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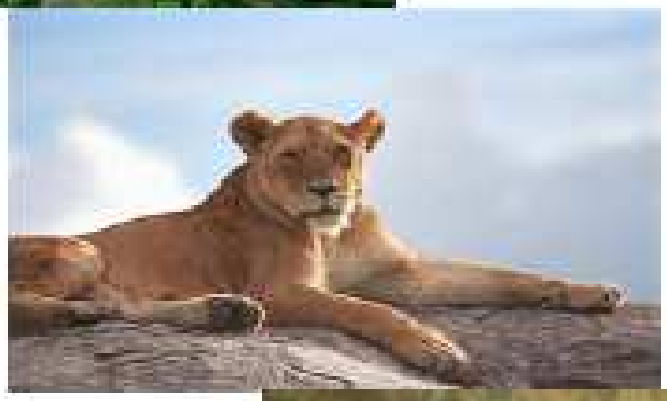

Volume 10 Number 2
September 2013

An International Peer Review Multidisciplinary Journal Publishing Original Research Involving the Use of Animals and Animal Products

ISSN: 5197-3115 **Website: www.zoo-unn.org**

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Prof. JE Eyo
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IN VIVO AND *IN VITRO* EVALUATION OF THE INHIBITORY EFFECT OF SOME MEDICINAL PLANT EXTRACTS ON HAEMOZOIN CONCENTRATION

[1]DIBUA, Uju M. Esther, [2]KALU, Adindu, [3]ATTAMA, Anthony A., [4]ESIMONE, Charles Okechukwu and [5]EYO, Joseph Effiong

[1,2]Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[3]Department of Pharmaceutics, University of Nigeria, Nsukka, Enugu State, Nigeria.
[4]Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.
[5]Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Corresponding Author: Dibua, U. M. E. Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria. **Email:** Oby2112@yahoo.co.uk **Phone:** +234 8037792951

ABSTRACT

The resistance of current drugs against malaria parasite is increasing, thus the need for evaluation of the haemozoin (HZ) concentration in malaria parasite as a novel strategy for malaria control. Haemozoin load in the blood of patients was measured after taking antimalarials or plants extracts. The tested plant extracts were established to reduce HZ concentration in vivo. Haemozoin was extracted from the blood samples of all the malaria positive patients studied by centrifugation and the concentration analyzed spectrophotometrically at 400 nm wavelength. Comparative anti-malaria activity of some conventional drugs: Maldox, Halfan, Artecxin, Amatem, Mefloquine (quinolines) and Malmed, the leaf and stem back extracts of Sarcocephalius latifolius and Alstonia boonei, containing potent pyhytochemicals including tannins, flavonoids, saponins, alkaloids, was evaluated to establish the most effective agent for haemozoin reduction and subsequently, malaria therapy. Each agent was administered to patients in each malaria episode, and the absorbance of haemozoin produced determined at 4000 nm wavelength. Packed cell volume (PCV) was estimated to establish the proportion of red blood cells before and after haemozoin production, using microhaematocrit reader. All the chemical antimalarial drugs used effected reduction in haemozoin concentration. However, Mefloquine (Quinolines) showed the highest activity with significant difference of 0.01 ($p<0.05$). The plant extracts similarly exerted significant reduction in the haemozoin concentration. Nevertheless, Alstonia boonei extract was the most effective in haemozoin reduction at 0.00 significant level ($p<0.05$). Of all the therapeutants (chemical and plant extracts) tested, Alstonia boonei stem back extract most significantly reduced haemozoin production ($p<0.05$), indicating its potential for use in novel anti-plasmodium and anti-malaria drug formulation.

Keywords: Haemozoin, Antimalarial agents, Haemoglobin degradation, *Plasmodium falciparum*

INTRODUCTION

Haemozoin is a disposal product formed from the digestion of haemoglobin present in the erythrocytes by blood-feeding malaria parasites by means of its lysate enzymes such as plasmepsins, falcipains and dipeptidylpeptidase. During malaria infection, the parasite ingests

abound 5mM of haemoglobin as a source of amino acids. As it grows, free toxic haeme, which is capable of generating oxygen radicals are released together with globulin (protein component) after haemoglobin catabolism in an acidic lysosome like organelle. The digestion of haemoglobin releases toxic monomeric α-haematin called haeme, which is capable of generating oxygen radicals (Lew *et al.,* 2003).

A haeme is a prosthetic group that consists of reactive iron which forms a unique iron-oxygen coordinate bond found in the centre of a heterocyclic porphyrin ring, as a result of this, it is also called iron protoporphyrin, or ferriprotoporphyrin (fePPTX), which is toxic to the cells of the malaria parasites *Plasmodium falciparum* (Pagola *et al.,* 2000). For the malaria parasite to survive it therefore detoxifies the toxic haeme by secreting haeme polymerase enzyme which converts the toxic haeme into non-toxic, insoluble and chemically-inert ß-haematin crystals called haemozoin, otherwise called the malaria pigment (Egan *et al.,* 2001). The formation of haemozoin is thus known as bio crystallization (Bennett *et al.,* 2004). The primary function of haemozoin is to prevent oxygen radical-mediated damage to the parasite. Another vital function of haemozoin is essentially the removal of the reactive iron, haeme out of solution in the oxygen rich acidic environment of the vacuolar compartment where haemoglobin degradation occurs (Sallusto, 2001).

Many chemically used drugs are thought to act by inhibiting the formation of haemozoin in the food vacuole (Buller, 2004). This prevents the detoxification of the haeme released in this compartment and kills the parasite. The best understood examples of such haematin bio crystallization inhibitors are quinolines drugs such as chloroquine and mefloquine. These drugs bind to both free haeme and haemozoin crystal and therefore block the addition of new haeme units into the growing crystals (Dedios, 2003). Haemozoin formation during malaria infection results in the reduction of packed cell volume (PCV) of the patients due to the consumption of red blood cells by the merozoites. This reduction gives rise to anaemia which is one of the symptoms of severe malaria (Clarke, 2002). Hence, evaluation of packed cell volume of patients following malaria treatment in each episode of malaria attaches is an essential step in assaying for the effectiveness of the target drug.

Haemozoin formation is an excellent anti-malarial drug target, since it is a process that is essential for the survival of the malaria parasites (Gligorijevic, 2006). The drug target haematin is host-derived and largely outside the genetic control of the parasites, which makes the development of drug resistance more difficult (Jani *et al.,* 2006). Therefore anti-malaria drugs to be used or produced for therapeutic purposes are those that act by inhibiting the formation of haemozoin in the food vacuole of the parasite. This prevents the detoxification of the haeme released in this compartment, and thus, killing of the parasite (Pisciotta, 2006).

Each day, malaria disease claims the lives of over 3000 people, 90% of whom are children in sub-Sahara Africa (WHO, 1998). Among the more than 120 *Plasmodium species* that infect reptiles, birds, and mammals, four protozoan species of *Plasmodium* infect humans and *P. falciparum* is more common, causing over 90% of the deaths (Ekpeyong and Eyo, 2006). In tropical geographic areas such severe *Plasmodium falciparum* malaria morbidity and mortality affect all ages from children to adults (Kazmi and Pandit, 2001). Malaria is endemic throughout the regions of Nigeria. The World Health Organization (WHO) estimated that malaria mortality rate for children under five in Nigeria is at 729 per 100,000. The Ministry of Health of Nigeria further indicated that malaria was responsible for one out of ten deaths in pregnant women and costs the Federal Government of Nigeria over one billion naira annually (PRCU, 2005).

Increased multi-drug resistance of malaria parasites especially *Plasmodium falciparum* to conventional orthodox antimalarials in Nigeria has necessitated the search for a cost effective toxic free and highly active compound from natural sources for the management of malaria and associated anaemia. Quinine extracted from the bark of the

cinchona tree, has been used as an antimalarial agent in Nigeria for several years.

There is increasing resistance of malaria parasites to chloroquine, the cheapest and commonly used drug for malaria in Nigeria. Chloroquine, a quinine derivative took the pride of place in malaria control for several years, and the fear of the emergence of chloroquine – resistant strains of malaria parasites has led to the use of Artemisin, an Indian product whose active ingredient is derived from *Artemisia annua.* However, little has been done to further develop Nigerian local plants with antimalarial activity or enhance the activities of traditional or folkloric medicine in the advancement of the search for malaria cure.

Traditional medicine has nevertheless remained the most affordable and easily accessible source of treatment in the primary healthcare system of resource poor communities; the local therapy therefore, is the only means of medical treatment in such communities. The use of medicinal plants in the treatment of diseases has generated renewed interest in recent times, as herbal preparations are increasingly being used in both human and animal healthcare systems. The study observed that in spite of the advancement in modern medicine and healthcare programmes, many people in developing countries including Nigeria still rely on traditional healing practices and medicinal plants for their daily healthcare needs. Most of the antimalarial drugs currently in use were not developed on the basis of rationally selected targets, but by investigation of traditional medicinal plants (quinine and artemisinin), synthesis of analogues (CQ, mefloquine, primaquine, atovaquone), chemical modification of an active natural product (arteether, artemether, artesunate), or by assaying drugs that were used against other infectious pathogens (antifolates, antibiotics) (Fidock *et al.,* 2004). The herbal remedies are often prepared by pounding either the fresh or dried parts of the plants followed by either soaking or boiling in water, and the infusions or decoctions administered by drenching.

Sarcocephalius latifolius (African peach), of the family Rubiaceae, is a multi-stemmed tree or shrub up to 12 m. Reports of its medicinal value include its effectiveness in the treatment and management of malaria, constipation, dysmenorrhoea, abscesses, vomiting and threatened abortions stomach disorder, cough, fever and jaundices. The aqueous root bark extracts of *S. latifolius* demonstrated protection against liver toxicity induced by CCl_4 in a dose dependent manner and this was linked to the phytochemical constituents. The terminal event in the attack on the liver by CCl_4 was the production of highly reactive radicals leading to lipid peroxidation and inhibition of calcium pump of the microsome giving rise to liver lesions (Gamzi *et al.,* 1999). Several secondary metabolites have been shown to have wide ranges of antimicrobial activities. Similar, *S. latifolius,* was observed as one of the sixty-four extracts assayed from twenty-one plants used in the Malian traditional medicine that were found to be significantly active against the intracellular forms of Leishmania major. It was further showed to exhibit antiplasmodial activity against *P. falciparum* (Abreu and Pereira, 2001).

Alstonia boonei de Wild (Apocynaceae) is a large deciduous tree up to 45 m tall, which consists of 50 species widely distributed in Africa and beyond. It is called, Egbu in Igbo and is widely used by the natives for the treatment of various ailments. The stem bark of *A. boonei* has anti-inflammatory, analgesic and antipyretic activities and is commonly used against malaria (Olajide *et al.,* 2000). An infusion of the bark is used as antivenom for snake bites. It is also used in treating painful menstruation and rheumatic conditions (Azuzu and Anaga, 1991). Other documented scientific reports validating the medicinal values of *A. boonei,* include, the presence of phytochemicals such as alkaloids which possess analgesic, antispasmodic, antiplasmodial and bactericidal effects; flavonoids, potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity as well as lower the risk of heart diseases. Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing. Tannins hasten the healing of wounds and inflamed

mucous membrane. Cardiac steroids are widely used in the treatment of congestive heart failure. They help in increasing the force of contraction of the heart (Okwu, 2004).

The daily increasing high rate of mortality and morbidity in the sub-Sahara Africa and especially in Nigeria as a result of multi-drug resistant malaria parasites has reached an alarming proportion. This has therefore led to a search for an alternative therapeutic regimen for effective management of malaria parasitemia especially in the malarious areas of the south-east Nigeria, the site of this research, which aims at extracting haemozoin from the blood samples containing malaria parasites, screening for, and comparing both conventional antimalarial drugs and plant extracts for antimalarial activity.

MATERIALS AND METHODS

Study Location: The study was carried out for a period of 14 months at the Bishop Shanahan Hospital (BSH), Nsukka, Enugu State, Nigeria. This hospital serves the health needs of the local community and beyond and is basically a point of healthcare for the poor and underprivileged. People from nearby rural and peri-rural communities including the border towns of Kogi and Benue states, especially those who cannot afford high hospital bills of the private and teaching hospitals in the states troop in here for attention and medicare. One of the major concerns here for which routine tests and treatment is sought is malaria, a serious scourge in this area beset with malnutrition, poverty and attendant anaemia, unhygienic conditions and drug abuse.

Ethical Issues: The approval to undertake the study was granted by University of Nigeria ethical committee. Anther approval was obtained from Bishop Shanahan Hospital Management Board to under take the study. Written consents of willingness to participate in the study as subject were obtained from 48 participants listed for the study. All listed subject were symptomatic malarial cases managed by BSH. All ethical issues involving the identity, compensation and management of the subjects followed the approved guidelines.

Plant Collection: An ethnobotanical survey of Nigerian medicinal plants commonly used in folkloric medicine for the management of malaria carried out among traditional practitioners and herbalist who both treat and sell dried and fresh plant materials at Nsukka Local Government Area, Enugu State, reveal that *S. latifolius* and *A. boonei* were commonly used singly or in combination for the treatment of malaria in the locality. This informed the selection of these plants for the study. Both plant were collected from the Botanical Garden, University of Nigeria, Nsukka and identify to species level by the curator. Voucher specimens SL-2134 and Ab-2135 were stored in the herbarium of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Plant preparation and Extraction: The fresh leaves of *S. latifolius* and fresh stem back of *A. boonei* were used for the investigation. Five hundred grammes (500g) of *S. latifolius* leaves were washed and boiled in 450 cm^3 of water for 45 minutes. Three hundred grammes (300g) of the stem bark of *A. boonei* was thoroughly washed and pulverized in a mechanical home blender and soaked in 150 cm^3 of water for 6 days as practised by the traditional healers. The resulting concoctions were separately filtered and store in sealed plastic 1 litre bottles pending administration. All extracts were freshly administered.

Phytochemical Screening: Aqueous extracts of the tested plants were subjected to phytochemical analysis for the qualitative determination of phytochemical constituents using standard procedures (Sofowora, 1983).

Determination of the Effect of Selected Conventional Antimalarial Drugs and Plant Extracts on Haemozoin Concentration: The effect of conventional chemical antimalarial agents, Maldox, Halfan, Mefloquine, Amatem, Arteccin, Malmed was evaluated on 48 individuals by administration of the drugs to

each malarial patient during each malaria episode according to the prescription of the physicians in attendance. Patients were strictly followed up during the 14 months study and monitored for malaria symptoms during which prescribed doses of the different drugs were given at different malaria episodes which varied among patients: some came down with malaria faster than others; some within 2 weeks and others within 1 – 4 months intervals depending on patients' resistance to malaria attack, exposure to mosquito bites and effectiveness of each test drug on individual patient. Criteria used for drug administration included the classical malaria symptoms: fever, malaise, pains on the joints, severe weakness and in minor cases vomiting; marked reduction in PCV and presence of haemozoin in patients' blood samples. However, effort was made to administer all test drugs to each patient at different malaria episodes and the PCV and haemozoin concentration estimated simultaneously at 24 hours interval for 96 hour post administration. To ensure compliance, patients were not allowed to take drugs home but encouraged to take them in the presence of the physician and the investigators.

Administration of Plant Extract Concoctions: Concoctions of the test antimalarial plant extracts, were administered to the same malarial patients in subsequent episode of malaria (with 4 weeks interval after administration of chemotherapeutants), following examination of their blood smears for malaria parasites and evaluation of haemozoin level. Consequently, a cup (50 cm^3) of *S. latifolius* concoction was administered orally thrice daily (morning, midday and night) to patients for 5 days with concurrent estimation of their PCV and haemozoin concentration. Furthermore, patients were each monitored for a period of 2 months after which their blood samples were collected and screened for malaria parasites. All those who came down with malaria based on established criteria were subjected to the next regimen of *A. boonei* concoction; consequently, 50 cm^3 of the concoction was given by oral administration to patients twice daily (morning and night) for 3 days. Haemozoin concentration and patients' PCV were estimated following each administration at 24 hourly intervals for 96 hours. The malarial patients without the antimalarial agents served as a control experiment for both the PCV and haemozoin concentration determination.

Collection of Blood Samples: Approximately 2ml of venous blood samples were collected from malarial symptomatic patients at the Hospital and dispensed into ethyl diamine-tetra acetic acid (EDTA) vacutainer tubes.

