



A RARE CASE OF EQUINE HAEMOTROPIC *MYCOPLASMA* INFECTION IN NIGERIA

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SUMMARY

Equine haemotropic mycoplasmosis (EHM) is a condition rarely reported worldwide. A horse presented with unspecific clinical findings and non-response to treatment to the common and endemic haemoparasitic and bacterial infections, warranted a thorough molecular investigation of suspected haemoparasitic infection given the fluctuating parasitaemia and the low sensitivity and specificity of Light Microscopy (LM) detection of haemoparasitic infections. Blood collected from an adult horse, domiciled at the University of Ibadan Veterinary Teaching Hospital, Ibadan, Nigeria was screened by LM and PCR techniques for haemo-parasites. The 16S rRNA gene of pan-*Hemoplasma* spp was targeted amplified and sequenced using Sanger automatic sequencing techniques. This case shows the very first molecular evidence of EHM in Africa and Nigeria, and the third case in the World. Microscopic examination of the horse's blood smear presented with signs of lethargy, inactivity, anorexia and moderate emaciation, showed numerous coccoid-shaped epierythrocytic parasites. Subsequent 16S rRNA sequence data and phylogenetic analyses confirmed the presence of a haemotropic mycoplasma ('*Candidatus* M. haemocervae'-like) in the horse. The hemoplasma sequence obtained falls in the same clade with some *Candidatus* Mycoplasma haemocervae sequences with which it shared more than 98.7% homology. This finding suggests that horses in this geographical region may also be suffering from EHM and calls for the need of epidemiological surveillance of equine hemoplasmosis with emphasis on their clinical, economic, performance and zoonotic implications in the sub-region.

Keywords: Nigeria, Horse, Haemotropic mycoplasma, '*Candidatus* M. haemocervae'-like

INTRODUCTION

Haemotropic mycoplasmas (hemoplasmas) are small, pleomorphic, non-cultivable obligate blood cell parasites that infect a wide range variety of vertebrates throughout the world. Hemoplasmas infect mammalian hosts such as pigs (Hoelzle, 2008; Groebel *et al.*, 2009), cattle (Hoelzle *et al.*, 2011), sheep (Adejinmi *et al.*, 2004), cats (Messick, 2004) and dogs (Aquino *et al.*, 2016; Happi *et al.*, 2018) including several reports of human infection (Maggi *et al.*, 2013). Hemoplasmas have characteristics and morphologic features of rod, coccoid, and ringed-shaped structures found individually or in chains on the red blood cell (Messick, 2004). Animal infections with hemoplasmas are clinically marked either by an overt live-threatening haemolytic anaemia or a subtle chronic anaemia, ill thrift, infertility, and immune suppression (Hoelzle, 2008; Messick, 2004).

Reports of equine hemoplasma are rare. The first incidence of equine hemoplasma infection was provided by light microscopic finding four decades ago in a case of equine “hemobartonellosis” in Niger Republic (Gretillat, 1978). Affected horses exhibited clinical signs including fever, apathy, lymphadenitis, circulatory disorders, and pale mucosa. Blood smears from these animals revealed bacteria of approximately 0.3 μm on the surface of red blood cells (Gretillat, 1978). Hemoplasma species, closely related to ‘*Candidatus Mycoplasma haemobos*’, was also detected in two horses from Germany suffering from poor performance, apathy, weight loss, and anaemia (Dieckmann *et al.*, 2010), and a survey of horses at a single breeding farm in Northern Germany recorded a prevalence of

26.5% of hemoplasmas (Dieckmann *et al.*, 2012). However, in Nigeria, there are scanty reports of hemoplasmosis described in animals and human except a very few in dogs (; Leeftang and Ilemobade, 1977; Aquino *et al.*, 2016; Happi *et al.*, 2018) and cattle (Obi and Anosa, 1980; Happi *et al.*, 2019), Sheep (Adejinmi *et al.*, 2004) and pigs (Dipeolu *et al.*, 1982; Ola-Fadunsin, 2017). The paucity of reports of hemoplasma infections may be due to the low sensitivity of the commonly used microscopic detection method for diagnosis (Happi *et al.*, 2018) in most veterinary hospitals in the country or the microscopist experience.

