the nested PCR is a more sensitive diagnostic tool than microscopy and has the ability to detect very low infections which concurs with the findings of Waldenström et al. [5]. The result showed that the avian haemosporidian parasites in this study belonged to the unspecified *Plasmodium* species, *Haemoproteus columbae* and *Leucocytozoon schoutedeni* respectively. The two strains of undefined *Plasmodium* species found in village chickens in this study with both lineages (MAID30F, MAID71F) formed cluster with other unspecified *Plasmodium* species reference sequences from strains reported in Japan (JF279955) [33] in Uganda (MG018671) [34], in Iran (HM179147) [35] and in China (JX975222) [36] in free range chickens. The most common lineage, MAID30F, was identical to the JF279955 and MG018671 lineage found on the MalAvi database in GenBank, which has been previously identified in scavenging chickens in Japan and Uganda respectively by Yoshimura et al. [33] and Nakayima et al. [34]. The present study may likely have detected a novel genotype of *Plasmodium* species in village chickens in the study area. Based on phylogenetic analysis, it is likely that this genotype represents a new species of *Plasmodium*. However, it is still premature to be definite about the status of this novel genotype as species designation can only be afforded once detailed morphological characteristics have been obtained. Finding of novel haemosporidian genotypes are on the rise with advances in molecular detection techniques and gene sequencing. Over 2118 unique lineages of this group of parasites have been detected and curated at the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi/index.html) as reported by Bensch et al. [37]. In addition, it is evident that cryptic speciation occurs in the avian haemosporidians [38] and it is likely that new species will constantly be discovered as new avian hosts are examined in different parts of the globe. Previous analyses on the *cyt-b* gene among avian haemosporidians have concluded that even a single or a few base pair differences may reveal cryptic and novel species as suggested by earlier workers [24,39]. The distinct nature of the clustering away from other known species of *Plasmodium* suggests that the *Plasmodium* species identified in this work may represent a novel and undescribed species. Although this novel genotype must await morphological description, it is interesting to note that the phylogenetic analysis placed them within the cluster and range acceptable for species designation. Therefore, a genome-wide analysis involving comparism of various molecular markers and coding genes, in tandem with morphological descriptions, may be necessary before *Plasmodium* species isolated in this study could be conclusively designated as a novel *Plasmodium* species infecting village chickens in Gombe State. It has been established that mixed infections of *Plasmodium* and *Haemoproteus* and also mixed infection of various *Plasmodium* species occurs in avian host.

The findings of this study showed that *Haemoproteus* haplotypes clustered very tightly with *Haemoproteus columbae*. This is of interest because it is known that *Haemoproteus columbae* are highly specialized haemosporidian parasites, they usually infect various members of the Columbriformes such as the pigeons and doves pigeons and doves and would not infect chickens from a different order as reported by Valkiûnas and Iezhova [40]. However, when using molecular protocol to diagnose haemosporidian parasites it is necessary to have this in mind, since this technique can detect the presence of parasite DNA, but no confirm the infection. *Haemoproteus columbae* (MAID15F) lineage in village chickens isolated in the present study clustered with one distinct clade, consisting of lineage unique to feral Columbriformes previously reported by Gimba [41], in scavenging village chickens and jungle fowls in Malaysia (KU963212). This may represent a new host for *Haemoproteus*

columbae, but more importantly points to the possibility of cross species transmission, from feral pigeons to village chickens. This is possible because in most households in present study area, it is common to find village chickens being reared with pigeons. So, it is likely that hippoboscid flies which were infected by *Haemoproteus columbae* were also feeding on the chickens. During the feeding process, the sporozoites would be injected in the chickens, which served as template for parasite DNA amplification, but does not imply necessarily in parasite development. Unless blood or tissue stages of *Haemoproteus columbae* can be found in the infected chickens. This opens a new ground for the study of the epidemiology and transmission dynamics of this haemosporidian species. Although at present, *Haemoproteus columbae* was recovered from village chicken, it is not remote to suggest the possibility of transmission to other poultry species, especially those reared in households with pigeons reared in close proximity to other poultry species.