Screening for Malaria Parasites

The test was carried out using standard Giemsa staining techniques, by thin and thick smears. The working solution was prepared by diluting 1 ml of standard Giemsa stain with 19 ml of phosphate buffer (pH 7.2). The solution was prepared prior to use (John and Petri, 2006). The test was carried out by examination of thick and thin Giemsa stained blood smears, prepared in duplicates.

Preparation of thin film: Thin films (smears) of blood samples were made on clean, greaseless slides and fixed by immersing in absolute ethyl alcohol for 30 seconds. The films were allowed to air dry, immersed in Giemsa working solution for 30 minutes and then further immersed in phosphate buffer for 10 seconds, after which they were air-dried in a vertical position. After drying, the slides were then examined at x 40 objective lens before re-examination in oil immersion (x 100 objective lens). The percent of infected RBCs was determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs. A minimum of 500 RBCs was counted. Percent infected RBCs = Number of infected RBCs ÷ Total number of RBCs counted × 100.

Preparation of thick films: Duplicate slides of prepared thick blood films were allowed to air dry completely for 1 hour or more depending on the thickness of the film. The films were immersed in phosphate buffer solution for 30

seconds and air dried in vertical position. The microscopic examination was done as described above. The number of parasites/µl of blood was determined by enumerating the number of parasites in relation to the standard number of WBCs/µl (8000). Number of parasites per µl of blood = Number of Parasites × 8000 ÷ Number of WBCs counted.

Qualitative evaluation of parasitemia: A modified qualitative three point scale of classification from CDC (2003) five point scale classification of parasitemia was adopted thus: + (1) 10 – 10,000 parasites/µl of blood = mild parasitemia, ++ (2) 10,001 – 100,000 parasites/µl of blood = severe parasitemia and +++ (3) > 100,000 parasites/µl of blood = very severe parasitemia.

Determination of Packed Cell Volume (PCV): To determine the level of haemoglobin degradation before and following administration of drug, packed cell volume (PCV), which is the amount or proportion of red blood cells present in a given sample of blood was assayed using the Microhaematocrit Reader. Approximately 5 ml of blood samples were collected from patients using sterile syringes and needles and dispensed into EDTA vacutainer tubes (Rubio *et al.,* 2001). An aliquot of the blood samples was pipetted into capillary tubes by capillary movement and centrifuged for 5 minutes using haematocrit centrifuge after which the capillary tubes containing the sediment samples were placed in a Microhaematocrit Reader and the packed cell volumes (PCV) read.

Haemozoin Extraction: A 2ml EDTA blood samples were centrifuged for 5 minutes using bucket centrifuge (Model 80-2). The supernatant was discarded and the pellets suspended in normal saline (NaCl) and further centrifuged for 5 minutes and the supernatant discarded. About 0.5ml of phosphate solution, pH 7.6 was added to each tube and vigorously shaken mechanically for 2 seconds to haemolyse the erythrocytes. The tubes were then kept on ice for 10 minutes to avoid excess haemolysis and then centrifuged for 5 minutes before discarding the supernatant. Approximately 1 ml of Tris buffered solution of pH 7.2 was dispensed into the pellets in the tubes, centrifuged for 10 minutes, and the supernatant discarded. The insoluble pellets were re-suspended in 0.5ml of 2.5% Sodium dodecyl sulphate solution (SDS), buffer with Tris buffer solution, pH 7.8 and kept at room temperature for 1 hour before centrifuging for 10 minutes. The supernatant was again discarded and the pellets once more re-suspended in 0.5ml of 2.5% Sodium dodecyl sulphate (SDS) solution buffered with Tris buffer solution pH 7.8 and kept at room temperature for 1 hour. The suspension was then centrifuged for 10 minutes, and the supernatant discarded before harvesting the SDS insoluble pellets (haemozoin) as previously described (Orji, 2001).

Determination of Haemozoin Concentration by Spectrophotometry: The weight of extracted haemozoin was determined using the Mettler weighing balance and the various masses obtained recorded. The concentration of haemozoin was calculated by completely dissolving known masses of haemozoin (in mg/ml) in 0.5ml of diluted sodium hydroxide, and the solution of haemozoin analyzed spectrophotometrically using Spectrophotometer S23A, at 400nm wavelength (Bohle *et al.,* 2005).

Statistical Analysis: Data were analysed by one-way or two way analysis of variance where appropriate at 95% Confidence intervals between means.

RESULTS

Phytochemical composition of Tested Plant Extracts: Analysis of the phytochemical constituents of the test plant extracts *S. latifolius* and *A. boonei* indicated the presence of alkaloids, saponins, tannins, steroids, terpennoids, flavonoids, cardiac glycosides with steroidal ring. However, there was absence of anthroquinones in both plants (Table 1). Highly active compounds including alkaloids, tannins, and flavonoids were more in abundance in *A. boonei* than in *S. latifolia.*

Haemozoin Extraction: A brown crystalline pigment, haemozoin in form of insoluble crystals in the sodium dodecyl sulphate (SDS) solution used for the extraction was obtained from all the malaria positive blood samples at different levels of malaria parasitemia.

Table 1: Phytochemical constituents of *S. latifolia* and *A. boonei* aqueous extracts

Phytochemicals	*S. latifolia*	*A. boonei*
Alkaloids	+	++
Saponins	+	+
Tannins	+	+++
Anthroquinones	-	-
Steroids	+	+
Terpenoids	+	+
Flavonoids	+	++
Cardiac glycosides with steroidal ring	+	++

Legend: *+ = moderate, ++ = abundant, - = absent*

Parasitemia, PCV and Haemozoin Concentration: There was significant reduction in the number of patients corresponding with increased parasitemia. Patients with mild parasitemia (19) being significantly higher than those with very severe parasitemia (13) (Table 2). Negligible and insignificant increase in the PVC of patients corresponding to increasing levels of parasitemia was observed. Proportionate increase in haemozoin concentration with increase in the level of malaria parasitemia among subjects was observed (Table 2).

Effect of Administered Conventional Antimalarial Drugs on Haemozoin Concentration: All the antimalarial medications administered to the malarial patients showed a decrease in haemozoin concentration at 400nm wavelength at 24 hourly intervals with simultaneous increase in patients' PCV values. The differences were statistically significant at 0.00 ($p<0.05$)**.** However**,** mefloquine belonging to quinoline group exerted the highest decrease on the haemozoin concentration. The difference was statistically significant ($p<0.05$). Nonetheless, Maldox a combination of sulfadoxine and pyrimethamine showed the lowest reduction in haemozoin concentration at significant level of 0.02 ($p<0.05$) (Figures 1 and 2).

Extracts of Administered *Sarcocephalius latifolius* Leaf and *Astonia boonei* Stem Bark on the Haemozoin Concentration in the Malaria Patients at 24 hour Intervals: The two anti-malarial test plant extracts, *S. latifolius* leaf extract and *A. boonei* stem back extract exerted appreciable decrease on the haemozoin concentration at 24 hour intervals. The differences were statistically significant ($p<0.05$). However, *A. boonei* extracts caused the highest decrease in haemozoin concentration with a significant increase in the PCV values ($p<0.05$) (Figures 3 and 4).

Comparative effect of Mefloquine and Alstonia boonei on Haemozoin Concentration in the Malaria Patients: Comparison of the most effective medicine, mefloquine and the plant extracts, in haemozoin reduction is presented in Figure 5. *A. boonei* nevertheless exerted much higher decrease in haemozoin concentration than the medicine, mefloquine ($p<0.05$).

DISCUSSION

Haemozoin formation is reckoned a vital step in the survival of *Plasmodium falciparum*, the malaria parasite. Haemozoin, variously identified as a black-brown by-product of haemoglobin degradation by the process of biocrystallization is a unique diagnostic tool in the malaria parasite identification and thus a laboratory marker of malaria infection. Its formation has therefore become a subject of intensive scientific research as interest in the search for efficient therapeutic regimen for malaria broadens. Due to its pivotal role in the survival of the malaria parasite, haemozoin formation by *P. falciparum* is therefore considered an essential target in the malaria drug discovery, and remains the ultimate goal of novel malaria research.

In spite of the stringent global efforts to fight and eliminate malaria, it is still recorded as one of the greatest human killers, causing almost one million deaths per year (mainly small children in Africa) and 300-400 million infections annually (WHO, 2010).

Table 2: Parasitemia, packed cell volume (PCV) and haemozoin concentration of malaria patients in Bishop Shanahan Hospital, Nsukka

Parameters	Number of patients	Blood PCV	Haemozoin concentration mg/ml	Absorbance
Parasitemia		**+ (Mild)**		
Sub total	19	7.17	0.79	12.57
Mean	-	0.38 ± 0.01	0.04 ± 0.01	0.66 ± 0.03
Parasitemia		**++ (Severe)**		
Sub total	16	6.03	1.31	12.33
Mean	-	0.38 ± 0.01	0.08 ± 0.004	0.77 ± 0.01
Parasitemia		**+++ (Very severe)**		
Sub total	13	5.05	1.9	10.5
Mean	-	0.39 ± 0.02	0.15 ± 0.01	0.81 ± 0.01

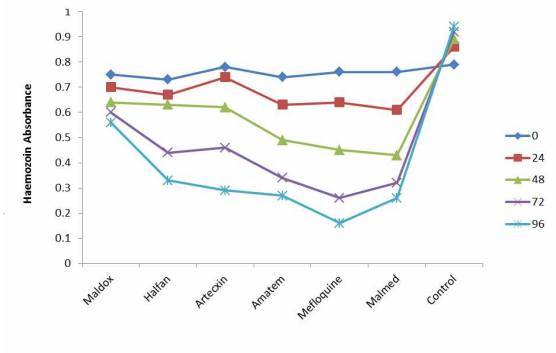

Figure 1: Effects of test anti-malarials on haemozoin concentration of patients in Bishop Shanahan Hospital, Nsukka

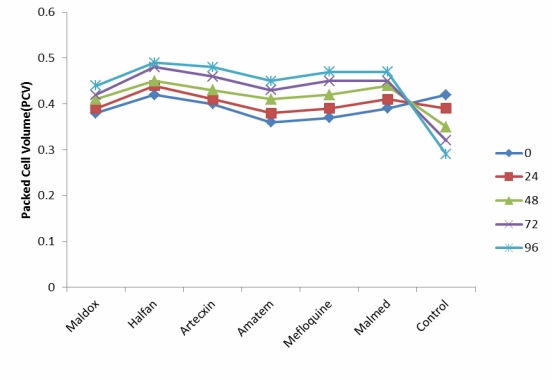

Figure 2: Effects of test anti-malarials on PCV of patients in Bishop Shanahan Hospital, Nsukka

Several approaches have been envisaged by Nigerian government in their roll back malaria strategy, and these include the mosquito larval control (vector control) strategies, indoor residual spraying (IRS), intermittent preventive treatment in infants (IPTi) and using drugs to protect infants. Currently, the cornerstone of malaria control across the globe and especially in Nigeria is the exploitation of effective and inexpensive drugs particularly those from plant sources. Hence, inhibition of haemozoin biocrystallization in the *Plasmodium* using natural products of plant origin which compares favourably with currently used antimalarial drugs (Mepacrine, Fansidar, Maloprim, Halofantrin *(Halfan),* and Artemisinins) might be the answer to the age-old quest for malaria eradication and the roll back malaria (RBM) objective in Nigeria.

This study therefore attempted the extraction of haemozoin from the blood samples of malaria patients, as well as elucidation of the antimalarial activity and/or comparative inhibitory effect of some indigenous plant extracts on haemozoin formation in *Plasmodium falciparum,* the malaria parasite. The evidence presented in the study, and especially the clinical trial data, clearly validates the claim outlined here, on the haemozoin extraction as well as the inhibitory effect of the elucidated chemical antimalarials and some esteemed natural plant extracts: *S. latifolia* and *A. boonei* on haemozoin formation in malaria parasites. Malarial pigment has long fascinated malariologists, with Meckel first having observed pigment in the blood and spleen during an autopsy of an insane person, though he failed to make the connection to malaria (Meckel, 1847). In his description of blood film examinations from 70 patients, William Osler later related the pigment to malaria and instituted routine blood smear analyses to diagnose malaria in febrile

patients at Johns Hopkins Hospital (Osler, 1889). Haemozoin formation in malaria parasite is therefore observed as a unique diagnostic tool and/or surrogate marker of malaria infection. Results of this study finds credence in the reports on haemozoin extraction and is further supported by the reports which identified haemozoin as a brown pigment in malaria infected blood samples (Orji, 2001; Urban and Roberts, 2003).

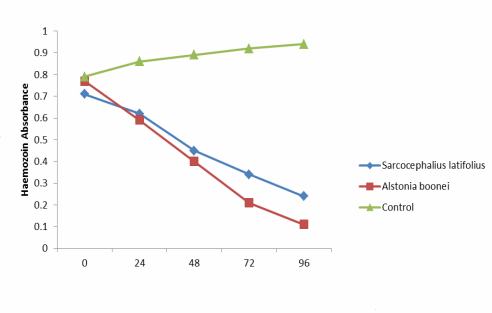

Figure 3: Effects of *Sarcocephalius latifolius* leaf extracts and *Alstonia boonei* stem bark extracts on haemozoin concentration of patients in Bishop Shanahan Hospital, Nsukka

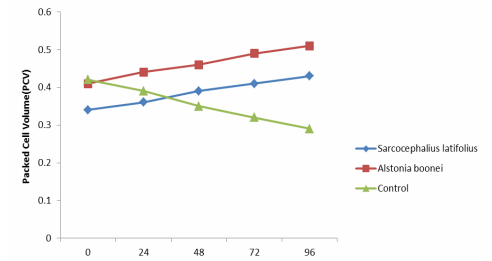

Figure 4: Effects of *Sarcocephalius latifolius* leaf extracts and *Alstonia boonei* stem bark extracts on PCV of patients in Bishop Shanahan Hospital, Nsukka

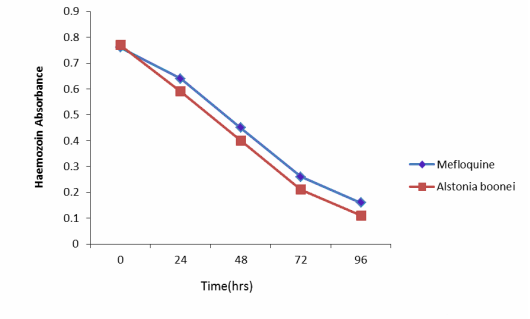

Figure 5: Comparison of effect of mefloquine and *Alstonia boonei* on haemozoin concentration of patients in Bishop Shanahan Hospital, Nsukka

Reduction in packed cell volume of malaria infected people was observed in the study, and this could be attributed to the consumption of red blood cells by the actively feeding merozoites in malaria infected persons during haemozoin formation. The dogma concerning haemoglobin degradation is that the parasite is reliant on the amino acids in haemoglobin for incorporation into its own amino acid pool. It was further indicated that the most conclusive evidence of this reliance on haemoglobin degradation is that the specific inhibition of haemoglobinases is fatal for the parasite (Francis *et al.,* 1997). Recently, Ginsburg reported that the parasite invades an erythrocyte containing 300 mg/ml protein, 95% of which is haemoglobin at a concentration of 5mM; this correlates to 30 pg (or 0.5 mol) of haemoglobin per cell. The parasite ingests more than two-thirds of the haemoglobin (20 pg), yet only uses 3 pg for its own amino acids the resultant effect of this therefore is acute anaemia in infected individual.

A direct relationship between haemozoin production and haemoglobin depletion or reduction in packed cell volume established in this study is thus confirmed by similar findings in which reduction of packed cell volume (PCV) of malaria infected individuals, with resultant anaemia, a symptom of severe malaria was reported (Clark, 2002). Orji further opined that haemoglobin catabolism is switched on during the ring stage in the food vacuole of *P. falciparum* where Fe^{3}-porphyrin, a by-product of haemoglobin digestion, is incorporated into β-haematin, the principal pigment of haemozoin (Orji *et al.,* 1994). In addition, haemozoin was identified as a by-product of haemoglobin degradation during malaria infection, and attributed haemozoin production to haemoglobin digestion by *P. falciparum* (Liu *et al.,* 2006). This study therefore provides clinical evidence of the association of haemozoin formation with anaemia, the major health impact of malaria, and the view is further supported by the findings on the impact of haemozoin production on malaria and related anaemia, as well as the observation that haemozoin was able to inhibit differentiation and maturation of human monocyte derived

dendritic cells in patients with malaria during malaria outbreak in the United States of America in 1990 (Palucka *et al.,* 1998).

