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MOLECULAR DETECTION AND IDENTIFICATION OF PIROPLASMS IN SEMI-INTENSIVELY MANAGED CATTLE FROM ABEOKUTA, NIGERIA

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ABSTRACT

Piroplasmosis is a tick-borne haemolytic disease caused by different species of the *Babesia* and *Theileria* genera. Data on the prevalence of bovine piroplasms and their genetic diversity are scanty in Nigeria. Hence, this study reported the detection of some piroplasms in the blood of cattle in Abeokuta, Nigeria by the polymerase chain reaction (PCR). Blood samples were collected from 252 cattle and subjected to DNA extraction followed by PCR amplification of the partial region of 18S rRNA of the haemoprotozoans. Selected positive amplicons were unidirectionally sequenced and compared to the reference sequences from the Genbank. A total of 220 (87.3 %) cattle were positive for *Theileria velifera* and/or *Babesia bigemina*. The *T. velifera* was detected only in 163 (64.7 %) cattle, while 7 (2.8 %) cattle had a single infection with *B. bigemina*. Fifty cattle (19.8 %) had mixed infections with both parasites. There were no significant differences in piroplasm infections between the ages of cattle for both parasites. There were no significant differences in infection rates between the sexes for *T. velifera*, while the males had a significantly higher ($P < 0.05$) rate of infection for *B. bigemina* than the female cattle. The molecular detection of *Babesia* and *Theileria* species of cattle are reported for the first time in cattle in Abeokuta, Nigeria. This study, which confirmed the endemic nature of the parasites in cattle in the study area, stresses their importance in livestock health and production in Nigeria.

Key words: Abeokuta; *Babesia bigemina*; cattle; Nigeria; piroplasm; 18S rRNA; *Theileria velifera*

INTRODUCTION

Apart from the fact that bovine piroplasmosis poses a threat to cattle production worldwide with a negative impact on protein supply, it is also an important emerging zoonosis affecting human health [15]. Piroplasmosis is a tick-borne disease of domestic and wild animals. The disease in cattle is caused by an intracellular protozoan parasites of the genus *Babesia*, that infect red blood cells (RBC) only,

and *Theileria* species which may be found in white blood cells in addition to RBC [37]. Piroplasms are transmitted by the members of the family *Ixodidae* [36].

Bovine babesiosis is caused mostly by *B. bovis*, *B. bigemina* and *B. divergens* [6, 25]. Other important *Babesia* spp. includes *B. orientalis*, *B. major*, and *B. ovata*. Babesiosis is prevalent all over the world with over half of the global cattle population at risk [7]. *Babesia* species are transmitted by the tick of the genus *Boophilus* (*Rhipicephalus*) spp. [6, 29]. The disease is characterized by fever and severe intravascular haemolysis, leading to anaemia, icterus, haemoglobinuria, neurologic signs in some subjects and death if not attended to in time [21]. The fever during infections may cause pregnant cattle to abort and bulls to show reduced fertility lasting six to eight weeks [9]. The pathogenicity of the parasite is dependent on strain, species, and breed of the infected animal; however, animals that survive from the infection generally become carriers of the parasite and serve as reservoirs for transmission [10]. Theileriosis in cattle is caused by the intracellular protozoan of the genus *Theileria*, especially *Theileria parva* and *Theileria annulata* that cause the complex syndromes known as East Coast Fever (ECF) and tropical (Mediterranean) theileriosis, respectively [34]. *Theileria taurotragi*, *T. mutans* and *T. velifera* are also responsible for bovine theileriosis in Africa [22]. The *Theileria* spp. are closely related to the *Babesia* spp. phylogenetically [5].

In Nigeria, ruminants including cattle contributes more than seventy percent of protein consumed by the populace and singly contributes about 12.7 % of the agricultural Gross Domestic Product (GDP) [19], but the production and establishment of profitable and sustainable cattle ventures is greatly hampered by haemoparasitic diseases among which piroplasmosis is the second most important disease after trypanosomosis [15, 16]. This has made the introduction, adaptation, and multiplication of exotic breeds of cattle in Nigeria a challenge.

None-the-less, there is insufficient data on the prevalence and genotypes of piroplasms that exist in different geographical regions of the world [8]. There is a dearth of information on bovine piroplasmosis in Ogun, a state that shares borders with Benin Republic through which animals are grazed from Burkina Faso, Niger Republic, Mali, Togo, and Cote d'Ivoire into Nigeria. Hitherto, diagnosis and epidemiological research on bovine piroplasmosis in Nigeria were commonly done using conventional parasitological techniques [2, 3, 27, 30], which most of the time could not differentiate different forms of piroplasms.

To date, only one study [22] applied the Polymerase Chain Reaction technique for detection of piroplasms in cattle in the northern part of Nigeria. Also, H a p i et al. [13] recently reported the detection of *Babesia* and *Theileria* species in the blood of cattle sampled from an abattoir in Ibadan, a city in the southern part of Nigeria. To the best of our knowledge, no study has described the molecular characteristics and the genetic diversity of piroplasms present in the blood of cattle in the study area. Here, the prevalence and molecular characteristics of piroplasms in the blood of apparently healthy cattle in the environs of Abeokuta, a south-western city in Nigeria were assessed.

MATERIALS AND METHODS

Sample area, animal, and sample collection

This study was approved by the Ethical Committee, College of Veterinary Medicine, Federal University of Agriculture Abeokuta, Nigeria, before embarking on this project. The ethical clearance number is FUNAAB/COLVET/CREC/2012/032.

This study was carried out in the environs of Abeokuta, Ogun State, South West Nigeria. It borders Lagos State and Atlantic Ocean to the south, Oyo and Osun States to the North, Ondo State to the east and the Republic of Benin to the west. The State lies approximately within latitude 7° 31.98' N and longitude 3° 49.65' E in the humid tropical lowland region [26].

A total of 252 cattle of different sexes and ages were examined and randomly sampled between January and March 2013. All animals were owned by small holder farmers and were apparently healthy at the time of sampling. Based on rostral teeth development [20], the animals were classified as young (less than 2 years old), and adult (two years and above). Animals with a history of treatment with anti-babesial drugs less than two weeks prior to sampling were excluded from the study. Blood samples were collected aseptically from the jugular vein of each cattle into 5 ml tubes containing disodium ethylene diamine tetraacetic acid (EDTA) as an anticoagulant using a sterile needle and syringe. The blood samples were transported on ice packs to the laboratory and were stored at 4 °C prior to analysis.

Genomic DNA extraction and detection of piroplasms by nested Polymerase Chain Reaction

Genomic DNA was extracted from the EDTA-blood using Quick-gDNA™ MiniPrep (Zymo Research Corporation, Irvine, CA 92614, U.S.A.) as previously described [31]. The eluted DNA in 1.5 ml microcentrifuge tubes were stored at –20 °C until use. Three sets of previously published primers targeting the 18S rRNA gene were selected for optimization [14]. The PCR was performed in a 20 µl final reaction volume containing the equivalent of 20 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primers and 1unit of *Thermus aquaticus* DNA polymerase (Bioneer, USA). The reactions were placed in MJ MiniT-Mpersonal cycler (BIORAD, USA). The reaction conditions were as follows: Conventional PCR for piroplasms using primers AF (5'-ACCTGGTTGATCCTGCCAG-3') and BR (5'- CCATTTATTAGCTTTGTTGC-3') involved an initial denaturation at 94 °C for 30 sec followed by 45 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 15 sec, and an extension step at 68 °C for 2 min. This was then followed by a final extension step at 68 °C for 7 min. Nested PCR for *Theileria* spp. using primers AN (5'-GCTTGTCTTAAAGATTAAGCCATGC-3') and BN: (5'CGACTTCTCCTTCCTTTAAGTGATAAG-3') involved an initial denaturation at 94 °C for 30 sec, followed by 45 cycles of denaturation at 94 °C for 15 sec, annealing at 60 °C for 15 sec, and extension at 68 °C for 2 min. This was then followed by a final extension at 68 °C for 7 min. Nested PCR for *Babesia* spp. using the primers BB (5'-GCGTTTATTAGTTCGTTAACC-3') and BN (5'- CGACTTCTCCTTCCTTTAAGTGATAAG-3') involved an initial denaturation at 94 °C for 30 sec, followed by 45 cycles of denaturation 94 °C for 15 sec, annealing at 56 °C for 15 sec, and extension at 68 °C for 2 min. This was then followed by a final extension at 68 °C for 7 min. Ten microliters of the PCR products were electrophoresed through 1.5 % agarose gel in 1 × TAE (40 mM TRIS-acetate and 1 mM EDTA) at 90 V for 45 min. along with 10 µl of biological marker, GENEMateQuanti-Marker, 100 bp DNA ladder (BioExpress, UT, USA). Gels were stained with GelRed(R) Nucleic Acid Stain (PHENIX Research Product, Candler, NC, U.S.A) at 5 µl/100 ml of the agarose gel suspension. Then PCR products were visualized using an ultraviolet transilluminator (SpectrolineR TC 312 E).

Sequencing and phylogenetic analysis

To confirm our results, five PCR products showing expected band sizes for *Babesia* and *Theileria* spp. respectively, were randomly selected and unidirectionally sequenced using the forward primers (AN and BB for *Babesia* and *Theileria* respectively) in a commercial molecular laboratory (Sequetech, Mountain View, California, USA). The obtained sequences were assembled and edited manually using BioEdit® (version 7.0.9.0.) [12]. Search for homologous sequences in Gene bank were performed using BLASTn (www.ncbi.nlm.nih.gov/BLAST/). The sequences were aligned with each other and published 18S rRNA gene sequences of *Babesia* spp. and *Theileria* spp. using the Molecular Evolutionary Genetic Analysis (MEGA 5.05) software [32]. Phylogenetic trees were constructed using the Maximum Likelihood (ML) and Unweighted (UPGMA) algorithms of the phylogeny program of MEGA 5.05 [32]. *Plasmodium falciparum* (AJ250700) was used as the out group. The bootstrap confidence interval of the tree was determined based on l000 replicates.

Statistical analysis

The raw data were entered into a Microsoft Excel spread sheet and descriptive statistics used to summarize the data. SPSS 16.0 was used for the data analysis. The prevalence of piroplasm infection was compared using chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

Animals

Of the 252 cattle sampled, males were 95, while females were 157. As regards age, 117 cattle were young while 135 cattle were adults.

Detection of piroplasms by PCR

The electrophoresed DNA products after amplification with the sets of primary and secondary primers revealed amplicon sizes of 1467 bp and 1588 bp corresponding to expected band sizes of *Babesia* and *Theileria* species 18S rRNA respectively [14]. Out of 252 blood samples examined, piroplasm DNA was detected in the blood of 220 (87.3 %) of the cattle examined. Of these, 163 (64.7 %) accounted for *Theileria* spp. only, 7 (2.8 %) for *Babesia* only and 50 (19.8 %) had co-infection with both *Theileria* and *Babesia*.

Age, and gender variations among cattle infected with piroplasms

Table 1 reveals the effects of age and sex on the prevalence of the parasites. As regards age, the young cattle had a significantly higher *Babesia* infection than adults while there was no significant difference in *Theileria* infection between the two age groups. Male cattle were more predisposed to infection than females for *Babesia* while no difference was observed between the sexes for *Theileria*.

Table 1. Effect of age and sex on the prevalence of *Babesia* and *Theileria* in cattle

Parameters	Number of cattle	*Babesia* species Number positive [%]	*Theileria* species Number positive [%]
Age			
Young	117	33 (28.2)[a]	102 (87.2)[a]
Adult	135	24 (0.18)[b]	111 (82.2)[a]
Sex			
Male	95	32 (33.7)[a]	84 (88.4)[a]
Female	157	25 (15.9)[b]	129 (82.2)[a]
Total	**252**	**57 (22.6)**	**213 (84.5)**

In each parameter, the values with different superscript in each column show significant differences ($P < 0.05$)

Sequences and phylogenetic analysis

Three and four readable sequences of *Babesia* and *Theileria*, respectively were analysed in this study. The homology search revealed the partial sequences of 18S rRNA gene of *Theileria* spp. from this study had 97 % to 98 % homology with *T. velifera* with accession numbers KU206307 and FJ869897, respectively from Uganda and Mozambique. That of *Babesia* spp. had homology scores of between 95—98 % with those sequences of *B. bigemina* available in the GenBank database. The partial sequences lengths of *T. velifera* ranged from 618 to 985 bp with a mean G/C content of 44.43 %.

Phylogenetic analysis carried out involved those partial sequences of 18S rRNA gene obtained from GenBank database. These included: *Theileria velifera* (AF097993, JN572705) *Theileria ovis* (AY508455), *Theileria cervi* (AY735122), *Theileria* spp. (U97054), *Theileria capreoli* (AY726011), *Theileria* spp. Giraffe (FJ213582), *Theileria mutans* (FJ869898, AF078815, KU206320 and JN572698), *B. bigemina* (HQ264113, JN714975, HQ840959, AY603402, FJ426361, EF458191, JQ437264, EF458206 and DQ785311) and *B. bovis* sequences (L19078 and L19077). The tree was rooted on *Plasmodium* 18S rRNA gene sequences as an out-group.

Alignment and phylogenetic analysis of the sequences of *T. velifera* and *B. bigemina* from this study and those from GenBank revealed that the two genera clustered to form two separate clades (Fig. 1). The phylogenetic trees inferred by both algorithms exhibited almost the same topology placing the sequences of *T. velifera* isolates from this study in the same clade with *T. velifera* sequences from Africa (AF097993 and KU206307) with the *T. mutans* separated into different cluster. All the sequences of *B. bigemina* from this study clustered in the same clade with those from Europe (DQ785311, FJ426361), USA (HQ264113, EF458206) and Asia (JN714975, HQ840959). Also, one each of the autochthonous *B. bigemina* and *T. velifera* sequences clustered into separate taxa with very high nodal value within the clades formed by *B. bigemina* and *T. velifera*.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (>50 % cut-off). *B. bigemina* (ABKN 1-3) and *T. velifera* (ABKN 2-5) are those piroplasms detected in this study.

DISCUSSION

Limited data exist on the epidemiology of piroplasms of cattle in Nigeria, despite being a major disease of cattle. This study provided some useful data on the prevalence and genetic characteristics of some haemoparasites of cattle in Abeokuta, Nigeria. The presence of *Babesia* and *Theileria* in the blood of cattle in Abeokuta were detected using conventional and nested PCR protocol and sequences analysis of amplified partial region of 18S rRNA gene.

The White Fulani breed of cattle accounted for most of the sampled animals. The high numbers of the breed could be due to their tolerance to the highly endemic trypanosomosis coupled with their high reproductive and feed conversion rate [28, 33].

The prevalence of piroplasmosis due to *Babesia* spp. and or *Theileria* spp. reported in this study is higher than previously reported in Nigeria [2, 3, 18, 27]. These investigators employed microscopy techniques for their study. Differentiating piroplasms based only on morphology

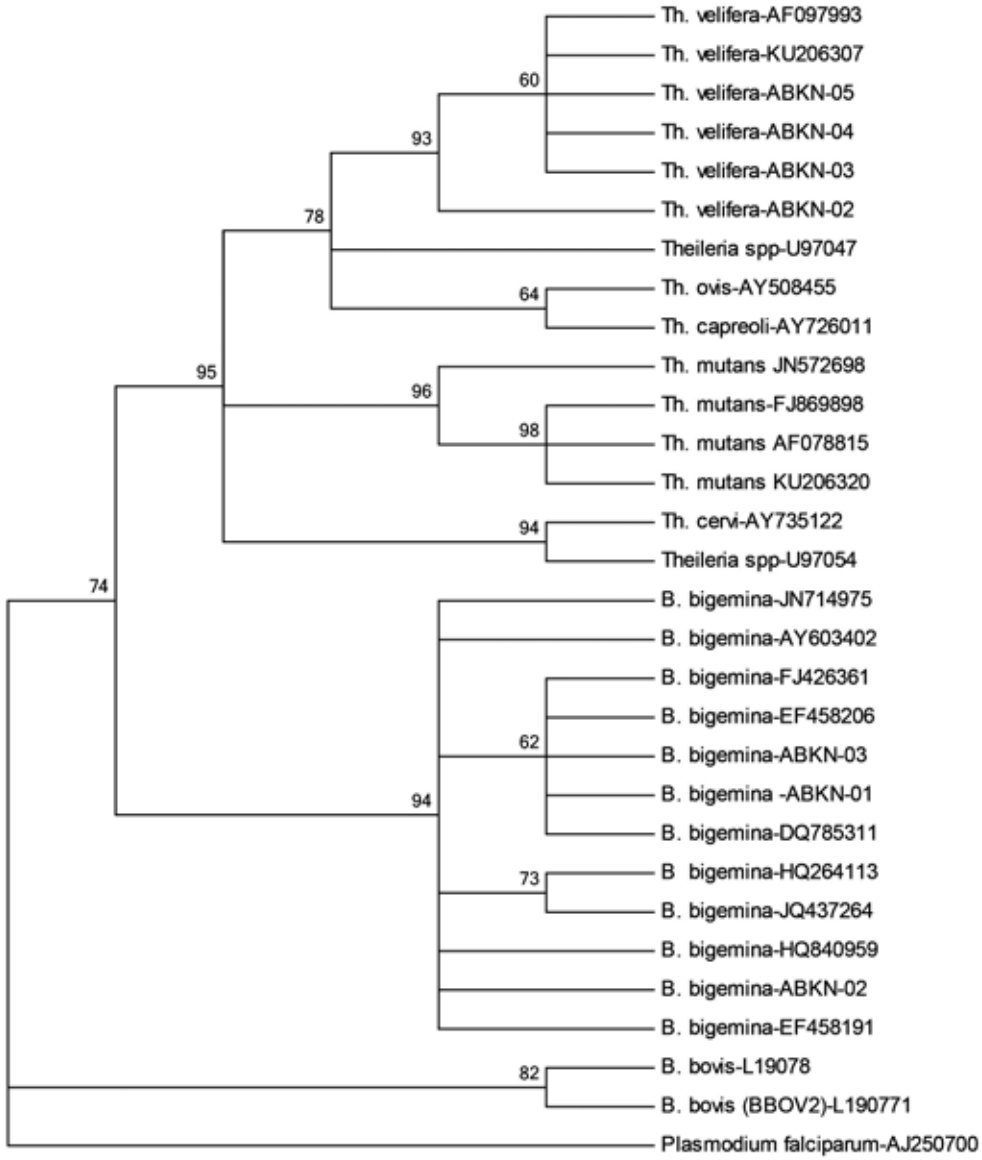

Fig. 1. Evolutionary relationship of piroplasms (*B. bigemina* and T. *velifera*) detected in cattle from Abeokuta, Nigeria, using partial sequences of 18S rRNA gene inferred with Maximum Likelihood (ML) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in MEGA 5.05. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (> 50 % cut-off). *B. bigemina* (ABKN 1-3) and *T. velifera* (ABKN 2-5) are those piroplasms detected in this study

using microscopy is often challenging because these two parasites share a similar morphology, making identification particularly difficult if mixed infections occur. Also, the identification of *Babesia* parasites is difficult at the onset of the disease and in carrier animals due to the low number of the parasites present [23] which may lead to a false negative diagnosis [4]. In comparison to our findings, L o r u s s o et al. [22] recorded a lower prevalence of *B. bigemina* (7.9 %) and *T. velifera* (52.4 %) in cattle from the northern part of Nigeria. In another study, H a p i et al. [13] amplified the 18S rRNA region of piroplasms detected in the blood of slaughtered cows in Ibadan, Nigeria and recorded a prevalence of 46 % for *Babesia*/*Theileria* spp. The high prevalence of *T. velifera* in this study, and that of L o r u s s o et al. [22], supports the assertion of the latter workers that the parasite is endemically established or stable in Nigeria.

In the present study, young cattle (less than two years old) had a significantly higher rate of infection with *Babesia* spp. compared to the adult cattle. As for the infection with *Theileria*, no significant association with age was noticed. In contrast to our findings, most studies suggest that

younger cattle (less than 1 year old) have lower infection rates than older ones, probably due to the protective effects of maternal immunity [1, 13, 22]. The wider age group in this study might have obfuscated the effect of age on the prevalence of *Babesia* species.

Male cattle had a higher rate of *Babesia* species infection in this study which agrees with the report of H a p p i et al. [13] who also noted a higher prevalence of piroplasms (*Babesia/Theileria*) in male cattle. Unlike *Babesia*, there was no significant difference in infection with *Theileria* spp. between males and females in our study.

In our study, the overall prevalence of piroplasms was 87.3 %. The high prevalence may reflect a high endemic level of blood parasites of cattle in the Abeokuta environs. This could indicate that cattle in the studied area were exposed to a high and continuous challenge with piroplasms or that these species were harboured for long periods at detectable levels post-infection. Endemic (or enzootic) stability refers to a state of high level of challenge with haemoparasite-infected ticks and a concurrent low incidence of clinical disease in the host [17]. It may also mean that the vectors of the parasite breed well in this environment. During our study, ticks were present on most of the cattle sampled.

The polymorphism shown by the multiple alignment analysis of the autochthonous sequences may indicate continuous genetic exchange between these parasites in the tick gut or a gene reassortment within the mammalian host in which asexual reproduction takes place [11, 24]. Although 18S rRNA gene has been studied extensively to understand the genetic diversity of piroplasms around the world, this is, to the best of our knowledge, the first time it is being used to shed light on the genetic diversity of bovine piroplasms in Abeokuta, Nigeria. None-theless, for a better understanding of this diversity among the piroplasms circulating in the cattle population in Nigeria, there is a need to employ the analysis of other gene regions such as cytochrome C oxidase subunit III (COX3) and Mitochondria cytochrome B (COB) genes which have been claimed to give an improved phylogeny of piroplasms in China [35].

Although the pathogenicity of *T. velifera* has not been studied extensively in Nigeria, the suggestion of more than one strain by phylogenetic analysis calls for further studies to elucidate the strain distribution and associated pathogenicity in cattle in Nigeria.

CONCLUSIONS

This study confirmed the presence of piroplasms, notably *B. bigemina* and *T. velifera* in the blood of cattle in the study area with *T. velifera* being more prevalent. The results of our study have provided additional molecular data on the epidemiology of piroplasms of cattle in Nigeria. The information may be useful for the diagnosis and effective formulation of control strategies. Further studies covering all regions of Nigeria should be conducted to generate and co mpare the prevalence data for the distribution of tick transmitted parasites in livestock, especially cattle in the country.