Light microscopy diagnosis is neither sensitive nor specific (Happi *et al.*, 2018; Dieckmann *et al.*, 2012). Development of sensitive PCR assays capable of discriminating between various hemoplasmas has greatly enhanced the diagnosis of these parasites and has led to identification of several new haemotropic *Mycoplasma* spp (Vandervoort *et al.*, 2001; Iso *et al.*, 2013; Meli *et al.*, 2010). Thus, PCR-based assays are suggested as the diagnostic methods of choice (Happi *et al.*, 2019; Santos *et al.*, 2009; Tasker *et al.*, 2010). To the best of our knowledge, this report provides the first molecular evidence and characterization of hemoplasma infection in horses in Africa as a whole and Nigeria in particular.

PRESENTATION AND METHODS

Case History and Presenting Signs

An adult male Dongola horse (stallion) domiciled at the Veterinary Teaching Hospital (VTH), Faculty of Veterinary Medicine, University of Ibadan, Ibadan,

Nigeria, and that has been used for two years for students' teaching was reported to be lethargic, inactive, anorexic and moderately emaciated. It was reported to have been in a slightly inactive state for over a month. The animal was examined physically and blood samples were collected for haematology, serum chemistry and haemoparasite screening. After the first blood collection, the horse was treated for trypanosomosis/babesiosis using diaminazine aceturate injection with multivitamin and dextrose saline as supportive therapy, despite negative result by microscopy and because of the endemic nature of those diseases in this environment and their fluctuating parasitaemia. However, there was no improvement. Ten days later, a second blood sample was taken and examined similarly. However, the result of equine hemoplasmosis was refuted as a condition not found in horses by the treating clinician. Afterward, the horse became gradually more emaciated, weak, recumbent and succumb 6 weeks later. Unfortunately, the necropsy findings were not reported nor shared with us.

Sample Preparation and Laboratory Evaluation

Giemsa-stained thin blood and buffy coat smears were examined under light microscopy at x 100 oil immersion. The haematological analysis was done manually as described by Jain, (1986) and the findings were compared with the reference values of Duncan and Prasse (Latimar, 2011) as previously reported by Happi *et al.* (2018). Similarly, serum chemistry analysis was performed using manual standard methods and the values of the analyte evaluated were

compared with the reference values of Duncan and Prasse (Latimer, 2011).

From the second EDTA blood sampled, 500 µl were aliquoted into two other tubes and stored at -20°C for DNA extraction and molecular investigation by polymerase chain reaction (PCR).

DNA extraction from whole blood and PCR detection of hemoplasma

Genomic DNA was extracted from 200µl of whole blood using QIAmp DNA mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To detect hemoplasma in the sample, a set of primers was used for the amplification of the entire group of hemoplasma employing a slightly modified conventional PCR described by Nishizawa *et al.* (2010). About 175-195 bp nucleotide fragment of the 16S rRNA gene *Hemoplasma* spp was amplified using the universal primers HEMO-F (5'- ATA TTC CTA CGG GAA GCA GC -3') and HEMO-R (5'- ACC GCA GCT GCT GGC ACA TA -3') (Ootake *et al.*, 2010). Positive controls were also used as previously reported by Happi *et al.* (2018). The PCR reaction was performed using the PuReTaq Ready-To-Go™ PCR Beads from GE Health care as previously reported by Happi and colleagues (2019), briefly, the PCR program was set with an initial denaturation cycle at 94°C for 30 seconds, followed by 39 cycles at 95°C for 5 seconds for subsequent denaturation, 57°C of annealing for 20 seconds, initial extension at 72°C for 15 seconds and a final extension of 72°C for 10 minutes as described by Nishizawa *et al.* (2010) with a slight modification in an Eppendorf AG Vapo-Protect Master Cycler 6321. Five (5) µl of

amplicon was visualized by ethidium bromide staining after electrophoresis in a 2% agarose gel. The PCR amplification of the DNA extracted from this sample was performed three (3) times.

In addition, aliquots of purified DNA were subjected to two series of reactions targeting a 460-520 bp DNA fragment of the 16S rRNA gene for the detection of *Anaplasma/Ehrlichia* spp and 460-540 bp of the 18S rRNA gene for the detection of *Babesia/Theileria* spp as previously described by Happi *et al.* (2019).