Malaria parasites detoxify haeme by forming crystalline haemozoin. Many clinically-used drugs are thought to act by inhibiting the formation of haemozoin in the parasite food vacuole; this prevents the detoxification of the haeme released thereby causing parasite death due to haeme build up. The formation of haemozoin by *P. Falciparum* is therefore an essential process in the survival of malaria parasite. This assumption pre-supposes that an efficient control strategy against the parasite must necessarily be directed towards inhibition of the process. Many clinically-used drugs have been reported to exert inhibitory effect on haemozoin formation by malaria parasites, thereby preventing the detoxification of the haeme released in the food vacuole which is otherwise toxic and lethal to the parasite. Therapeutic doses of quinoline drugs such as chloroquine and mefloquine were reported to inhibit haemozoin production in human erythrocytes, and the inhibition caused monomeric haeme to accumulate and kill the parasites (Jani et al., 2006; Pisciotta, 2006). This view is further buttressed by the reports of the present study which highlighted the inhibitory effect of the test chemical therapeutants: Maldox (sulfadoxine and pyrimethamine), Halfan (halofantrine), Malmed (amodiaquine and artesunate), Mefloquine (quinolines), Artecxin (dihydroarteminisin) and Amatem (artemether and lumefantrine). The effectiveness of Mefloquine, a quinoline drug in exerting inhibition on haemozoin formation observed in this report is attributed to the activity of its functional group, which is reckoned as the best haematin bio-crystallization inhibitor, and therefore effective in the management of chloroquine resistant malaria (Wood and Eaton, 1993). The study similarly indicated the remarkable inhibitory activity of Amatem, a derivative of arteminisin, also known to inhibit haemozoin formation. Artecxin of the group dihydroarteminisin and piperaquine phosphate and trimethoprin, were also observed to significantly reduce haemozoin concentration in malaria infected persons. The arteminisin drugs produced from the plants arteminisia have previously been shown to significantly reduce the malaria pigment (haemozoin) produced by malaria parasite (Orji *et al.,* 1994). The monoalkylated (HA) and dialkylated (HAA) haeme derivatives of artemisinin have been implicated in binding to PfHRP II (*P. falciparum* histidine-rich protein II), and thereby inhibiting haemozoin formation. Artemisinins therefore owe their antimalarial activity to the presence of an endoperoxide bridge, since deoxyartemisinin, which lacks the bridge, is devoid of antimalarial activity. Several studies have also suggested that the haeme-promoted cleavage of the peroxide in artemisinin, leading to the formation of C-radicals which alkylate some proteins of the malaria parasite, also contributes to its antimalarial action. In addition, specific reactions of artemisinin with TCTP (translationally controlled tumour protein), inhibition of the SERCA (sarcoplasmic /endoplasmic-reticulum Ca^{2+}-ATPase) orthologue (PfATP6) of *P. falciparum* and inhibition of *P. falciparum* cysteine proteases have also been suggested to contribute to the drug's activity (Eckstein-Ludwig *et al.,* 2003). The observed inhibitory activity of these drugs is ultimately linked to their ability to bind to both free haeme and haemozoin crystals, and thereby blocking the addition of new haeme units onto the growing crystals (haeme polymerization). The resultant effect therefore was a remarkable increase in the average packed cell volume (PCV) of the patient's blood.

The increasing resistance of *Plasmodium falciparum* to quinoline-based drugs and the absence of an effective vaccine against malaria have made the management of malaria in endemic areas of Nigeria highly problematic, and therefore the development of newer and more potent pharmacophores from natural products of plant origin has become crucial to the control and management of malaria. Consequently, novel drug targets and pathways controlling the unique life cycle of the parasite, such as haemozoin formation was studied and the inhibitory effect of *S. latifolia* and *A. boonei* on the product formation elucidated. Comparative evaluation of the activity of both

test chemical antimalarials and plant extracts on haemozoin reduction indicated the efficacy of the test plant extracts, notably *A. boonei* over the chemical therapeutants in the reduction of haemozoin formation. This was observed as a significant increase in the PCV values of all patients administered the concoction, thus highlighting their inestimable value in folkloric medicine as highly efficient and cost effective anti-malarial agents.

Several scientific reports lend credence to the antimalarial efficacy of *S. Latifolia*. Alkaloids present in *S. Latifolia* are known to have numerous beneficial pharmacological effects. *S. latifolius* was observed to display antiplasmodial activity against *P. falciparum.* Phytochemical investigation of the root extract of *S. latifolius* led to the isolation of the new indole alkaloids -methylstrictosamide aglycone and ethylstrictosamide aglycone, together with strictosamide, angustine, nauclefine, angustidine, angustoline, ethylangustoline, naucleidinal, -epi-naucleidinal, quinovic acid-3 beta-O-beta-D-fucopyranoside, quinovic acid-3 beta-O-alpha-L-rhamnopyranoside, scopoletin, and beta-sitosterol. Strictosamide displayed moderate antiplasmodial activity against *P. falciparum* (Guede *et al.,* 2005). Furthermore, *S. latifolius* was reported to have significant antimalarial properties. These findings corroborates with the results of this study on the efficacy of the plant resulting from its phytochemical constituents, chiefly alkaloids, tannins and flavonoids.

The various species of Astonia are highly rich in alkaloids, steroids and triterpenoids, and phenolic compounds which contribute to their toxicity. The antimalarial efficacy of the root back extract of *A. boonei* was previously reported (Sofowora, 1983). However, their relationship to haemozoin reduction was not studied Result of this study, establishing the remarkable efficacy of *A. boonei* in haemozoin reduction and consequently its unique antimalarial activity is in consonance with similar on the anti-malaria efficacy the plant. The high activity of *A. boonei* on malaria parasite which has been well elucidated in this study and also cited in scientific literature could be alluded to its chemical constituents including alkaloids, terpenes and steroids; over 90% of the isolated chemical constituents are alkaloids (Kweifo-Okai *et al.,* 1995). *In vitro* antiplasmodial activity of the alkaloids against both drug sensitive and resistant strains of *P. falciparum* and *P. berghei* in mice was well reported. Though study on the specific class of alkaloid implicated was not done, however, all the known classes have been reputed to exert good activity on malaria parasites (Kirandeep *et al.,* 2009). This study observed that, *Alstonia boonei* was the most efficacious inhibitor, exerting a significant decrease in the haemozoin concentration from the initial average absorbance of 0.77 to 0.11, with a correspondingly high increase in the packed cell volume (PCV), making it a choice antimalarial agent among all the therapeutants (chemical and plant extracts) tested, thereby underscoring its potential for use in anti-malaria drug formulation as previously demonstrated (Majekodunmia *et al.,* 2009).

Conclusion: The emergence of multi-drug resistant strains of the malaria parasites especially in the sub-Saharan Africa is reducing the therapeutic arsenal for the treatment of malaria at a rate that is barely balanced by the development of novel effective drugs. Haemozoin formation was in this study established as an attractive target for developing antimalarial drugs; its reduction by antimalarial drugs particularly, the plant extracts *A. boonei* was similarly ascertained. The inhibitory activity of test anti-malaria agents, chemicals and plants on haemozoin formation, was therefore demonstrated: all the therapeutants tested exhibited varying degrees of activity in terms of reduction in haemozoin concentration, with Mefloquine showing the highest decrease among the chemical agents, while *Alstonia boonei* stem back extract, was the most efficacious among all the test therapeutants. The potential use of *Alstonia boonei* stem back extract as an effective antimalarial is therefore evident from the study in consonance with its use in folkloric medicine, therefore should be further exploited the search for a novel natural source of therapeutant in the antimalarial drug discovery.

ACKNOWLEDGEMENTS

The authors appreciate the technical assistance given by the staff and patients of the hospital from where the samples were collected. The authors declare no competing interests.

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AN EXPOSITORY ON MALARIA DIAGNOSTIC TECHNIQUES IN THE TROPICS

AMALU, Christopher Tochukwu and OKAFOR, Fabian Chukwuemenam
Animal Parasitology and Public Health Research Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Corresponding Author: Amalu**,** C. T., Animal Parasitology and Public Health Research Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Enugu State, Nigeria. **Email:** cityamalu@yahoo.com **Phone:** +234 803 7240369,

ABSTRACT

The need for new perspectives for malaria diagnostic techniques in the tropics is on the increase due to rising prevalence rate of malaria mortality and morbidity. The microscopic examination of venous thin and thick blood films is the current "gold standard" for routine laboratory diagnosis of malaria. Other available methods include:- Fluorescence microscopy, which has improved the sensitivity, but not the specificity of diagnosis, Polymerase Chain Reaction (PCR) diagnosis, which is sensitive for all four species of human Plasmodium spp. and can be expected to exceed the sensitivity of microscopy; but poses time and technical problems, Immunochromatographic dipsticks that offer the possibility of more rapid, non-microscopic methods for malaria diagnosis, thereby saving on training, time and Self-diagnosis. Kits for self-diagnosis by travellers in remote areas has been developed.

Keywords: Microscopy, Immunochromatographic dipsticks**,** *Plasmodium* spp.**,** Polymerase chain reaction, Diagnostic techniques, Gold standard, Mortality, Morbidity

INTRODUCTION

Malaria threatens more than 40% of the world's population. Every year, there are 300 – 500 million cases of malaria and it kills 1.5 – 2.7 million people worldwide, with pregnant women and children being more vulnerable (WHO, 2000a). Malaria does not only affect the developing world. Isolated, locally transmitted cases still occur in North America, and a growing number of western travellers are developing malaria. There are 12, 000 malaria cases per year in Western Europe (WHO, 2000a). Deaths linked to malaria in Africa are on the increase due to changes in climate, movement of populations arising from political instability and civil strife, resistance of malaria to common and inexpensive medicines, resistance of mosquitoes to insecticides, and limitations in national services (WHO, 2000b).

Malaria keeps Africa's people poor. It prevents adults from working and children from attending school. Each year a family spends several months' earnings on malaria treatment and prevention. Malaria turns pregnancy, a normal life process into a nightmare, in which the mother may die or her baby born too small to survive (WHO, 2000b).

South and Central America, South and East Asia, the Caribbean, Oceania, Central Asia and Middle East are all affected by malaria, but Africa bears the heaviest malaria burden. Ninety percent (90%) of all malaria deaths occur in the tropical Africa, and in some parts of the continents, sickness and deaths due to malaria have been increasing steadily due to resurgence of resistant *Plasmodium* species and *Anopheles* species vectors. The burden of malaria in Africa is particularly dangerous, causing 900,000 deaths, and every 30 seconds an African child

dies. Everyday, at least 1000 Africans die of malaria and at least 20% of all are children under five. The morbidity is high with about 500,000 African children suffering from cerebral malaria and high incidence of mortality during pregnancy and neonatal deaths (within the first 28 days of birth), about 8 – 14% low birth weight and 3 – 8% of all infant deaths in endemic areas of Africa (Akukwe, 2004).

The disease is transmitted in human by a protozoan parasite of genus *Plasmodium,* which include *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*; in order of decreasing virulence.

Malaria can be suspected based on a patient's symptoms and physical findings at examination. However, for a definitive diagnosis to be made, laboratory tests must reveal the malaria parasites or their components (CDC, 2005).

Prompt and accurate diagnosis is the key to effective disease management, one of the main interventions of the Global Malaria Control Strategy. Poor diagnosis hinders effective malaria control. This is due to a combination of factors, including non-specific clinical presentation of the disease, high prevalence of asymptomatic infection in some areas, lack of resources and insufficient access to trained health care providers and health facilities, and widespread practice of self-treatment for clinically suspected malaria.

A major contributing factor, however, is that the laboratory diagnosis of malaria has up till now relied nearly exclusively on microscopy, which is a valuable technique when performed correctly, but unreliable and wasteful when poorly executed. A better utilization of microscopy and the development of alternative diagnostic techniques could substantially improve malaria diagnosis. Such objectives prove particularly relevant to the Roll Back Malaria initiative. According to WHO (1991), the most commonly used technique for blood examination is the stained blood films. Giemsa stain (one of the Romanwosky stains) is usually used to stain the films. Field's stain is an alternative where rapid diagnosis is required.

The development during the past ten years of rapid diagnostic tests (RDTs) for malaria using immunochromatographic test strips that offers a valid alternative to or complement microscopy. Various RDT kits have been tested in clinical and field situations, marketed and have found limited use in some malaria control programmes, as well as in special situations such as complex emergencies, epidemics and the diagnosis of malaria in returning travellers. The overall results have been encouraging, and several manufacturers are currently developing improved kits for the global market. The viable market for such kits is illustrated by the fact that one manufacturer alone has introduced 3 – 6 million test kits to date.

The time has come for serious consideration of how RDTs can most effectively be applied to the very diverse situations of malaria occurrences. To that effect, an informal consultation was convened in Geneva on 25 – 27 October 1999, bringing together the developers, manufacturers and potential users of RDTs, representatives of other interested agencies, and other stakeholders to discuss future actions to ensure their optimal deployment to control malaria (WHO, 2000b).

MATERIALS AND METHODS

A comprehensive search was made from the Internet, various journal articles and textbooks of reports on malaria diagnostic techniques in the tropics. Such articles were assembled and studied and this opinion report developed.

RESULTS

Different Types of Malaria Diagnostic Techniques

Clinical diagnosis: This is based on the patient's symptoms and on physical findings at examination. The characteristic feature of malaria is fever caused by the release of toxins (when erythrocytic schizonts ruptures) which stimulate the secretion of cytokines from leucocytes and other cells. In the early stages of infection the fever is irregular or continuous. As schizogony cycles synchronize, fever begins to recur at regular intervals particularly in quartan

malaria (every 72 hour), vivax and ovale malaria (every 48 hour). This method of diagnosis is widely practiced by physicians and some health workers including the indigenous medicine practitioners in remote areas due to absence of microscopy diagnostic facilities and unavailability of rapid diagnostic dipstick tests. The diagnosis of malaria is based upon detecting the asexual forms of the parasites, in the blood smear stained with Giemsa or Field's stain. In cases where the laboratory diagnosis is unavailable or unreliable, a history of exposure within the last year, particularly within the preceding 10 weeks and the suggestive clinical picture should prompt the physician to start anti-malarial treatment. The need to recognize severe malaria in both adults and children is important as this allows the immediate institution of optimal treatment in the hospital - either with increased nursing care in a general ward or preferably by admission to an intensive care unit (ICU) or high dependency unit (HDU), where requisite monitoring, nursing and therapeutic facilities are available (Njuguna and Newton, 2004).

Typical malaria fever attack: The first symptoms of malaria (most often fever, chills, sweats, headaches, muscle pains, nausea and vomiting) are often not specific and are also found in other diseases (such as the "flu" and common viral infections). Likewise, the physical findings are often not specific (elevated temperature, perspiration, tiredness). It starts with a cold stage (rigor) in which the patient shivers and feels cold, even though his or her temperature is rising. A hot stage follows in which the temperature rises to its maximum, headache is severe, and there are back and joint pains, vomiting, and diarrhea. The final stage known as paroxysm is when the patient perspires, the temperature falls, the headache and other pains are relieved, and the patient feels exhausted. Splenomegaly occurs in forms of malaria with repeated attack causing a great enlarged spleen. Anaemia and jaundice are also features of malaria, particularly *P. falciparum* malaria. In severe malaria (caused by *P. falciparum*), clinical findings (confusion, coma, neurologic focal signs, severe anemia, respiratory difficulties) are more striking and may increase the suspicion index for malaria. Thus, in most cases the early clinical findings in malaria are not typical and need to be confirmed by a laboratory test.

Presumptive diagnosis and treatment: In highly endemic areas (particularly in Africa), the great prevalence of asymptomatic infections and lack of resources (such as microscopes and trained microscopists) have led peripheral health facilities to use "presumptive treatment". Patients who suffer from a fever that does not have any obvious cause are presumed to have malaria and are treated for that disease, based only on clinical suspicion, and without the benefit of laboratory confirmation. This practice is dictated by practical considerations and allows the treatment of a potentially fatal disease. But it also leads frequently to incorrect diagnoses and unnecessary use of antimalarial drugs. This results in additional expenses and increases the risk of selecting for drug-resistant parasites.