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MOLECULAR DETECTION AND GENETIC ANALYSIS OF *THEILERIA EQUI* DETECTED IN APPARENTLY HEALTHY HORSES IN NIGERIA

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ABSTRACT

Equine theileriosis, an apicomplexan debilitating tick-borne parasitic disease of horses has caused considerable havoc to equine production all over the world. There is a dearth of information on the molecular characteristic of the parasites, *Theileria equi* Laveran, 1901, in Nigeria. Thus, in this study microscopy techniques and PCR were used to detect the *T. equi* of horses in Ogun, Oyo and Lagos States of Nigeria. We also characterized the partial region of 18S ribosomal RNA gene by sequencing and sequences analysis. One hundred and two horses consisting of Argentine 34 (33.3 %), Sudanese 21 (20.6 %) and local breeds 47 (46.1 %) including 2 females and 100 males were randomly sampled from the Polo Clubs in Ibadan, Lagos and from privately owned horse stables in Abeokuta. Blood samples were collected from the jugular vein, thin smears were prepared and stained with a field stain. The DNA was extracted from the blood and a partial region of the 18S ribosomal RNA gene was amplified. The amplified products were sequenced unidirectionally and subjected to phylogenetic analysis with those sequences obtained from the GenBank. Of the 102 horses tested, 12 (11.7 %) were positive for *T. equi* by microscopy which included 9 (19.1 %) local breeds, 2 (5.8 %) Argentine breed and 1 (4.8 %) Sudanese breed. In contrast, 7 (6.8 %) were positive by the PCR method; out of which 5 (10.6 %) of these samples were from the local breed of horses while the remaining 2 (5.8 %) were from the Argentine breed. The Packed

Cell Volume (PCV) of the infected and non-infected horses did not show any significant ($P < 0.05$) difference. The sequences lengths obtained were 311 bp and they had 97.43—98.07 % homologies with available sequences in the GenBank. The phylogenetic analysis of the sequences suggested that the strain of *T. equi* detected in the study area formed a new genotype different from the established genotypes around the world. In conclusion, the prevalence of *T. equi* was very low in the study area and one strain of the parasite may be in circulation among the studied horses.

Key words: equine theileriosis; horse; Nigeria; polymerase chain reaction; *Theileria equi*

INTRODUCTION

Equine theileriosis is an apicomplexan debilitating protozoan parasitic disease of horses, mules, donkeys and zebras. It is caused by a tick-borne intra-cellular protozoan parasite *Theileria equi* Laveran 1901, (formerly *Babesia equi*) [18, 22]. The parasite is transmitted horizontally by ticks of the genera *Rhipicephalus, Dermacentor*, and *Hyalomma*, [25]. *Theileria equi* in their mammalian host exhibits a biphasic life cycle which involves an intra-leucocytic developmental phase that is followed by an intra-erythrocytic stage [20, 24]. During the later phase, the disease is characterized by: fever, anaemia, icterus, hepatomegaly, splenomegaly, intravascular haemolysis, petechial haemorrhages of the mucous surfaces, haemoglobinaemia, and haemoglobinuria [5, 18].

Equine theileriosis has a cosmopolitan distribution, being endemic in most tropical and subtropical areas of the world, as well as in some temperate climatic zones. Once a horse is infected, it becomes a carrier and thus serves as a potential reservoir for the dissemination of the parasite [6, 31] to susceptible animals. The diagnosis of equine theileriosis can be made from a combination of clinical signs, examination of stained blood in a susceptible animal [16], PCR, [19, 31] and serological techniques [1, 8, 31]. While the clinical signs of equine piroplasmosis due to *T. equi* and *Babesia caballi* are not distinguishable, microscopy has been reported to fail in detecting *T. equi* in low parasitaemic horses. These, as well its microscopical techniques failure to differentiate artefacts from the small sized *T. equi* in blood smears makes microscopy not suitable for adequate diagnosis of equine theileriosis. On the other hand, serological methods are generally known not to be able to differentiate between active and previous infections and thus may lead to a false positive diagnosis [12].

Several studies have alluded to the superiority and sensitivity of molecular techniques in the detection and characterization of *T. equi* in the family equidae [4, 9, 15, 17, 21, 28, 29, 30]. In a recent report, W a n g et al. [30] have been able to establish the existent five genotypes (A, B, C, D, E) of *T. equi* around the world but no *T. equi* sequences from Nigeria were included in the analysis.

Equine piroplasmosis due to *B. caballi* and *T. equi* has been detected and reported in Nigeria using microscopic [7, 13, 26] and serological [8] methods but no research effort has been made for the detection and characterization of the species and genotype of *T. equi* in Nigeria using a molecular approach.

This study was conducted to assess the prevalence and molecular characteristic of *T. equi* in naturally infected horses in Nigeria by analysis of partial sequence of 18S ribosomal RNA gene (18S rRNA).

MATERIALS AND METHODS

Study area

The study was carried out in the South West zone of Nigeria which included Abeokuta, Ibadan and Ikeja of Ogun, Oyo and Lagos States, respectively (Fig. 1). Ogun State borders Lagos State to the south and Oyo State to the north. The vegetation pattern of Oyo state is that of rain forest in the south and guinea savannah in the north, Ogun Sate is a swampy rain forest and derived forest vegetation while that of Lagos is mainly a mangrove swamp forest. The average rainfall in the three States are 1468.2 mm, 1470.0 mm and 1631.8 mm for Ogun, Oyo and Lagos States, respectively, with average temperatures of 35.5 °C, 31.5 °C and 30.7 °C, respectively [3].

Samples

One hundred and two horses were randomly selected from two polo clubs, one each from Ibadan and Lagos, and from privately owned horse stables from Ogun State. The breed, sex and the age of the horses were recorded. Blood samples were collected from the jugular vein of each ani-

Fig. 1. Map of Nigeria showing the states where samples were collected from horses

mal into vials containing EDTA as the anticoagulant. The blood was stored and transported in an ice pack to the Veterinary Parasitology Laboratory, Federal University of Agriculture, Abeokuta for analysis. The aliquots of the blood samples meant for PCR were store in –20 ºC freezers until their use for DNA extraction.

Haematology and *Theileria equi* detection by microscopy

The packed cell volume (PCV) of each blood sample was determined by the haematocrit centrifugation techniques. The buffy coat from each capillary tube was expressed on to a clean slide, covered with a slip and checked for heamoflagellate with the x 40 objective lens. A thin blood smear was made, air dried and fixed with methanol after which the slides were reverse stained with Field stains A and B. The stained slides were examined under the x100 objective with oil immersion with the light microscope (Olympus, USA). Demonstrations of pyriform or round shape piroplasm in the red blood cells were taken to be positive for *Theileria* species in the blood samples collected.

DNA extraction

The DNA was extracted from each blood sample using the "Macherey-Nagel's DNA blood kit" (Macherey-Nagel, Germany). The extraction was done as described by the manufacturer as follows: Blood samples were removed from the freezer and allowed to thaw at room temperature. Thereafter, 200 µl of blood and 25 µl of proteinase K were pipetted into 1.5 ml microcentrifuge tubes, after which 200 µl of genomic lysis buffer (Buffer B3) was added to the samples and vortexed vigorously for 10 seconds. The mixtures were incubated at 70 °C for 10 min, after which 210 µl of ethanol was added to each sample and were all vortexed again. For each mixture, one NucleoSpin® Blood Column was taken and placed in a collection tube and the samples were loaded and centrifuged for 1 minute at 11,000 × g, after which the collection tube with flow-through were discarded. The NucleoSpin® Blood Column was placed into a new collection tube (2 ml) and 500 µl binding buffer (Buffer BW) was added and centrifuged for 1 min at 11,000 × g. Again the flow through was discarded and the collection tube reused. The NucleoSpin® Blood Column was placed back into the collection tube and centrifuged for 1 min at 11,000 × g to remove residual ethanol. After the removal of the residual ethanol, the NucleoSpin® Blood Column was placed in a 1.5 ml microcentrifuge tube and 100 µl preheated elution buffer (Buffer BE) was dispensed directly onto the silica membrane and incubated at room temperature for 1 min, after which it was centrifuged for 1 min at 11,000 × g. The eluted DNA concentration was measured using a nanodrop US spec (USA) and stored in –20 °C freezers until use.

***Theileria* DNA detection by PCR**

The 18SrRNA gene of *Theileria* species was targeted for amplification using the primer set designed by A l h a s s a n et al. [2] and modified by S l o b o d a et al. [23]. The forward primer TBM: CTTCAGCACCTTGAGAGAAATC and the reverse primer for *Theileria equi* BE R: TGCCTTAAACTTCCTTGCGAT (Bioneer Inc, South Korea) were used to amplify an approximately 360 bp region of the 18S rRNA gene of *T. equi*. The PCR amplification was carried out in a total volume of 25 µl containing 12.5 µl of 2× master mix (BioLabs, New England USA), 0.5 µl (10 µM) each of the reverse and forward primers, 9 µl nuclease free water and 2 µl of the DNA samples. The amplification was carried out using MJ Mini 48-Well Personal Thermo Cycler

(Bio-Rad Inc., USA) with the following program: an initial denaturation at 94 °C for 5 min, 35 cycles of denaturation, annealing and extension at 94 °C, 58.5 °C and 72 °C for 30 s, 60 s, and 45 s, respectively, and the final extension at 72 °C for 10 min. The DNA extracted from the blood of horse that tested positive for *T. equi* by microscopy and nuclease free water were used as the positive and negative controls in all the reactions, respectively.

Gel electrophoresis, sequencing and sequence analysis

The PCR products were resolved in 1 % ethidium bromide stained agarose dissolved in Tris-acetate-EDTA (TAE) buffer for 60 min at 90 volts and documented under an ultraviolet transilluminator to determine the positivity of the samples. The PCR products that showed the expected band sizes for *T. equi* were unidirectionally sequenced using the reverse primer in Cornell University core laboratory facility, Ithaca, New York. The sequences obtained were manually cleaned, aligned using ClustalW in the software of BioEdit Sequence Alignment Editor and the phylogenetic tree constructed using Neighbour Joining algorithm in MEGA 5.0 software. The obtained sequences reported in this study were deposited and available in the NCBI data base under the accession numbers MN785125—MN785128.

Statistical Analysis

Data obtained for haematology were presented as means ± standard deviations. The differences in mean PCV values were compared using the student T-test in SPSS version 19.

RESULTS

Animals sampled and microscopy

One hundred and two horses consisting of Argentine (34), Sudanese (21) and local (47) breeds were sampled from the polo clubs in Ibadan and Lagos, and from privately owned horse stables in Abeokuta. The privately owned horses were ceremonial and dressed horses; 100 (98.04 %) and 2 (1.96 %) were male and female, respectively. The *Theileria* spp. were detected in thin smears of 12 (11.76 %) of the horses which include 9 (19.1 %) local breeds, 2 (5.8 %) Argentine breed and 1 (4.8 %) Sudanese breed. In contrast, 7 (6.8 %) were positive by PCR, out of which 5 (10.6 %) of these samples were from the local breed of horses, while the remaining 2 (5.8 %) were from the Argentine breed. The horses from Abeokuta had higher incidences and all of the positive samples were from the male horses.

Determination of PCV

The PCV of the horses ranged from 29.6 % to 45.1 %. The Argentine had an average PCV of 32.7 ± 0.6179 %, Sudan had 31.1 ± 0.2526 % and Nigerian local breed had 30.7 ± 0.1035 %. The infected and non-infected horses were not significantly different (P > 0.05) in their PCV values. In general, the mean PCV values of those horses that tested positive and negative for *T. equi* by microscopy were 35.08 % and 32.97 %, respectively and those detected molecularly were 32.86 % and 30.08 %, respectively. These values were not significantly different between those infected and non-infected by *T. equi*.

Molecular detection and sequence analysis

The PCR products from 7 (6.9 %) horses (Table 1) revealed a band size of about 360 bp which was the expected band size for *T. equi* and no sample was positive for the

Table 1. Prevalence of *Theileria equi* in naturally infected horses from three western states in Nigeria detected by the polymerase chain reaction

	Argentine Horses (%)		Local Horses (%)		Sudanese Horses (%)		Total
	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	
Abeokuta	0 (0)	3 (13.6)	4 (18.2)	13 (59.1)	0 (0)	2 (9.1)	22
Ibadan	1 (2.1)	17 (36.2)	1 (2.1)	17 (36.2)	0 (0)	11 (23.4)	47
Lagos	1 (3.0)	12 (36.4)	0 (0)	12 (36.4)	0 (0)	8 (24.2)	33
Total	**2 (1.9)**	**32 (31.4)**	**5 (4.9)**	**42 (41.2)**	**0 (0)**	**21 (20.6)**	**102**

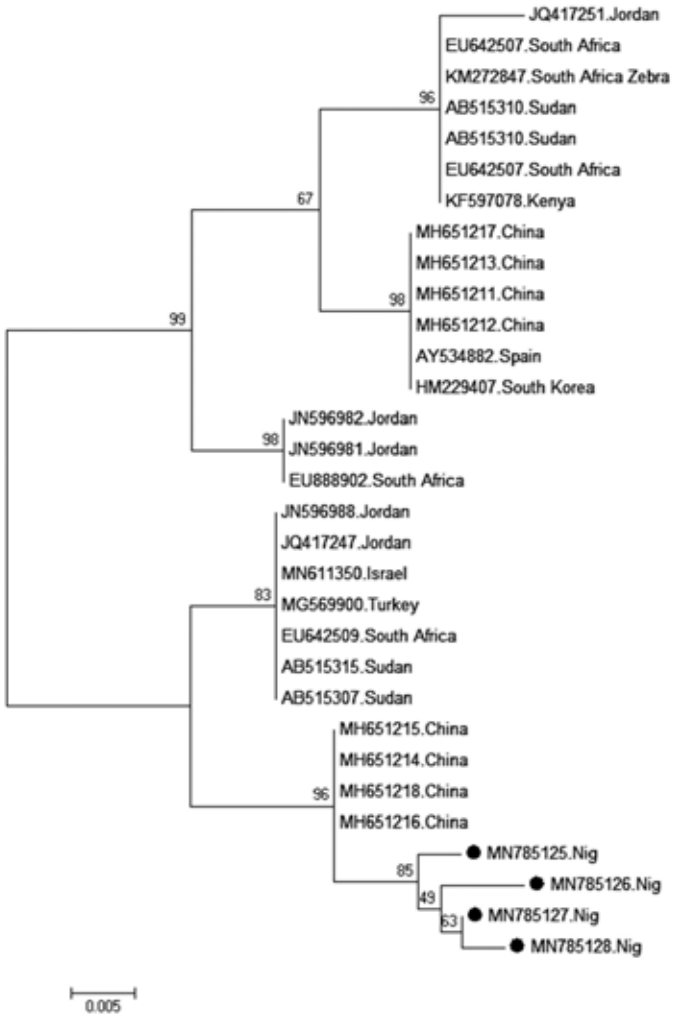

Fig. 2. Phylogenetic analysis of *Theileria equi* 18S rRNA gene partial sequences detected in naturally infected horses from three south western states of Nigeria

The tree was inferred using Neighbour joining algorithms. Numbers above the branches represent bootstrap support from 1000 replications. The sequences from this study are indicated in bold cycle and others from the GenBank database are shown with Accession No., and country of their geographical origin

DNA of *B. caballi*. The sequences obtained were subjected to a BLAST search in the GenBank and the four sequences had 97.43—98.07 % similarity with those available from the 18S rRNA gene sequences in the GenBank (MN611348 and MG052915). Also, pair wise comparison of the sequences from this study revealed a similarity of 99.03—99.35 %. The aligned sequences were less polymorphic except at point 13 where sample 3 and 37 had alteration of A → G and A → C, respectively. The tree inferred by the Neighbour Joining algorithm in the phylogenetic analysis of 18S rRNA gene partial sequences placed the sequences of *T. equi* from this study in a single or monophylectic clade that was completely separated from other sequences around the world, but situated closer to some of the sequences of *T. equi* detected from the Gansu Province in China. Also, to mention that some sequences of Africa origin (Kenya, Sudan, South Africa) are distantly separated from the autochthonous sequences (Fig. 2).

DISCUSSION

Equine theileriosis, a debilitating parasitic disease of horses has been studied extensively using microscopical and serological techniques in Nigeria [8, 13, 14, 27]. Whereas, no study yet assessed the prevalence of equine

theileriosis in the south western part of Nigeria, none has used molecular methods for the detection and genetic characterization of *T. equi* in Nigeria. This study therefore, detected by microscopy the *T. equi* of horses in Ogun, Oyo and Lagos States of Nigeria and characterized the partial region of 18S rRNA gene sequences by sequences analysis. In our study, we confirmed an 11.7 % prevalence of equine theileriosis in the investigated horse population. Our finding was within the range of prevalences that were variously reported in Northern Nigeria [13, 27] but much lower than 77 % and 57 % prevalences reported by M s h e l i a et al. [13] and E m e m u et al. [8], respectively using serological techniques in Nigeria. The higher prevalences by serology may be due to lingering antibodies produced against invading pathogens that had been cleared from the circulation. In contrast, the molecular techniques from this study revealed a prevalence of 7 (6.9 %) that was lower than the value detected by microscopy. The region of the gene targeted for the detection of this parasite has been adjudged to be a very sensitive region for the detection of *T. equi* [10, 11, 23], and as such would be expected to be more sensitive than the microscopy method. Hence, the PCR prevalence result that was lower than that of microscopy may only suggest that those positive samples by microscopy that became negative by PCR were other species of piroplasm in the horse population sampled other than the *T. equi* and *B. caballi* screened in this study. Therefore, future screening for piroplasm of horses should be widened to check for other species. The detection of more *Theileria* in horses from Abeokuta may suggest that horses from polo clubs get more attention than those from privately owned horses in term of regular cleaning, ectoparasites control and grooming by the animal handlers.

The infected horses were slightly anaemic and this observation was in line with our previous report of slightly anaemic horses presented to the Veterinary Teaching Hospital in the study area in Nigeria by T a k e e t et al. [26] and that of Z o b a et al. [32] in Sardinia, Italy. The obtained partial sequences of *T. equi* 18S rRNA gene formed a monophyletic clade that is well separated from other clades on the phylogenetic tree and as such may suggest that only one strain of the parasite was present among the horse population in the study area. Also, the analysis of autochthonous sequences with those analysed by W a n g et al. [30] suggested that the genotype formed by the sequences from this study may be different from the established genotypes around the world [30] and as such assigned genotype F (Fig. 2). This suggestion was strongly supported by the inferred phylogenetic tree that exhibit clustering together by those sequences from this study. Although the number of samples collected in this study may not be robust enough to conclude that only one strain is available in Nigeria, there may be a need to increase the sample population in order to be able to draw an inference with a larger scale.

CONCLUSIONS

In conclusion, this study was the first report of the molecular detection of *T. equi* in Nigeria. The prevalence of *T. equi* was low in the study area and the assessment of the 18S rRNA gene sequences suggested that only one strain of *T. equi* was in circulation among the horse population studied.

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ANAPLASMOSIS IN ANIMALS

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ABSTRACT

Anaplasmosis is a vector-borne, infectious and non-contagious disease. The disease is caused by various pathogens of the genus *Anaplasma*. The different species cause different types of anaplasmosis depending on which cells that are infected in the mammalian host. Anaplasmosis has a wide host range, including humans, and it is distributed worldwide. The zoonotic potential of some species is of great importance in regards to public health concerns. This review presents information about anaplasmosis in animals and its prevalence in Europe, and other countries in the world.

Key words: anaplasmosis; infectious disease; re-emerging; vector-borne disease; zoonosis

INTRODUCTION

Anaplasmosis is caused by bacteria of the genus *Anaplasma*, order *Rickettsiales* which was formed after a merging of the families *Anaplasmataceae* and *Rickettsiaceae* [17]. *Anaplasma* spp. contains several individual species including: *Anaplasma phagocytophilum, Anaplasma platys, Anaplasma marginale, Anaplasma bovis, Anaplasma ovis*, and *Anaplasma centrale*. A newly discovered species which has not yet been officially recognized as a separate species is named *Anaplasma capra* [45]. The zoonotic potential of the bacteria is of great importance in consideration of public health concerns [30]. Anaplasmosis is a vector-borne disease which is usually transmitted by ticks, but other mechanisms do also exist [50].

Bacteria in the *Anaplasma* spp. are obligate intracellular pathogens found inside vacuoles in the cytoplasm of the infected eukaryotic host cells. The mammalian host cells that become infected are variable depending on the species of *Anaplasma* spp., and includes granulocytes, erythrocytes, endothelial cells and platelets. The bacteria differ from other gram-negative bacteria by not having a cell wall which make them sensitive to mechanical stress. They are enveloped, but lack thickening of the leaflets and are without peptidoglycan layers or lipopolysaccharides (LPS) [30]. The bacteria replicate inside the host cell, forming microcolonies called morulae [45].

Anaplasma phagocytophilum is the causative agent of granulocytic anaplasmosis in horses, humans, canines and felines. In ruminants the disease is known as tick-borne fever [3]. *Anaplasma platys* cause infectious cyclic thrombocytopenia in dogs mainly. Individual cases have also been found in cats, humans and cattle. Although different strains of *Anaplasma platys* with variable pathogenicity have been detected [64], it is considered as a canine pathogen [30, 45]. *Anaplasma marginale* cause the "classical" anaplasmosis called erythrocytic anaplasmosis in ruminants [45]. It is the cause of economic losses in the cattle industry. *Anaplasma bovis, Anaplasma ovis* and *Anaplasma centrale* are closely related to *Anaplasma marginale*, but typically cause a milder disease [1, 59]. *Anaplasma capra* has been detected as an emerging bacterium which infects ruminants and humans [39]. S e o et al. [59] states that if this bacterium is determined to be pathogenic to humans and other animals, it may be an essential health risk.

Anaplasma spp. are commonly transmitted through ticks. The different *Anaplasma* spp. have different tick species for transmission. The most common genus of ticks that function as vectors is *Ixodes* spp. Other species include *Dermacentor* spp., *Rhipicephalus* spp., *Hyalomma* spp., and *Haemaphysalis* spp. [45]. For the tick to be infected and be able to transmit the pathogen, the tick needs to live in a habitat where there are mammals present that can serve as reservoirs for the *Anaplasma* spp. [18]. Biological transmission by ticks is the most common way of transmission of *Anaplasma* spp. In general, transmission of *A. phagocytophilum* by ticks starts within a few hours after attachment but establishment of infections was observed only when ticks attached for greater than 48 hours [26]. Furthermore, other mechanisms such as mechanically by biting flies and by blood-contaminated fomites, have also been recorded in *A. phagocytophilum* and *A. marginale* [37, 63]. The wide variety of reservoir hosts of *Anaplasma* spp. varies with the species and the geographical location, but typically include wild ruminant species [42, 71]. Migrating birds that can carry long-range ticks may have an essential role in the spreading of the pathogen [66, 67].

The incubation period is in the range from 5 to 14 days. The clinical signs of diseases caused by *Anaplasma* spp. show a lot of similarities across the different species and infected hosts, although there are some variations both in what type of signs that occur, and in the degree of severity of the clinical signs. Diseases caused by *Anaplasma* spp. commonly cause non-specific febrile illness in the infected mammalian host. Acutely infected animals lose condition. The next most described clinical signs are distal limb oedema, a reluctance to move, inappetence, decreasing of milk production, loss of coordination and breathlessness. Ruminants that are infected with *Anaplasma* spp. tend to develop more severe disease. Death from infection by *Anaplasma* spp. has the highest occurrence in infected ruminants that develop a progressive disease [5, 37, 54]. In addition, ruminants become persistent carriers and reservoirs for *Anaplasma* spp. [42]. Horses, dogs, cats, and humans rarely succumb to the disease. In most cases, the diseases are self-limiting [58, 64]. The severity of clinical signs tends to increase with the age of mammalian host infected with *Anaplasma* spp. [42, 58, 63]. The most common laboratory findings in diseases caused by *Anaplasma* spp. are thrombocytopenia, leukopenia, and anaemia [30]. When the pathogen comes in contact with the eukaryotic host cells, the pathogen adheres to the surface of the cell. It enters the cell by endocytosis and forms vacuoles within the cytoplasm of the host cells. Once inside the cells, they begin to replicate by binary fission after which the specific morulae is formed [30].