DNA sequencing and Phylogenetic Analysis for species differentiation

The three (3) PCR products obtained from each of the reactions were purified using a Qiaquick PCR purification kit (QIAGEN) and sent for sequencing by Eton Bioscience, Inc, (56 Roland Street, Suite 306 Floor 3R Boston, MA 02129 USA). They were sequenced in both forward and reverse directions, and a consensus sequence was derived from the alignment of all sequences. Chromatogram of each sequence was viewed using **Geneious** (Kearse *et al.*, 2012) and manual base calling was carried out for regions of ambiguities and consensus sequence was obtained. Following manual base call, consensus nucleotide sequence was analyzed by a BLASTn search on GenBank data base to identify the organism. The consensus nucleotide sequence was subsequently submitted to the NCBI database with accession number MN462840.

Following BLASTn analysis, the sequence (H15) was aligned with thirty-four (34) representative sequences of the top blast hits (*Mycoplasma ovis*, *Candidatus Mycoplasma*

haemocervae, and *Mycoplasma wenyonii*) obtained from the National Centre for Biotechnology Information (NCBI) database using **Clustal W** version 2.1 (Larkin *et al.*, 2007) with further adjustment made manually as necessary in **Geneious**. A maximum likelihood phylogenetic tree was constructed using IQ-tree software version 1.6.3 (Nguyen *et al.*, 2015). IQtree Model Finder (Kalyaanamoorthy *et al.*, 2017) selected TN+F+G4 as the best-fit model according to Bayesian Information Criterion (BIC) for the dataset and ultrafast bootstrap (Minh *et al.*, 2013) with 1000 replicates was carried out. The tree was viewed and manually edited using **FigTree** (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS

Clinical, Haematological and Microscopic Findings

The clinical examination revealed weight loss, rough hair coat, severe dehydration, anorexia, pale oral and conjunctival mucous membranes, and a few ticks on the neck, thorax, inguinal and scrotal regions amidst normal respiratory rate, heart rate and rectal temperature. Haematological analysis revealed PCV values of 29% and 30% within the 10-day period. In addition, other red blood cell parameters (Hb, RBC) were low, while MCV, MCH and MCHC were high, slightly high/normal and low, respectively, with mild difference between the two sampling periods (Table1). There was a mild macrocytic hypochromic anaemia at both times, but an haematologic diagnosis of a mild regenerative anaemia was inferred. However, the platelet counts, leukocyte values, plasma protein and fibrinogen levels were within normal

reference intervals at both time-points. There were no remarkable deviations in the levels of the serum chemistry analytes evaluated except for the mild hypoalbuminaemia recorded at both sampling periods (Table 1). However, as limitations the reference intervals used were neither breed nor laboratory specific, and could alter interpretation.

The thin blood and buffy coat smears revealed numerous small epierythrocytic coccoid to rod-shaped parasites (Figure 1), some of which were found around and in the neutrophil cytoplasm (Figure1). No other parasites were observed. A tentative diagnosis of equine hemoplasmosis was made. The horse was placed on oxytetracycline antibiotics and other supportive therapy for 7 days. The clinician reported a transient improvement in the horse condition by a slight increase in activity, appetite and alertness for about three weeks. Afterward, the horse became gradually more emaciated, weak, recumbent and died 6 weeks later. It was also reported that the horse suffered from regular insufficient food supply due to inadequate of resources.

PCR, DNA Sequencing and Phylogenetic Analysis

The PCR results confirmed the microscopic finding of hemoplasma positivity with about 195bp DNA fragment (Figure 2). The horse tested negative for *Babesia/Theileria* spp and *Anaplasma/Ehrlichia* spp.

Sequencing of the PCR product was successful, and alignment of the consensus sequence (Figure 3) using BLAST showed that it shares more than 98.7% homology

with *Mycoplasma ovis*, *Candidatus Mycoplasma haemocervae* and *Mycoplasma wenyonii*. On the phylogenetic tree, the hemoplasma sequence obtained from the horse falls in the same clade with some *Candidatus Mycoplasma haemocervae* sequences. It shared a most recent common ancestor with one suggesting a high level of relatedness. However, it branched out singularly on its own (Figure4) in the Wenyonii (or Heamominutum) cluster. Furthermore, BLAST alignment of our sequence with other *Mycoplasma haemocervae* sequences from the database shows the presence of four insertions, which are non-synonymous as they resulted in amino acid changes.