Advantages of clinical diagnosis: The advantages of clinical diagnosis include; prompt and/ or presumptive treatment in malaria endemic regions, especially in rural areas lacking health facilities, cost effective or cheap, diagnosis is based on signs and symptoms, except in some asymptomatic individuals, diagnosis and treatment are based on indigenous knowledge and no special training and rigid protocols are adhered to.

Disadvantages of clinical diagnosis: This may result in a delay before the correct diagnosis is made and which may lead to death before appropriate treatment can be initiated. It increases the dangers of self-medication. It may result to drug abuse, over dosage, under dosage, and consequently the insurgence of wide drug resistance to the resistant strain of *P. falciparum.* Small outbreaks of malaria may occur in countries considered free of the disease, such outbreaks are most likely the result of an infected person entering the country asymptomatic and where suitable mosquito vectors are present. No measurable sensitivity and specificity may be achieved and diagnosis is

based on assumptions, because signs and symptoms could be mimic as in the case of black fever.

Routine Laboratory Diagnosis / Microscopic Examination

Advantages of microscopy

Sensitive: When used by skilled and careful technicians, microscopy can detect densities as low as 5–10 parasites per µl of blood. Under general field conditions, the detection capabilities of a typical microscopist can be more realistically placed at 100 parasites per µl of blood.

Informative: Observed parasites are can be characterized in terms of their species (*P. falciparum, P. vivax, P. ovale*, and/or *P. malariae)* and of the circulating stage (e.g. trophozoites, schizonts, gametocytes). Occasionally, expert microscopists can detect morphological alterations induced by recent drug treatment. Also, the parasite densities can be quantified (from ratio of parasites per number of leukocytes or erythrocytes). Such quantifications are needed to demonstrate hyperparasitaemia (which may be associated with severe malaria) or to assess parasitological response to chemotherapy.

Relatively inexpensive: Cost estimates for endemic countries range from about 0.12 USD to 0.40 USD per slide examined. Such figures do not reflect the true cost to the health system or to the patient, which may be substantially higher. The cost per test will increase if utilization is low, or if microscopy in the health facility is used only for malaria diagnosis. It is a general diagnostic technique that can be shared with other disease control programmes, such as those against tuberculosis or sexually transmitted diseases. It can provide a permanent record (the smears) of the diagnostic findings and be subject to quality control.

Disadvantages of microscopy: During preparation of the thin and thick films, the parasites may appear distorted making species identification difficult. It is labour-intensive and time-consuming, normally requiring at least 60 minutes from specimen collection to result.
Thin and thick films preparations depend absolutely on good techniques, reagents, microscopes and, most importantly, well trained and well supervised technicians. Unfortunately these conditions are often not met, particularly at the more peripheral levels of the health care system. Thus, microscopic diagnosis may be becoming riskful an unreliable tool that uses up scarce resources for doubtful results. Long delays in providing the results to the clinician, that decision on treatment are often taken without the benefit of the results. False-positive results may result due to improper collection, preparation, examination, and identification of parasite density and species differentiation. Extremely difficult to find or recognize malaria parasites in blood films within few hours of treatment (expect in drug resistance situation). In patients who live in malarious areas and who are partially immune to the disease, malaria infections may be asymptomatic and of little clinical significance. Repeated blood films may be necessary to detect malaria parasites in non immune patients, in whom symptoms can arise from very low parasitemia. Danger of transmitting other diseases, such as AIDS or hepatitis B, through the use of contaminated lancets, the collection of blood samples raises significant biosafety issues. The transportation and examination of blood slides for epidemiologic surveys can be both cumbersome and logistically difficult, particularly in remote areas. There is a backlog of slides to be examined, and results may not be available for many months. High cost of materials, reagents and microscopes.

Fluorescence Microscopy

Before the introduction of rapid immunodiagnostic test strips for diagnosing of malaria, two fluorescence techniques were developed to improve the sensitivity and speed of detecting parasites using microscopy. These are: Becton Dickinson Quantitative Buffy Coat (QBC) system using the ParaLens objective to

obtain incident light fluorescence and Kawamoto AO Interference Filter system using transmitted light fluorescence.

QBC system: In the QBC system, malaria parasites are concentrated by centrifuging blood in a special capillary tube. The tube is coated with acridine orange and an anticoagulant. It contains a small plastic cylinder (float). Following centrifugation, the white cells, platelets, and upper layer of the red cells which contain the malaria can be found in the capillary. When examined by fluorescence microscopy at X600 (using a ParaLens objective), the acridine orange stained malaria parasites fluoresce green-yellow against a dark red-black background with the nucleus (chromatin dot) of trophoziotes and meroziotes of schizonts fluorescing bright green. Schizonts and gametocytes can be seen in the white cell layers. Although more rapid than examining thick stained blood films, field evaluations of the QBC system have shown it to be less sensitive than examining thick stained blood films in detecting low parasitaemia (<100/ul) and less sensitive than ParaSight F.

Kawamoto AO interference filter: This system, designed by Dr. F Kawamoto, requires an interference filter specially designed for the fluorochrome AO and located in the sub stage filter holder of an ordinary transmitted light microscope, a barrier filter (inserted in the eyepiece) and a 150 W or 200 W halogen lamp. The nuclei of malaria parasites and white cells fluorescent bright green and the cytoplasm of the parasites fluoresces red (particularly the cytoplasm of gametocytes). Compared with the QBC system, the Kawamoto fluorescence system is inexpensive. Most field evaluation of the system have found it to be as sensitive, but more rapid than Giemsa stained thick films in detecting *P. falciparum* parasites. Blood films must be prepared and stained correctly.

Advantages of fluorescence microscopy: Useful in survey work for screening blood donors and reduces wastage. The AO stained films can be washed with methanol and restained with Giemsa.

Disadvantages of fluorescence microscopy: They are of little value in the "acute" malaria situation. The equipment required for the QBC system is very expensive. The special disposable capillary tubes are also more than can be affordable by most hospital laboratories. Although more rapid than examining thick stained blood films, field evaluations of the QBC system have shown it to be less sensitive than examining thick stained blood films in detecting low parasitaemia (< 100/µl) and less sensitive than ParaSight F. Species differentiation is difficult thus identity of the actual cause of malaria is problematic. Considerable skill and experience are required to process and examine the tubes correctly and confidently. Microcoagulation, the adverse effects of heat and humidity, strong background fluorescence, scattered lyzed white blood cells and the incorrect positioning of the float, have been reported as making it difficult to examine some tubes. The tubes must be centrifuged promptly. Over staining or under staining make the parasites difficult to be recognized

Molecular Diagnosis

Polymerase chain reaction (PCR): Another relatively new method is the polymerase chain reaction (PCR) which uses a non-isotopically labelled probe following PCR amplification. PCR may yet prove to be a valuable addition to the examination of blood films for the diagnosis and speciation of malaria (Moody, 2002).

Advantages of PCR: It is possible to detect <10 parasites per 10uL of blood. PCR is a valuable addition to the examination of blood films for the diagnosis and it is useful in speciation of malaria parasites.

Disadvantages of PCR: The special equipment required precludes practical field application for rapid diagnosis of malaria. Some researchers have claimed that PCR (and Elisa) techniques are as sensitive as blood films, however they are infinitely more expensive, requires specialized equipment and takes a longer time to complete.

Rapid Diagnostic Test (RDT)/Immunochromatographic Tests (ICT)

A new generation of easy to perform tests has been developed to diagnose *falciparum* malaria rapidly and reliably without the need of a microscope. The three main groups of antigens detected by commercially available RDTs are: (1) histidine-rich protein 2 (HRP2), specific to *P. falciparum (2) Plasmodium* lactate dehydrogenase (pLDH), currently used in products that include *P. falciparum*-specific, pan-specific, and *P. vivax*-specific pLDH antibodies and (3) aldolase (pan-specific).

The targeted antigens of commercially-available malaria rapid diagnostic tests include; HRP2, pLDH, aldolase, *P. falciparum*-specific, Pan-specific (all species) and *P. vivax*-specific. Most of the commercial products include antibodies such as: HRP2 alone (*P. falciparum*), HRP2 and aldolase (distinguishing *P. falciparum*/mixed infection from non-falciparum alone), Falciparum-specific pLDH and pan-specific pLDH (distinguishing *P. falciparum* / mixed infection from non-falciparum alone), HRP2 and pan-specific pLDH, HRP2, pan-specific pLDH and vivax-specific pLDH or Pan-specific aldolase only. RDT detecting both falciparum-specific and non-falciparum (or pan-specific) target antigens are commonly called combination or 'combo' tests.

The products come in a number of formats such as: plastic cassette, card, dipstick and hybrid cassette-dipsticks. Cassettes tend to be simpler to perform than dipsticks, and this is likely to affect test accuracy (WHO, 2005).

The most recently developed tests can also diagnose vivax malaria. Three blood tests are available for diagnosing falciparum malaria based on the immunochromographic detection of antigen HRP 2 (histidine-rich protein 2) or specific pLDH (parasite lactate dehydrogenase). Both HRP 2 and pLDH are produced by the parasites during their growth and multiplication in red cells. The tests are ParaSight F and ICT Malaria pf (both producing antigen HRP 2) and OptiMAL (producing antigen pLDH).

HRP 2 antigen: This is produced by *P. falciparum* and is released from parasitized cells into the circulation. Following successful treatment, HRP 2 can be found in the blood several days after parasites have cleared. In heavy infections, HRP 2 may persist for up to 14 days or more. According to Cheesbrough and Precott (1998), a small number (2 – 3%) of locally found *P. falciparum* strains in Mali were naturally lacking the gene which produces HRP 2 (HRP 2 gene) and therefore gave false ParaSight F test results.

Performance of ParaSight F: ParaSight F was first rapid immunochromographic malaria test to be developed. It has been evaluated extensively in tropical and developing countries, in stable and unstable areas of malaria transmission and in both laboratory and non-laboratory situations including village health workers. The evaluations have shown ParaSight F to be sensitive and specific, performing as well as, and sometimes better than microscopy in field situations.

ICT combined test to diagnose falciparum and vivax malaria: ICT diagnostics have developed a test that can diagnose both falciparum and vivax malaria, and can be performed as easily as the ICT Malaria pf test. The new combined test is expected to be available in 1998.

ICT Malaria pf for diagnosing falciparum malaria: ICT malaria pf kit contains 25 tests and has a shelf-life of one year. ICT Diagnostics also manufactures 2 and 4 test kits for travellers visiting falciparum malaria endemic areas. These kits are called Malapachol.

ICT Combined Test to Diagnose Falciparum and Vivax Malaria: ICT Diagnostics have developed a test that can diagnose both falciparum and vivax malaria, and can be performed as easily as the ICT Malaria pf test.

pLDH antigen test to diagnose falciparum and vivax malaria: The metabolic malaria parasite enzyme pLDH is actively produced by

all human parasite species during their growth in red cells. It is found in the blood and although present in urine, it is not sufficiently constant to be useful. Differentiation of malaria species in the OptiMAL test is based on antigenic differences between pLDH isoforms. Unlike HRP 2, pLDH does not persist in the blood, but clears about the same time as the parasites following successful treatment.

OptiMAL test to diagnose falciparum and vivax malaria: This test, based on the detection of pLDH is the most recently developed of the immunochromographic rapid malaria strip tests. It is able to monitor responses to drug therapy and detect drug-resistant malaria because pLDH reflect the presence of viable malaria parasites in the blood. pLDH levels follow closely parasitaemia. *OptiMal* test detects pLDH enzyme with a series of monoclonal and polyclonal antibodies against *Plasmoduim* species. Studies so far carried out indicated that the tests were sensitive and able to differentiate between *P. vivax* and *P. falciparum*.

Sensitivity: This has been reported as between 84.20 – 96.60. Most studies have found the lower limit of detection for ParaSight F to be equivalent to 25 – 60 parasites /µl detected in thick film. Although an experienced microscopist working under optimal conditions is able to detect as few as 10 – 20 parasites/µl in a thick blood film, this level of sensitivity is rarely achieved in most district laboratories. Crag and Sharp (1997) found a sensitivity of 84 parasites /µl for Giemsa thick films and a sensitivity of 30 parasites/µl for ParaSight F.

Specificity: Most evaluations have estimated this to be between 81 – 99.5% with variations being found in different areas of malaria transmission. Positive predictive values ranged from 80 – 98.7% although lower values have been found in hypoendemic areas. Negative predictive values ranged from 72 – 100%.

Rapid Malaria Strip Tests: The cost of the new non-microscopical malaria tests in developing countries (in 1997, 1.230 – 2.25 USD or more, depending on country) is more than the cost of microscopy to diagnose falciparum malaria. In the many places where microscopy is not possible and malaria is diagnosed presumptively from clinical symptoms (shown repeatedly to be unreliable)**,** the savings in drug costs more than offset the cost of using HRP 2 or pLDH malaria test, particularly in areas of drug resistance where expensive second line antimalarial drugs may be need for used. In a recent study in Zimbabwe the use of ParaSight F reduced mistreatment for malaria, relative to clinical diagnosis, by up to 81%, especially in a hypoendemic area.

Besides the financial savings from unnecessary treatments, the use of non-microscopical rapid malaria tests is of value in the early investigation and management of malaria epidemics. The rapid tests are also of value in the diagnosing malaria in those who have taken antimalarial drugs (can make microscopical diagnosis difficult). Most important, extending and improving the accuracy of malaria diagnosis with subsequent correct use of drugs will reduce significantly the spread of parasite resistance to antimalarials.

Advantages of RDT: Rapid diagnostic test provides speedy result. The accuracy of diagnosing *P. falciparum,* particularly in non specialized laboratories where inexperienced or junior staff may be involved makes RDT preferable for diagnosis and mass chemotherapy associated with malaria. Very little training is required for these techniques. Dipstick kits are very useful in screening or confirmatory tests, especially when there is difficulty in identifying scanty ring forms in blood films. ParaSight F is available in tropical countries; kits contain 20 tests or 100 tests. It does not require refrigeration, but the strips and reagents must be kept out of direct sunlight and the strips kept dry. The kits have a 2 year shelf-life. The test is easy to perform and takes about 7 minutes.

Disadvantages of RDT: However dipstick methods are unable to indicate parasite load. In some countries, the cost may be prohibitive. A potential problem with these methods is that

the circulating antigen may be detected for many days (up to 2 weeks in our laboratory) after the elimination of viable parasites from the circulation. It must therefore be remembered that a positive test may not always be due to an active infection.

Other Diagnostic Techniques: Daily measurement of haemoglobin level especially at heavy parasitaemia, particularly in children under 5 years, when anaemia resulting from haemolysis, and anoxia, can be fatal. Blood urea or serum creatinine sensitivity test in suspected renal damage. Urine test for haemoglobin if black water fever is suspected. Haemoglobin sensitivity in Glucose 6 Phosphate Dehydrogenase (G6PD) deficiency. Liver Function Test (LFT), if liver cell is damage is suspected. Measurement of serum bilirubin, albumin and aspartate aminotransferase is a clue. Platelet count and measurement of plasma fibrinogen, FDPs (fibrin/fibrinogen degradation products), Activated Partial Thromboplastin time Test (APTT) and P0rothrombin Time (PT) if Disseminated Intravascular Coagulation (DIC) or other abnormal bleeding is suspected (Cheesbrough, 1987). An Indirect Haemagglutination Antibody (IHA) Test has also been used in epidemiological survey, but compared with the IFA test, it lacks sensitivity and specificity.

Serology: Serology detects antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serology does not detect current infection but rather measures past experience. Also are the Enzyme Linked Immunoabsorbent Assay (ELISA) (Voller, 1979) and Radioimmunoassay (RIA). These tests may become more important than the IFA test when monoclonal antibodies are used to produce specific purified antigens. Such tests enable large number of samples to be tested rapidly (Sulzer and Wilson, 1972).

Drug Resistance Tests: Drug resistance tests are performed in specialized laboratories to assess the susceptibility to antimalarial compounds of parasites collected from a specific patient. Two main laboratory methods are available:

In vitro tests: The parasites are grown in culture in the presence of increasing concentrations of drugs; the drug concentration that inhibits parasite growth is used as endpoint.