The epizootic history of the patient together with the clinical signs and laboratory findings is essential in confirming infection by *Anaplasma* spp. [18, 30]. The microscopic detection of the morulae in infected cells by Giemsa or Wright's staining is a fast and cost-effective diagnostic tool; PCR, IFA and ELISA are also commonly used [1, 30, 67].

Treatment of individuals with diseases caused by *Anaplasma* spp. depends on the severity. In individuals where the disease is commonly self-limiting, and the clinical signs are mild, the patient is usually left without any specific treatment. If the disease is more progressive, the typical treatment is antibiotics [1, 30, 63, 67]. One should also have in mind that the intensive use of antibiotics increases the chance of microorganisms becoming resistant to treatment by antibiotics [37]. The most important preventive measure to avoid infection and spreading of *Anaplasma* spp. is tick control [1]. There are no current vaccines that are available against *Anaplasma* spp. except for *Anaplasma marginale* [36, 65]. There have been some thoughts that recovery from the disease caused by *A. phagocytophilum* can provide immunity, but the period of protection is variable [1].

The aim of this study was to describe anaplasmosis in animals. This includes describing the different *Anaplasma* spp. with their characteristics, how they affect their host and their distribution worldwide. More cases have been reported during the latest years. This increase can be due to more transportation, climate changes and overall more research on the topic.

OCCURRENCE OF *ANAPLASMA* SPP. IN EUROPE

Anaplasmosis was detected in many European countries in various animal species with the prevalences ranging from 1.09 % to 97.9 % (Table 1). The variability of the prevalences could be due to the specific geographical area. High prevalence may be in association with the region having suitable environment for ticks, with appropriate vegetation and high mammalian host densities. The tick species *I. ricinus* is the main tick species in Europe, as well as a main vector for *Anaplasma phagocytophilum* transmission. Although in Europe several of the *Anaplasma* spp. are present and they vary in prevalence between the geographical areas, *Anaplasma phagocytophilum* was the most detected subspecies. However, the reason may also be the focus on this species due to its zoonotic potential. It has been identified in domestic species such as dogs, small mammals and horses but also in a variety of wild ruminants, as well as humans [54]. Ruminants have also been affected across Europe. *A. phagocytophilum, A. marginale* and *A. ovis* has been detected in Mediterranean countries, Central Europe and Sweden.

In general, higher prevalences seems to be in wildlife animals which could suggest the important role of wildlife as the possible reservoir. The prevalence may also vary with the different ecotypes or strains as showed in the study by J a h f a r i et al. [34]. As climates and habitats are changing, so does the distribution and movement of these wildlife species and the ticks with them. Norway function as a northern limit to the tick distribution due to the colder climate. In recent years there have been reports about the limit of ticks being pushed further north [54]. Tick-borne diseases such as anaplasmosis has been reported most frequently along the coast of Norway where there are high densities of ticks [31]. In Norway anaplasmosis is listed as a C-disease in a ranging system of infectious diseases so there is no need to report the disease [40, 41, 63]. The variation in prevalence across Europe may be explained in association with a few main factors like variation in tick density, and density of vertebrate hosts. Furthermore, the disease is not reportable, meaning there is no database on the occurrences. It also presents with non-specific signs which can lead to it being overlooked. And in addition there has been a lack of attention to the disease, as it is not common to test for it, and there is a lack of specific diagnostic methods [24].

OCCURRENCE OF *ANAPLASMA* SPP. IN OTHER COUNTRIES

Anaplasmosis has spread throughout the world (Table 2). Other than Europe, anaplasmosis has been found in America, Africa, Asia, and Australia [1]. In the USA, the disease caused by *A. phagocytophilum* in humans is needed to be reported. The CDC shows an increase in reports from 2000 until 2017 [9]. Similarly, a significantly increasing trend for *A. phagocytophilum* seroprevalence in the canine population was recently observed by D e w a g e et al. [15] in the eastern USA. Furthermore, cats, cattle, rabbits and small mammal were investigated for the presence of *Anaplasma* spp.

New studies show a higher geographical range of the ticks with a corresponding increased number of cases of human granulocytic anaplasmosis in Canada. Although anaplasmosis is now considered as endemic only in several areas in Canada [11], U m i n s k i et al. [66] suggested that the disease should be made reportable. In Central and South America, *A. platys* in canines have been more frequent than in North America [30]. In recent years, there have been also more reports on *Anaplasmosis* spp. in South America [16, 48].

In Africa and the Middle East most of the *Anaplasma* spp. have been detected. However, mostly ruminants and ticks have been studied, especially in North Africa. Other domestic species, such as horses, dogs, and also humans have not been studied as much. This interest in studying ruminants may be due to the importance of livestock in the economy in these areas. The *Anaplasma* spp. that are studied include: *A. marginale, A. ovis, A. bovis*, and *A. platys*. Only small amount of information about *A. phagocytophilum* and its distribution in this area exist [8]. In Tunisia, studies there have been conducted on cattle with sev-

Table 1. The prevalence of *Anaplasma* spp. in various animal host species in selected European countries
AO —*A. ovis*; AP —*A. phagocytophilum*

Country	Anaplasma species	Host species	No. tested	Prevalence [%]	Source
Austria	AP	Cattle	140 081	3.60	[6]
Czech Republic	AP	Cattle	55	5.45	[14, 33, 53]
		Sheep	109	1.09	
		Horse	40	5.00	
			96	73.00	
Hungary	AP	Dog	1305	7.90	[23, 32]
		Fallow deer	33	72.70	
		Red deer	48	97.90	
		Roe deer	65	60.00	
		Mouflons	16	6.30	
		Wild boars	17	39.20	
Germany	AP	Dog	111	43.20	[35, 38]
			522	43.00	
Italy	AP	Horse	20	15.00	[3, 19, 20, 21, 51]
			135	17.03	
			160	25.62	
			479	15.03	
		Fallow deer	67	46.26	
		Red deer	52	46.15	
		Cattle	137	16.78	
		Sheep	102	12.74	
		Goats	72	4.16	
		Dog	1232	8.76	
			40	7.50	
Norway	AP	Moose	111	70.00	[43, 54, 62]
		Red deer	141	94.00	
			37	81.10	
		Roe deer	28	82.00	
			104	88.10	
Poland	AP	Horse	76	1.3	[60]
Slovakia	AP	Sheep	202	16.16	[14]
	AO	Sheep	202	32.67	
		Goat	12	58.30	
Spain	AP	Cattle	456	3.07	[4]
		Sheep	389	0.51	
		Goat	207	0.48	
		Horse	46	6.52	
Sweden	AP	Sheep	20	45.00	[29]

Table 2. The prevalence of *Anaplasma* spp. in various animal host species in selected countries of the world
AB—*A. bovis*; AC—*A. centrale*; ACa—*A. capra*; AM—*A. marginale*; AO—*A. ovis*; AP—*A. phagocytophilum*; ASp—*A.* spp.

Country	Anaplasma species.	Host species	No. tested	Prevalence [%]	Source
Brazil	AP	Horse	98	17.40	[16, 47, 55, 56]
			91	12.00	
			97	11.34	
			20	65.00	
China	AB	Dog	243	4.10	[12, 52, 70, 71]
		Sheep	435	24.40	
	ACa	Sheep	435	18.20	
		Sheep	1453	8.90	
		Goat	943	9.40	
	AO	Dog	243	6.20	
		Sheep	435	5.70	
	AP	Sheep	49	42.90	
		Goat	91	38.50	
		Yak	158	32.30	
		Cattle yak	20	35.00	
		Dog	243	0.40	
		Sheep	435	28.00	
Iran	AB	Cattle	150	2.66	[46, 72]
	AM, AO	Sheep + goat	370	27.50	
Canada	AP	Dog	86 251	0.19	[57, 68]
		Horse	376	0.53	
Malaysia	AM	Cattle	1045	72.60	[49]
Mexico	ASp	Dog	1706	9.90	[44, 48]
	AP	White-tailed deer	25	20.00	
		Mazama deer	4	50.00	
Mongolia	AO	Sheep	1179	69.00	[22]
		Goat	871	71.30	
Nicaragua	ASp	Dog	329	28.60	[61]
Niger	AO	Sheep	33	69.70	[13]
South Korea	AP	Cat	33	18.20	[10, 59]
		Horse	92	2.20	
		Cattle	129	0.80	
	ACa	Cattle	1219	1.00	
Tunisia	AM	Cattle	232	25.40	[7, 42]
			328	24.70	
	AC		232	15.10	
	AB		232	3.90	
	AP		328	0.60	
Turkey	AO	Sheep + goat	343	60.00	[73]
USA	AP	Small mammal	2121	15.24	[2, 25, 27, 69]
		Rabbits	41	7.32	
		Cats	175	9.70	
	AB	Rabbits	41	36.58	
	AM	Cattle	247	21.86	

eral *Anaplasma* spp. including: *A. marginale, A. centrale, A. bovis*, and *A. phagocytophilum* [7, 42]. Several studies on sheep and goats have been conducted as well. Also, in Turkey, Iran and Niger, the breeding of small ruminants has significant economic importance. In these countries, *A. ovis* and *A. marginale* were detected in sheep and goat with variable prevalences [13, 72, 73].

In Asia and Australia, there has been more reports in recent years. China has reported several different *Anaplasma* spp. including the newly detected *Anaplasma capra*. *A. capra* has been detected in humans, sheep, and goats in China and in cattle in South Korea. The tick species *Ixodes persulcatus* is thought to be a possible vector for *A. capra* [39, 52, 59, 71]. *A. phagocytophilum, A. ovis, A. bovis*, and *A. capra* have been studied on several occasions across China. These *Anaplasma* spp. have been discovered in multiple host species [12, 52, 70, 71]. They have been detected mainly in ruminants, probably due to the high impact of livestock production, but the occurrence of *Anaplasma* spp. were also investigated in dogs. Studies that were done on sheep, goat, and cattle in Mongolia and Malaysia show a relatively high prevalence of *A. marginale* [22, 49]. In Australia, the only detected *Anaplasma* spp. has been on the strains of *A. marginale, A. centrale*, and *A. platys*, which have been introduced to the country by imported animals. However, they recently detected a unique *A. bovis* strains in native Australian ticks. This is genetically different from other variants worldwide [28].

CONCLUSIONS

Anaplasmosis is a disease caused by the bacteria of the *Anaplasma* genus. They affect a variety of host species with varying prevalences around the world. The explanation for the variability can be the size of the population tested, the density of ticks and reservoir hosts which usually depend on the geographical area and climate, and the different strains of *Anaplasma* spp. that are present. To fully understand how this is all connected and to be able to conduct proper preventive measures, more research is needed on anaplasmosis caused by the different *Anaplasma* spp.

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EFECTS OF COCONUT OIL (*COCOS NUCIFERA*), AVOCADO OIL (*PERSEA AMERICANA*), MELON SEED OIL (*CITRULLUS COLOCYNTHIS* L.) ON GROWTH PERFORMANCE, BLOOD, BIOCHEMICAL, HAEMATOLOGICAL PARAMETERS, AND TOTAL MICROBIAL LOADS OF NOILER BIRDS

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ABSTRACT

This study was carried out to examine the comparative effects of coconut oil (CO), avocado oil (AO), and melon seed oil (MSO) on the growth performance, blood, biochemical, hematological parameters, and total microbial loads of Noiler birds. A total of 120 Noiler birds with an average weight of 50.3 ± 0.13 g were randomized into four treatment groups with 3 replications (10 per pen) for six weeks of fattening. Weekly body weight gain and daily feed intake of the birds were recorded for six weeks, after which average weight gain and feed conversion ratios were calculated. At the end of the feeding trials, blood samples were collected for biochemical and hematological parameter assessments, and the digesta from the colon and ileum were collected for their intestinal total microbial load analysis. The average weight gains and feed conversion ratios (FCR) of the birds supplemented with CO (1229.40 ± 15.00) and MSO (1232.66 ± 43.18) were observed to be significantly higher (P < 0.05), compared to the birds supplemented with AO (1110.73 ± 18.29) and the birds fed feed only (1034.79 ± 2.04) having the least weight gained. The biochemical parameters of the birds across the treatment were not significantly different (P > 0.05). White blood cells, packed cell volume, red blood cells and lymphocytes were significantly higher in the CO group compared to the birds supplemented with the avocado oil and melon seed oil. There was no significant difference (P > 0.05) in the weight of the spleen, bursa and gall bladder among the birds. The *Lactobacillus* spp. in the colon of birds supplemented with coconut oil (6.43 ± 0.56) and melon oil (6.25 ± 0.65) were significantly higher. It can be concluded that coconut oil and melon seed oil have the potential to serve as growth promoters for chicken production.

Key words: comparative study; digesta; growth; haematology; intestinal microbes

INTRODUCTION

The poultry industry can produce very high-quality

proteins for human nutrition as well as a source of income for the community in many countries, hence poultry production has a very important role in economic development of any country [44]. Noiler chicken is a dual purpose breed of chicken developed by Amo Farm Sieberer Hatchery Limited for small holders to address the challenges of food insecurity and financial dependency among the rural populace, especially women. Noiler was bred to survive on low quality feedstuffs to provide good quality meat and egg [37].

Antimicrobials are sometimes given to food animals at low doses in order to promote faster growth [7]. The mechanism of action of the antimicrobial growth promoter is not fully understood but the potential of the intestinal microbiota in increasing feed efficiency has been shown [8, 19]. However, repeated use of these drugs in small doses in food animals, such as for the purposes of growth promotion, feed proficiency enhancement, and prophylaxis has been identified as a significant contributing factor to increasing antimicrobial resistance [12, 46]. Because of this, many countries have already taken action to reduce the use of antibiotics in food-producing animals [47, 48].

Following this action, alternative substances and strategies for animal growth promotion and disease prevention are being investigated, among which phytogenic and natural products have received attention since they have acquired more acceptability among consumers as natural additives [18, 45].

Fats especially from plant-based sources have shown positive alterations in the gut microbiota in both *in vivo* and *in vitro* studies [26]. Coconut oil is a highly saturated oil (about 90 %), and 60 % of its total fatty acid composition are medium chain length of 6 to 12 carbon atoms [10], which are absorbed directly into the portal circulation without re-esterification in intestinal cells [22]. This is in contrast to the longer-chain saturated fatty acids which are more typically found in animal fats; medium-chain fatty acids (MCFA) are more easily metabolized [41]. Recent data from a human trial suggest that coconut oil, at least in daily doses of 30 ml, may not have the same dyslipidemic properties and proinflammatory properties as other saturated fats [6]. In addition, virgin coconut oil contains high concentrations of polyphenols and may therefore have antioxidant, as well as anti-inflammatory effects, as demonstrated in some animal models [15].

Avocado is a fruit grown mainly in warm temperate and subtropical climates throughout the world. The pulp of this fruit contains about 60 % oil, 7 % skin, and approximately 2 % seeds [43]. The lipid content of avocado oil, mainly of monounsaturated fatty acids (MUFA), is associated with cardiovascular system benefits and anti-inflammatory effects [5]. Avocado oil has a high content of monounsaturated fatty acids (69.4 %) and a lower amount of polyunsaturated fatty acids (PUFA) and saturated fatty acids, which were 16.6 % and 14 %, respectively [16]. The presence of compounds with nutritional interest, such as unsaturated fatty acids (MUFA and PUFA), as well as compounds with biological activity, such as tocopherols, tocotrienols, phytosterols, carotenoids, and polyphenols, have made avocado oil of growing interest for research on the possible biological effects of avocado oil, with the aim of preventing and treating diseases through the diets of the population [17].

Citrullus colocynthis has a significant history of medicinal, pharmaceutical, nutraceutical and nutritional use [35]. The oil from melons is rich in fatty acids; the oil contains five main fatty acids: palmitic, stearic, oleic, linoleic and linolenic acids, but the most abundant fatty acid is linoleic; a polyunsaturated fatty acid [13, 14, 20]. Monounsaturated fatty acids of melon seed oil was reported to be 18.2 %, while polyunsaturated fatty acids and saturated fatty acids contributed 53 % and 21.2 % w/w respectively [38, 39]. Research has shown that melon seed oil contains antibacterial, anti-diabetics, and antimicrobial activity [4].

Therefore, this study investigated the effects of coconut oil, avocado oil, and melon seed oil on the growth performance, biochemical, hematological parameters and total microbial loads of Noiler chickens.

MATERIALS AND METHODS

Experimental location

The experiments were carried out at the Poultry Unit of the Teaching and Research Farm, Faculty of Agriculture, Obafemi Awolowo University, Ile-Ife, Osun State.

Experimental design

A total number of 120 Noiler day old chicks were purchased from a standard and reputable hatchery in Aawe, Oyo state. The birds were transported in a perforated day old chick's carton during the cool weather condition for

proper aeration. Pens were thoroughly cleaned and disinfected against germs and bacteria prior to the arrival of the birds. The birds were grouped using a completely randomized design and allocated to four separate treatments with ten birds per treatment and replicated thrice. The birds were first acclimatized for a week and then the experiments lasted for six weeks.

Experimental diet and feeding

The birds were fed with chick mash from day old to three weeks of age (18 % CP and 2800 kcal); at week four grower mash 17 % CP and 3100 kcal were fed to the birds. The feeds were weighed and supplied to the birds in the morning and remnants were removed in the evening and weighed before fresh feed of known quantity was supplied, this was done in order to determine the feed intake of a birds per day for the efficient calculation of the Feed Conversion Ratio and Average Daily Weight Gain.

Treatment 1 were administered the control experimental diet without supplements.

Treatment 2 were administered the avocado oil supplemented diet at 10 $ml.kg^{-1}$ of feed.

Treatment 3 were administered the melon oil supplemented diet at 10 $ml.kg^{-1}$ of feed.

Treatment 4 were administered the coconut oil supplemented diet at 10 $ml.kg^{-1}$ of feed.

Table 1. Ingredient composition of diets

Ingredients	Chick mash	Growers' mash
Maize	64.9	64.9
Wheat offal	4.0	4.0
Soya bean meal	26.0	14.5
Full fat soya	1.6	13.1
Bone meal	2.5	2.5
Chick premix	0.25	0.25
Salt	0.25	0.25
Methionine	0.3	0.3
Lysine	0.2	0.2

Extraction of oils

Coconut oil

Mature coconuts were purchased from Sabo market, Ile Ife, Osun state, located in the South West of Nigeria. The coconuts were cut open and the kernel meats (endosperm) were removed and washed clean. The kernel meats were grinded by a bur mill and poured into a bowl. Hot water was added to it and the mixture was sieved to remove the shaft. The milky solution obtained was kept in the refrigerator for a few hours until a cake crust was formed on top of the solution. The cake was then scraped and heated until a clear, transparent liquid was obtained.

Avocado oil

Ripe avocado was purchased from Sabo market, Ile-Ife, Osun state, located in the South West of Nigeria. The skin of avocado was peeled off, while the fleshy part was cut into smaller pieces, and blended into a smooth paste. The paste was spread out in a thin layer and sun dried for a period of 28 hours which resulted into dark-brownish green dried crumbs. The oil was exhaustively extracted by Soxhlet extraction according to the method described by Santana et al. [40].

Melon seed oil

The oil from the melon seeds were extracted by the continuous extraction in Soxhlet apparatus (chemglass) for 8 hours using petroleum ether (60—80 °C boiling range) as a solvent according to the method described by AOAC [2]. At the end of the extraction, the extraction solvent was evaporated in a rotary evaporator [37].

Data collection and analyses

The birds were weighed weekly and at the end of the experiment, the feed conversion ratios were calculated to measure the efficiency with which the bodies of the livestock converted animal feed into the desired output. It was calculated as: average feed intake per daily weight gain. Blood samples (six per treatment) were collected by severing the jugular vein of the neck, the blood was collected directly into both blank and EDTA sample bottles. The sample inside the plain sample bottle was centrifuged at 3000 rpm for 10 minutes after which the serum was separated into clean bottles. The digesta from the colon and ileum were collected for microbiological analysis according to the method of Olutiola et al. [36] and all organs were weighed. The immune organ index was calculated using the formula, immune organ weight (mg)/live weight (g).

Packed Cell Volume (PCV)

Microcapillary tubes were filled with uncoagulated blood and the end was sealed with plasticine. The tubes were centrifuged in a micro hematocrit centrifuge. The plasma at the top of the capillary tube could be used for determining the plasma protein and fibrinogen and for assessing plasma colour and turbidity. The level of the settled blood cells was read by placing the tube on the hematocrit graphic reader.

Erythrocyte counts

The red blood cell count per million was done using blood containing anticoagulants by the haemocytometer method. A red cell pipette was used to draw blood up to 0.5 mark and was dropped on a glass slide, smeared and was covered with a cover slip. This was viewed under the microscope. The erythrocytes in the five lower squares were counted. The volume counted was calculated using the formula below:

RBC = Total number of cell count × dilution factor/0.02 mm^3

White blood cell count

The total white blood cells were counted under the microscope using the haemocytometer. A white blood cell pipette was used to draw blood to 0.5 mark and diluted to 1.2 mark with the aid of a white cell diluting fluid. The leucocytes in the four lower squares were counted. The number of cells counted was calculated using the formula below:

WBC = Total number of cell count × dilution factor/0.4 mm^3 [32]

Microbial analysis of crop and ileum samples

Bacterial detection was carried out on samples recovered from the crop and ileum. The double fold dilution method was conducted for each sample and thereafter pour-plated using several media. The media which include nutrient agar and de Man Rogosa and Sharpe agar (MRS) were assessed to determine the total heterotrophic bacteria count and *Lactobacillus* spp. respectively [36].

Biochemical parameters

To determine the biochemical parameters, blood samples were collected into plain sample bottles and allowed to clot and centrifuged for 15 minutes at 2200—2500 rpm to separate the sera. The sera samples were used for the biochemical parameters determination of total protein, albumin, total cholesterol, HDL, LDL and triglyceride, according to the enzymatic colorimetric method described by Schmidt, Schmidt [42] using a commercial diagnostic reagent kits (Randox Laboratories Ltd., UK). The globulin was calculated by subtracting the albumin value from the total protein.

Statistical analysis

The statistical software program SAS was used to analyze the data obtained from the experiment. The data were subjected to analysis of variance (ANOVA), while the means were separated using the Duncan Multiple Range Test.

RESULTS

The growth performance indices of Noiler birds supplemented with different oils are shown in Table 2. The Noiler birds fed with coconut oil and melon oil supplements showed final body weights which were significantly higher ($P < 0.05$) compared to the birds on the avocado oil supplemented diet. Birds supplemented with the three natural oil had significantly lower feed conversion ratios (FCR) compared with the control birds.