DISCUSSION

Haemotropic mycoplasmas rarely reported in horses (Vieira *et al.*, 2015a; Vieira *et al.*, 2015b). This case reports the first molecular characterization of haemotropic mycoplasma from a horse in Nigeria and Africa as a whole. Before now, a microscopic finding of hemoplasma infection in a horse in Niger was reported by Gretillat 41 years ago (Gretillat, 1978). World-wide, the first molecular proof of hemoplasma in horses was not reported until 2010, when hemoplasmas were detected using the SYBR Green qPCR assay and the 16S rRNA gene sequencing in two horses from Germany in 2010 (Dieckmann *et al.*, 2010). Subsequently, a prevalence of 26.5% of hemoplasmas in horses from one breeding farm in Northern Germany was recorded (Dieckmann *et al.*, 2012). A recent study reported the occurrence (6.77%) of hemotropic *Mycoplasma ovis*-like species in horses in some localities in Iran (Kalantari *et*

al., 2019). Other molecular investigations of hemoplasma infection in horses from Brazil tested negative (Vieira *et al.*, 2015a; Vieira *et al.*, 2015b).

The clinical signs in this case were in some respects similar to those found with the 40-

year-old case report of equine hemobartonellosis in Niger (Gretillat, 1978) and also very similar to the 2010 cases of hemoplasmosis in horses in Germany (Dieckmann *et al.*, 2010).

Table 1. Haemogram and serum chemistry findings of a horse infected with *Hemoplasma spp.*

Parameters	First sample Values	Second sample Values	Reference values*
Hb (g/dl)	8.7	7.7	10.1 - 16.1
PCV (%)	29	30	27- 43
RBC (x10 ⁶ /µl)	4.36	4.26	6.0 – 10.43
MCV (fl)	67	70	37 - 49
MCHC (g/dl)	30	32	35.3 – 39.3
MCH (pg)	19.9	18.1	13.7-18.2
Platelets (n/µl)	223,000	224,000	100000 – 600000
Total WBC (n/µl)	6800	7000	5600-12100
Segmented Neutrophil (n/µl)	3536	4343	2900 - 8500
Band Neutrophils (n/µl)	0	0	0 - 100
Lymphocytes (n/µl)	3060	2310	1160 – 5100
Monocytes (n/µl)	68	210	0 – 700
Eosinophils (n/µl)	136	140	0 – 780
Basophils (n/µl)	0	0	0 – 330
Albumin (g/dl)	2.15	2.3	2.6 – 4.1
AST (u/l)	200	200	160 – 412
Total Bilirubin (mg/dl)	-	0.7	0.0 – 3.2
Conjugated Bilirubin (mg/dl)	-	0.5	0.0 – 0.4
Creatinine (mg/dl)	1.8	1.5	0.4 – 2.2
Urea-BUN (mg/dl)	16	14	11 - 27

- Reference values from Duncan and Prasse’s Veterinary Laboratory Medicine: Clinical Pathology 5th Edition (Latimer, K.S., 2011).

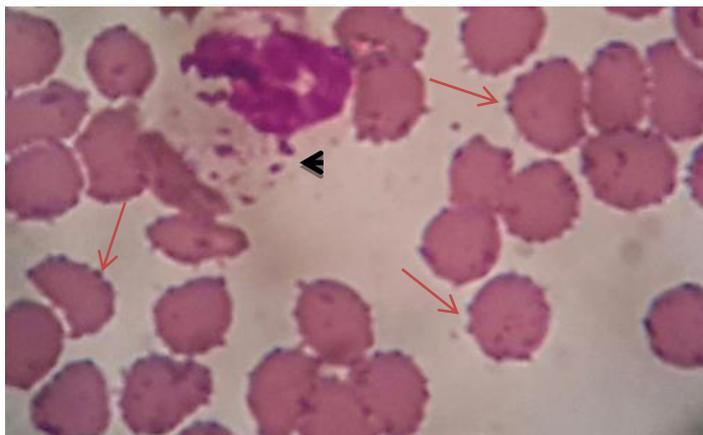


Figure 1. Giemsa-stained blood smear of an adult Dongola horse showing numerous small coccoid and rod-shaped epierthrocytic hemoplasmas (arrows) some of which are attached on the surface and in the cytoplasm of neutrophil (black arrow head) (oil Immersion)