Molecular characterization: molecular markers assessed by PCR or gene sequencing allow also the prediction, to some degree, of resistance to some drugs; however, the predictive values of these molecular tests are still being evaluated.

Sensitivity and specificity: These are ratios comparing test results to True disease, which means test results from individuals known to have the particular condition are compared with test result of individual known not to have the condition. Actually, tests are used the other way round. Specimens are taken from individuals whose disease state is not known. It is the testy that is used to predict whether the individual have the disease, hence the term predictive value. Predictive value depends upon sensitivity, specificity and upon the disease in the population being tested.

DISCUSSION

A marked improvement in diagnosis would greatly enhance the applicability of the tests and/or their reliability: an increase in sensitivity, aiming at 100% sensitivity for densities of >100 parasites per µl blood in all four species; reduction or suppression of time-critical steps, or development of methods for self-timing; improvement in stability at high temperatures and against short temperature surges; improvement in the robustness of the test kits; reduction in the number of steps and test components; improvement in the readability of the tests (applies to better signal intensity as well as to the avoidance of mix-ups); development of safer methods of blood handling; and development of non-blood-based immunological tests (e.g. saliva and urine). It is suggested that the body itching and the

characteristic odour that occur during severe malaria may be due to acidosis and hypoglycemia as proposed by researchers and thus are symptoms for clinical diagnosis. Intradermal blood film provides a more complete picture both of the stage of infection and total body parasite burden than examining the peripheral blood alone, but it is unlikely to replace peripheral blood film as the primary tool for malaria diagnosis, as they fail to pick-up the none-sequestering *P. vivax.* Meanwhile, they may turn-out to be useful adjunct to the peripheral blood film for both diagnosis and provision of prognostic information either alone or more probably in combination with examination for more phagocyte pigment. However, venous blood films are the best for diagnosis because of the availability of free parasitized red cells in the circulating blood and with the pressure exerted by the syringe in drawing sequestered red cells from the capillaries during blood collection. In-addition, placental histological microscopy is the best diagnostic method for malaria in pregnancy. *If one is asked, identify the "*gold standard" *against which malaria diagnostic tests should be assessed?* While microscopy is acknowledged to be an imperfect diagnostic tool, it has practically always been used as the standard against which other tests such as RDTs are assessed. This work emphasizes that these dipstick methods are regarded as useful additional tests to the long established method of examining thick and thin blood films which is still regarded as the "gold standard", NOT as replacement methods. The highest density of malaria occurs in countries least able to afford sophisticated and expensive diagnostic tools. Tests such as PCR are more sensitive and specific, but may not reflect accurately the presence of live parasites. The identification of a better "gold standard" would not only provide an improved tool for the development of new diagnostic tests, but might also offer a better understanding of the biology of malaria in the human host. The main research questions are as follows. Which of the currently available methods should be used as the "gold standard"? Is there a combination of diagnostic findings that might yield a better approach to a "gold standard"? Can the same "gold standard" be used for all epidemiological situations?

Conclusion: It is suggested that a more proactive approach by government and stakeholders in equipping the laboratories in the tropics with diagnostic facilities that can give 100% sensitivity and specificity at a reduced cost and to ensure availability. Currently, the use of venipunture in microscopy is highly advocated for a better diagnostic result and as a "gold standard" to malaria diagnosis. There should be regular reporting of the malaria burden to health authorities to increase the opportunities of getting support for prevention and intervention in the control of malaria in the tropics. Malaria is still curable, the key to this, is prompt identification of the parasite and early institution of anti-malarial therapy.

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REPRODUCTIVE EFFICIENCY OF AN INDIGENOUS IRANIAN GOAT (*Capra hircus*)

[1]MOHAMMADZADEH, Saeid, [2]ABASI, M., [3]TAVAFI, Majid and [4]KIANI, Ali.

[1]Department of Science and Technology, Lorestan University, Khorramabad, Iran.

[2]Department of Basic Sciences, School of Veterinary Medicine, University of Lorestan, Khorramabad, Iran.

[3]Department of Anatomy, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran.

[4]Department of Animal Science, Faculty of Agricultural Sciences, University of Lorestan, Khorramabad, Lorestan, Iran.

Corresponding Author: Mohammadzadeh, S. Animal Science Group, Department of Science and Technology, Lorestan University, Khorramabad, Iran. **Email:** mohammadzadeh.s@mail.lu.ac.ir **Phone:** +98-661-6200071

ABSTRACT

Goats (Capra hircus) are economical animal in livestock production especially in rural areas. It is well known that goat can tolerate harsh conditions; however there are little information about its reproduction functions such as testis histomorphometry and efficiency of sertoli cells. This study aimed to estimate germ cell types and number per sertoli cell of an indigenous Iranian goat (Lori goat). Semen was collected from five Lori goats by means of an artificial vagina. Semen volume, concentration, normality and motility of spermatozoa were determined. After removing both testes, tissue paraffin section (5µ thickness) were prepared and stained using Haematoxylin and Eosin method. Mean weight of testis was 114.40 ±27.00 grams. Means of seminiferous tubule diameter and tubule length per gram testis were 197.20 ± 1.80 µm and 9956.00 ± 93.00 m, respectively. The mean number of spermatids per each tubule, spermatogonia and spermatocytes at stage I were 163.00 ± 99.9, 140.30 ± 28.60 and 146.40 ± 19.80, respectively. From this study it can be concluded that the Lori goat has high reproductive potentials probably due to high number of sertoli cell per germ cells.

Keywords: Lori goat, Testis, Sertoli cell, Seminiferous tubule, Spermatocytes, Spermatogonia, Spermatozoa, Semen

INTRODUCTION

The goat is an economically important species in livestock production in rural areas. Goats are able to tolerate high rainfall areas and other harsh conditions associated with the semi-arid climate. Goats can efficiently ingest low quality feedstuff especially fibrous materials; they are favorite animal in rural area of most sub-tropical countries (Green and Baker, 1996; Adams *et al.,* 1997). Although it is well known that goat can tolerate harsh conditions, our knowledge is limited about indigenous goat reproduction functions such as testis morphometry and efficiency of sertoli cells. Goats have been used as a model for spermatogonial transplantation and a number of studies have described spermatogenesis in goats (Gordon, 1997). Even though goat fertility has been studied in different environmental conditions (Ahmed *et al.*, 1997; Al-Ghalban *et al.*, 2004; Martemucci *et al.*, 1998), published data on testis function

and spermatogenesis (Courtens and Loir, 1981; Bilaspuri and Guraya, 1984; Oke *et al.,* 1984; Onyango *et al.,* 2000) is limited. Efficiency of spermatogenesis (i.e. estimated number of spermatozoa produced per gram of testicular parenchyma) is highly correlated to germ cells supported by single sertoli cell (Russell and Peterson, l984; Franca and Russell, 1998). Efficiency of reproduction is also highly correlated with density of seminiferous tubules, number of sertoli cell per gram of testis and the duration of the spermatogenic cycle (Russell *et al.,* 1990; Sharpe, 1994; Neves, 2001; Leal, 2004). Thus it has been proposed that daily sperm production can be accurately determined from the total number of sertoli cell (Franca and Russell, 1998; Franca and Godinho, 2003).

In Iran, there are about 25 million goats playing critical and economical roles for their producers in rural area. About 2 millions of the Iranian indigenous goats are Lori goats and are reared in west of Iran mainly in Lorestan province. Lori goat is highly economic for goat producers in terms of meat production. Therefore, governmental strategies such as breeding and estrus synchronization programs have been started to protect these beneficial animals and their producers. However, there is a lack of knowledge about reproduction characteristics of the Iranian indigenous goats in the country. Zamiri and Heidari (2006) studied the reproductive characteristics of Rayani goat in central area in Iran. There are no published information about the reproductive characteristics and testis histomorphometry of the Lori goat. Therefore this study aimed to investigate reproductive characteristics of Iranian indigenous goat (Lori goat) that is mainly reared in west of Iran.

MATERIAL AND METHODS

Five mature Lori bucks (2 – 3 years old) were selected. An artificial vagina was used to collect semen. Semen volume, concentration of sperm per milliliter, sperm normality and motility were determined using the methods of Sorensen (1979). Goats were slathered and testis were trimmed and weighed. Length and width of testis were determined by means of digital caliper. Testicular volume were determined applying the following equation; 4/3 πABC, where A, B and C were half width, half thickness and half length, respectively (Mascarenhas *et al.,* 2006). Testis volume was directly converted into gram, since density of mammalian testis has been established to be close to one (Johnson *et al.,* 1981; Paula, 1999). The weight of testis parenchyma was calculated after exclusion of the tunica albuginea and mediastinum (~10%) from the total testis volume (Becker-Silva, 2000). Slabs of testis (25 mm^2) were sampled randomly and fixed in 10% formalin, and stored at room temperature for testicular histological study. Fixed slabs of testis were histologically processed and embedded in paraffin. Histological sections (5µm thickness) were prepared using a rotary microtome (4060 cut SLEE). Sections were stained by routine Haematoxilin-Eosin (H&E) method. The mean diameter of seminiferous tubules, lumen and height of seminiferous tubule epithelium (Figure 1) were measured using Motic Image Plus 2 software.

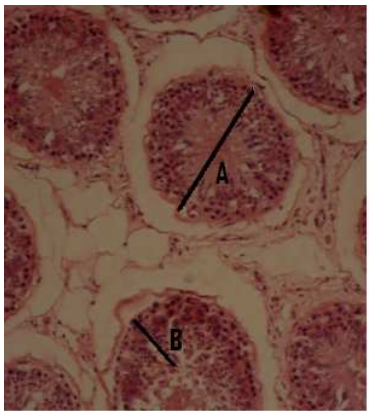

Figure 1: Transverse section of semniferous tubules in Lori goat. A. diameter of tubule. B. height of epithelium.

Sixty seminiferous tubules were randomly selected and measured. The numbers of spermatogonia, spermatocytes and sertoli cell were morphometrically estimated. The loss in spermatogenesis was not significant (Johnson *et al.*, 2000) thus; spermatid reserves of the testis (SRT) were calculated on the basis of the round spermatid populations (Amann, 1962; Berndtson, 1997) as follows: SRT = (Round spermatid × % seminiferous tubules × total volume of the testis parenchyma)/(seminiferous

tubule area in transverse section × cut thickness × 100). Transversal section area of the seminiferous tubule was calculated using the equation πR^2 where R was ray obtained from average of 60 seminferous tubules diameter per animal. The length density of seminiferous tubules (length per unit volume) was estimated stereologically (Howard and Reed, 2010). Briefly microscopic image of testis (total magnification of 25×) was displayed on a computer monitor and superimposed with a 10 × 10 cm frame. The tubules that felled within the frame and do not cross the lower and left lines of the frame were counted (Figure 2).

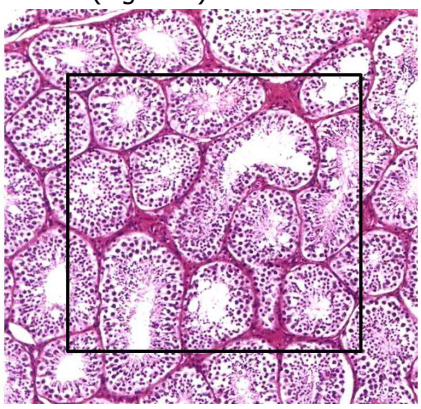

Figure 2: Method of random selection of seminiferous tubules for estimation of length tubules of an Iranian indigenous goat (Lori goat).

The length of tubules was calculated using the formula: Lv (tubule/testis) = 2.∑Q/ (a/f.∑p), where ∑Q = total number of tubules counted, a/f = area per frame = frame area/magnification^2 and ∑p = total number of frames applied (Howard and Reed, 2010). The data were analyzed statistically for their central tendencies by using SPSS 11.5. Data are presented as means ± standard divisions of means.

RESULTS

Average of semen volume in each ejaculation was 2-2.5 ml with 1.28×10^9 sperm per ml. Motility and normality of sperm were estimated to be 90 and 95 percents, respectively. The means of spermatogonia, primary and secondary spermatocyte, spermatid (Figure 3) and sertoli cell (Figures 4 and 5) per each tubule section were 140.30 ± 28.60, 146.40 ± 019.80, 163.00 ± 99.9 and 5.50 ± 2.40, respectively (Table 1). Each sertoli cell was sustained approximately 24.4 germ cells.

The average diameter of seminiferous tubules and epithelial height was 197.00 ± 1.80 and 39.00 ± 1.00 µm, respectively. According to section thickness (5 µm) and number of seminiferous tubules per area and length of seminiferous tubules were estimated to be 87.00 ± 1.30 and 9956 ± 93 meters per gram and per testis, respectively (Table 2).

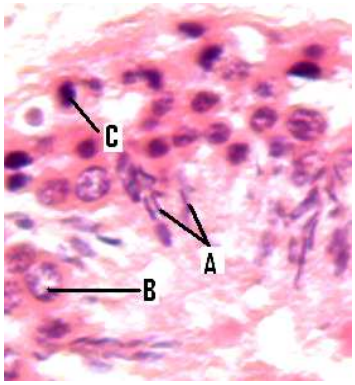

Figure 3: Transverse section seminiferous tubules, different type of germ cells spermatogonium (C), spermatocyte (B) and spermatozoid (A) of an Iranian indigenous goat (Lori goat).

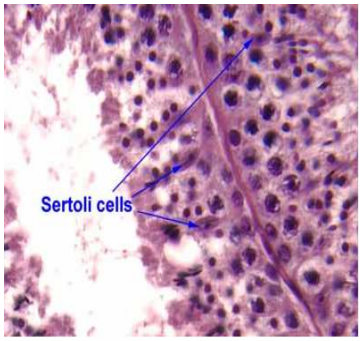

Figure 4: Transverse section of seminiferious tubules of an Iranian indigenous goat (Lori goat). Sertoli cells shown in tissue section.

DISCUSSION

The average of testis weight for Lori goat was about 114 ± 27 grams. In Lori goat, density of testis parenchyma estimated to be about 86.5%. The density of testis parenchyma of 80 – 85 % has been reported (Oke *et al.,* 1984, Yadav and Sharma, 1994; Nishimura *et al.,* 2000). This was in agreement with the finding of Russell *et al.* (1990) that seminiferious tubules occupy between 70 – 90 % of testis parenchyma in most mammals. The recorded tubular diameter of 197.20 ± 1.80 µm in this

study was in agreement with the range 180 – 350 µm for tubular diameter of most mammals (Roosen-Runge, 1973; Setchell *et al.,* 1994). However, the value of 197.2 µm was smaller that values of 237.00 ± 3.00 µm reported by Franca and Russell (1998) and Leal *et al.* (2004) for Alpine bucks. The differences may be due to excessive shrinkage in long term storage of samples in formalin. Spermatogenesis is a complex process involving mitotic, meiosis cell divisions and the process of spermiogenesis (De Krester *et al.,* 1998). Related to testis morphometry, for calculating the testis parenchyma, the mediastinum and Tunica albuginea are reduced from the gonadal mass, because these sections do not directly participate in the spermatogenesis and androgenic function (Johnson et al., 2000).

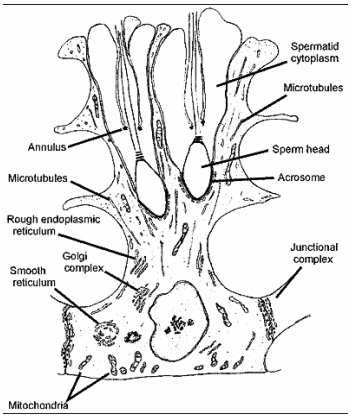

Figure 5: Schematic diagram of sertoli cell in testis of an Iranian indigenous goat (Lori goat).

The volumetric proportion of the testicular albuginea and mediastinum has been reported to be around 10% of testis weight (Franca and Russell, 1998). In African lion, tunica albuginea and mediastinum were calculated to be 18% of testis weight (Barros *et al.,* 2004). In present study tubular length per gram of testis parenchyma was estimated 87.00 ± 1.30 meter. This value was more than 4 times higher that the values reported previously (Setchell *et al.,* 1994; Franca and Russell, 1998; Leal *et al.,* 2004). Leal *et al.* (2004) found that the length of seminiferous tubules per gram testis parenchyma was 20 meter. Seminiferous tubular length in goat was about 85% of testis parenchyma and occupied (Yadav and Sharma, 1994). The differences may due to variation in goat breeds and can also be attributed to variations in methodological approaches utilized for histological processing of testis (Okwun *et al.,* 1996). Seminiferous epithelium in Lori goat was estimated to be 39.2 ± 0.98 µm, this parameter in goat has previously been reported to be 74.4 ± 1.0 µm (Leal *et al.,* 2004). During spermatogenesis, apoptosis normally occurs (Blanco-Rodriguez, 1998). The kinetic of spermatogenesis wasn't investigated in this study, but in goats it has been shown that 30% cell loss occurred during the second process of meiotic division (Leal *et al.,* 2004).