The biochemical parameters of Noiler chicken presented in Table 3 showed that there were no significant differences ($P > 0.05$) in the serum parameters of the birds in all of the treatment groups. There were significant increases ($P < 0.05$) in red blood cell, haemoglobin and packed cell volume (PCV) of animals supplemented with coconut oil, when compared with other groups of experimental birds (Table 4). The birds fed the coconut oil supplemented diet had significantly higher WBC (18691.7 ± 238.19) and lymphocyte (68.83 ± 0.95) compared to animals fed the avocado oil and melon oil supplemented diet. Animals in the control group showed higher heterophils values (31.83 ± 2.39), followed by animals in the avocado oil group (28.83 ± 3.05), melon oil group (25.67 ± 0.56), and coconut oil group (24.33 ± 1.09). However, there were no significant differences in platelet, monocyte, eosinophil, and basophil count across all of the treatments.

The organ parameters presented in Table 5 showed that there were significant differences ($P < 0.05$) in: the weights of the heart, liver, gizzard, right kidney, left kidney, large

Table 2. Growth performance of Noiler chickens fed different oil supplemented feeds

Parameters	T1	T2	T3	T4
Initial weight [g]	50.42 ± 0.15	50.37 ± 0.09	50.39 ± 0.07	50.35 ± 0.13
Final weight [g]	1085.14 ±1.83^{b}	1161.21 ±18.08^{b}	1283.03 ± 43.10^{a}	1279.76 ± 14.98^{a}
Weight gain [g]	1034.79 ± 2.04^{b}	1110.73 ± 18.29^{b}	1232.66 ± 43.18^{a}	1229.40 ± 15.00^{a}
Feed intake [g]	2173.20 ± 35.76^{b}	2063.78 ± 3.62^{c}	2315.23 ± 7.38^{a}	2291.57 ± 4.56^{a}
FCR	2.10 ± 0.03^{a}	1.86 ± 0.03^{b}	1.83 ± 0.07^{b}	1.86 ± 0.02^{b}

$^{a, b, c}$—means with different superscript in the same row are significantly different at $P < 0.05$; T1— birds on control diet; T2—birds supplemented with avocado oil; T3—birds supplemented with melon oil; T4—birds supplemented with coconut oil; FCR —feed conversion ratio

Table 3. Serum biochemical parameters of Noiler chickens fed different supplemented feeds

Parameter	T1	T2	T3	T4
Total protein [g.dl^{-1}]	7.03 ± 0.28	7.07 ± 0.08	7.57 ± 0.45	7.13 ± 0.18
Albumin [g.dl^{-1}]	2.47 ± 0.31	2.40 ± 0.15	2.58 ± 0.37	2.42 ± 0.21
Globulin [g.dl^{-1}]	4.87 ± 0.08	4.67 ± 0.11	4.98 ± 0.23	4.72 ± 0.05
A/G ratio	0.47 ± 0.07	0.50 ± 0.05	0.48 ± 0.08	0.48 ± 0.05
Total cholesterol [mg.dl^{-1}]	192.00 ± 7.26	199.3 ± 3.70	203.17 ± 9.22	194.17 ± 3.54
Trig [mg.dl^{-1}]	78.67 ± 8.11	71.83 ± 3.71	85.50 ± 7.93	78.19 ± 6.81
HDL [mg.dl^{-1}]	103.3 ± 2.42	96.67 ± 2.01	102.17 ± 3.55	98.33 ± 3.33
LDL [mg.dl^{-1}]	72.93 ± 6.42	88.27 ± 2.24	83.90 ± 5.11	80.20 ± 3.11

T1—birds fed feed only; T2—birds supplemented with avocado oil; T3—birds supplemented with melon oil; T4—birds supplemented with coconut oil; A/G Ratio—albumin/globulin ratio; Trig—triglyceride; HDL— high density lipoprotein; LDL—low density lipoprotein

Table 4. Haematological parameters of Noiler chickens fed different supplemented feeds

Parameter	T1	T2	T3	T4
PCV [%]	31.67 ± 2.30^{b}	31.33 ± 2.53^{b}	33.33 ± 0.21^{b}	37.00 ± 1.03^{a}
Hb [g.dl^{-1}]	10.40 ± 0.69^{b}	10.42 ± 0.85^{b}	10.92 ± 0.10^{b}	12.33 ± 0.33^{a}
RBC [10^{12}.l^{-1}]	3.36 ± 0.11^{ab}	3.16 ± 0.25^{b}	3.49 ± 0.04^{ab}	3.67 ± 0.05^{a}
WBC [10^{9}. l^{-1}]	16.43 ± 0.80^{b}	17.51 ± 0.54^{ab}	17.32 ± 0.23^{ab}	18.69 ± 0.24^{a}
Platelet [10^{9}. l^{-1}]	143.00 ± 129.79	137.00 ± 94.90	157.833 ± 74.09	138.167 ± 141.83
Lymphocyte [%]	61.7 ± 2.46^{b}	63.67 ± 3.06^{ab}	67.17 ± 0.60^{ab}	68.83 ± 0.95^{a}
Heterophils [%]	31.83±2.39^{a}	28.83±3.05^{ab}	25.67±0.56^{ab}	24.33±1.09^{b}
H/L ratio	0.51	0.45	0.38	0.35
Monocyte [%]	2.83 ± 0.31	3.17 ± 0.48	3.33 ± 0.44	3.00 ± 0.52
Eosinophils [%]	4.67 ± 0.56	4.17 ± 0.91	3.83 ± 0.87	3.83 ± 0.54
Basophil [%]	0.17 ± 0.17	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00

$^{a, b, c}$—means with different superscript in the same row are significantly different at $P < 0.05$; T1—birds fed feed only; T2—birds supplemented with avocado oil; T3—birds supplemented with melon oil; T4—birds supplemented with coconut oil; H/L ratio—heterophil/lymphocyte ratio; Hb—haemoglobin; RBC—red blood cell

Table 5. Weight of organs of Noiler chickens fed different supplemented feeds

Parameter	T1	T2	T3	T4
Gizzard [g]	–	27.36 ± 1.65^{b}	28.03 ± 2.01^{a}	28.75 ± 2.49^{a}
Heart [g]	2.99 ± 0.25^{b}	3.58 ± 0.19^{b}	3.69 ± 0.32^{a}	3.73 ± 0.31^{a}
Liver [g]	11.17 ± 1.55^{b}	16.97 ± 1.69^{a}	14.59 ± 0.94^{ab}	16.97 ± 1.34^{a}
Spleen [g]	1.62 ± 0.14	1.74 ± 0.11	1.85 ± 0.13	1.88 ± 0.12
Bursa [g]	1.66 ± 0.07	1.72 ± 0.26	2.32 ± 0.33	0.94 ± 0.11
IOI (Spleen) [mg.g^{-1}]	1.68 ± 0.16	1.33 ± 0.03	1.50 ± 0.15	1.32 ± 0.13
IOI (Bursa) [mg.g^{-1}]	1.60 ± 0.10	1.47 ± 0.48	1.31 ± 0.08	1.51 ± 0.06
Right kidney [g]	2.11 ± 0.28^{b}	2.79 ± 0.18^{a}	2.58 ± 0.10^{ab}	2.55 ± 0.24a^{b}
Left kidney [g]	1.98 ± 0.22^{b}	2.89 ± 0.23^{a}	2.87 ± 0.18^{a}	2.74 ± 0.20^{a}
Right lung [g]	1.78 ± 0.12^{b}	2.12 ± 0.13^{ab}	2.37 ± 0.09^{a}	2.18 ± 0.14^{a}
Left lung [g]	1.78 ± 0.14^{b}	2.29 ± 0.07^{ab}	2.83 ± 0.26^{a}	2.75 ± 0.25^{a}
Large intestine [g]	4.15 ± 0.16^{a}	1.65 ± 0.06c	3.79 ± 0.56^{ab}	3.01 ± 0.43bc
Small intestine [g]	29.88 ± 4.17^{b}	43.51 ± 4.27^{a}	34.75 ± 2.63^{ab}	36.82 ± 2.59a^{b}
Large intestine [cm]	8.08 ± 0.94^{a}	5.93 ± 0.29^{b}	7.83 ± 0.59^{a}	6.27 ± 0.63a^{b}
Small intestine [cm]	109.95 ± 5.09^{c}	122.70 ± 4.89^{bc}	139.83 ± 6.41^{a}	127.57 ± 4.52a^{b}
Gall bladder [g]	1.52 ±0.20	1.26 ± 0.13	1.45 ± 0.08	1.31 ± 0.11

$^{a, b, c}$— means with different superscript in the same row are significantly different at $P < 0.05$;
T1—birds fed feed only; T2—birds supplemented with avocado oil; T3—birds supplemented with melon oil;
T4—birds supplemented with coconut oil

Table 6. Microbial loads of Noiler chickens fed different supplemented feeds

Parameter	T1	T2	T3	T4
		Colon		
THB [CFU.ml^{-1}]	5.73 ± 0.55	5.11 ± 0.29	4.65 ± 0.19	4.84 ± 0.19
LSC [CFU.ml^{-1}]	4.66 ± 0.13^{b}	5.69 ± 0.50^{ab}	6.25 ± 0.65^{a}	6.43 ± 0.56^{a}
		Ileum		
THB [CFU.ml^{-1}]	5.73 ± 0.55	5.16 ± 0.15	5.32 ± 0.32	5.21 ± 0.42
LSC [CFU.ml^{-1}]	4.66 ± 0.13	4.88 ± 0.08	5.63 ± 0.52	5.58 ± 0.56

$^{a, b, c}$—means with different superscript in the same row are significantly different at $P < 0.05$; T1—birds fed feed only;
T2—birds supplemented avocado oil diet; T3—birds supplemented melon oil diet; T4—birds supplemented coconut oil diet;
THB—total heterotrophic bacteria; LSC—*Lactobacillus* spp. count

intestine, small intestine, right lung, and the left lung; also the length of the large intestine, and small intestine. However, there was no significant difference ($P < 0.05$) in the weights of spleen, bursa, and gall bladder.

Table 6 shows the intestinal microbial loads collected from the colon and ileum of Noiler birds. In the colon of birds supplemented with natural oil diets, Table 6 showed that there was no significant difference ($P > 0.05$) in the total heterotrophic bacteria, while there was a significant difference ($P < 0.05$) in the *Lactobacillus* spp. count across the treatment which was seen to be higher compared in oil supplemented birds compared to the control birds.

DISCUSSION

In recent years, attention has been focused on the dietetic and health effects of fat. This study was conducted to evaluate the efficacy of the utilization of coconut, melon seed and avocado oils as natural feed additives on dual purpose Noiler chickens. The improved feed intake and weight gain noticed in birds supplemented with coconut and melon seed oil could be due to dietary fat reduced passage rate of the digesta through the gastrointestinal trait, allowing for better nutrient absorption and utilization [24]. The addition of fat to the diet, besides supplying energy, improved the absorption of fat-soluble vitamins, diminishes the pulverulence, and increased the palatability of the rations [38]. The improvement in growth performance could be due to an increase in the efficiency of nutrients utilization; especially crude protein or antibacterial properties of medium-chain fatty acids (MCFA) [11, 49]. The carcass and organ weights of this study agrees with the reports of T u l e u n, I g b a [46] that the organ weights of broilers are the results of their live weight, since the surface area determines the amount of feathers and visceral organs required.

The non-significant levels of serum parameters of the experimental birds were in agreement with N a n d a k u m a r a n et al. [30] who found no difference when coconut oil was fed to rats. K h a w et al. [23] however found a non-significant increase in LDL but a significant increase in HDL values while L i m [25] reported a significant increase in the serum levels of both HDL and LDL cholesterol in humans consuming coconut oil for over two weeks. N e v i n, R a j a m o h a n [31]; A r u n i m a, R a j a m o h a n [3] reported that the supplementation of virgin coconut oil beneficially modulated hepatic lipid metabolism in rats and coconut oil, in particular, lowered total cholesterol, lipoproteins, and phospholipids. The reason for this was that the coconut oil consisted predominantly of medium chain fatty acid that was metabolized rapidly in the liver to energy and did not participate in the biosynthesis and transport of cholesterol. Moreover, M u r r a y et al. [28] reported that the increased concentration of HDL-C protected against the development of atherosclerosis in rodents and humans possibly as a result of enhanced reverse cholesterol transport (RCT), i. e. transfer of cholesterol from the arterial wall to the liver, followed by excretion into the bile, thus, reducing the plasma cholesterol levels. O l u b a et al. [33, 34] reported that melon oil with a rich content of polyunsaturated fatty acids produced a significant reduction ($P < 0.05$) in the serum total, free and esterified cholesterol and triglyceride concentrations in rats. Broilers supplemented with avocado non-saponifiable fatty had HDL-C fraction increased significantly ($P < 0.05$) with a decrease in LDL-C [9].

The increase in haematological parameters of Noiler chickens supplemented with coconut oil was in agreement with A k i n j a y e j u, A d e b o l u [1] who reported an increase in haematological parameters of coconut oil supplemented rats. This result was however in contrast to the findings of N a n d a k u m a r a n et al. [30] and with E l – A b a s y et al. [11] that reported that oral coconut oil administration to pregnant rats and rabbits did not have any effect on the hematological parameters.

The heterophil to lymphocyte ratio (H/L) is a widely established measure of stress particularly in the medical and veterinary literature [27]. Increases in heterophil to lymphocyte ratios (H/L) caused by heterophilia have been reported to be an indicator of chronic stress [9]. The heterophil/lymphocyte ratio was lowest in birds supplemented with coconut oil. This ability of coconut oil to reduce stress in chickens may be due to the presence of phenolic compounds, such as caffeic acid, p-coumaric acid, ferulic acid and catechin which improved its antioxidant capacity and the ability to fight diseases associated with oxidative stress [21]. Coconut oil also may enhance the immune system activity through several fatty acids, which are a potent immune stimulant [11].

The intestine provides the primary supply of nutrients: the sooner it achieves its functional capacity, the sooner the young birds can utilize dietary nutrients, efficiently grow at their genetic potential and resist infectious and metabolic diseases [26]. The fats from plant-based sources such as nuts, or vegetable oils have shown positive alterations in the gut microbiota biodiversity both in *in vivo* and *in vitro* studies [29]. The increase in lactobacillus count could be due to the antimicrobial effects of natural oil, as suggested by E r h a n, B o l u k b a s i [13] who reported that citrus peel oil supplementation increased lactobacilli counts in the jejunum of broiler chickens. It is well documented that the mode of action of *Lactobacillus* consists in competitive exclusion against harmful bacteria in the gut, in favor of beneficial microbial populations, leading to improvement in nutrients utilization of feed and this could have been responsible for the weight advantage of coconut oil over the control birds [50].

CONCLUSIONS

It can be concluded that supplementing the feed of chickens, especially with coconut oil, has beneficial effects on the growth and stress response of Noiler birds and has no deleterious effect on the serum cholesterol.

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EFFECTS OF LONG-TERM FEEDING OF TREATED RAPESEED MEAL ON GROWTH PERFORMANCE, BLOOD MINERAL PROFILE AND FATTY ACID COMPOSITION OF BACK FAT IN PIGS

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ABSTRACT

This experiment was conducted to evaluate the effects of replacing soybean meal (SBM) with treated rapeseed meal (tRSM) on the growth performance, blood mineral levels and fatty acid composition of back fat in growing-finishing pigs. A total of 12 crossbred pigs (Slovakian White × Landrace) with an initial live weight of 40.82 ± 2.69 kg were divided into two dietary treatments. The experimental period lasted 84 days; 38 days in the growing period and 46 days in the finishing period. The dietary treatments were as follows: control, a SBM-based diet (growing and finishing), and experimental, a treated RSM-based diet (growing and finishing). The rapeseed meal was treated with a product that neutralizes the negative physiological effects of anti-nutritive glucosinolates contained in rapeseed products for livestock feeding. Our analysis showed minor, no significant negative effects of tRSM on live weight, average daily gain and feed conversion ratio. Replacing SBM with tRSM had no significant effects on the proportion of the total saturated, monounsaturated and polyunsaturated fatty acids of back fat samples, as well as concentrations of analysed serum minerals except zinc level. In conclusion, the inclusion of 25 % and 18 % of treated RSM in growing and finishing pig diets had no negative effects on the growth performance, blood mineral profile or fatty acid composition of back fat.

Key words: growth performance; minerals; pigs; rapeseed meal

INTRODUCTION

Cereal/soybean meal-based diets are typical in pig farms located especially in countries where soybean meal is affordable [20]. Soybean meal (SBM) is the most widely fed protein source in pig diets [29] due to its content of highly digestible essential amino acids (lysine, but also threonine, tryptophan and isoleucine). SBM is characterised by high protein content (from 43 to 53 % as fed) and low crude fibre content. But increasing dietary supplementation levels of SBM caused an increased feed cost in pig diets [12, 27]

and also the potential health issues of genetically-modified (GM) soybean and other GM foods is a matter of considerable debate.

The worldwide increasing demand for proteins as animal feeding leads to a growing interest on other alternative protein sources for animals. Rapeseed is an alternative crop that can be grown in cold climates, and its cultivation has increased during recent years due to a growing demand for oil for biofuel production [14]. Rapeseed meal (RSM) is a by-product of rapeseed after the removal of its oil and is a cost effective protein source in pig diets and also an alternative ingredient to SBM [22]. Rapeseed meal generally contains 33 % to 40 % protein. The RSM has lower lysine but is richer in sulphur-containing amino acids (methionine + cysteine) compared to SBM [4]. It has been used in animal diets over an extended period of time, but due to a high fibre content, and anti-nutritional factors such as glucosinolates, tannins, and erucic acid, the inclusion of RSM in pig diets usually has been kept low [13]. Nevertheless, the beneficial effects of RSM dietary fibre on the intestinal health and overall well-being of pigs have been demonstrated [15]. The glucosinolates, anti-nutritional factors, have been demonstrated to impair growth and feed intake [1, 8]. The anti-nutritional factors in rapeseed can be reduced by a number of techniques including physical, chemical, biological and crop breeding. But their efficacy varies from one treatment method to the other [17].

This fact motivated us to determine the effects of long-term feeding of treated rapeseed meal as the protein source in diets on growth performance, blood mineral profiles and fatty acid composition of the back fat in growing-finishing pigs.

MATERIALS AND METHODS

Animals, diets and feeding

A total of 12 crossbred pigs (Slovakian White × Landrace) with an average body weight (BW) of 40.82 ± 2.69 kg were used for a 12-week trial (84 days). Pigs were divided into two groups (6 pigs in each group). Both groups contained equal numbers of females (3) and castrated males (3).

The same basic ingredients for the control and the experimental groups were used in the study. The diets were formulated based on corn, wheat, barley, soybean meal or treated rapeseed meal, vitamin + mineral premix and synthetic amino acids. Dietary treatments were as follows: control, a SBM-based diet (growing and finishing) and experimental, a treated RSM-based diet (growing and finishing).

The rapeseed meal was treated with 2.5 % LinaropAgri® LRA (LINAGRI s.r.o., Czechia); the product that neutralizes the negative physiological effects of anti-nutritive glucosinolates contained in rapeseed and rapeseed products for livestock feeding. The nutritional characteristics of the treated RSM (97.5 % rapeseed meal + 2.5 % LRA) were the following: dry matter 897 $g.kg^{-1}$, crude protein 349 $g.kg^{-1}$, ether extract 16.1 $g.kg^{-1}$, crude fibre 122 $g.kg^{-1}$, neutral detergent fibre 197 $g.kg^{-1}$, calcium 6.1 $g.kg^{-1}$, phosphorus 1.08 $g.kg^{-1}$, magnesium 4.6 $g.kg^{-1}$, sodium 0.15 $g.kg^{-1}$, potassium 1.05 $g.kg^{-1}$, copper 8.9 $mg.kg^{-1}$ and zinc 49.7 $mg.kg^{-1}$ as feed basis. All diets met or exceeded the requirement of the National Research Council NRC [16]. The composition and analyses of diets are shown in Table 1.

All pigs were fed twice per day and the animals were given free access to water. Water was also provided directly in the trough during meals. Feed consumption and pigs' weight were recorded weekly to determine the average daily gain (ADG), and feed conversion ratio (FCR). The investigation was carried out in the animal quarters of the Institute of Animal Nutrition and Dietetics at the University of Veterinary Medicine and Pharmacy in Košice in compliance with the EU regulations concerning the welfare of animals.

Diets analysis

The diets were analysed for their dry mater (DM), crude protein (CP), crude fibre (CF), neutral detergent fibre (NDF) and aether extract (EE) by the AOAC [2]. The starch content of the diets was determined using a fully automatic polarimeter. The feed samples were analysed for the presence of minerals (except phosphorus) using the flame method of an atomic absorption spectrometer. The determination of total dietary phosphorus was performed using the photometric method. The amino acids (AA) content in the diets were calculated according to the program for formulation of diets for pigs from the AA composition of feeds and the addition of synthetic amino acids.

Table 1. Ingredients (%) and chemical composition (g.kg^{-1}, as feed basis) of diets for growing (G) and finishing (F) pigs

Ingredients [%]	Control diet		Experimental diet	
	G	F	G	F
Corn	36.6	35	37.65	35
Wheat	14	15	27	29.88
Barley	28	34.35	7	14
SBM, CP 46 %	18	12.5		
tRSM			25	18
Premix VM	3	3	3	3
Lysine	0.20	0.08	0.24	0.09
Methionine	0.08	0.03		
Threonine	0.12	0.04	0.11	0.03
Analysed content [g.kg^{-1}]				
DM	896.7	891.7	891.5	894.8
CP	160.5	140.8	161.2	141.3
EE	28.8	23.3	30.8	25.1
CF	25.6	35.2	27.9	42.5
NDF	106.4	125.0	108.1	130.8
Starch	466.3	488.9	460.9	478.2
Ca	6.8	6.38	7.2	6.8
Total P	5.4	5.0	5.3	4.7
Calculated content [g.kg^{-1}]				
Lys	11.2	8.4	11.21	8.45
Thr	7.25	5.6	7.3	5.6
Met + cys	6.5	5.06	6.5	5.92

SBM—soybean meal; tRSM—treated rapeseed meal; DM—dry matter; CP—crude protein; EE—etheric extract; CF—crude fibre; NDF—neutral detergent fibre; Lys—lysine; Thr—threonine; Met+cys—methionine+cysteine; Ca—calcium; P—phosphorus

Blood mineral profiles

Blood samples were collected from all pigs from the control and the experimental group and were obtained via the anterior *vena cava* at the end of the growing period (day 38). The concentrations of copper (Cu), zinc (Zn), calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) in the blood serum were determined using the flame method of an atomic absorption spectrometer (Unicam Solar 939, UK).

Fatty acid composition of back fat

The samples of the back fat from the 12 pigs were obtained at the end of the experiment (day 84). Samples for the determination of the fatty acid profiles were kept at –20 ± 2 °C for up to three weeks. The fatty acid composition of the back fat samples was determined by an evaluation of their methyl ester content via gas chromatography, according to Čertík et al. [7].

Statistical methods

All data were reported as the mean ± SD (standard deviation). The differences between means were determined according to the unpaired t-test using the Graph-Pad Prism statistical program (Graph Prism software, USA). By conventional criteria, differences ($P < 0.05$; $P < 0.01$; $P < 0.001$) were considered to be statistically significant.