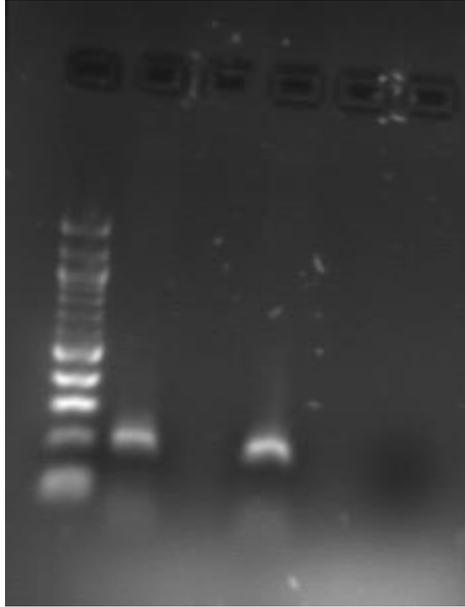


Figure 2. Agarose gel image of equine hemoplasma PCR positive blood obtained from the horse at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Ibadan. Lane 2 shows band at 195 bp (equine hemoplasma +ve (H+)); lane 1 is the DNA ladder while lanes 4 and 6 are the positive (PC) and negative (NC) controls, respectively.

(Kalantari *et al.*, 2019) but show no clinical sign. The hypoalbuminaemia recorded in this case could be ascribed to anorexia and insufficient food supply. This suggests that the insufficient food supply contributes to worsening the condition.

The PCR and sequencing analysis confirmation was important to erase any doubt and the confusion that could be caused by artifact and other haemoparasites with similar light microscopy morphological characteristics (*Theileria* spp, *Babesia* spp).

The origin of this rare *M. haemocervae* that infected this horse in Nigeria remains unknown. Microscopic findings from blood smear also revealed bacteria on the surface of the red blood cells as described previously by Gretillat (1978). The similarities recorded in the physical presentation (weight loss, rough hair coat, anorexia, lymphadenopathy) and haematological findings of the horse in this case and the German cases suggest that equine hemoplasmosis a disease associated with a debilitating state of affected horses. The haematological findings characterized by mild macrocytic hypochromic anaemia were recorded at the two periods of collection. Given the severity of the anaemia (mild), the history more than 2 weeks of illness) and the physical finding (weight loss), it was inferred that it was a mild regenerative anaemia yet chronic. In addition, extravascular haemolytic anaemia could not be ruled out in this case. Haemoplasmosis has also been described as a disease, which ranges from overt life-threatening haemolytic anaemia to subtle chronic anaemia, ill thrift and infertility in some animals (Messick, 2004; Hoelzle, 2008). In a previous study, only young horses infected with hemoplasma showed a significantly lower hematocrit value compared to adult and non-infected horses, which had their values within normal reference intervals, while in the overall infection, hemoplasma positive horses were not anaemic (Dieckmann *et al.*, 2012). However, Iranian horses infected with *M. ovis*-like species showed a mild anaemia compared to the non-infected group

It was also necessary to ascertain infection, given that hemoplasmosis in horses is sparingly reported.

The BLAST results showed that the sequence shared 98.7% pairwise identity with *Mycoplasma ovis*, *Candidatus Mycoplasma haemocervae* and *Mycoplasma wenyonii*. Interestingly, phylogenetic analysis (Figure 3), showed that the sampled sequence branched out singularly on its own, but shared a most recent common