Sertoli cells play an important role in spermatogenesis. Sertoli cells are mainly responsible for transport of spermatogenic cells from basal seminiferous tubules to the lumen. Sertoli cells are also involved in the phygocytosis of degenerated cells, as well as nutrition and protection of germ cells (Kelly *et al.,* 1984; Jurado *et al.,* 1994). Sertoli cells represent the structural framework that supports the developing germ cells in the seminiferous tubules (Yin *et al.,* 2006). In ruminant, sertoli cells have prominent structure (multivesicular nuclear body) and differ from non-ruminant species (Wroble and Schimmel, 1989). The number of sertoli cell determines the sperm production in sexually mature animals (Orth *et al.,* 1988, Franca *et al.,* 2000). Efficiency of spermatogenesis is usually positively correlated with the number of germ cells supported by each sertoli cell (Russell and Peterson, l984; Sharpe, 1994; Neves, 2001). It is therefore likely that sertoli cells play a significant role in sequestration and degradation of residual bodies in the goats and sheep after spermiation (Onyango *et al.,* 2000), thus it has been proposed that the number of sertoli cells and their size are important parameters for determination of spermatogenic efficiency in mammals (Franca *et al.,* 2002). Present study showed that the number of sertoli cells per gram in Lori goat was about 8 $\times 10^6$. This value for was lower than those found in Alpine goat and other domestic mammals. In Alpine goat the number of sertoli cell per gram of testis were found to be 21.4 $\times 10^6$ (Leal *et al.,* 2004). The number of sertoli cell per gram testis of bull, stallion, rabbit and boar were reported to be 29, 28, 25 and 20×10^6, respectively (Franca and Russell, 1998). Lori goat had nearly the

same number of sertoli cell as in ram 8 – 12×10^6 (Franca and Russell, 1998). In each tubule section, the number of sertoli cell was estimated to be 5.5 ± 2.4. This specifically may play a role in sperm release (Ross, 1976) during different seasons. The seasonal variations mainly occur in response to a decline in the levels of FSH and LH and testosterone (Michael and Bonsall, 1997; Elsayed, 2008). In Lori goat, spermatic reserve of the testis (SRT) was estimated to be 1.7×10^9. Thus it seems that high reproductive efficiency of Lori goat can be related to the long seminiferous tubules and the number of sertoli cell. However, further studies are needed on the kinetic of spermatogenesis in Lori goat.

ACKNOWLEDGMENTS

The authors would like to thank Lorestan University for financial support of this study. Likewise we are thankful to all the various Departments in Lorestan University for use of equipments, reagents laboratory space and other technical supports.

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EFFECTS OF GRADED LEVELS OF ALPHAMUNE G ON PERFORMANCE OF GROWING PULLETS TO LAYING HENS

OLONIJOLU, T. O., BABAWALE, O. I., BOLU, S. A. and MALOMO, G. A.
Department of Animal Production, University of Ilorin, Ilorin, Kwara State, Nigeria.

Corresponding Author: Bolu, S. A. Department of Animal Production, University of Ilorin, Ilorin, Kwara State, Nigeria. **Email:** bolusao2002@yahoo.co.uk **Phone:** +234 806 024 0049

ABSTRACT

The study was conducted to investigate the effects of Alphamune G fed to growing pullets on their performance during laying. A total of one hundred and forty four (144) commercial black Harco pullet chickens made up of seventy-two (72) each of age 19 weeks old (group A) as well as age 15 weeks old (group B) were fed four graded levels of Alphamune G (0.00, 0.04, 0.05 and 0.06 % inclusion level). A factorial arrangement of four levels of dietary Alphamune G and two age groups were the treatments in a Complete Randomized Design. The study was conducted for 17 weeks, when the birds of each of group A and B attained age 36 weeks and 32 weeks, respectively. Laying pullets fed the control diet 0.00% had the highest value of egg weight, shell thickness and feed intake. Highest values for feed to gain ratio, feed per dozen eggs and feed cost per dozen eggs were observed in laying pullets fed 0.04% inclusion level of Alphamune G. The haugh unit, yolk index and weight gain were observed to have the highest value in layers fed 0.05%. Highest values was observed for % hen day production for laying hens fed 0.06% inclusion level of Alphamune G. In conclusion, among laying hens fed dietary levels of Alphamune G, birds fed inclusion levels of 0.06% performed the best in terms of production characteristics, egg quality and economic value compared to 0.04% and 0.05%.

Keywords: Alphamune G, Growing pullets, Laying hen, Production characteristics, Egg quality, Economic value

INTRODUCTION

The egg industry is enjoying increased production as consumers have become more educated about the nutritive value of eggs. Eggs are relatively inexpensive per unit of protein and energy, therefore egg consumption has continued to increase in developing countries (Leeson and Summers, 2005). For several decades, antibiotics and chemotherapeutics in prophylactic doses have been used in animal feed to improve animal welfare and to obtain economic benefits in terms of improved animal performance and reduced medication costs. Disease control accounts for over 10% of the total cost of Isa-brown production (Kabir and Haque, 2010).

There are increasing concerns about the risk of developing cross-resistance and multiple antibiotic resistances in pathogenic bacteria in both humans and livestock linked to the therapeutic and sub therapeutic use of antibiotics in livestock and pets (Bolu *et al.,* 2009a). The European Union has banned all in-feed use of antibiotics from 2006 and the use of antibiotics in feed is being considered for elimination in other parts of the world. This perspective has stimulated nutritionists and feed manufacturers to search for new and safe alternatives (Hajati and Rezeal, 2010). Several

products with different modes of actions have been proposed, including prebiotics, probiotics, acidifiers and phenolic compounds.

Alphamune G is an alternative to antibiotics growth promoter (Alpharma Animal Health, 2004). It is one of the products that have a beneficial impact on the overall health of the gut of different species of poultry birds. Alphamune G is a spray dried and granulated product produced after the autolysis of food grade yeast (*Saccharomyces cerevisae*). It contains a unique combination of (1-3, 1-6) β-glucans and mannan oligosaccharides. The β-glucans have been shown to be involved in enhancement of the immune system (Huff *et al.,* 2006). Mannan oligosaccharides on the other hand have prebiotic effect when fed to biological systems (Józefiak *et al.,* 2004). Yeast has been used in fermentation technology for production of alcohol and in food industry. *Saccharomyces cerevisae* one of the most widely commercialized types of yeast, has long been fed to animals. Addition of live yeast to animal feed has been known to improve the nutritive quality of feed and performance of animals (Martin *et al.,* 1994). In addition, mannan oligosaccharide derived from the cell wall of the yeast *S. cerevisae* has shown promise in suppressing enteric pathogens and modulating the immune in studies with poultry (Iji and Tivey, 1999; Santin *et al.,* 2001). Additionally, there are trials showing that enrichment of diets with yeast could favorably improve the feed efficiency and growth rate (Ghasemi *et al.,* 2006) and carcass percentage (Vargas *et al.,* 2002). The additional cost of Alphamune G to the ration yield a superior financial performance in previous study using 500g per ton of ration (Alpharma Animal Health, 2004). Bolu *et al.* (2009 a,b) reported 0.04% and 0.06% dietary inclusion of Alphamune G gave better performance in broiler and cockerel chicks, respectively. The use of Alphamune G in growing to laying pullets is expected to reduce cost incurred on medication, improve feed and production efficiency thereby giving the farmers more turn-over. This study was conducted to investigate the effects of feeding graded levels of Alphamune G on laying performance as well as the optimum time and level at which Alphamune G can be administered to diet for optimum production from growing pullets to laying hens.

MATERIAL AND METHODS

A total of 144 commercial black Harco pullet chickens made up of seventy-two (72) each of age 19 weeks old (group A) and 15 weeks old (group B) were used for study. The study lasted for a period of 17 weeks when the birds in each of the groups (A and B) attained 36 and 32 weeks old, respectively. The birds were housed in battery cages. Routine management and vaccination programme necessary for pullets and layers production were followed. Feed and water were given *ad-libitum* to the birds throughout the experiment.

Treatments consist of four graded levels of dietary Alphamune G (0.00, 0.04, 0.05 and 0.06 %) incorporated into the diets (Table 1). The pullets in each group were weighed and randomly allocated to each of the four levels of Alphamune G in 6 replicates of 3 birds. Pre-lay diet was fed to birds for twenty-one days of the experiment after which the layer diet was fed till the end of the experiment.

Production Parameters Recorded

Weight gain: The initial weights of the birds in grams were recorded at the onset of the experiment using a weighing scale balance and subsequent weights of the birds were recorded on weekly basis. The difference in weight for each week was used to determine the weight gain record.

Feed intake: The feed intake recorded in grams was calculated from the difference between the weight of the feed offered and the weight of the left over for each respective replicate on weekly basis.

Feed to gain ratio: The feed to gain ratio for each respective replicate was determined by the ratio of feed intake to weight gain on weekly basis.

Age at first egg lay and hen day production (HDP): Age of first egg lay for each treatment was recorded and hen day production was recorded for each replicate;
% HDP = Number of eggs produced × 100 / Number of hen-days; where hen-day = Number of hens × number of days in lay.

Feed utilization efficiency: This was obtained as the total feed consumed to the produce a dozen eggs.

Mortality: This was computed as the ratio of dead to stock birds in percentage thus; % Mortality = Total number of dead bird × 100 / Total number of birds stocked.

Egg Assessment

Average egg external and internal qualities i.e. shell thickness, egg weight, yolk and albumen width as well as yolk and albumen height were determined at age 28 to 32 weeks old for each group of birds at an interval of two weeks.

Egg weight: Eggs were weighed on the weighing scale balance and recorded. This was used in calculating the Haugh unit.

Albumen height: Blunt steel was used to crack the shell to avoid rupturing of the albumen in breaking the egg. A tripod spherometer marked 0.1mm was used to take the height of the albumen at the mid-point.

Albumen width: The albumen width was measured with the venier caliper.

Haugh unit: The albumen height and egg weight records were used to compute the haugh unit. The haugh unit is calculated from the formula: $H.U = 100 \log (H - G) \sqrt[2]{(30W^{0.37} - 100)} + 1.9/100$, where HU= Haugh unit, H= Albumen height (mm), G = 32.2, W = Weight of the egg (g).

Yolk index: This was obtained from the formula: Yolk Index = Yolk height / Yolk diameter (width). Yolk height was measured with the spherometer while the yolk diameter was measured with the venier caliper.

Shell thickness: This was measured with the aid of the micrometer screw gauge. The egg shell thickness was measured at three points namely, the broad region, the equatorial region and narrow region. The average was used.

Nutrient retention: Twelve (12) pullets of average body weights from each groups (A and B) at 32 weeks old were selected respectively and placed in a separate battery cage compartments to serve as metabolic cage. Each selected birds was fed the dietary treatments for a period of three (3) days after which the weight of feed offer, left over and feacal output were recorded. Feed intake record was deduced from the difference between the weights of feed offer and left over. Proximate analysis (i.e. crude protein, crude fat, crude fiber and ash) of the experimental diets and feacal sample was carried out according to the Association of Official Analytical Chemist (AOAC, 1990).

Statistical Analysis

Data obtained were subjected to Analysis of Variance (ANOVA) using Genstat 5, Release 3.2 (2nd Edition) statistical software. Means were separated by Duncan New Multiple Range Test (Duncan, 1955).

RESULTS

Average feed to gain ratio, % production, weight gain, egg weight, feed per dozen egg, feed cost per dozen egg and the haugh unit were not influenced by dietary inclusion levels of Alphamune G ($p>0.05$) . However, feed intake, yolk height, egg shell thickness and yolk index were significantly affected by the treatments ($p<0.05$) (Table 2). Between groups, % hen day production and feed cost per dozen egg were significantly different ($p<0.05$) (Table 3). There were significant differences ($p<0.05$) in the interaction effects of Alphamune G and age groups on egg thickness, haugh unit, feed to gain ratio and feed intake (Table 4).

Laying hens in group A fed the control diet had the highest value for average weight gain, average egg weight and average shell thickness.

Table 1: Ingredients and nutrient composition experimental diets fed to growing pullets to laying hen administered graded levels of Alphamune G

Ingredients	Prelay Diet	Layer Diet
Maize	46.42	58.00
Corn Bran	12.50	5.00
Wheat Bran	11.00	7.00
Palm Kernel Cake	6.00	0
Fish Meal 68%	1.50	2.00
Soyabean Meal	15.00	18.00
Oyster Shell	4.65	2.00
Bone Meal	2.20	7.30
Vitamin Premix	0.25	0.25
Lysine	0.1	0.1
Methionine	0.1	0.1
Salt	0.27	0.25
Total	100	100
Calculated Nutrient Composition (%)		
Metabolizable Energy(Mekcal/Kg)	2661	2761
Crude Protein	16.1	16.1
Lysine	0.72	0.78
Methionine	0.27	0.29
Calcium	2.5	3.46
Phosphorus	0.83	0.77

Birds in group B fed 0.04% Alphamune G had the highest values for average feed intake and average feed cost per dozen eggs, while average percentage hen day production was the least at this level. Laying hens in group A fed 0.05% Alphamune G had the least value in average weight gain and average feed to gain ratio, while those of 15 weeks at the onset of the experiment fed similar dietary level of Alphamune G had the highest value in average feed to gain ratio, average feed per dozen eggs and yolk index. Yolk index value was lowest in birds of 19 weeks old fed 0.04% Alphamune G, while the least value for average feed intake value was observed in laying pullets of 15 weeks old at the onset of the experiment fed 0.05% Alphamune G. Birds of 19 weeks old at the inception of the experiment fed 0.06% Alphamune G had the highest value in % hen day production as well as the least values for average feed per dozen egg and average feed cost per dozen egg. The lowest value for average egg weight was observed in birds of 15 weeks old at the onset of the experiment fed 0.06% Alphamune G and birds of 19 weeks old fed 0.05% Alphamune G for average shell thickness. Haugh unit value was highest in birds of 15 weeks old fed 0.04% Alphamune G and the least value for similar birds fed 0.06% Alphamune G (Table 4).

There were significant differences ($p<0.05$) for all nutrient retention parameters analyzed in the experiment. The control diet had the highest value in % crude protein retention, % crude fat retention, % crude fibre retention and % ash retention. Lowest value of % crude protein retention was observed in 0.06% Alphamune G while 0.04% Alphamune G had the least values in % crude fat retention, %crude fibre retention and % ash retention (Table 2). For all the nutrient parameters analyzed, growing to laying pullets of age 15 weeks old at the onset of the experiment had higher value than those of age 19 weeks old at the onset of the experiment (Table 3). It was also observed that birds of age 15 weeks old at the onset of the experiment fed the control diet had the highest values in all % nutrient retention parameters for the interaction of Alphamune G and age at which Alphamune G was fed while birds of age 19 weeks old fed 0.04% Alphamune G diet had the least values for % crude fibre retention, % crude fat retention and % ash retention. Laying pullets of age 19 weeks old fed 0.05% Alphamune G had the least value for % crude protein retention. Percentage crude protein retention had the highest value in laying pullets of age 15 weeks old at the onset of the experiment fed the control diet while the least value was recorded in birds of age 19 weeks old fed 0.05% Alphamune G diet (Table 4).