RESULTS

Growth performance

The live weights at the end the growing, the finishing and also the overall period were not significantly affected by the dietary inclusion of the treated RSM as an alternative to SBM. Although the animals from the experimental group achieved the higher final live weight at the end of the growing and finishing periods, there were no statistically significant differences between the experimental and the control group ($P > 0.05$). The experimental period lasted 84 days; 38 days in the growing period and 46 days in the finishing period. During the growing, finishing and overall period, ADG was slightly lower in pigs fed the tRSM diet (experimental) than those fed the SBM diet (control), but there were no statistically significant differences in ADG between groups in the all periods ($P > 0.05$). The feed conversion ratio (FCR) was a little poorer in pigs fed the tRSM diet in the growing (–0.04 kg feed per kg gain) and in the overall period (–0.03 kg feed per kg gain), whereas there were no differences in the FCR between groups in the finishing period (Table 2).

Blood mineral profile

The levels of selected mineral elements detected by atomic absorption spectrometry pointed to small changes in the amounts of mineral present in the blood serum (Table 3). On day 38 of the experiment, calcium (Ca),

Table 2. Growth performance of growing-finishing pigs fed diets based on soybean meal (SBM) and treated rapeseed meal (tRSM) (means ± SD)

Diet	Control (SBM)	Experimental (tRSM)
Number of pigs	6	6
Live weight [kg]		
Initial	40.68 ± 2.60	40.95 ± 2.78
End of the growing period	76.25 ± 5.58	75.55 ± 5.91
End of the finishing period	114.9 ± 8.15	113.7 ± 8.71
Growing period		
Days in the experiment	38	
ADG [g]	935.9 ± 96.6	910.5 ± 103
FCR, kg feed per kg gain	2.44	2.48
Finishing period		
Days in the experiment	46	
ADG [g]	840.2 ± 58.9	829.7 ± 69.3
FCR, kg feed per kg gain	3.13	3.13
Overall period		
Days in experiment	84	
ADG [g]	883.5 ± 74.2	866.3 ± 83.3
FCR, kg feed per kg gain	2.75	2.78

ADG—average daily gain; FCR—feed conversion ratio; SD—standard deviation

magnesium (Mg), sodium (Na), potassium (K) and copper (Cu) levels were similar between two treatments ($P > 0.05$). The serum zinc levels were significantly higher ($P < 0.05$) in pigs fed the tRSM diet (experimental group) than those fed the SBM diet (control group). The detected amounts of calcium, magnesium, sodium and potassium in the blood serum of pigs from both groups were within the physiological range (Ca 2.4—3; Mg 0.5—1.3; Na 140—160; K 4—5 mmol.l^{-1}, respectively) [11]. The blood levels of Cu and Zn in both groups were also maintained within the normal physiological range (Cu 20.4—47.2 and Zn 10.7—22.9 μmol.l^{-1}, respectively) [21].

Fatty acid composition of back fat

The results of the back fat fatty acid profile are shown in Table 4. The increase in α-linolenic content in the back fat samples for the experimental group was significant ($P < 0.05$). The proportion of palmitoleic acid in the back fat of the experimental group (tRSM diet) increased ($P < 0.01$) compared to the control group (SBM diet). The proportion of total saturated FA, monounsaturated FA, polyunsaturated FA and also the proportion of other individual FA were not affected by the dietary inclusion of the treated RSM as protein feed in the diet of experimental group.

Table 3. Mineral profile of the blood serum samples (means ± SD)

Mineral	Control group	Experimental group
Calcium [mmol.l^{-1}]	2.80 ± 0.25	2.67 ± 0.10
Magnesium [mmol.l^{-1}]	0.87 ± 0.26	0.79 ± 0.08
Sodium [mmol.l^{-1}]	159.90 ± 2.91	158.94 ± 1.49
Potassium [mmol.l^{-1}]	4.92 ± 0.33	4.72 ± 0.32
Copper [μmol.l^{-1}]	25.95 ± 6.91	22.38 ± 4.87
Zinc [μmol.l^{-1}]	17.35 ± 2.03^{A}	21.07 ± 3.05^{B}

$^{A, B}$—significant differences ($P < 0.05$); SD—standard deviation

Table 4. Fatty acids profile [g.100 g^{-1}] of back fat samples (means ± SD)

FA	Control group	Experimental group
C14:0 (myristic)	1.20 ± 0.07	1.15 ± 0.06
C16:0 (palmitic)	26.22 ±0.66	25.74 ± 0.33
C16:1 n-7 (palmitoleic)	1.60 ± 0.10A	2.10 ± 0.30C
C18:0 (stearic)	17.41 ± 0.90	16.80 ± 1.08
C18:1n-9 (oleic)	42.43 ± 0.81	43.29 ± 0.95
C18:2n-6 (linoleic)	6.46 ± 0.44	6.11 ± 0.36
C18:3n-3 (α-linolenic)	0.31 ± 0.04A	0.37 ± 0.03B
C20:0 (arachidic)	0.27 ± 0.03	0.28 ± 0.03
C20:1 (eicosenoic)	1.24 ± 0.15	1.19 ± 0.12
C20:2 (eicosadienoic)	0.39 ± 0.05	0.37 ± 0.04
C20:3 (eicosatrienoic)	0.02 ± 0.01	0.03 ± 0.01
C20:4 (arachidonic)	0.10 ± 0.02	0.12 ± 0.01
SFA	45.10 ± 1.05	43.97 ± 1.10
MUFA	45.28 ± 1.01	46.60 ± 1.15
PUFA	7.09 ± 0.61	7.00 ± 0.42

FA—fatty acids; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; $^{A, C}$—significant differences ($P < 0.01$); $^{A, B}$—significant differences ($P < 0.05$); SD—standard deviation

DISCUSSION

A number of studies have shown that an increased use of RSM in pig diets holds a great potential to improve sustainability and self-sufficiency [25, 30]. In our study, we reported the effects of a twelve-week dietary replacement of SBM with treated RSM on growth performance, blood mineral profiles and fatty acid composition of back fat in growing finishing Slovakian White x Landrace pigs. The rapeseed meal was treated with the product (intercalation complex), where the anti-nutritive compounds (e.g. glucosinolates) undergo a chemical conversion, the result of which is that the formed compounds are absorbed to a lesser degree and therefore may improve the digestibility of rapeseed meal.

Rapeseed meal, called canola meal (CM) in North America, Australia and some other countries, is the by-product of the extraction of oil from rapeseed. In several previous experiments, it has been demonstrated that between 15 % and 30 % of CM can be included in the diets fed to growing-finishing pigs without impairing their growth performance [10, 23]. Growing-finishing pigs are expected to be more tolerant to the inclusion of high RSM levels than weanling pigs in terms of the effects on their growth performance [13].

In the results of the current study, live weight, ADG and FCR were not significantly affected by dietary inclusion of treated RSM in growing and finishing diets (25 % and 18 %, respectively). However, the effects of RSM or CM in pig diets has been reported to be inconsistent in many reports [12, 23, 24].

The result of the Choi et al. [6] study indicated that RSM could be supplemented to growing-finishing diets up to 9 % without detrimental effects on the growth performance of growing-finishing pigs. In that study, body weight, ADG, and gain/feed ratio were affected by dietary RSM supplementation levels up to 12 %.

Yun et al. [32] reported the inclusion of 4 % of RSM or CM in finishing pig diets had no negative effects on the growth performance, nutrients digestibility, faecal noxious gas emission, blood characteristics, or meat quality.

A new study of Skugor et al. [26] was performed to investigate the effects of dietary inclusion of 20 % rapeseed meal (RSM) as an alternative to soybean meal (SBM) in a three-month feeding experiment with growing finishing pigs. The dietary alteration affected the growth performance, and several carcass traits, but did not affect the measured meat quality traits.

The meta-analysis of Hansen et al. [9] showed minor, but significantly negative effects of RSM on ADG and feed conversion, while regression analyses revealed no differences in the growth performance with increasing levels of RSM in the diets. Overall, the results suggested that low glucosinolate RSM can be used as an alternative feed resource without adverse effects on the growth performance if used in well-balanced diets for weanling and growing-finishing pigs [9].

The content of mineral elements in the protein sources does not meet the minimal nutritional requirements of pigs at the dietary levels of the protein supplements used in a balanced diet. It is usually met by adding a commercial trace mineral premix to the diet. Rapeseed meal is a better source of calcium, selenium and zinc than soybean meal, but a poorer source of potassium and copper. Its high phytic acid and fibre contents reduce the availability of many mineral elements [5]. In our study, blood mineral levels in both treatments were maintained within the normal physiological range. Cu and Zn are involved as co-factors in a myriad of metabolic enzyme systems within the pig [28].

The fatty acid profile of the pig back fat directly reflects the fatty acid profile of the pig diet [19]. Rapeseed oil is rich in MUFA and has higher levels of n-3 PUFA while soybean oil has a high C18:2 (linoleic) content and moderate levels of C18:1 (oleic) and C18:3 (linolenic acid). In pigs, dietary FAs are absorbed unchanged from the intestine and incorporated into tissue lipids [31]. Hence, dietary fat influences the FA profile of the adipose tissue [3]. Solvent-extracted rapeseed meal contains small amounts of residual oil (about 3 % DM). In solvent-extracted soybean meals, the oil content is typically lower than 2 %. The dietary fat (ether extract) content in our diets varied between 23.3 and 30.8 $g.kg^{-1}$. In our study we did not observe any differences between the total proportion of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) among the groups. There were a significantly higher level of monounsaturated palmitoleic acid ($P < 0.01$), and polyunsaturated α-linolenic acid ($P < 0.05$) in the back fat samples of pigs from the experimental group (tRSM diet).

Nevrkla et al. [18] in the study with pigs of hybrid combination of Large White × Landrace × Duroc × Pietrain pigs, which is very popular in meat production,

analysed fatty acid composition in the back fat. Their results showed a higher content of C18:3 n-3 (α-linolenic) (0.97 g.100 g^{-1} of total fatty acids) and comparable content of C16:1 n-7 (palmitoleic) (2.3 g.100 g^{-1} of total fatty acids) in the back fat samples compared to our results.

CONCLUSIONS

The current analysis showed minor and no significant effects of treated rapeseed meal as an alternative feed resource on growth performance in growing-finishing pigs. The concentrations of serum minerals were not influenced by dietary inclusion of treated rapeseed meal in the growing diet. Both protein feeds (soybean meal and treated rapeseed meal) did not have significant effect on the total proportion of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) in the back fat of pigs.

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THE FIRST RECORD OF FACULTATIVE PARASITISM OF *MEGASELIA* SPP. (DIPTERA: *PHORIDAE*) IN A HONEYBEE COLONY IN SLOVAKIA

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ABSTRACT

The current global climate warming trend leads to a shift in animal-habitats northwards. According to the Slovak Hydrometeorological Institute, summer 2018 was extremely hot and long throughout Slovakia. This was probably the fact that resulted in the detection of the presence of *Megaselia* spp. (Diptera: *Phoridae*) in one honeybee colony at the University apiary located in Rozhanovce (48° 46' 27.24" N; 21° 22' 26.01" E; eastern Slovakia). The first warning signal after opening the hive was the changed odour. During closer inspection, there were observed small parasitoid phorid larvae that emerged from the sealed bee brood; further examination revealed that the parasitized bee larvae and pupae contained emptied body cavities. Vice-versa, parasitisation was not detected in adult honey bees. Our knowledge of Diptera being responsible for parasitizing (even facultative) the honey bee (*Apis mellifera*) is still incomplete and needs to be investigated further in more details.

Key words: climate warming; facultative parasitoid; honey bee; *Megaselia* spp.; phorid fauna

INTRODUCTION

Honey bees (*Apis mellifera* L.; Hymenoptera: *Apidae*) belong to the most important commercial pollinators, providing valuable pollination services worldwide. That is why their good health status is crucial in this role. Global climate change has an impact on the numerous pathogens of honey bees, the interactions with their host-species have not been observed previously [14]. Migration of new pathogens northwards will lead to new encounters with their hosts. It is therefore very important that we understand the current pathogen-bee interactions and prevalence in southern regions of Europe, where the subspecies of *A. mellifera*, adapted to a hot climate, is native [16].

Our knowledge of Diptera responsible for parasitizing the honey bee is still a challenge for scientists; e. g. the detection of *Apocephalus borealis* (Diptera: *Phoridae*) was tied to honey bee parasitoidism, and considered as a potential indirect cause of colony collapse disorder [3].

Megaselia spp. insects belong to *Phoridae* (Diptera)—the family of small, hump-backed flies (1—6 mm lengths) which resemble the fruit flies. They can be recognised according to their escape habit of running rapidly across

a surface (rather than taking to the wings) and their alternate name "scuttle fly" based on this behaviour [20]. The family *Phorid* (Diptera) has very heterogeneous larval food requirements and adaptable feeding behaviours such as scavenger, phytophagous and entomophagous [2].

In this paper, we present *Megaselia* spp. as a legitimate agent of facultative honey bee parasitoidism in Slovakia.

MATERIALS AND METHODS

During the summer of 2018, four queen-right nuclei prepared on June 4, 2018, each consisting of 6 frames (one foundation frame, two store frames plus three brood frames), were maintained in a normal bee-keeping manner in small six-frames hives, with open access to natural food sources. By the regular weekly inspection on July 10, 2018, there was one colony detected as suspicious. By opening the hive, a typical "fetid odour" of old mushrooms was the first warning signal. The strength of the colony was comparable to the other three colonies prepared on the same time, but closer inspection showed, that the suspected nucleus had shifted from the front side of the frames more backwards (Fig. 1) and the sealed brood was left without any bee care. Moreover, there was visibly presented large numbers of small whitish larvae in the brood area (Fig. 2). Capped cells were perforated (Fig. 3) and many larvae were hatching from some of the cells (Fig. 4). The cells in the area where the nucleus had shifted was full of bee eggs (Fig. 5), showing that the nucleus had left the previous brood nest full of parasitoid larvae and tried to set a new nest more backwards on the same frames.

No larvae were detected in the dead or living adult honey bees by necropsy done *in situ*. After stereomicroscopic examination of the larvae in the laboratory, two specimens of the suspected brood were introduced into a plastic box and maintained at a temperature of 24 ± 2 °C and humidity of 60 % (Fig. 6). In 2—3 days after placing the pieces of the comb containing the suspicious brood into plastic containers, larvae pupated and later, after next 7 days emerged as the adult stage. At the beginning, the pupae were whitish and yellowish-brown and in the end of their pupal stage they changed to a dark brown colour.

The dissected bee larvae/pupae were examined using a stereo laboratory zoom-tool on July 11, 2018. On July 21, 2018, the flies that emerged were examined under a stereoscopic microscope at a magnification of 5—100 times for the systemic determination using a more recent key to European genera [7] and an updated guide to the identification literature for each genus by the same author [8].

RESULTS AND DISCUSSION

The examination of the dissected bee larvae and/or pupae respectively under stereo laboratory zoom-tool indicated that parasitoid phorid larvae had fully developed in the bee larvae/pupae body cavities by consuming their internal organs. Inspection of the parasitized bee larvae/pupae remnants revealed the complete destruction of the body cavity structures. In the case of the pupae, the thorax and abdomen had been completely emptied and dismembered. No parasites were detected in the adult bee specimens.

Based on the examination of the emerged adult flies under the stereoscopic light-microscope, the insects were identified as *Megaselia* spp. (Insecta: Diptera: *Phoridae*). Due to the difficult taxonomy of the *Megaselia* spp. genus (the list of *Megaselia* species is only tentative), the exact species identification was not possible, as the taxonomy of this giant genus counts more than 1400 species worldwide and is still insufficiently known [8, 13]. The species-richness of the phorid fauna is well known in the Czech Republic but not so well in Slovakia (pers. communication with RNDr. Mocek/entomologist).

Detailed clinical signs of *Megaselia rufipes* infestation have been documented under controlled conditions in laboratory-reared Hemiptera of the cockroach species [6] and in other insects, such as blowflies (Diptera: *Calliphoridae*) [1], and Noctuid moths [15]. *M. rufipes* infestation begins with egg deposition on the abdomen of adult bee and the ensuing embryonic development. After hatching, the first instar larvae penetrate the intersegmental membranes of the abdomen. At completion of the instar life, larvae leave their host in search of suitable pupation sites. While such parasitoid behaviours have often been observed and reported in species of the *Megaselia* genus, some species of the genus *Megaselia* are known to beekeepers as cleptoparasites, which are dependent on stored pollen and other organic remains found on the hive bottom [9]. The most common is *Megaselia scalaris* and has been documented as a scavenger in bee colonies in Spain [11], in Central-Southern Italy [17] and in Cameroon [4] in the last decade.

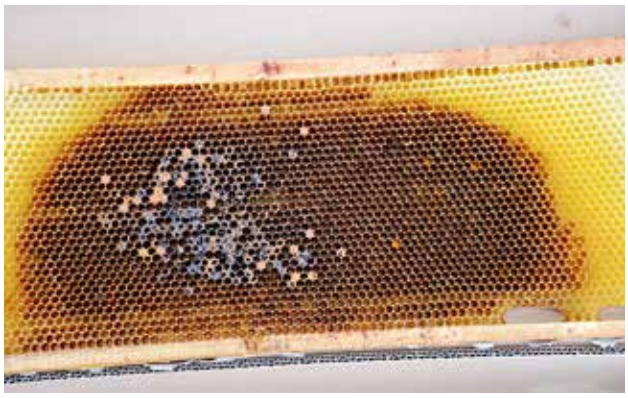

Fig. 1. Suspected frame

Fig. 2. Small larvae on the brood area

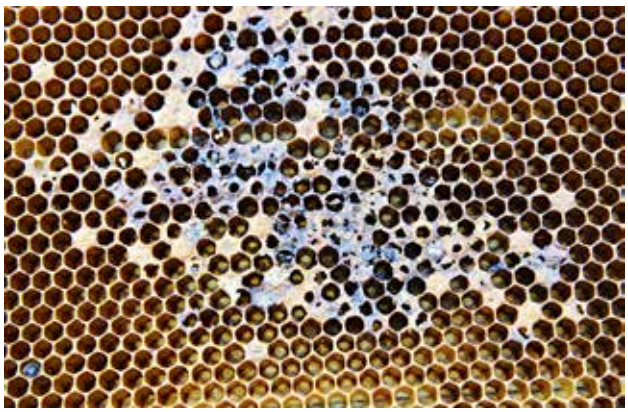

Fig. 3. Perforated capped cells

Fig. 4. Hatching larvae from cells

Fig. 5. New nest shifted backwards of the frames (area of fresh laid bee eggs)

Fig. 6. Hatched adult insects

Moreover, the presence of *Megaselia rufipes* and *Megaselia praeacuta* (both Diptera: *Phoridae*) were recorded from samples of dead honey bee colonies during 2017 in the Republic of North Macedonia [12]. *M. rufipes* is suggested to be a facultative parasitoid of *A. mellifera* that exploits the movement difficulties of deformed-winged bees [10]. Similar behaviours were observed in *Megaselia scalaris* in both the laboratory cockroach cultures and in the field [18].

Aside from honey bees, the broad host range of these phorid flies also makes them a considerable danger to wild

pollinators [5]; phorids have also shown an attraction to injured ants [3, 19]. Phorid larvae *Apocephalus borealis* discovered in dead honey bees are strongly suspected of colony collapse disorder, despite that it is reported that this parasitoid fly affects bumblebees and wasps [5]. The adult fly lays eggs on the bee's abdomen, later hatch and feed on the bee; affected honeybees show hive abandonment behaviour, leaving their hives at night and dying shortly outside the hive.

Our case showed that *Megaselia* spp. (Insecta: Diptera: *Phoridae*) can be considered a facultative parasitoid of *A. mellifera*. Except for the matter of fact, that this is the first record in central Europe, its detection also opens new scenario in the role of *Phoridae* in honey bee colony health for the future in this geographical latitude.

CONCLUSIONS

In this paper, we presented *Megaselia* spp. (Insecta: Diptera: *Phoridae*) as a legitimate agent of facultative parasitoidism, recorded for the first time in bee colony in central Europe/Slovakia. As this is the first report, the situation needs to be monitored due to the potential adverse/negative impact of *Megaselia* spp. on bee colonies survival in the future.

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VITAMIN E AND SELENIUM DECREASED CORTISOL IN CATTLE

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ABSTRACT

Twenty dairy cows with left abomasal displacement were used to investigate the effects of vitamin E and selenium treatment on blood cortisol in dairy cows stressed by omentopexy. The cows were randomly divided into two groups. Ten hours before surgery 6 g of DL-α-tocopheryl acetate (6 mg.kg^{-1}) and 67 mg of natrium selenite (0.1 mg.kg^{-1}) in a volume of 40 ml were administered subcutaneously to 10 cows; the control animals (n = 10) received an equivalent volume of injectable water (40 ml). The injection of vitamin E and selenium produced a rapid rise (P < 0.05) in blood α-tocopherol and selenium concentrations. The serum vitamin E increased several times ten hours after vitamin E and Se injection and raised continuously to the highest average concentration of 21.6 mg.l^{-1} at hour 24 after the surgery. The highest selenium concentration was seen ten hours after selenium administration with holding the increased concentrations in comparison to initial ones during the whole study. Serum cortisol increased in both groups after surgery. The highest cortisol concentrations were reached at one hour after surgery in the experimental and control group (56.7 ± 28.8 and 65.3 ± 26.1 μg.l^{-1}, respectively). A return to the levels similar to the initial ones was recognized 24 hours after the surgery. The ANOVA revealed a significant effect of vitamin E and selenium injection on serum cortisol (P < 0.05). In conclusion, we have demonstrated that abdominal surgery resulted in typical stress changes with a weaker cortisol response to the abdominal surgery in animals treated with vitamin E and selenium.

Key words: cortisol; dairy cows; selenium; surgical stress, vitamin E

INTRODUCTION

Peroxidative processes of lipids, proteins, carbohydrates as well as nucleic acids and their products can exert severe negative effects on cell membranes as well as biochemical pathways. Peroxidative processes and their products may regulate and influence the efficiency of an antioxidative

system. An antioxidative system represented by enzymes and non-enzymatic antioxidants is responsible for maintaining the balance between production and neutralization of reactive oxygen species [12]. Lack of antioxidants can result in various pathological processes like myopathy, cardiomyopathy, embryonic degeneration, liver necrosis, and encephalomalacia [17]. Increased health problems around parturition are particularly problematic as they may greatly impact the productivity of dairy cows in the ensuing lactation [9]. Therefore, it is not surprising that considerable research efforts have focused on defining factors that may contribute to immune dysfunction during this critical period in the production cycle of dairy cows [26].

It has been demonstrated that a number of manipulations including transport [10], therapeutic manipulation [22], and surgery [2], increased secretion of cortisol from the adrenal cortex in cattle. Several studies demonstrated that the stress reaction has enhancing effects on free radical production, thus contributing to an increased lipid peroxidation in animals [21]. Although stress reactions are organised to protect the homeostatic state of animals, they contain elements that may either enhance or diminish susceptibility to the disease process; in many instances, however, stress reactions themselves may induce pathologic change [3].