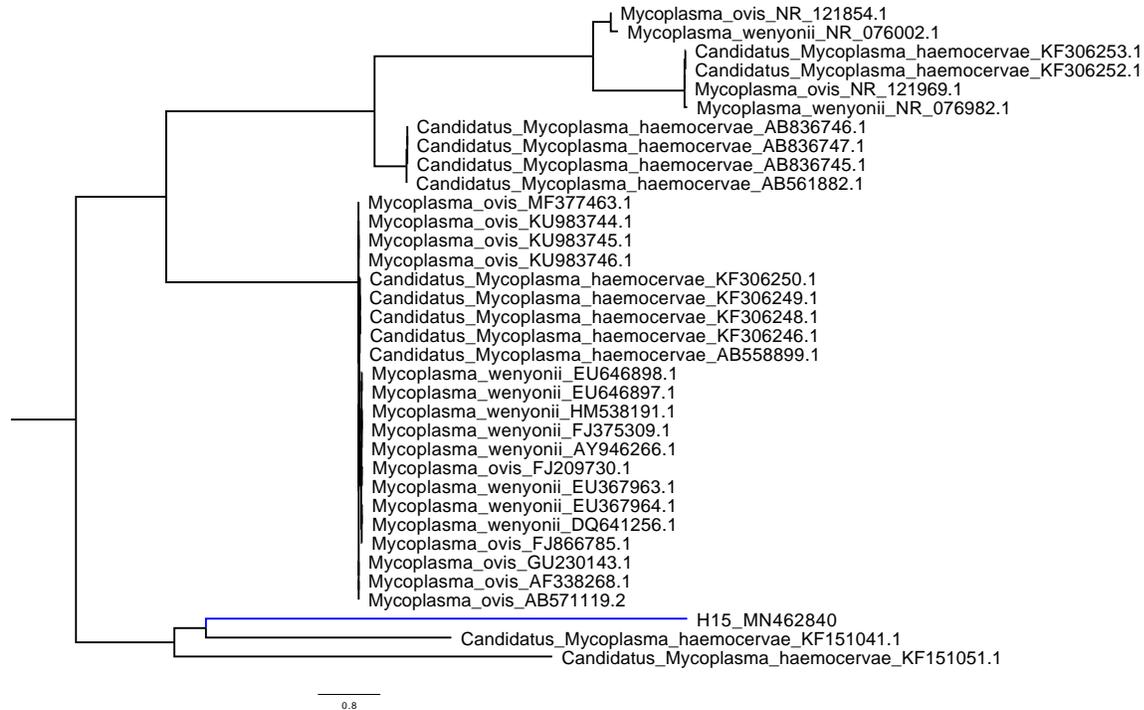


Figure 3: Maximum likelihood tree showing relationship between the sequence from this study (coloured blue) and representative sequences of the top BLAST hits obtained from the NCBI database (coloured black).

ancestor with a *Candidatus Mycoplasma haemocervae* sequence from the United Kingdom, showing that it is more related to *M. haemocervae* than to *M. ovis* or *M. wenyonii*. In addition, the phylogenetic analysis revealed that the parasite sequence from our horse sample is different from the previous hemoplasma detected in two horses in Northern Germany as they fall in different clades on the tree. BLAST alignment of our sequence with other sequences from the database shows the presence of four insertions, which are non-synonymous as they resulted in amino acid changes thereby indicating a higher degree of genetic change. In position 41, there was a gap in the database sequences and insertion of a Guanine nucleotide base in our sequence resulted in the formation of a new amino

acid Arginine (Arg); in position 123, insertion of a Thymine nucleotide base resulted in the formation of a new amino acid Serine (Ser) where a gap once existed in the database sequences; in positions 150 and 151, insertions of two Adenine nucleotide bases resulted in the formation of a new amino acid Isoleucine (Ile).

The hemoplasma sequences previously reported from the two horses in Germany (which we also include in our phylogenetic analysis) were closely related to “*Candidatus Mycoplasma haemobos*” and *Mycoplasma haemofelis*, while the Iranian horses had *M. ovis*-like species and fell in the *Haemofelis* cluster. The sequence in this case however shared a most recent common ancestor (MRCA) with a ‘*Candidatus Mycoplasma haemocervae*’ sequence

showing that it is more related to it than to *M. ovis* and *M. wenyonii*. Previous studies have shown ‘*Candidatus M. haemocervae*’ and ‘*Candidatus M. erythroceruae*’ in sika deer (*Cervus nippon centralis*) in Japan (Peters *et al.*, 2008; Watanabe *et al.*, 2010; Tagawa *et al.*, 2013) but none has been reported in horses.

Given the short nucleotide length (195bp) of the hemoplasma sequenced the actual specie cannot be ascertained. However, the light microscopy and the sequence evidence of mycoplasma species in the here with reported horse case strongly indicates the occurrence of hemoplasmosis horses in Nigeria. Thus, molecular epidemiological investigation and genomic characterization of hemoplasma among a larger population of horses in Nigeria should be envisaged. The pathogenic significance of hemoplasma in horses is still unclear as well, and needs further studies in a larger sample population with the conduct of experimental infections, which will be useful to study the pathogenicity of the infection in horses.

This case presents the first molecular proof of equine hemoplasmosis in Africa and the third molecular evidence of hemoplasma infection in horse worldwide. Our sequenced hemoplasma although different, showed the highest homology to ‘*Candidatus Mycoplasma haemocervae*’, member of the haemominutum group within the hemoplasma cluster. Therefore, there is a need for a large-scale investigation of horses in Nigeria, as that is critical for epidemiological surveillance, accurate diagnosis, successful treatment and control of the disease with resultant improvement in performance.

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