DISCUSSION

Results on weight gain were different from the report of Bolu *et al.* (2009 a,b). However, the recommended 0.04% dietary inclusion of Alphamune G also gave the highest weight gain

Table 2: Effects of Alphamune G and age on production characteristics, egg quality traits and nutrient retention of growing pullets to laying hen

Parameters	Age Difference	
	A	B
Average Feed Intake(g/bird/day)	86.83 ± 0.73	85.59 ± 0.86
Average Weight Gain (g/bird/day)	4.63 ± 0.74	4.39 ± 0.62
Feed To Gain Ratio	13.10 ± 3.02	16.30 ± 2.86
%HDP	47.00 ± 2.46^{b}	37.80 ± 2.11^{a}
Feed(g)/Dozen Egg	2.34 ± 0.56	2.51 ± 0.95
Feed Cost(₦)/Dozen Egg	159.00 ± 2.46^{a}	215.00 ± 4.82^{b}
Egg Weight(g)	56.91 ± 0.42	55.92 ± 0.70
Shell Thickness (mm)	0.31 ± 0.02	0.32 ± 0.01
Yolk Index	0.42 ± 0.04	0.45 ± 0.03
Haugh Unit	78.70 ± 1.87	79.30 ± 1.42
Protein Retention (%)	48.25 ± 3.28^{a}	56.72 ± 2.56^{b}
Fat Retention (%)	44.58 ± 4.64^{a}	54.35 ± 2.86^{b}
Fibre Retention (%)	-3.40 ± 2.20^{a}	20.30 ± 3.48^{b}
Ash Retention (%)	-45.13 ± 1.42^{a}	-18.65 ± 2.26^{b}

a,b-means followed by the same superscript letter in the same row; are not significantly different (P>0.05).

Table 3: Effects of graded levels of Alphamune G on production characteristics, egg quality traits and % nutrient retention of growing pullets to laying hen

Parameters	Alphamune G Levels			
	0.00%	0.04%	0.05%	0.06%
Feed Intake(g/bird/day)	86.53 ± 1.03^{a}	88.80 ± 10.10^{b}	83.87±2.54^{a}	85.64±1.26^{a}
Weight Gain (g/bird/day)	5.30 ± 2.46	5.47 ± 1.88	3.60±2.16	3.67±2.12
Feed to Gain Ratio	11.8 ± 4.26	16.9 ± 2.24	13.8±3.24	16.4±2.18
Hen Day Production (%)	42.7 ± 2.36	40.2 ± 3.56	39.8±5.48	47.0±4.38
Feed(kg) Per Dozen Eggs	2.65 ± 1.22	2.84 ±1.35	2.77±1.26	2.43±1.34
Average Feed Cost(₦)/Dozen Egg	180.00 ± 20.34	215.00 ± 23.12	188±22.56	165±23.15
Egg Weight(g)	57.39 ± 0.93	56.22 ± 0.56	56.77±0.64	55.29±0.72
Shell Thickness(mm)	0.34 ± 0.01^{b}	0.31± 0.01^{a}	0.31±0.02^{a}	0.31±0.01^{a}
Yolk Index	0.43 ± 0.05	0.43 ± 0.04	0.48±0.05	0.41±0.04
Haugh Unit	78.3 ± 4.58	80.20 ± 2.56	81.9±2.65	75.6±3.86
Protein Retention (%)	68.30 ± 4.52^{b}	47.55 ± 2.55^{b}	48.60±2.48^{b}	45.50±2.24^{a}
Fat Retention (%)	63.80 ± 3.86^{c}	39.45 ± 4.56^{a}	41.70±3.36^{a}	52.90±5.44^{b}
Fibre Retention (%)	35.20 ± 3.38^{d}	-8.40 ± 2.12^{a}	0.0±0.02^{b}	6.8±2.56^{c}
Ash Retention	9.65 ± 2.34^{c}	-50.40 ± 1.01^{a}	-41.85±3.28^{b}	-42.95±2.56^{b}

a,b-means followed by the same superscript letter in the same row are not significantly different (P>0.05).

in this study was similar to that reported by Bolu *et al.* (2009a) in broiler chicks. Cockerel chicks fed similar dietary inclusion level had best performance at 0.06% (Bolu *et al.,* 2009b). Increase in weight gain in laying birds fed higher doses of Alphamune G from this study agreed with the report that cumulative weight gain is a function of nutrition and that Alphamune G, in the same vein, yeast cell complex has been reported to improve feed conversion efficiency and increased body weight in chickens (Zhang *et al.,* 2005; Bolu *et al.,* 2009 a, b). Body weight, although not considered as an important laying parameter, controls feed intake and egg size. Body weight has a dramatic effect on egg size; large birds at maturity can be expected to produce large eggs throughout their laying cycle (Leeson and Summers, 2005). Results on effects of age at Alphamune G supplementation on feed intake and % hen day production agreed with the report by Leeson and Summers (2005) indicated that feed intake vary with age and egg size of the birds. However, Applegate *et al.* (1999), Schafer *et al.* (2006) and Yasmeen *et al.* (2008) reported that age of laying birds did not affect feed intake.

Table 4: Interactions of Alphamune G and Age on production characteristics, egg quality traits and percentage nutrient retention of growing pullets to laying hen

Alphamune Levels (%)	Age	Parameters						
		Average feed intake (g/bird/day)	Average weight gain (g/bird/day)	Feed To gain ratio	% HDP	Feed (g) per dozen egg	Feed cost(₦) per dozen egg	Average egg weight (g)
0.00	**A**	87.40^{b}	6.12	15.6^{a}	44.0	2587	176	59.90
	B	85.67^{a}	4.48	7.9^{a}	41.5	2717	185	54.88
0.04	**A**	86.30^{a}	5.35	16.7^{a}	48.5	2175	148	55.20
	B	91.30^{b}	5.60	17.1^{a}	31.9	9523	283	57.23
0.05	**A**	85.47^{a}	2.40	0.60^{a}	42.4	2572	175	55.68
	B	82.28^{a}	4.80	27.1^{b}	37.2	2970	202	57.85
0.06	**A**	88.17^{b}	4.67	19.6^{a}	53.1	2044	139	56.85
	B	83.12^{a}	2.68	13.3^{a}	40.8	2820	192	53.73
SEM		1.462	1.240	6.04	4.92	1917	32.7	1.309
Alphamune Levels (%)	**Age**	**Average shell thickness (mm)**	**Average yolk index**	**Average Haugh unit**	**% crude protein retention**	**% crude fat retention**	**% crude fiber retention**	**% crude ash retention**
		0.3617^{b}	0.4317	79.9^{a}	67.5^{b}	59.6^{b}	30.3^{b}	4.5^{b}
	B	0.3217^{a}	0.4300	76.7^{a}	69.1^{b}	66^{b}	40.2^{b}	14.8^{b}
0.04	**A**	0.2967^{a}	0.4117	73.1^{a}	36.5^{a}	26.1^{a}	-32.2^{a}	-82.5^{a}
	B	0.3300^{b}	0.4633	87.3^{b}	58.6^{b}	52.8^{a}	15.4^{b}	-18.3^{b}
0.05	**A**	0.2950^{a}	0.4433	78.0^{a}	35.9^{a}	32.2^{b}	-25.2^{b}	-77.4^{b}
	B	0.3283^{b}	0.5167	85.9^{b}	61.3^{b}	51.2^{b}	25.4^{b}	-6.3^{b}
0.06	**A**	0.3200^{a}	0.4233	83.7^{b}	53.1^{b}	60.4^{b}	13.66^{b}	-25.1^{b}
	B	0.3167^{a}	0.4150	67.5^{a}	37.9^{b}	45.4^{b}	-14.6^{b}	-64.8^{b}
SEM		0.00985	0.02433	3.47	0.329	0.317	0.25	0.298

a,b-means followed by the same superscript letter in the same column are not significantly different (P>0.05). A-Denotes growing to laying pullets of 19 weeks old at the onset of the experiment. B- Denotes growing to laying pullets of 15 weeks old at the onset of the experiment.

In an earlier study *Lactobacillus* (Lacto) administered at 8.8×10^8cfu/g in feed of laying birds significantly improved ($p<0.05$) body weight gain for corn–soybean meal and barley–corn–soybean meal diets by 24% and 21%, respectively, but had no effects on feed consumption and conversion (Nahashon *et al.,* 1994). Combination of *L. acidophilus, L. casei, B. bifidum, A. oryzae, S. faecium and Torulopsis* spp. (probiotics) used as probiotics administered at 2.7×10^5 and 5.4×10^5 cfu/g in laying birds gave a similar effect in body weight gain and feed conversion ratio in terms of feed to gain and feed per dozen eggs (Panda *et al.,* 2003). Similar trend was reported by Nahashon *et al.* (1996) that *Lactobacillus* administered at $4.84\times^{107}$ cfu/g in feed of laying birds significantly increased daily feed consumption by 2.5% during the laying phase. Result of feed intake agreed with the report of Belavi *et al.* (2001) that administering *E. faecium* at 0.5×10^6, 1.0×10^6 and1.5×10^6 cfu/g in feed of laying birds significantly reduced feed consumption and feed conversion. Aghaei *et al.* (2010) reported that both egg production and feed consumption did not differ significantly when dried whey (prebiotics) and probiotics were fed to laying birds. Khan *et al.* (2008) reported that average egg weight was not affected by dietary inclusion of 0, 2, 6 or 8% garlic powder or by 0, 2, 4, 6, 8 or 10% inclusion of garlic paste, over the six-week study period, respectively. In contrast, Yalcin *et al.* (2006) found that egg weight increased when laying hens were fed 5 and 10 g/kg garlic powder supplementation. Yoruk *et al.* (2004) and Panda *et al.* (2003) reported a contrary result of statistical significant increase of egg production in leghorn laying hens during whole laying period. Nahashon *et al.* (1996) showed that using vital biomass of probiotics supplements affect the egg weight significantly. Chen *et al.* (2005a) demonstrated an elongation of both small and large intestine in laying hens receiving fructan supplementation. This was associated with concomitant increased egg production and improved feed efficiency. Moreover, fructan supplementation increased skeletal and plasma calcium levels, resulting in increased egg shell strength (Chen and Chen, 2004) and reduced yolk cholesterol concentrations without affecting yolk weight (Chen *et al.,* 2005b). Alpharma Animal Health (2004) reported that layers fed Alphamune G produced a higher number of eggs and first quality egg than the control. Many factors have been reported to affect haugh units such as storage, time, temperature, age of birds, strain, nutrition and disease (Toussant and Latshaw, 1999). Verheyen and Decuypere (1991) observed on the contrary that haugh unit decrease with increase in age of bird. Atteh (2004) stated that egg size is influenced by strain of the bird, age at first egg, environmental temperature, age of the bird, adequacy of methionine and/or linoleic acid in the ration.

Protein and fat retention results were not similar to the report by Bolu *et al.* (2009) of no statistical significance as well as in the report that protein and fat retention values increased than the control. Once the bird starts to produce eggs, its ability to build fat reserves is greatly limited (Leeson and Summers, 2005).

Conclusion: From the experiment, it was concluded that in term of production characteristics, % nutrient retention and egg quality of experimental birds fed with inclusion levels of Alphamune G performed better than the control except for some variation in results on weight gain and feed to gain ratio. Among those fed inclusion Level of Alphamune G, birds fed inclusion levels of 0.06% performed the best in terms of production characteristics, egg quality and economic value compared to 0.04% and the recommended standard 0.05%. Age at which Alphamune G was supplemented did not have pronounced effects on the parameters measured. Mortality was observed to be least in growing to laying pullets fed diet containing 0.06% Alphamune G. From the result it can be recommended that the use of Alphamune G at a higher level of 0.06% can still give a better result in terms of egg production, egg quality, livability and economic value. It is also recommended that Alphamune G can be included in diet of growing pullets at 15 weeks old.

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GROSS AND HISTOMORPHOLOGICAL ASSESSMENT OF THE OROPHARYNX AND TONGUE OF THE GUINEA FOWL (*Numida meleagris*)

IGWEBUIKE, Udensi M. and ANAGOR, Tochukwu A.
Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria.

Corresponding Author: Igwebuike, U. M. Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria. **Email:** abuchi2002@yahoo.com or udensi.igwebuike@unn.edu.ng **Phone:** +234 8038726150

ABSTRACT

The study investigated the morphology of the oropharynx and tongue of the guinea fowl using gross anatomical and histological techniques. The results showed that the mouth and pharynx of the guinea fowl lacked a definite line of demarcation, and so formed a common oropharyngeal cavity. The roof of the oropharynx was formed by the hard palate and the choana. The hard palate was characterized by a broad v-shaped rostral mucosal swelling, a median palatine ridge that bifurcated caudally into left and right lateral palatine ridges, and para-median rows of caudally pointed conical papillae. The tongue of the guinea fowl was located on the floor of the oropharynx, but did not extend to the full limits of the lower beak. The caudal and rostral parts of the tongue were demarcated by a v-shaped row of papillae, the papillary crest. Histologically, the dorsal surface of the tongue was lined by a non-keratinized stratified squamous epithelium that contained intraepithelial taste buds, while the lining on the ventral surface of the tongue was a keratinized stratified squamous epithelium. Other features include a wide sub-epithelial connective tissue layer containing lingual glands, and a core of striated muscles. The lingual glands of the guinea fowl consisted of tubular secretory units made up of mucus-secreting cells. These findings may be important in nutritional and medical management of guinea fowls especially under the intensive system of production. Furthermore, our study has provided a foundation for recognition of pathology in the oropharynx and tongue of the guinea fowl.

Keywords: Guinea fowl, *Numida meleagris,* Gross anatomy, Oropharyngeal cavity, Hard palate, Tongue, Lingual gland

INTRODUCTION

Guinea fowl production is gaining some attention among smallholder farmers as an alternative source of meat protein, eggs and income (Abubakar *et al.,* 2008; Obike *et al.,* 2011). This is particularly because the guinea fowl has been reported to have some advantages over the chicken. Such advantages include greater disease resistance, greater ability to scavenge for food and higher meat-to-bone ratio (Kozaczynski, 1998). Intensive management of guinea fowl production is a relatively new enterprise in Nigeria, and efforts to position this emerging industry on a sound financial basis may be hamstrung by lack of basic knowledge of the biology of these birds. In the present time, most scientific studies are conducted on the chicken, while the same physiological responses are assumed in the guinea fowl. However, accumulation of knowledge on the biology of the guinea fowl,

especially data on the morphology of components of the digestive tract would prove useful in relation to the nutritional and medical management of these birds. Furthermore, specific information on the anatomy of the oropharynx and tongue of the guinea fowl is important to identify structural features that may influence food intake and ingestion, as well as to provide a foundation for the recognition of pathology in this region of the bird.

Although many studies have attempted to describe the morphology of the avian oropharynx and tongue (Jackowiak and Godynicki, 2005; Crole and Soley, 2008; Igwebuike and Eze, 2010; Tivane *et al.,* 2011; Erdogan and Alan, 2012), specific information on the anatomy of the oropharynx and tongue of the guinea fowl is yet very scanty. The objective of the present study is to investigate the morphology of the oropharynx and tongue of the guinea fowl using gross anatomical and light microscopic techniques.

MATERIALS AND METHODS

Experimental Animals: All procedures involving animals were conducted according to the guidelines for the protection of animal welfare in the University of Nigeria, Nsukka. The ten adult guinea fowls used for this study were obtained from local small-holder farmers in Enugu-Ezike, Igbo-Eze North Local Government Area, Enugu State, Nigeria. The birds were sacrificed by euthanasia using overdose intravenous injection of ketamine (4 ml of ketamine per bird).

Gross Anatomy: Following death, components of the digestive tract located within the head region were dissected and studied in terms of their shape, physical appearance and *in-situ* topographical relationships. Gross photographs were captured using a Yashica 7.1 mega pixels digital camera.

Histological Preparations: Specimens of the tongue were cut and fixed by immersion in Bouin's fluid for 48 hours. Later, these specimens were dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin wax. The 5 µm thick sections were cut, mounted on glass slides, and stained routinely with Haematoxylin and Eosin (H&E) for light microscopy (Nickel *et al.,* 1977). Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic China Group Limited).

RESULTS

Gross Anatomy: The mouth and pharynx of the guinea fowl did not show any definite line of demarcation, but formed a common oropharyngeal cavity. The oropharyngeal cavity was bounded dorsally by the oropharyngeal roof comprising the hard palate and choana, and ventrally by the tongue and floor of the oropharynx. The maxillary ramphotheca formed the lateral borders of the roof of the oropharyngeal cavity (Figure 1).

The right and left maxillary ramphotheca merged rostrally to form the maxillary rostrum. Immediately caudal to the maxillary rostrum was a broad v-shaped mucosal swelling on the hard palate. The two arms of the v-shaped mucosal swelling extended caudally to mark the lateral boundaries of the rostral ⅓ of the palate. A median palatine ridge situated in the median plane, separated the left and right arms of the v-shaped mucosal swelling.