The present trial was aimed to study the effects of vitamin E and selenium treatment on blood cortisol in dairy cows stressed by omentopexy.

MATERIALS AND METHODS

Twenty Holstein-Frisian lactating dairy cows, mean age 4.41 ± 1.34 years (x ± SD), admitted for treatment of left abomasal displacement, were used in the study. All of them were within first six weeks after calving. Their mean body weight was 586 ± 65 kg (x ± SD). They were randomly divided into two equal groups (n = 10) according to the order of admission to the clinic. The surgery followed on the subsequent day to correct the abomasal displacement. Ten hours before surgery 6 g of DL-α-tocopheryl acetate (6 $mg.kg^{-1}$) and 67 mg of natrium selenite (0.1 $mg.kg^{-1}$) in a volume of 40 ml were administered subcutaneously to 10 cows; the control animals (n = 10) received an equivalent volume of injectable water (40 ml). Abdominal surgery (omentopexy) was performed in a standing position 16—24 hours after admission. The mean duration of preparation for surgery lasted approximately 30—40 minutes, and the surgery approximately 40 minutes. Procaine-hydrochloride (2 %) was used for local anaesthesia. All experimental animals were housed in pens with straw bedding and were fed hay *ad libitum*. Concentrates were offered on the day after the surgery according to milk yield and had free access to water. No additional health disorders were diagnosed in the experimental animals and no supportive treatment was necessary. All cows recovered and left the clinic on day 4 or 5 after the omentopexy.

Blood samples were drawn from the jugular vein before vitamin E/Se injection, just prior to surgery, immediately after surgery, then 15, 30, 60 minutes, and 2, 5, 10, and 24 hours after surgery. The blood samples were stored at 4 °C maximally for two hours before centrifugation. The serum samples were obtained and then stored frozen at –80 °C until analysis. The α-tocopherol concentrations in the serum were determined in saponified samples by high performance liquid chromatography (HPLC) using a fluorescent detector. The concentrations of selenium in the serum were measured using the fluorimetric method of Rodriguez et al. [25]. The serum cortisol concentrations were determined by chemiluminescent enzyme immunoassay (Immulate®/Immulate® 1000 Cortisol immunoassay, DPC, L.A., USA).

Statistical analysis was carried out by a two-factorial analysis of variance (one repeated factor: time, one grouping factor: treatment) with the *post hoc* Bonferroni test (IBM SPSS Statistics 23, 2015). Significance was declared at $P < 0.05$.

RESULTS

The subcutaneous injection of vitamin E and selenium resulted in a rapid increase ($P < 0.05$) in blood α-tocopherol and selenium concentrations (Table 1). The serum vitamin E increased to sixfold values ten hours after administration in the treated animals and rose continuously to the highest average concentration of 21.6 $mg.l^{-1}$, 24 hours after the surgery. There were no changes in serum Vitamin E concentration in the control group during the study. The serum selenium concentrations of the experimental group showed a similar dynamic like α-tocopherol (Table 1). The highest selenium concentration was seen ten hours after

Table 1. Concentrations of blood α-tocopherol, selenium, and cortisol in operated dairy cows after vitamin E/Se or placebo treatment (mean ± SD)

Sampling time	Group	α-tocopherol [mg.l^{-1}]	Selenium [mmol.l^{-1}]	Cortisol [mg.l^{-1}]
Before injection	T	2.38 ± 1.71	0.75 ± 0.19	8.13 ± 4.60
	C	2.27 ± 1.28	0.91 ± 0.22	7.87 ± 4.14
Before surgery	T	13.4 ± 5.16*	1.25 ± 0.20*	10.9 ± 7.10
	C	2.33 ± 1.23	0.91 ± 0.23	13.7 ± 11.6
Immediately AS	T	15.9 ± 3.85*	1.12 ± 0.20*	48.0 ± 24.8
	C	2.02 ± 0.85	0.88 ± 0.21	58.9 ± 41.0
15 min AS	T	15.2 ± 4.15*	1.10 ± 0.20	43.8 ± 20.7
	C	2.01 ± 0.90	0.89 ± 0.19	59.7 ± 30.6
30 min AS	T	16.5 ± 3.60*	1.18 ± 0.22*	44.5 ± 19.4
	C	2.05 ± 0.79	0.86 ± 0.18	61.1 ± 28.7
60 min AS	T	16.7 ± 3.81*	1.16 ± 0.22*	56.7 ± 28.8
	C	2.18 ± 1.00	0.84 ± 0.17	65.3 ± 26.1
2 hours AS	T	17.6 ± 3.18*	1.09 ± 0.26*	26.6 ± 16.5*
	C	2.13 ± 0.84	0.90 ± 0.21	49.9 ± 21.7
5 hours AS	T	19.0 ± 3.08*	1.08 ± 0.19*	15.8 ± 7.97
	C	2.11 ± 0.91	0.88 ± 0.21	21.8 ± 11.1
10 hours AS	T	20.6 ±2.53*	1.06 ± 0.16*	9.04 ± 5.19
	C	2.66 ± 2.53	0.95 ± 0.25	12.6 ± 8.02
24 hours AS	T	21.6 ± 2.60*	1.10 ± 0.15	9.41 ± 9.70
	C	2.51 ± 1.57	0.98 ± 0.25	10.7 ± 5.43
Group effect		**P < 0.05**	**P < 0.05**	**P < 0.05**
Time effect		**P < 0.05**	**P < 0.05**	**P < 0.05**

T —Treatment group (Vit E/Se); C—Control group (placebo); AS—after surgery;
*—means within sampling times differ significantly (P < 0.05) (Bonferroni test)

selenium administration with holding the increased levels in comparison to initial ones during the whole study.

Serum cortisol concentrations increased in both groups after surgery (Table 1). The highest values were reached at one hour after surgery in both groups. A return to concentrations similar to the initial ones were observed 24 hours after the surgery. The ANOVA revealed a significant effect of vitamin E and selenium injection on plasma cortisol concentrations ($P < 0.05$).

The initial serum concentrations of vitamin E were low in both groups but not clearly deficient [29]. The concentrations of vitamin E measured in our study were similar to those found by Pontes et al. [23] in dairy cows around calvings. The serum concentrations of α-tocopherol found ten hours after vitamin E and selenium injection were approximately as high as those in cattle reported by Hidiroglou and Laflamme [14] who used DL-α-tocopheryl acetate for intramuscular injection in

a similar dosage (4500 IU per 250—30 kg body weight). Thus, it can be assumed that concentrations of vitamin E, reached in the experimental animals within the surgical procedure, were high enough to be effective on a lipid peroxidation or other physiological reactions associated with the stress response in animals.

Similarly, the subcutaneous administration of selenium elevated the blood selenium in the experimental dairy cows within the complete experimental period (24 hours) which could create a different metabolic condition in animal tissues affecting multiple biochemical processes and reactions. These dynamics were similar to that previously reported by Chorfi et al. [7] after subcutaneous Se injections of 0.13 $mg.kg^{-1}$ in feedlot heifers. In dairy cows receiving Se injections in our study, the mean concentrations of serum Se observed prior to the injection in the experimental and control group (0.75 and 0.91 $\mu mol.l^{-1}$, respectively) were in the range of reference intervals for dairy cows [13]. Selenium is well known to be effective in reducing oxidative stress and the severity of several proinflammatory-based dairy cattle diseases such as mastitis and metritis [18]. Most of the antioxidant functions of Se were based on the reducing capacity of selenoproteins including the glutathione peroxidase (GPX) and thioredoxin reductase (TrxR) families [26].

Hopster et al. [15] concluded that 69.4 % of 307 baseline cortisol samples had concentrations below 3 $\mu g.l^{-1}$, whereas 13.7 % of the samples contained 6 $\mu g.l^{-1}$ cortisol or more. The relatively higher mean cortisol values prior to surgery in our study (higher than 7.5 $\mu g.l^{-1}$) may be due to sickness and transport stress of the animals. The effect of surgery on plasma cortisol was significant, however, cortisol concentrations fell near to pre-surgery values by the end of the trial. Thus, the pattern of cortisol response to surgery was similar to the pattern seen in 5—6 month-old cattle after amputation dehorning [27]. Comparable to some other studies, a significant effect of vitamin E and selenium administration on cortisol levels could be observed in our study. The reduction effect of vitamin E on the production of cortisol has been shown in calves [24], cattle [6, 11], and mice [16]. Dietary compounds with antioxidant activity reduce activation of the hypothalamicpituitary-adrenocortical axis in animals [8]. This may be due to an effect of vitamin E suppressing phospholipase A2 activity [5]. The enzyme phospholipase A2 is involved in ACTH secretion [1]. Taniguchi et al. [28] observed that broiler chickens supplemented with vitamin E and injected with ACTH had reduced corticosterone content in the adrenal glands. However, there was no inhibiting effect of vitamins E and C on the release of cortisol from bovine adrenocortical cells when stimulated with ACTH *in vitro* [19]. In addition, maternal Se supplementation of dams resulted in lower circulating cortisol concentrations in lamb offspring compared with lambs born from non-supplemented ewes [4]. In an experiment with a transportation stress in sheep, a depressive effect of trace element supplementation, including selenium, could be seen on cortisol levels in ewe lambs [20]. It was speculated that Se can act directly on blood cortisol by affecting the free radical-antioxidant capacity balance.

In conclusion, we have demonstrated that the single injection of 6 g of DL-α-tocopheryl acetate and 67 mg of natrium selenite significantly increases the serum vitamin E and Se within 10 hours. In addition, the blood cortisol was reduced in the treated cows. This decrease in blood cortisol may suggest that vitamin E and selenium supplementation may be an effective method to minimize the stress response in dairy cows.

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OCCURENCE AND ANTIMICROBIAL RESISTANT PATTERNS OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AMONG PRACTICING VETERINARIANS IN KEBBI STATE, NIGERIA

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an emerging zoonotic pathogen incriminated in causing multiple disease conditions in humans and livestock. Studies have shown relationships between livestock rearing and increased MRSA colonization risk among farm workers, and also suggest that livestock may serves as reservoirs of the bacteria and could also infect humans via close contact and consumption of contaminated animal products. The aim of this study was to investigate if practicing veterinarians with significant livestock contacts are at risk for MRSA colonization. Therefore, a non-randomized survey was conducted to establish the presence of MRSA among veterinarians practicing in Kebbi State Nigeria, using both cultural characteristics and molecular detection of the resistant gene (mecA). Forty-one (41) nasal swabs were aseptically collected. The detection rate of MRSA in the veterinarians was 14.6 %. The study revealed a high occurrence rate of MRSA among veterinarians in the study area. The relatively high prevalence recorded among veterinarians in this study could be attributed to the poor understanding of MRSA as a disease, its mode of transmission and its status in the country which have contributed immensely to the little/no awareness of MRSA among veterinarians and hence making it favourable for the bacteria (MRSA) to spread.

Key words: mecA; MRSA; Nigeria; practicing veterinarians; *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is a gram-positive opportunistic bacterium affecting both humans and other animals globally. The emergence of antimicrobial resistant strains of the bacteria has developed into a cause of concern as a serious public health problem [33]. Several species of staphylococci

have been recognized and some of them have been isolated as commensals in humans with enormous pathogenic potentials [37]. On mannitol salt agar, *S. aureus* grows as golden yellow colonies [30]. *S. aureus* is a facultative anaerobe, growing well under aerobic conditions within 24 hours [12]. It is found in people and frequently colonizes the nares, the armpit, the perineum, skin folds and the vagina without giving rise to disease [23]. It is a major opportunistic pathogen that is incriminated in a range of chronic and acute infections of humans and other animals worldwide [36]. A fundamental biological property of *Staphylococcus aureus* is its ability to colonize both healthy and hospitalized people asymptomatically; most often involving the anterior nares [11]. *Staphylococcus aureus* is a highly pathogenic organism capable of causing multiple diseases in humans. Its success as a commensal organism and pathogen is because of its versatility and rapid adaptability [31]. Mobile genetic elements (MGEs) as a means for the transfer of genetic information between and within bacterial species play a critical role in its adaptation process [24].

As a "superbug", *S. aureus* has high morbidity and mortality rates due to its ability to mutate, therefore endowing it with considerate levels of resistance to different classes of antibiotics recommended for their treatment [15]. Antimicrobial resistance to *S. aureus* is a major public health threat, compounded with the emergence of vancomycin and daptomycin resistant strains which are the last line of antimicrobials [19]. There has been a global increase of MRSA over the past few decades and livestock associated methicillin resistant *Staphylococcus aureus* (LAMRSA) has remained endemic in certain groups of workers with direct contact to live animals [41]. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been reported in different species of animals and their products [44]. Animals can serve as reservoirs and also as a source for the emergence of new clones of methicillin-resistant *Staphylococcus aureus* (MRSA) in humans [16]. Recently, a new sequence type belonging to the clonal complex 398 has emerged in pigs and calves worldwide causing invasive disease in humans [38]. And since its emergence in the early 2000s, the new strain of MRSA has increasingly become the cause of human infections most especially in those with livestock exposure [35].

The presence of LA-MRSA in farm workers is a major threat to public health and the health care system [45]. The prevalence of MRSA in humans has been shown to be strongly associated with its prevalence in animals and the intensity of contact with animals positive for methicillin-resistant *Staphylococcus aureus* [17]. MRSA can be transmitted between humans and their pets, although the route of transmission and risk factors for transmission are not well understood [45]. B r o n w y n et al. [8] described a case of human-to-dog transmission of MRSA. Several researchers postulate that MRSA infection and colonization in household pets strongly suggests that pets tend to be infected or colonized with MRSA strain types from community human populations [23, 45]. Also, researchers believed that MRSA in pets are closely linked to MRSA infection in humans and that infected or colonized humans may often be the source of MRSA in household animals [23]. N w o r i e et al. [29] reported a prevalence of 23 % of *S. aureus* amongst meat sellers in Abakaliki metropolis, Ebonyi State, Nigeria. Nonetheless, the presence of methicillin resistant *Staphylococcus aureus* has been documented in Nigeria among farm animals and farm workers [30, 42].

Despite the MRSA colonization rate in animals and their potential to transmit the infection to veterinarians, there has not been any molecular detection of MRSA among practicing veterinarians in Kebbi State, Nigeria, nor a documentation of its involvement in staphylococcal infections in livestock in the State. Therefore, this study was aimed at investigating the presence of MRSA among veterinarians in Kebbi State, Nigeria.

MATERIALS AND METHODS

Study area

The sampling locations for this study were the Zonal Veterinary Clinics in Kebbi State, Nigeria. The state is located in North-Western Nigeria, which lies between 12.45° N latitude and 4.2° E longitude.

Sample collection

A cross sectional study approach using non-randomized sampling was adopted. Considering the zoonotic potential of the disease, gloves were used during the collection of the samples. After seeking the veterinarian's consent, a nasal swab was taken using a sterile swab and labelled appropriately. Forty-one (41) veterinarians were sampled across the state. The study was conducted between April and June 2019.

Ethical clearance

All the sampling procedures were done in accordance with the Animal Research Ethics Committee of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University Sokoto (UDUS/FAREC/03/2019).

Bacteriological culture and isolation

Mannitol salt agar (OXOID) was prepared according to the manufacturer's instructions. The prepared agar was then poured into petri dishes and the prepared plates were then incubated at 37 °C for 24 hours to ascertain the sterility of the plates before inoculation of the samples (sterility test). The swab stick containing the sample was used to inoculate the plates by creating a primary well and then a sterile wire loop was used for streaking in order to obtain discrete colonies. The streaked plates were incubated at 37 °C for 24 hours. Presumptive colonies of *Staphylococcus aureus* species appearing as small, smooth, golden shiny convex colonies with golden yellow zones on Mannitol salt agar were picked and inoculated onto nutrient agar slants, and further incubated at 37 °C for another 24 hours.

Biochemical analysis

All of the colonies grown on nutrient agar medium were subjected to the following conventional biochemical tests such as catalase, and coagulase tests for the identification of *Staphylococcus* species.

The catalase test was carried out as described by Ochei and Kohlhatkar [30], while the slide agglutination test to determine coagulase positive isolates was carried out as described by Cheesbrough [12].

Phenotypic characterization of MRSA

Oxacillin resistance *Staphylococcus aureus* broth (ORSAB; Oxoid) was prepared according to the manufacturer's instructions. Presumptively, *S. aureus* colonies based on biochemical test were inoculated on prepared ORSAB plates. The presumptive MRSA (oxacillin resistant strains) colonies appeared as bright blue on the media.

Antimicrobial susceptibility test

The Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility of the isolates on Mueller Hinton agar using a panel of antimicrobials including; erythromycin, oxytetracycline, neomycin, penicillin, sulphonamides, gentamycin and vancomycin. The procedure and interpretations were based upon the recommendations of the Clinical and Laboratory Standards Institute [14]. Resistance or susceptibility of the isolates against the drugs tested were determined based on zones of inhibition measured to the nearest millimetre using a calliper. The sizes of the zones of inhibition were interpreted by comparing with the breakpoints as described by Clinical and Laboratory Standard Institute [14].

GENOTYPIC CHARACTERIZATION OF MRSA

Genomic DNA extraction

Genomic DNA was extracted using the traditional boiling method as described by Chen et al., [13] with slight modifications. A loop-full suspension of overnight grown cultures on nutrient agar plate was transferred into a 1.5 µl microcentrifuge tube containing 200 µl of sterile distilled water. The suspension was first incubated at room temperature for 5 min, and then heated in a dry water bath at 96 °C for 10 min. This was then followed by centrifugation at 12,000 g for 5 min., and the supernatant was then collected in a new 1.5 µl tube using a micropipette and then used as the DNA template.

MecA gene amplification

The DNA of all of the positive ORSAB *S. aureus* isolates were subjected to amplification by polymerase chain reaction (PCR) for the presence of the 163 bp fragment of the mecA gene using the following primers; mecA1 5'AAAATCGATGGTAAAGTTGGC-3'(forward), mecA2 5'AGTTCTGCAGTACCGGATTTGC-3'(reverse) [26]. The PCR was performed using the Qiagen Master mix kit in a 25 µl reaction mixture containing 3 µl of nuclease free water, 5 µl of the DNA template, 1 µl of each forward and reverse primer, 2.5 µl of coral load (loading dye, Qiagen) and 12.5 µl of the master mix (Qiagen). The PCR amplification protocol comprises of 35 cycles of amplification with an initial denaturation of 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min and then final extension at 72 °C for 7 min (GeneAmp PCR System 9700). The PCR products were visualized after electrophoresis for 45 min at 90 volts in a 1 % agarose gel. The bands were then viewed under a UV trans-illuminator.

Table 1. Antibiotic susceptibility profile of MRSA isolates (n = 10) from veterinarians in Kebbi State, Nigeria

Antibiotics	Breakpoints [mm]	Resistant	Inter. mediate	Susceptible
Erythromycin	14—22	5	5	–
Oxytetracycline	14—15	5	5	–
Neomycin	13—14	6	4	–
Penicillin	17—18	10	–	–
Sulphonamides	13—16	6	3	1
Gentamycin	13—17	7	3	–
Vancomycin	15—19	–	–	10

Note: The breakpoints (in millimetres) are based on the CLSI standard (2008)

Table 2. Antibiotic resistant pattern for MRSA isolates from veterinarians in Kebbi State, Nigeria.

Antibiotic resistance pattern	Number of isolates
Ery-Oxy-Neo-Pen-Sul-Gen	4
Ery-Oxy-Neo-Pen-Gen	1
Ery-Pen-Sul-Gen	1
Ery-Pen-Gen	1
Oxy-Neo-Pen-Sul-Gen	1
Oxy-Neo-Pen-Sul	1
Neo-Pen-Sul-Gen	1

Ery—Erythomycin; Oxy—Oxytetracyclin; Neo—Neomycin; Pen—Penicillin; Sul—Sulfonamide; Gen—Gentamycin

Data analysis

The results obtained in this study which were presented in tables and percentages, were computed using Microsoft Excel programme version 2010.

RESULTS

Among the 41 samples cultured, 17 (41.5 %) had colonial growth and biochemical characteristics synonymous to *Staphylococcus* sp. However, only ten (10) out of the 41 (24.4 %) samples were MRSA positive based on the phenotypic characterization and bright blue pigmentation on the ORSAB media.

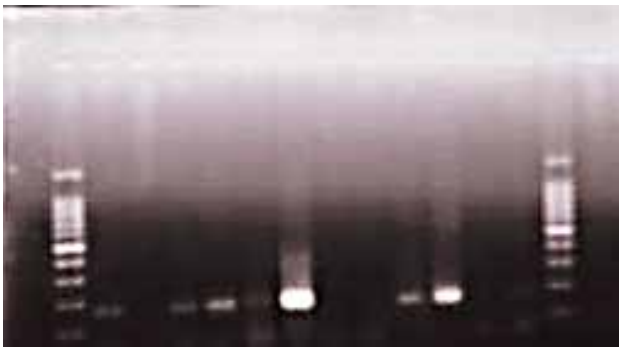

Fig. 1. Agarose gel electrophoresis image showing amplification of mecA gene (163bp). Lane L—100 bp ladder; lane P—positive control; lanes N—negative control; lanes 1, 2, 3, 4, 7, 8—positive samples; lanes 5, 6, 9, 10—negative samples

The results for the antimicrobial susceptibility tests revealed that the ORSAB positive isolates exhibited varying level of resistance against the antimicrobials tested, with five (50 %) showing resistance to erythromycin and oxytetracycline, six (60 %) of the isolates were resistant to neomycin and sulfonamides, and 7 (70 %) of them were resistant to gentamycin. Additionally, all of the MRSA isolates were seen to be resistant to penicillin, while all of the isolates were susceptible to vancomycin on the other hand (Table 1). However, four of the isolates were found to show multidrug resistance (MDR) with Ery-Oxy-Neo-Pen-Sul-Gen being the most common pattern (Table 2).

Similarly, PCR amplification of all the ten (10) positive samples on ORSAB for the detection of mecA gene showed that 60 % (6/10) of the isolates possess the mecA gene (Fig. 1). Hence, the overall molecular detection rate of MRSA among the veterinarians studied is 14.6 % (6/41).