The result of the present study revealed various combinations of avian haemosporidian parasites in infected village chickens, the most common concurrent infection are mixed infections involving *Plasmodium* species and *Haemoproteus columbae*. In addition, triple infection with *Plasmodium*, *Haemoproteus* and *Leucocytozoon* was also encountered in the infected village chickens during PCR amplification. This is probably the first report of such infection in village chickens in the study area. The occurrence of mixed avian haemosporidian infection in village chickens could be due to the fact that these birds are usually reared under the free range management in environments which support a high numbers of vectors that could transmit the parasites, as reported by Idika et al. [42] and Ayeh- Kumi et al. [43]. This study also reports molecular detection of *Haemoproteus columbae* in village chickens in the study area, before this work, microscopy was the method of detection of the genus *Haemoproteus* in village chicken in Northeastern Nigeria as reported by Lawal et al. [19]. Although, previous reports by Karamba et al. [30] has demonstrated the presence of *Haemoproteus columbae* in domesticated pigeon using molecular typing, it is likely that further examination of avian hosts using molecular techniques, will shed more light on the occurrence and geographical distribution of *Haemoproteus* in village chickens and other domesticated poultry in Nigeria. As pointed out earlier the husbandry practice of village poultry farmers where different species are reared together (Figure 4) might be responsible for the detection of *Haemoproteus columbae* in village chickens. This practice may facilitate the transmission of *Haemoproteus columbae* in the presence and abundance of infected arthropod vectors.

The presence of *Leucocytozoon schoutedeni* in village chickens in the study area confirmed by sequencing and phylogenetic analysis is noteworthy, as this parasite has not been reported previously in village chickens in Nigeria. Although, this species is known to occur in some African countries with sporadic prevalence reported in America and Taiwan by Valkiûnas and Iezhova [40]. Microscopic techniques used previously may have missed identifying *Leucocytozoon schoutedeni* in stained blood films from village chickens. This also is further supported by the fact that the molecular tools are efficient in detecting specific species of avian haemosporidian parasites and that the *cyt-b* gene is a good marker for fishing out and characterizing genotypes. The *Leucocytozoon schoutedeni* lineages identified in this study appeared to exhibit varying levels of specificity. Six lineages from this study (MAID6F, MAID19F, MAID20F, MAID28F, MAID75F and MAID77F) clustered with those from other village chickens hosts which were identical to the lineage found on the MalAvi database in GenBank as *Leucocytozoon schoutedeni* lineages (DQ676824) from scavenging chickens in Uganda reported by Sehgal et al. [44] and KT290937 from village chickens and Jungle fowls in Malaysia reported by Gimba [41] where chicken leucocytozoonosis was endemic. As a vector-borne disease, the occurrence of chicken leucocytozoonosis in village chickens in this study area is strongly associated with the presence of its suitable arthropod vectors. Previous studies using microscopy have not been able to detect the presence of this species of haemosporidian parasite in domestic birds in Nigeria [32,19]. The identity of this species was confirmed by sequencing the amplified *cyt-b* gene and comparing with sequences of others deposited in the GenBank. Sequence alignments revealed 100% and 87% identities with published *Leucocytozoon schoutedeni* sequences [44]. This finding is of interest as *Leucocytozoon schoutedeni* is known to occur mainly on the African continent of which Sehgal et al. [44] reported the presence of *Leucocytozoon schoutedeni* from village chickens in Uganda and Cameroon with a prevalence of 18.3%. The detection of *Leucocytozoon schoutedeni* in this study may spark new interests on the evolution and zoogeographical distribution of this species of haemosporidian parasite in village chickens.

Figure 4: Village chickens in household mingling with other pigeons during feeding.

Conclusion and recommendation

Molecular detection was found to be more sensitive in the detection of avian haemosporidian parasites in healthy village chickens than the microscopy technique. Avian haemosporidian parasites infecting village chickens in Gombe State include unspecified *Plasmodium* species, *Haemoproteus columbae* and *Leucocytozoon schoutedeni*. Rearing village chickens with other poultry species may lead to cross transmission of the blood parasites to the chickens. It is therefore, recommended that village chickens farmers should be enlightened on the existence of avian haemosporidian parasites, so as to establish the appropriate control and prevention methods.

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