Caudally, the median palatine ridge bifurcated into left and right lateral palatine ridges. A choanal slit (Figure 1) was apparent as a long single opening that extended from the middle to the caudal aspects of the oropharyngeal roof. This opening, which was in the median plane, was characterized by a narrow tubular rostral portion and a broad rounded caudal portion. The lumen of the choanal slit was partially demarcated into left and right compartments by a median ridge. Para-median rows of caudally pointed conical papillae occurred on the palatine mucosal surface between the choanal slit and the lateral palatine ridges. There were usually five or six rows of papillae on the left and right sides. The last row (the most-caudal row) contained numerous papillae that were especially large and prominent.

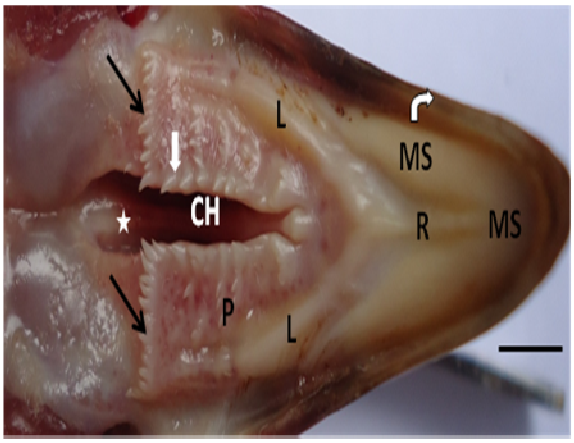

Figure 1: Roof of the oropharynx of a guinea fowl showing the maxillary ramphotheca (curved arrow), broad v-shaped rostral mucosal swelling (MS) on the palate, median palatine ridge (R), and lateral palatine ridges (L). The choanal slit (CH) is partially demarcated into two compartments by a median ridge (star). Caudally pointed papillae (white arrow) occur on the margins of the choanal opening. Note the rows of paramedian conical papillae (P). The most caudal row (black arrows) consists of especially prominent papillae. Scale bar = 3 cm.

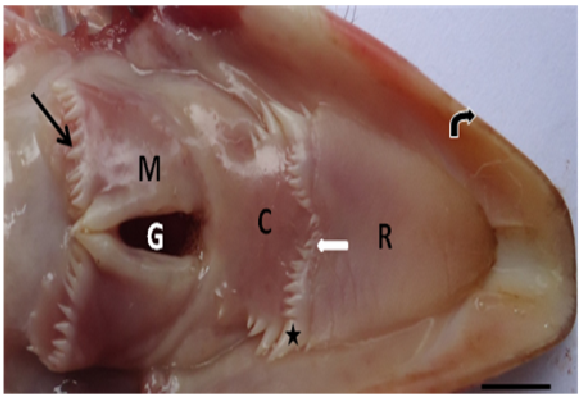

Figure 2: Floor of the oropharynx of a guinea fowl showing the mandibular ramphotheca (curved arrow), glottis (G) and laryngeal mound (M) whose caudal border exhibits a row of conical papillae (black arrow). The caudal (C) and rostral (R) parts of the tongue are separated by a v-shaped row of conical papillae (white arrow). Note different sizes of lateral lingual papillae (star) associated with the caudal part of the tongue. Scale bar = 3 cm.

Similarly, the margins of the tubular rostral portion of the choanal opening exhibited caudally pointed papillae, but these were absent in the broad, rounded caudal part of the choana.

The floor of the oropharyngeal cavity of the guinea fowl presented as a concave depression between the rami of the lower beak (Figure 2).

The mandibular ramphotheca that formed the lateral boundaries of the oropharyngeal floor followed the contours of the mandibular rami, and converged rostrally to form the mandibular rostrum. The tongue of the guinea fowl was located on the floor of the oropharynx. It was a relatively small organ that did not extend to the lateral and rostral margins of the lower beak. A v-shaped transverse row of caudally pointed conical papillae demarcated the rostral and caudal parts of the tongue. Whereas the rostral tongue body appeared arrow-shaped and lacked lingual papillae, the lateral margins of the caudal part of the tongue exhibited prominent conical lingual papillae of various sizes. The largest of these lateral lingual papillae were the most-rostral, while the smallest were the most-caudal (Figure 2).

The laryngeal mound was situated caudal to the tongue, and it was associated with a large opening, the glottis. A prominent row of caudally pointed papillae was evident on the caudal border of the laryngeal mound.

Histomorphology: The dorsal surface of the tongue of the guinea fowl was lined by a non-keratinized stratified squamous epithelium that exhibited many intraepithelial taste buds (Figure 3). In contrast, the epithelial lining on the ventral surface of the tongue was keratinized stratified squamous epithelium (Figure 4). Underneath these epithelial linings on both the dorsal and ventral surfaces of the tongue was a dense irregular connective tissue that formed the lingual submucosa. The lingual submucosa beneath the dorsal surface epithelium showed presence of numerous lingual glands (Figure 5). Each gland was characterized by tubular secretory units, and opened onto the dorsal surface of the tongue via a duct. Condensed connective tissue sheath surrounded each glandular unit. Connective tissue septa from this sheath demarcated individual tubular secretory acini (Figure 5).

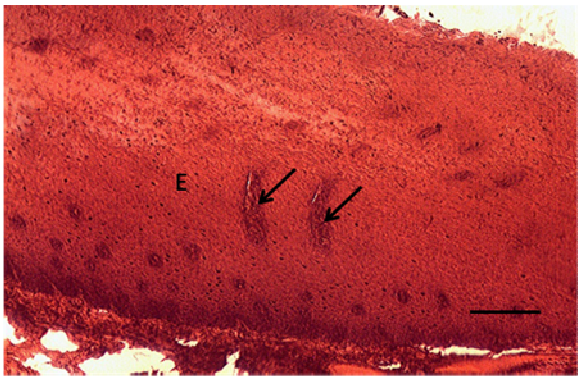

Figure 3: Non-keratinized stratified squamous epithelium (E) on the dorsal surface of the tongue in the guinea fowl. Note the occurrence of intraepithelial taste buds (arrows) in this epithelium. H&E stain, scale bar = 60 µm.

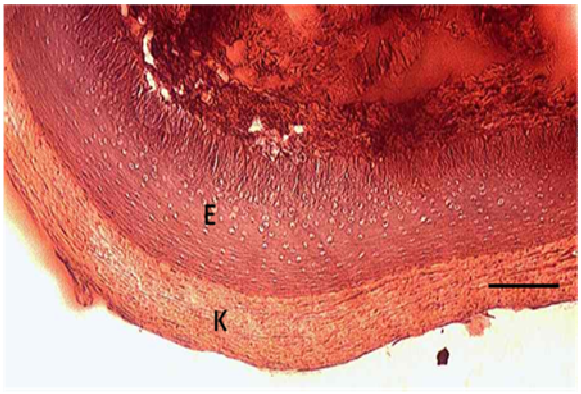

Figure 4: Presence of keratin (K) in the stratified squamous epithelial lining (E) of the ventral surface of the tongue in the guinea fowl. H&E stain, scale bar = 60 µm.

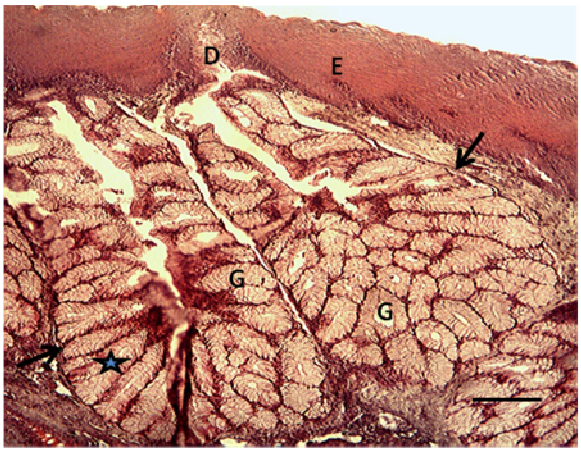

Figure 5: Lingual glands (G) are present in the sub-epithelial connective tissue beneath the dorsal surface epithelium (E) of the guinea fowl's tongue. The glands consist of tubular secretory units (star), and are surrounded by dense connective tissue sheaths (arrows). Note the ducts (D) that lead onto the surface of the tongue. H&E stain, scale bar = 60 µm.

DISCUSSION

Generally, phylogenetic relationships, adaptation to environmental conditions, dietary specialization and feeding habits are thought to determine morphological differences and variations in the anatomy of components of the avian digestive system including the oropharyngeal cavity and tongue. Occurrence of a common oropharyngeal cavity illustrated in the guinea fowl in this study reinforces previous reports in both domestic and wild species of birds (Bacha and Bacha, 2000; Gussekloo, 2006; Igwebuike and Eze, 2010). The ramphotheca is the stratum corneum of the epidermal covering of the beak (Clarke, 1993) and forms the lateral boundaries of the oropharyngeal cavity in the guinea fowl. Rostral convergence of the right and left maxillary ramphotheca in the upper beak, and a similar convergence of the left and right mandibular ramphotheca in the lower beak resulted in a bluntly tapered rostral extremity of the beak that functions as a prehensile organ, and may play significant roles in procurement, handling and incomplete break down of food materials.

The present study demonstrates a broad v-shaped mucosal swelling on the rostral part of the hard palate in the guinea fowl. This feature appears to be unique to the guinea fowl, and has not been reported in other birds. The median and lateral palatine ridges of the guinea fowl are similar to the palatine ridges of chicken (McLelland, 1979). In the rhea (Gussekloo, 2006), emu (Crole and Soley, 2010) and ostrich (Tivane *et al.,* 2011), only a prominent median palatine ridge is present and the lateral palatine ridges are absent.

Partial demarcation of the lumen of the single choanal opening of the guinea fowl into two compartments by a median ridge is comparable to the choana of ostrich (Tivane *et al.,* 2011), but differs from that of chicken (McLelland, 1979) and African pied crow (Igwebuike and Eze, 2010). However, unlike the ostrich (Tivane *et al.,* 2011), herons and ducks (McLelland, 1979) in which the choana is restricted to the caudal part of the roof of the oropharynx, the choanal slit in the guinea fowl

is long and extends from the middle to the caudal aspects of the oropharyngeal roof. Thus, it is similar to what has been described for most birds including bustards (Bailey *et al.,* 1997). Whereas the conical papillae present on the lateral margins of the rostral part of the choanal cleft may serve to protect the choanal entrance (Nickel *et al.,* 1977), the caudally pointed conical papillae on the mucosal surface of the hard palate are thought to aid in unidirectional movement of food bolus towards the pharynx. These papillae are typical in most avian species (McLelland, 1979; Igwebuike and Eze, 2010), but are absent in the rhea (Gussekloo and Bout, 2005) and ostrich (Tivane *et al.,* 2011).

The shape of the tongue in birds is characteristically related to the form of the lower beak and the feeding habits of the particular species (Parchami *et al.,* 2010a,b; Erdogan *et al.,* 2012a). The arrow-shaped tongue of the guinea fowl does not extend to the full limits of the lower beak, and so resembles the tongue of the chicken (Iwasaki and Kobayashi, 1986), quail (Parchami *et al.,* 2010a) and white-tailed eagle (Jackowiak and Godynicki, 2005). Moreover, like the tongue of the chicken (Iwasaki and Kobayashi, 1986), European magpie (Erdogan and Alan, 2012) and penguin (Kobayashi *et al.,* 1998), the guinea fowl's tongue lacks a median sulcus on its dorsal surface. The median sulcus is reported to be prominent in the tongues of some birds such as the white-tailed eagle (Jackowiak and Godynicki, 2005), black kite (Emura, 2008), nutcracker (Jackowiak *et al.,* 2010) and goose (Hassan *et al.,* 2010), but vague and short in the raven (Erdogan and Alan, 2012).

The conical papillary crest that demarcates the rostral and caudal parts of the tongue in the guinea fowl may serve to facilitate movement of food towards the oesophagus, and to prevent regurgitation. A similar explanation may also be adduced for the prominent caudally pointed conical papillae located caudal to the laryngeal mound. The row of papillary crest on the guinea fowl's tongue is characteristically v-shaped in arrangement. Likewise, v-shaped arrangement of the papillary crest is observed in the partridge (Erdogan *et al.,* 2012b), white-tailed eagle (Jackowiak and Godynicki, 2005), quail (Parchami *et al.,* 2010a), goose (Hassan *et al.,* 2010) and zebra finch (Dehkordi *et al.,* 2010), but not in the African pied crow (Igwebuike and Eze, 2010) and raven (Erdogan and Alan, 2012). In addition to the row of papillary crest, laterally located papillae formed by large and small conical papillae were seen in the caudal part of the guinea fowl's tongue in the present study. Similar structures have been demonstrated in magpie and raven (Erdogan and Alan, 2012), golden eagle (Parchami *et al.,* 2010b), black kite (Emura, 2008), chicken (Iwasaki and Kobayashi, 1986) and cormorant (Jackowiak *et al.,* 2006).

Our study shows that in the guinea fowl, the dorsal surface of the tongue is covered by a non-keratinized stratified squamous epithelium, while the ventral surface is lined by a keratinized stratified squamous epithelium. The extent of lingual surface keratinization varies greatly among avian species, and it has been suggested that this may be related to the habitat and type of food consumed by the particular bird (Iwasaki, 2002). Whereas both dorsal and ventral tongue surfaces are covered by keratinized epithelium in the penguin (Kobayashi *et al.,* 1998), only the ventral surface epithelium is keratinized in the Japanese quail (Warner *et al.,* 1967), white-tailed eagle (Jackowiak and Godynicki, 2005) and African pied crow (Igwebuike and Eze, 2010). In contrast, both dorsal and ventral surfaces of the tongue are non-keratinized in the emu (Crole and Soley, 2008) and ostrich (Jackowiak and Ludwig, 2008). Intraepithelial taste buds observed in the dorsal surface epithelium of the guinea fowl's tongue in this study is akin to the reports in chicken (Kudo *et al.,* 2008), bulbul (Al-Mansour and Jarrar, 2004), white-tailed eagle (Jackowiak and Godynicki, 2005), African pied crow (Igwebuike and Eze, 2010) and partridge (Erdogan *et al.,* 2012b). Thus, it is most probable that these birds may exhibit some degree of taste discrimination, which may play an important role in food selection. Although the acuity of taste may vary among avian species, their capacity for taste discrimination may be an important consideration when administering therapeutic drugs via the oral route.

Lingual glands of the guinea fowl are simple branched tubular glands, and the histological features of these structures, including the lightly stained 'foamy' cytoplasm of the secretory cells suggest that these are mucus-secreting glands. It is thought that the most common configuration of avian lingual glands is simple tubular, but branched tubulo-alveolar, alveolar and compound alveolar glandular profiles have also been reported (Crole and Soley, 2010). Moreover, sero-mucous-secreting units were observed in the little egret (Al-Mansour and Jarrar, 2007), while glands composed of serous and sero-mucous units were demonstrated in the chicken (Gargiulo *et al.,* 1991), quail (Taib and Jarrar, 1998; Liman *et al.,* 2001) and chukar partridge (Erdogan *et al.,* 2012b). Obviously, avian lingual glands contribute to the secretion of saliva that provides for a moist environment in the oropharyngeal cavity, and protects the cavity from the activities of microorganisms (Gargiulo *et al.,* 1991). Saliva owes its function to its mucin content (Liman *et al.,* 2001). It has been proposed that salivary mucins establish a barrier between the oral mucosa and bacterial flora, and so forms a protective layer on the oral cavity against desiccation, mechanical damage, external toxic substances and microbial toxins (Samar *et al.,* 2002; Crole and Soley, 2011; Sagoz *et al.,* 2012). Furthermore, secretions of lingual glands may aid in swallowing of food by lubricating the caudal part of the oropharynx and probably, the initial part of the oesophagus as reported in the African pied crow (Igwebuike and Eze, 2010).

In conclusion, our study has provided information on the morphology of the oropharynx and tongue of the guinea fowl, and this may be important in nutritional and medical management of these birds especially under the intensive system of production. In addition, this study has offered a foundation for recognition of pathology in the oropharynx and tongue of the guinea fowl.

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ANIMAL RESEARCH INTERNATIONAL

Volume 10 Number 2, September 2013

Global Impact Factor: 0.562

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