DISCUSSION

MRSA has been reported in different livestock including cattle, poultry and pigs [6, 21]. Several studies have indicated MRSA transmission from livestock to farm workers [20], but the exact extent of transmission between livestock and farm workers alongside with its clinical significance remain unclear; in the same vein whether individuals in walks of life other than animal rearing are at risk of becoming MRSA carriers remains unknown. The overall detection rate of MRSA among veterinarians in this study was 14.6 %. The prevalence recorded in this study were similar to the

prevalence reported by Middleton et al. [27] who recorded a prevalence of 14 % in veterinarians at a veterinary teaching hospital in the USA. Also, Loeffler et al. [23] reported a prevalence of 18 % among the veterinary staff of a small animal hospital in the UK. Weese [45] reported a prevalence of 13 % among veterinary personnel that came in contact with animals positive with MRSA in Canada. The similarities noticed in the prevalence above could be attributed to the same technique of detection and the sample size used in our study. Seguin et al. [39] reported a nasal carriage of MRSA in three (3) of five (5) veterinary staff during an outbreak of MRSA in an equine hospital in the USA. Our prevalence was much higher than that reported by Abudu et al. [1] and Maudsley et al. [25] that reported 1.5 % and 0.8 % respectively in a community survey conducted among the veterinary staff in the UK. This also can be partly attributed to the fact that the endemicity of MRSA was higher in Nigeria than the UK and also the irrational use of antibiotics (beta-lactam) in animals in Nigeria was higher than in the UK which can lead to increased MRSA spread and hence may explain the higher prevalences recorded. Wulf et al. [47] reported a prevalence of 3.9 % among veterinarians. Also, Akilu et al. [3] showed a prevalence of 7 % among veterinary staff at a veterinary hospital in Malaysia. The relatively high prevalence recorded among veterinary staff in our study could be attributed to the poor understanding of MRSA as a disease, its mode of transmission and its economic impact to healthcare in the country which have also contributed greatly to lack of awareness of MRSA among veterinarians and thus making it favourable for the bacteria (MRSA) to spread. Also, in Nigeria, antimicrobial agents are still readily available to people in local drug stores over the counter, a practice which has led to misuse of antibiotics with the associated high prevalence of antibiotic resistance among *Staphylococcus* [27]. Nuttall et al. [28] suggested that MRSA could be an occupational hazard to individuals in the animal health profession. Numerous studies have suggested that animals may serve as a reservoir for human infection with MRSA [20]. Hanselman et al. [18], Wulf et al. [47], Williams et al. [46] and Anderson et al. [5] reported that veterinarians have much higher MRSA colonization rates when compared with the general population. Ishihara et al. [20] suggested that in a veterinary hospital, the veterinary staff might be a primary source of MRSA infection for the animals.

Slight differences between phenotypic and genotypic detection techniques were also observed in our study. Notwithstanding, all isolates that appeared as bright blue on ORSAB were presumed to be MRSA because ORSAB has been commonly used for the detection MRSA with 99 % sensitivity in the detection of MRSA as reported by Becker et al., [7]. The gene responsible for the resistance (mecA) was detected in 60 % (6/10) of the isolates that were ORSAB positive in our study. Even though the presence of mecA has been recognized as the gold standard for MRSA detection as reported by Aliyu et al., [3], studies have shown that there exist mechanisms that are non-mec-dependent and they may contribute towards methicillin resistance in staphylococci strains [8, 9]. Aliyu et al., [4], Usman and Mustapha [43], Shamila-Syuhada et al. [40], and Pu et al. [36] all reported differences in phenotypic and genotypic detection of MRSA in *S. aureus*. So, these discrepancies noticed between the phenotypic and genotypic methods of detection could be attributed to the non-mecA-dependent methicillin resistance and heterogeneous expression of methicillin resistance in *S. aureus*. Also, the discrepancy between the results obtained in our study and those of the aforementioned researchers above, can be attributed to the postulation of Lee et al. [22], who opined that phenotypic resistance of *S. aureus* to methicillin could vary depending on cellular growth conditions. Phenotypic expression of resistance to methicillin in MRSA varies, and each strain has a characteristic profile of the proportion of bacterial cells that grow at specific concentrations of methicillin according to Plata et al. [34]. It has also been observed by Adesida et al. [2] that PCR detection of mecA gene does not always give indisputable results. Some MRSA strains have been found to be mecA-negative in PCR, but resistant to methicillin. Furthermore, some MRSA strains have also been reported to be mecA positive but susceptible to methicillin (Olonitola et al. [32]). The absence of mecA in MRSA strains could also be an indication of the potential presence of "modified *S. aureus*" (MODSA). MODSA possess modified penicillin-binding proteins (PBPs) which was a different classical mechanism of resistance to methicillin in MRSA according to Bhutia et al. [10]. In this study, a negative correlation was found between the phenotypic resistance to methicillin and PCR results for the detection of mecA gene that code for resistance to methicillin.

CONCLUSIONS

The overall prevalence of MRSA in veterinarians in our study was 14.6 %. This suggests that animal health workers could be at a higher risk of MRSA asymptomatic colonization and this could be attributed to occupational exposure with animals, therefore we concluded that, veterinarians caring for livestock have a high risk of being colonized by MRSA. The public health implication of our study was that veterinarians with professional contact with livestock are frequently colonized with livestock-acquired (LA) MRSA. As such, these individuals are presumably the source for LA-MRSA transmission to household members and other parts of the human population. The infection of these group of professionals leads to the introduction of LA-MRSA into the hospital and other healthcare facilities. Further studies are needed to determine the exact source of MRSA in both animal care providers and the livestock. More so, in Nigeria, the indiscriminate use of antibiotics in livestock production could have also contributed immensely to the patterns of antibiotic resistance recorded in our study although the sample size in our study was not large enough to make a generalization of the situation, we strongly believe that it provided evidence of the occurrence of MRSA in veterinarians in Kebbi State Nigeria and hence can serve as a baseline data for further investigations.

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GROSS MORPHOLOGY OF THE CEREBRUM AND BRAINSTEM OF THE ADULT AFRICAN GRASSCUTTER (*THRYONOMYS SWINDERIANUS*—TEMMINCK, 1827)

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ABSTRACT

In order to meet the increasing protein and income demand in Africa due to the rapid population growth, wildlife, such as the African grasscutter, is currently bred and domesticated as microlivestock. This study is one of the series on the brain morphology of this very large rodent, aimed at providing information that is lacking in the literature. Here, the gross anatomy of the cerebrum and brainstem in nine adult African grasscutters is described. The cerebral cortex was smooth, devoid of gyri and sulci, thus, placing the rodent in the lissencephalic group of mammals. However, blood vessels on the cortex created arterial and venous impressions. The cortex was asymmetrically-tapered oval in shape. The rostral and caudal colliculi were exposed through the cerebral transverse fissure. The rostro-caudal extent of the *corpus callosum* was from the mid-point of the frontal and parietal lobes, to a point just rostral to the occipital lobe. The rostral colliculi were grossly smaller than the caudal colliculi. The occulomotor and trochlear nerves emerged from the ventral midbrain, rostral to the pons. The pons was exceptionally large; it was pre-trigeminal. On either side of the ventral median fissure of the *medulla oblongata* were conspicuous pyramids. The trapezoid bodies were also conspicuous. These, and other findings, will be useful in future phylogenetic comparison of rodent brain morphology.

Key words: African grasscutter; brainstem; cerebrum; *medulla oblongata*; trapezoid body

INTRODUCTION

The current estimate of the global human population is 7 billion, and expected to be 9.3 billion by the year 2050, with Africa recording the highest value [3]. This demographic upheaval exerts pressure on food supply, including animal protein. According to Auzel and Wilkie [1], at least 20 % of the animal protein source in Africa is obtained through hunting and fishing. Thus, wildlife represents a substantial source of animal protein and income for a large part of the rural populations on the continent. The

African grasscutter is a wild rodent. However, to meet the current protein decline in Nigeria, the rodents are currently bred and domesticated as microlivestock. Unfortunately, the behaviour of the rodent is not well known, as the biology is still being studied. Knowledge of the biology of the African grasscutter is necessary to provide a near-natural habitat for the rodent in captivity. This is one of the reasons the brain of the African grasscutter was studied.

The morphology of mammalian brains has recorded wide diversity. The morphology of the cerebrum varies in mammals. In higher mammals, the cerebral cortex is convoluted, forming ridges called gyri and troughs called sulci. Such mammals with a convoluted cerebral cortex are said to be gyrencephalic, while those with smooth cerebral cortex are said to be lissencephalic. The convolutions permit more cortical surface areas to be contained in the same volume of the cranial vault [10]. Studies have revealed that the shape of the cerebral cortex vary in different animals, although spherical in humans. P e n g et al. [26] reported that the shape of the cerebral cortex of the African ostrich is an obtuse triangle. I b e et al. [13] observed a diamond-shaped cerebral cortex in the adult African giant pouched rat.

The brainstem is made up of the *medulla oblongata*, the pons and the midbrain, though some authors include the thalamus as part of the brainstem [12]. The thalamus serves as the gateway between the brainstem and the cerebrum, such that all incoming information (except olfaction) approaches the cerebrum through the thalamus [18]. The thalamus has also been linked to cerebral arousal [18]. The midbrain, which contains neurons that connect the cerebrum with lower parts of the brain and spinal cord, occupies only a small region in the brain of humans, but it is relatively larger in lower vertebrates [5]. The cerebral aqueduct separates it into the dorsal *tectum* and the ventral *tegmentum*. The midbrain narrows rostrally into the third ventricle; it is connected to the pons caudally. The *medulla oblongata* is the most caudal part of the brainstem. It is continuous with the spinal cord caudally and the pons rostrally. It lies in the cranial vault, rostral to the *foramen magnum*. In the Wistar rat, it begins about 3 mm rostral to the origin of the first cervical nerve [34], while in the one-humped camel, the level of the first cervical nerve is a convenient landmark for separation of the *medulla oblongata* from the spinal cord [21]. The *medulla oblongata* is the control centre for autonomic functions and a relay centre for impulses between the spinal cord and higher brain centres. It coordinates autonomic functions such as respiration, blood pressure and heart rate [9].

Gross morphological studies of the *corpora quadrigemina* [11], cerebellum [4, 15] and olfactory bulbs [14] of the African grasscutter are available in extant literature. Conversely, no detailed account of the cerebrum and brainstem is available and the purpose of this article is to provide descriptive information to fill this gap. This study will not only bridge the existing gap in available literature, but, it will also provide a reference for phylogenetic comparison of the rodent brain.

MATERIALS AND METHODS

Experimental animals and management

Nine adult African grasscutters were used for this study. The gender of the animals were not taken into consideration. They were purchased from a grasscutter farm in Elele, Rivers State, Nigeria. Rivers State is on latitude 4.7500° N and longitude 6.8333° E. Prior to purchase, the African grasscutters were physically examined under careful restraint and ascertained to be clinically healthy. They were transported by road, in metal cages, to the Histology/Embryology Laboratory of the Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria, for the study.

The animals were fed twice daily, at 8.00 am and 6.00 pm with fresh guinea grass (*Panicum maximum*), fresh cane grass (*Eragrostis infecunda*) and commercial rodent-pelleted concentrates. Drinking water was provided *ad libitum*. The feeding troughs and drinkers were sterilized daily using Milton®.

Ethical approval

The experimental procedure got the approval of the Research Ethics Committee, College of Veterinary Medicine, Michael Okpara University of Agriculture Umudike, Nigeria. Management of the experimental animals was as stipulated in the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Research Council, USA (National Academic Press, Washington, DC: http://www.nap.edu).

Extraction of the brain

Each animal was sedated by intraperitoneal injection of 20 $mg.kg^{-1}$ thiopental sodium (Rotexmedica, Trittau, Ger-

many) and immediately weighed using a digital electronic balance (Citizen Scales (1) PVT Ltd., South Patel Nagar, New Delhi, sensitivity: 0.01 g). Thereafter, the animal was placed on dorsal recumbency on a dissection table, and perfused, via the left ventricle, with 4 % paraformaldehyde fixative, as described by G a g e et al. [7]. Immediately after the perfusion fixation, each animal was decapitated at the atlanto-axial joint, using a pair of scissors and a knife. The skull was obtained after skinning and stripping off all the facial and cranial muscles. This was followed by brain extraction from the skull, using scalpel blades, thumb forceps and a pair of scissors. The meninges and underlying blood vessels were gently removed to expose the intact brain. A mid-sagittal longitudinal incision was done on 5 brain samples to access the mid-sagittal view of the brain parts for study. The rest of the brain samples were used for the extraction and study of the brainstem.

Extraction of the brainstem from the cerebrum and cerebellum required systematic removal of the cerebral halves, followed by severing of the cerebellum. In order to remove the cerebrum from the rest of the brain, the two cerebral halves were gently pulled apart at the occipital pole to expose the *corpus callosum*. The entire *corpus callosum* together with the *septum pellucidium* and the body and rostral commissure of the fornix were severed in the midline. This completely separated the cerebrum from the brainstem and cerebellum. In order to separate the cerebellum from the brainstem, the floculli of the cerebellum were manually raised to expose the cerebellar peduncles. These peduncles were then severed on both sides starting with the laterally located *brachium restiformis*, followed by the middle *brachium pontis* and then the *brachium conjuctivum*. Thereafter, the brainstem was freed from the *arachnoidea* and cranial nerves by simple trimming.

Morphological study

The extracted brain samples were studied grossly with the naked eyes and with the aid of a magnifying glass. These included the shape, size, surfaces, borders and angles of existing brain structures. The presence or absence of sulci, gyri, fissures or prominences were evaluated in each of the brain structures. Photographs of the surface of the dorsal and ventral views of the intact brain, the mid-sagittal view of the brain and the dorsal view of the extracted brainstem were obtained using a digital camera (Eastman Kodak® Model 14650, Rochester, New York, USA, 12.5 megapixels). Anatomical structures were defined based on standard rodent anatomy literatures [23, 25, 34]. *Nomina Anatomica Veterinaria* [22] was used for the nomenclature.

RESULTS

Morphology of the cerebrum

On dorsal view of the intact brain, the cerebral cortex was the largest brain part. It was distinctly divided by a longitudinal fissure (Fig. 1. White line arrow) and separated from the cerebellum by a transverse fissure (Fig. 1. Black line arrow). The cerebral cortex was smooth and devoid of prominent gyri and sulci. However, blood vessels on the surface of the cerebral cortex created arterial and venous impressions on the cortex (Fig. 1. White double arrow). The cerebral cortex presented a tapered frontal lobe (Fig. 1. B1) and broad temporal and occipital lobes (Fig. 1. B3 and B4). Thus, the cerebral cortex had an asymmetrically-tapered oval shape. The cerebral cortex presented a notch at the point of expansion of the temporal lobe, which was indicative of the distinctly large temporal lobe relative to the rostrally located frontal lobe (Fig. 1. Black arrowhead). The rostral and caudal colliculi were exposed through the transverse fissure (Fig. 1. C and D).

On the ventral view of the intact brain, the olfactory bulbs existed as rostral outgrowths of the olfactory cortex, with the olfactory tracts connecting the bulbs and the piriform cortex (Fig. 2. A, B1, B2, B3). The olfactory nerve fibres, which were rostral to the olfactory bulbs, could not be accessed due to their position in the cribriform plate of the ethmoid bones. The paired optic nerves (cranial nerve II) united at the optic chiasma and gave off optic tracts that ran on the rostral boarder of the mammillary body (Fig. 2. C1, C2, C3). The *infundibulum, cinerium tuber* of the hypothalamus and mammillary bodies were evident (Fig. 2. i, ii, D). The temporal lobe was the largest of the cerebral cortical lobes (Fig. 2. G). It was presented caudal to the piriform lobe (Fig. 2. E). The cerebral peduncles, relaying impulses to and from the cerebral cortex were very visible.

On a mid-sagittal view of the brain (Fig. 3), the absence of cerebral cortical sulci and gyri was further confirmed as no trough was evident on the dorso-ventral extent of the cerebrum. The cerebral lobes were thus, identified by their location, not by the presence of sulci and gyri. The frontal, parietal and occipital lobes were evident on this view

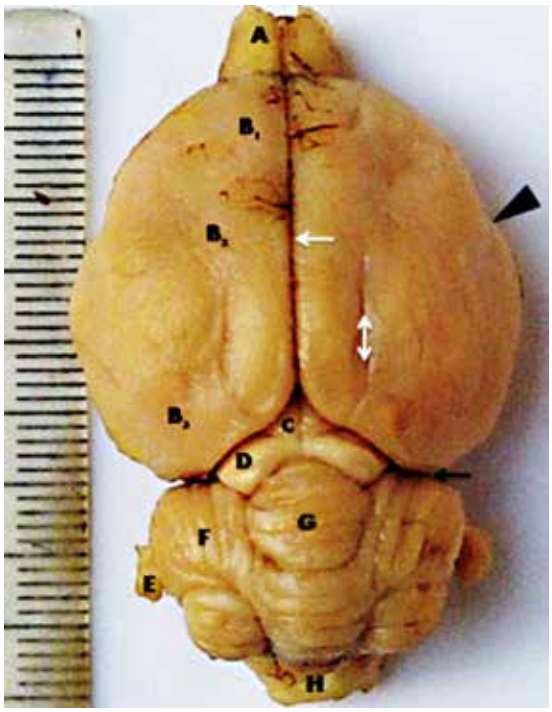

Fig. 1. Dorsal view of the brain of the adult African grasscutter

A—Olfactory bulb; B—Cerebral cortex (B1—Frontal lobe; B2—Parietal lobe; B3—Temporal lobe; B4—Occipital lobe); C—Rostral colliculus; D—Caudal colliculus; E—Paraflocculus; F—Flocculus; G—*Vermis*; H—*Medulla oblongata*; White line arrow—cerebral longitudinal fissure; Black line arrow—cerebral transverse fissure; White double head arrow—arterial impression on cerebral cortex. Magn. ×2.0

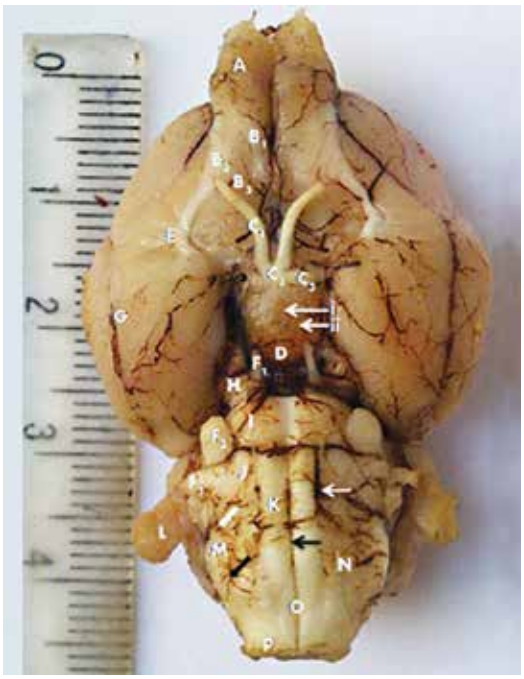

Fig. 2. Ventral view of the brain of the African grasscutter

A—Olfactory bulb; B1—Medial olfactory tracts; B2—Lateral olfactory tracts; B3—Middle olfactory tracts; C1—Optic nerve (CN II); C2—Optic chiasma; C3—Optic tracts; D—Mammillary body; i—Cinerium tuber; ii—Infundibulum; E—Piriform lobe; F1—Occulomotor nerve (CN III); F2—Trigeminal nerves (CN V); F3—Abduscence nerve (CN VI); G—Temporal lobe; H—Cerebral peduncle; I—Pons; J—Trapezoid body; K—Pyramid; L—Paraflocculus; M—Olivary body; N—*Medulla oblongata*; O,—Point of pyramidal decussation; P—Spinal cord; White line arrow—ventral lateral sulcus; White block arrow—pre-olivary sulcus (CN IX-XI); Black block arrow—post-olivary sulcus (CN XII); Black line arrow—ventral median fissure. Magn. ×2.0

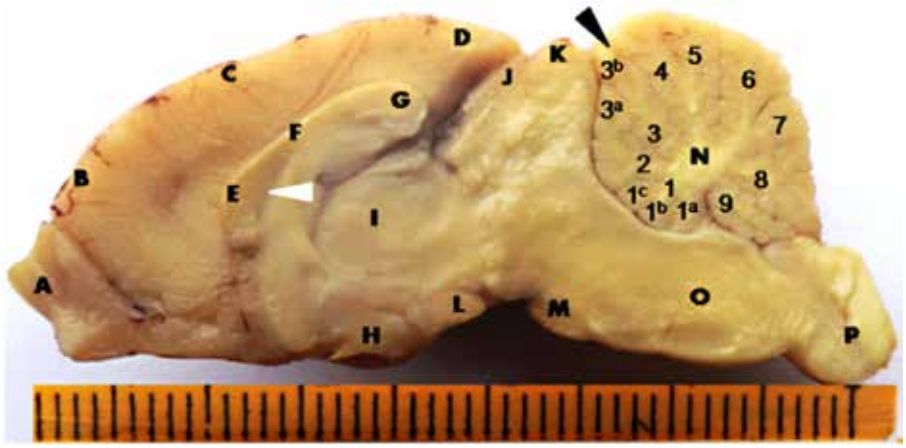

Fig. 3. Mid-sagittal view of the brain of the adult African grasscutter

A—Olfactory bulbs; B—Frontal lobe of cerebrum; C—Parietal lobe of cerebrum; D—Occipital lobe of cerebrum; E—*Corpus callosum* (Genu); F—*Corpus callosum* (Truncus); G—*Corpus callosum* (Splenium); H—Mammillary body; I—Inter-thalamic adhesion; J—Rostral colliculus; K—*Caudal colliculus*; L—Pituitary gland; M—Pons; N—*Arbor vitae*; O—*Medulla oblongata*; P—Spinal cord; 1—*Lingula*; 1a—*pars rostralis*; 1b—*pars medialis*; 1c—*pars caudalis*; 2—Central lobule; 3—Culmen; 3a—*pars rostralis*; 3b—*pars caudalis*; 4—Declive; 5—Folium; 6—Tuber; 7—Pyramis; 8—Uvula; 9—Nodulus; White arrowhead—lateral ventricle; Black arrowhead—primary fissure. Magn. ×3.0

(Fig. 3. B, C, D). Caudal to the occipital lobe were the visible rostral and caudal colliculi, sandwiched between the occipital lobe of the cerebrum and the cerebellum (Fig. 3. J, K). The inner white matter of the cerebrum, the *corpus callosum* lay between the cerebral cortex and the thalamus. The rostro-caudal extent of the *corpus callosum* was from mid-point of the frontal and parietal lobes, up to a point just rostral to the occipital lobe. The genu, truncus and splenium of the *corpus callosum* (Fig. 3. E, F, G), as well as the third ventricles (Fig. 3. white arrow-head) bounded by the *corpus callosum* were very evident.

Morphology of the brainstem

On a dorsal view of the brainstem, the third ventricle was flanked on either side by the thalamus (Fig. 4. i, B). The dorsal surface of the midbrain was characterised by the paired rostral and paired caudal colliculi (Fig. 4. D and F), and their respective geniculate bodies (Fig. 4. C and E). The rostral colliculi were grossly smaller than the caudal colliculi. The pair of rostral colliculi was separated from each other by a longitudinal *intracollicular sulcus* (Fig. 4. ii). The pair of caudal colliculi was also similarly separated. The rostral colliculi were separated from the caudal colliculi by a transverse *intercollicular sulcus* (Fig. 4. iii). Caudal to the midbrain was the open rostral part of the *medulla oblongata* which formed the floor of the fourth ventricle (Fig. 4. H). It was larger than the rostrally placed third ventricle and the lateral ventricles observed in the cerebrum. It was roughly rhomboid in shape. It was bounded rostrolaterally by the *sulcus limitans*, bilaterally by the stumps of the rostral, middle and caudal cerebellar peduncles and caudally by the obex. It was divided into two symmetrical halves by a dorsal median sulcus (Fig. 4. iv). This sulcus extended caudally to also divide the closed caudal part of the *medulla oblongata*. Flanked bilateral to the dorsal median sulcus on the closed caudal part of the *medulla oblongata* was the large *gracile fasciculus* (Fig. 4. J). The *fasciculus* continued rostrally on the ventro-lateral margins of the open part of the *medulla oblongata*. A dorsal intermediate sulcus (Fig. 4. vi) separated the conspicuous *gracile fasciculus* from a laterally placed, faintly visible *cuneate fasciculus*. The dorsal lateral sulcus was not grossly visible. A convex prominence, corresponding to the underlying vestibular nucleus, was evident between the *sulcus limitans* medially and the medial cerebellar peduncles laterally. The hypoglossal trigone and vagal trigone were not visible.

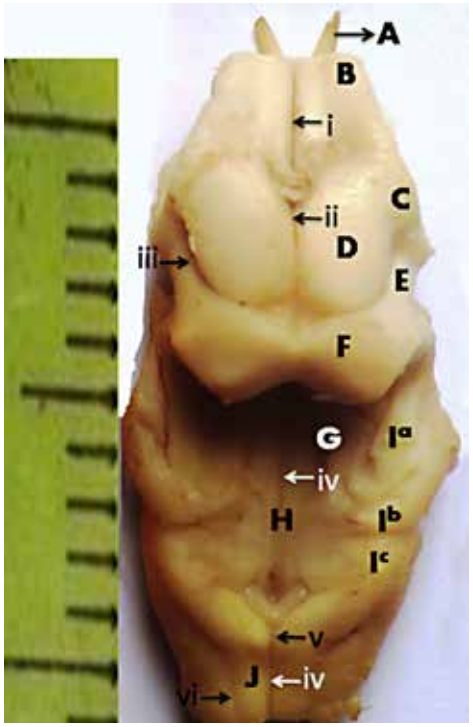

Fig. 4. Dorsal view of the brainstem of the African grasscutter

A—Optic nerve; B—Thalamus; C—Lateral geniculate body; D—Rostral colliculus; E—Medial geniculate body; F—*Caudal colliculus*; G—*Sulcus limitans*; H—Floor of fourth ventricle; Ia—Rostral cerebellar peduncle; Ib—Middle cerebellar peduncle; Ic—Caudal cerebellar peduncle; J—*Gracile fasciculus*; I—Third ventricle; ii—Longitudinal intracolliculi sulcus; iii—Transverse intercolliculi sulcus; iv—Dorsal median sulcus; v—obex; vi—Dorsal intermediate sulcus. Magn. ×3.0

On a ventral view of the brainstem, the thalamus marked the most rostral structure. The mammillary body and infundibulum preceded the pons. The occulomotor and trochlear nerves emerged from the ventral midbrain, rostral to the pons (Fig. 2. F1). The pons was exceptionally large, with a bony impression on its middle. It was pre-trigeminal. The trigeminal nerve (Fig. 2. F2) emerged from the pontomedullary junction. It was the largest cranial nerve on the brainstem. The *medulla oblongata* (Fig. 2. N) was symmetrically divided into two by a ventral median fissure (Fig. 2. black line arrow). On either side of the ventral median fissure were conspicuous pyramids (Fig. 2. K). The most rostral structure on each half of the *medulla oblongata* was the trapezoid body (Fig. 2. P). The olivary prominence was situated lateral to each of the pyramids, sandwiched between the pre-olivary and the post-olivary sulci. Nerve rootlets emerged from these sulci. Caudally, the *medulla oblongata* terminated at the prominent fossa, caudal to the pyramidal decussation at the level of the *foramen magnum*.

DISCUSSION

Result of the morphology of the cerebral cortex revealed a smooth cortex, devoid of gyri and sulci, and this finding places the African grasscutter in the lissencephalic group of mammals. Initially, R a k i c [28] proposed that gyrification was a phylogenetic trait; so that while lissencephaly was a primitive trait in lower mammals like rodents, gyrencephaly evolved in higher mammals, including primates. Recent molecular analysis has however, objected to this hypothesis [29], and proved that gyrencephaly is more closely related to variability in brain and body sizes in mammals. Thus, while gyrencephaly has been reported in large rodents such as the beaver and capybara [32], lissencephaly has been reported in small primates such as the marmoset monkey [8]. Although the adult African grasscutter is also a large rodent; the fourth largest extant rodent after the crested porcupine, beaver and capybara; the second largest rodent in Africa after the North African crested porcupine [6], there was no observable evidence of gyrification from the present study. One generally acclaimed reason for gyrification is the need to permit more cortical surface areas to be contained in the same volume of cranial vault. It has also been linked to increased neuronal connection and cognitive ability in higher mammals [35]. However, the functional significance of gyrified brain is still in debate, as individuals with severe disruptions of gyrification sequence still survive [33].

The asymmetrically-tapered oval shape of the cerebral cortex, with a tapered frontal lobe, and broad temporal and occipital lobes can be linked to apparently increased neuronal size which was more evident in the temporal and occipital lobes. This shape is different from the spherical cerebral cortex, observed in humans and most mammals. The common assertion is that the cerebral cortex is spherical, thus, each halve is referred to as a hemisphere. Studies (including the present study) have revealed that the shape of the cerebral cortex vary in different animals, although spherical in humans. P e n g et al. [26] reported that the shape of the cerebral cortex of the African ostrich is an obtuse triangle. I b e et al. [13] observed a diamond-shaped cerebral cortex in the adult African giant pouched rat. It is pertinent to mention that the terminology hemisphere ought to be restricted to humans and animals with spherical cerebral cortex. The increased size of the auditory cortex (temporal lobe), relative to other cerebral lobes, points to a good auditory ability in the African grasscutter.

Gross observation of the brainstem in our study revealed that the caudal colliculi were bigger than the rostral colliculi. This confers a more acute auditory ability in the African grasscutter. Gross morphometric and histological assessment of these colliculi have been reported by I b e et al. [11] and I b e et al. [15], respectively and the results point to a more acute auditory than visual sense in the African grasscutter. The optic lobe (or rostral colliculus in mammals) is present in all vertebrates, but exceptionally large in birds such as the Wild African parrot which rely on visual stimuli for much information about their environment [37]. The rostral colliculus is bigger than the caudal colliculus in the camel [21], rabbit [2], calf [30], donkey [24], pig [31] and dog [17], while the caudal colliculus is bigger than the rostral colliculus in the cetaceans [19] and African giant pouched rat [12].

In our study, the rostral and caudal colliculi were exposed through the transverse fissure separating the cerebrum and the cerebellum. In the African giant pouched rat, only the caudal colliculi were visible through the transverse fissure, with the rostral colliculi occluded by the occipital cerebral lobe [13]. From this disparity, it may be deduced that the caudal colliculi of the African giant pouched rat is relatively bigger than that of the African grasscutter, conferring a better acoustic sense in the African giant pouched rat than the African grasscutter. V o o g d and B a r m a c h [36] reported that in the platypus (*Ornithorhynchus anatinus*), marsupials and bats, the *tectum* is visible on a dorsal view of the brain; but in larger mammals, it is hidden from view by the cerebral cortex. The disposition of the *tectum* in the Wistar rat is also different from that of the African grasscutter from this study. According to S u c k o w et al. [34], the *tectum* of the Wistar rat is distinct with the exception of the tips of the caudal colliculi that are covered by the occipital portion of the cerebrum, the pineal body and rostral part of the cerebellum. In the guinea pig, the *tectum* is covered by the cerebrum and cerebellum [27], unlike the observation in our study.

The most rostral part of the *medulla oblongata* in our study was the trapezoid body. It was conspicuous, and this conspicuousness and size may be indicative of the development of the trapezoid nuclei which can be extrapolated to the acuity of the auditory system of the African grasscutter. The medial trapezoid nucleus of the trapezoid body sends ipsilateral auditory sensory impulses to the central nucleus of the caudal colliculus [16]. M a r s d e n

and R o w l a n d [20] opined that the trapezoid body progressively decreases in size relative to the pons in higher primates, so that in humans, it is completely covered by the pons and thus not visible on the ventral surface of the *medulla oblongata*. It is more prominent in our study than was reported in the African giant pouched rat [13].

The exceptionally large size of the pons of the African grasscutter in our study is worthy of note. The pons in the African giant pouched rat, as observed by I b e et al. [13] was small. The large size of the pons in our study may have some functional significance in the cortico-pontine and striatopontine pathways. The pons of the African grasscutter, from our study is pre-trigeminal, similar to that of the African giant pouched rat [13]. M a r s d e n and R o w l a n d [20] reported that the pons is post-trigeminal in monotremes and pre-trigeminal in marsupials and most species of lower eutherian orders. The pyramids observed in our study were also exceptionally large. This contradicts the report of V o o g d and B a r m a c h [36] which states that the pyramids are inconspicuous in monotremes, small in insects, rodents, michrochiroptera, *Cetacea* and ungulates and prominent in carnivores and primates.

CONCLUSIONS

The morphology of the cerebrum and brainstem of the adult African grasscutter was described. Despite the exceptionally large size of the rodent, there was no observable evidence of gyrification of the cerebral cortex, placing it in the lissencephalic group of mammals. The asymmetrically-tapered oval shape of the cerebral cortex, unlike the spherical shape of the human cerebral cortex, calls for caution in the use of the term "cerebral hemisphere" in animals. The large size of the temporal lobe of the cerebrum and caudal colliculi of the midbrain as well as the presence of a visible trapezoid body all point to a good auditory sense in the rodent. These, and other findings, will be useful in future phylogenetic comparison of rodent brain morphology.

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RETINAL DISEASES OF SENIOR DOGS

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ABSTRACT

Aging consists of a physiological decline of an organism's functional activity. During the aging process, the structural and functional changes of the retina can be observed. In most cases, progressive vision loss occurs due to the age related changes of the anterior segment. Retinal diseases, characteristic for senior dogs are: retinal detachment, hypertensive chorioretinopathy, sudden acquired retinal degeneration syndrome (SARDS), progressive retinal atrophy (PRA), glaucoma, retinopathy, cystoid degeneration and neoplasms. The examination of the retina in senior dogs is based on: ophthalmoscopic examination, electroretinography, spectral-domain optical coherence tomography (AD-OCT) and if necessary, histopathological examinations. Comprehensive knowledge regarding the senior dog's health, significantly increases their quality of life.

Key words: senior dogs; retina; vision loss; retinopathy

INTRODUCTION

An animal's life can be divided into four stages: paediatric, adult, senior (mature, middle age) and geriatric (called senior or super senior) [14]. The understanding of ageing varies between different authors [9, 14, 34]. The progress of ageing in the individual species depends upon its size, breed, genetics and conditions under which they exist. In 2012, Fortney published "The current human pet analogy chart", that helps to assess an animal's life stage, based on its weight and age. This chart has been widely used in many studies, which required assigning patients to different age groups [14].

The main reasons for vision loss of geriatric dogs are not only the commonly occurring cataracts or corneal diseases, but also retinal disorders. Retinal diseases in aging dogs are usually secondary. In many dogs, vision loss is the consequences of systemic diseases or other ophthalmic problems like retinal degeneration due to high intraocular pressure (IOP). The primary problem is typically cases affected with hereditary or congenital eye diseases with a progressive character.

The process of aging of the retina varies between species. Generally, during aging, the retinal inner limiting membrane becomes thick and vacuolated. In the inner and outer plexiform layers, cystoid spaces may be observed. The outer nuclear layer of the peripheral retina progressively thins [15]. According to a study performed in dogs using SD-OCT, the whole retinal thickness in geriatric dogs is thinner than in the middle aged dogs [6]. The understanding of the aging retina develops with every performed study, and requires further analysis [46].

The changes in the senses, including vision, hearing, and olfaction are common problems in ageing dogs causing a decrease ability to react to stimuli [9]. There are several "normal" aging changes which occur in dog's and cat's eyes like: lenticular sclerosis, iris atrophy, asteroid hyalosis, or endothelial degeneration of the cornea [11]. In many cases, the main reason for the ophthalmic consultations are problems with the vision caused by age-related diseases.

This report reviews some of the most common retinal problems observed in geriatric dogs.

Retinal detachment

Systemic hypertension is a common problem in ageing dogs and cats and are often the primary cause of retinal detachments. Although the prevalence of hypertension in dogs and cats is not known, an annual screening of cats and dogs older than 9 years old, is recommended [1]. Retinal detachments due to systemic hypertension, often occurs in patients with heart or kidney failure. The main sign of retinal detachment is partial or complete vision loss depending on the extent of the detachment. It can be diagnosed during ophthalmic examination using behavioural testing (menace response) and ophthalmoscopy. In electroretinography, the results may be normal, partially attenuated, or attenuated depending on the severity of the condition. Additionally, an ultrasound examination (USG) of the posterior eye segment can demonstrate the typical appearance of the condition called "seagull wings". During ultrasound, it can be visible that a detached retina remains attached in the area of the optic nerve head and *ora serrata* [43]. In some cases, retinal detachments cannot be seen with the ophthalmoscope or on USG. In these cases, optical coherence tomography (OCT) is required to scan the whole retinal thickness and detect the origins of the flat focal retinal separations [33]. A typical detachment occurs between the retinal pigmented epithelium and the photoreceptor layer due to its embryological development. In early eye development, the retina is made with two layers of epithelium of neuroectoderm origin. The outer epithelial layer differentiates into the retinal pigment epithelium, while the inner epithelial layer differentiates into the sensory neuroretina. These two layers may be separate due to accumulation of fluids in the subretinal space [35, 43]. Patients with retinal detachments require complete ophthalmic and physical examinations. The condition is not painful, but it may cause pain due to its primary cause like: trauma, inflammation, etc. The treatment of retinal detachments require emergency service as a detached retina needs metabolites and diffusion of oxygen from the separated choroid. The medical treatment is mainly based on the treatment of the primary cause. Progression of a partial detachment may be prevented by laser retinopexy. It is important to remember that a blind eye is not painful, does not require enucleation, and blindness is not a reason for euthanasia [43, 48].

Hypertensive chorioretinopathy

Ocular lesions are common in dogs with systemic hypertension [28]. Retinal and choroidal vascular diseases may be caused by systemic hypertension secondary to renal or endocrine diseases [47], e. g. *diabetes mellitus* which is a common problem in geriatric dogs [43]. The typical fundus lesions that can be observed in dogs with hypertensive chorioretinopathy are: papilledema, narrowing of the retinal vessels, retinal oedema, serous detachment, retinal and vitreal haemorrhages [54]. Systemic hypertension can be the main cause, not only of retinal detachment but also of retinal haemorrhages which usually develop as the disease progresses. It is possible to classify the depth of haemorrhages based on the ophthalmoscopy appearance: subretinal (bleeding from the choroidal vessels between the retina and choroid), intraretinal (usually manifest as focal petechiae bleeding from the deep capillaries of the retinal vessels), or preretinal (bleeding between the retina and vitreous form retinal vessels). Subretinal haemorrhage can lead to retinal detachments [43]. An analysis of 83 dogs diagnosed with punctate retinal haemorrhage revealed a mean age of the patients of 10 years ±3.8 [55]. A study performed on 42 hypertensive dogs revealed that 40 % of them had retinal haemorrhages [28]. In dogs affected with acute blindness due to initial clinical signs, such as intraocular haemorrhage, retinal detachment, or secondary glaucoma, hypertensive chorioretinopathy, should be taken under consideration [30].

Diabetic retinopathy

Diabetic retinopathy is a less common manifestation of systemic diseases. The typical ocular manifestation of *diabetes mellitus* (DM) in dogs are cataracts. In one study, 80 % of the dogs affected with DM developed cataracts within 16 months of the diagnosis [7]. According to Herring et al. [20], about 20 % of the diabetic dogs had retinal petechiae. Retinal haemorrhages involve: retinal capillary degeneration, micro aneurysms, or even can cause small retinal detachments that can be observed in the ophthalmoscopic examinations. These changes may lead to vision impairment, which often occurs in humans affected with *diabetes mellitus* [43]. DM tends to occur in middle aged to older dogs (5—12 years of age) [12]. Retinopathies in dogs occur less often than in humans [20].

Sudden acquired retinal degeneration syndrome (SARDS)

Sudden acquired retinal degeneration syndrome has only been diagnosed in dogs. The typical reported sign of the disease is an acute blindness with a normal-looking fundus [43]. SARDS affects middle-aged to elderly dogs and often moderately overweight, aged between 7—10 years [2, 3, 51, 53]. According to the studies, the majority of the affected dogs are spayed females [5, 24]. Until now, genetic predisposition has not been documented. An analysis of breed predisposition revealed that SARDS occurs most commonly in mixed-breed dogs. It has also been reported that SARDS occurs more often in small breed dog (Dachshund, Miniature Schnauzer, Pug, Brittany, Bichon Frise, Beagle, Maltese, American Cocker Spaniel, Pomeranian, and possibly Shihtzu) [5, 10, 19, 51, 53].

Typical for SARDS is that a rapid vision loss develops over a period of days to weeks; this has been reported in the USA since the 1980s. Previously, the condition had been known as "toxic metabolic retinopathy" or "silent retina syndrome". SARDS can be diagnosed using electroretinography (ERG). This examination helps to distinguish SARDS from central blindness, in which the manifestation of the disease is similar—acute blindness, but a normal looking fundus can be seen. In dogs with central blindness, the ERG is normal, while in SARDS cases, it is distinguished due to the loss of photoreceptor outer segments and the numbers of apoptotic nuclei in the outer nuclear layer can be seen in the histopathology examinations [43]. Similar findings were revealed after analyses of 10 dogs affected with SARDS using OCT [44]. Although the primary cause of the disease is still not known, endocrine disorders have been suspected due to the common history of SARDS patients, i. e. polyuria, polydipsia, polyphagia, lethargy, and obesity. Currently, autoimmune inflammatory processes and toxicity have been taken under consideration as a primal reason in case of sudden acquired retinal degeneration syndrome. Even today, there is no proven treatment for SARDS, so affected dogs remain blind, although systemic signs usually decrease over time [43].

Progressive retinal atrophy (PRA)

Progressive retinal atrophy is a broad general term that describes a number of inherited neuroretinal degenerations [8]. Although PRA can affect even young animals, very often it is a terminal stage, when the owner recognise eyesight problems are occurring in advanced age. The analysis of 31 dogs with PRA revealed that 48 % were diagnosed between 6—10 years and 26 % were diagnosed between 11—15 years of age [22]. PRA is a hereditary disease that initially affect rods, leading to loss of night-vision. Complete blindness occurs when the disease develops and cones starts to be affected [42]. Because the disease is incurable, it is important to determine the diagnosis before breeding [39]. It has been found that PRA is an autosomal recessive inherited disease, but X-linked forms and one autosomal dominant form has also been reported [40]. The diagnosis is based upon the clinical history and complete ophthalmic examinations with fundoscopy and electroretinography (ERG) [17]. During ophthalmoscopic examination hyperreflectivity that develops as a result of thinning neurosensory retina and the thinning of blood vessels can be seen as a characteristic for PRA. In terminal stages, the complete atrophy of the retina occurs. Abnormalities, like vitreous liquefaction (syneresis), asteroid hyalosis and cataracts are also observed in the later stages of PRA [18, 50].

Glaucoma related fundus changes

Glaucoma has many definitions. Generally, it is a group of diseases united by increased intraocular pressure (IOP). According to Maggio, it is a neurodegenerative disorder of the retinal ganglion cells and the optic nerve, causing blindness [31]. Glaucoma is a common ocular emergency in veterinary practice. The value of IOP is a consequence of balance between aqueous humour production and outflow

[31]; both decline with age [36]. Primary glaucoma is associated with increasing age [4, 25, 26], e.g. primary close angle glaucoma can occur in any age but most affected dogs are middle aged to older [16]. The value of intraocular pressure for certain species varies between different sources and different devices. According to Slatter's Ophthalmology, the normal range of IOP in dogs is 8—25 mmHg [37, 38] or 19.2 ± 5.5 mmHg or 12.9 ± 2.7 mmHg using applanation tonometry and 10.8 ± 3.1 mmHg or 9.1 ± 3.4 mmHg using rebound tonometry [31, 36]. In the case of glaucoma, an unresponsive dilated pupil can often be present [38]. Mydriasis or a poorly responsive pupil is a consequence of increased IOP that is causing ischemia, pressure induced paresis, or paralysis of the iris sphincter muscles [32]. Highly increased IOP for longer than 24 to 72 hours leads to irreversible blindness [36]. Vision loss occurs due to the damage of ganglion cell axons in the optic disc region. The damage is caused by increased IOP that is causing negative effects on the vascular supply of the optic nerve and axonal transport in optic nerve axons [32]. During the fundus examination, the optic nerve cupping and retinal and optic nerve atrophy can be observed. Cupping of the optic disc is due to the bowed outward optic nerve head. It is caused by an increased IOP on the *lamina cribrosa*. Another fundoscopic finding is the common hyperreflectivity due to retinal thinning and attenuation of the retinal vessels [32]. There are various types of glaucoma that should be managed according to aetiology, presence or absence of vision, and the stage of the disease [31].

Primary neoplasms of the retina—canine retinal gliomas (astrocytoma)

Although retinal glioma rarely occurr in canine patients, Glial Fibrillary Acid Protein-positive gliomas are the most frequently encountered primary retinal tumours in dogs. Gliomas most often arise in the central retina, near or continuous with the optic nerve. It can have a bland or an anaplastic cellular profile and can be extensively necrotic [13]. Gliomas in the central nervous systems usually occur in brachycephalic dog breeds [23, 29, 49]. According to studies, the affected dogs are middle-aged to old with the mean age of 9 years in which enucleation is performed, similarly to previous descriptions of gliomas of the central nervous system [23, 49, 52]. The diagnosis of glioma is reached based on a signalment and ocular examination with ultrasound examination of the eye globe, supported with other ocular imagining tests. A histopathology examination and eventually immunohistochemistry is necessary in order to confirm the diagnosis. In the literature there is an interesting clinical case which describes a 9.5 year old spayed female miniature Schnauzer with the history of a unilateral 1-week old ocular problem. Employing a B-mode ultrasonographic examination using 10 MHz, the authors demonstrated a mushroom-shaped, relatively homogenous hyperechoic mass arising from the mid-dorsal chorioretinal region. The histopathology examination of the mass revealed a retinal glioma [41]. Other investigators have described ocular changes of retinal gliomas such as: intra ocular haemorrhage, glaucoma, and retinal detachment with its typical clinical signs [27, 41]. The metastatic potential of canine retinal glioma (astrocytoma) is low, but ascending invasion into the ventral aspect of the brain should be considered [41].

Cystoid degeneration of the retina

The other rarely diagnosed ocular disease that occurs in senior dogs is cystoid degeneration [45]. However, an analysis performed on age-related changes in the eyes of 86 Beagle dogs revealed that 85 % of the animals were affected with cystoid degeneration of the retina. The cysts were single or multiple at or near the ora ciliaris retinae [21]. With age, the inner limiting membrane becomes thickened and increasingly vacuolated. The inner and outer plexiform layers can have cystoid spaces, which are formed within Mullerian cells [46]. Peripherally, the retina is adjacent to the ora ciliaris retinae; this region undergoes cystic changes with multiple cystic structures that protrude into the vitreous. These changes may lead to retinal detachment. Because the changes occur peripherally, they tend not to affect the vision [42].

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