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Case Report

Molecular and Pathological Diagnosis of Mixed *Rhizopus microsporus* and *Klebsiella pneumoniae* Infections in a Russian shepherd Dog

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ABSTRACT

Introduction: Laboratory investigations are essential for diagnosing and managing diseases. The present study aimed to detect *Rhizopus microsporus* (*R. microsporus*) and *Klebsiella pneumoniae (K. pneumoniae)* in a Russian shepherd dog using molecular, gross, and histopathology methods to confirm the disease entity in the clinical setting following treatment failure.
Case report: A 4-month-old female Russian shepherd dog weighing 16 kilograms was presented to the Veterinary Teaching Hospital, University of Jos, Nigeria, with signs of haematuria, melena, ascites, and anorexia. The dog exhibited severe icterus in its mucous membranes with ascites. Blood samples were collected for parasitological and haematological examinations. Haematological evaluations revealed mild leukopenia and a mild babesiosis, which was effectively treated before the dog died. Immediate therapy was started using imidocarb, ceftriaxone, furosemide, lactated Ringer's solution, B-complex, iron dextran, and piroxicam. Despite initial therapy, the dog died five weeks after treatment. In post-mortem examination, samples from the lung, heart, liver, kidney, and spleen were collected and fixed in neutral buffered formalin 10% and prepared for histological evaluation. The non-formalinized samples of liver and lung were processed for both bacteriology and mycology assessments. To determine the presence of mycotoxins, a urine sample was obtained. Aflatoxin B1 was also detected in the urine at a concentration of 10,000 ng/mL. Generalized icterus, pulmonary congestion, cardiomegaly, splenomegaly, multifocal nodular lesions on the liver, severe intestinal haemorrhages, and ascites were the gross lesions observed. *Rhizopus microsporus* and *K. pneumoniae* were detected in microbial cultures of liver and lung samples. The present findings were further supported by PCR and gene sequencing. These strains were genetically related, clustering with Asian strains on the phylogenetic trees.
Conclusion: The present study illustrated challenges in the clinical diagnosis of uncommon mixed microbial infections in veterinary practice, where laboratory diagnosis, after post-mortem examination, was essential in the diagnosis of aflatoxicosis along with systemic *R. microsporus* and *K. pneumoniae* infections, which caused nodular hepatopathy, toxic nephropathy, myocardial necrosis, and interstitial pneumonia.

1. Introduction

Infectious diseases, infertility and reproductive abnormalities, metabolic disorders, and dietary imbalances pose a persistent threat to animal health and productivity[1]. Throughout the twentieth century, laboratory-based

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diagnosis has become more common[1]. Laboratory-assisted diagnosis has always been directly impacted by developments in chemical, biochemical, biophysical, molecular, and cell biology concepts and techniques, along with bio, nano, and instrumentation technologies[1]. Infectious disease incidence is rising, disease-resistant pathogens are emerging, and the dynamics of already-existing pathogens are changing in different parts of the world due to evolutionary forces, shifting climate and weather patterns, high-density animal production operations, and agricultural practices[1]. Depending on the type of disease or infection to be diagnosed, several diagnostic principles and techniques are employed. The process of analyzing clinical diseases in an animal involves taking a detailed history, performing a physical examination, evaluating clinical signs and symptoms, and using specialized laboratory diagnostics analysis[1].

Rhizopus microsporus (*R. microsporus*) is an opportunistic fungus that belongs to the order mucorales[2,3]. It is widespread and may be found on plants, soil, and food[1,2]. *Rhizopus microsporus* infections can occur through ingestion, inhalation, or spore deposition on wounds or sensitive mucous membranes[4-6]. The main risk factor for *R. microsporus* infection in humans and animals is immunosuppression, particularly in those with ketoacidosis, neutropenia, and corticoids[4-6]. It has been shown that *R. microsporus* invades and grows inside blood vessel walls and lumens, causing thrombo-embolism that results in ischaemia and tissue necrosis[4-6]. Rhizoxins are pharmacologically potent, active metabolites with effective anticancer activity[7] that are produced by all four strains of *R. microsporus*. Nevertheless, two cyclic peptides known as rhizonins A and B, which are mycotoxins with significant hepatotoxic potential, have been discovered to be produced by all four strains of *R. microsporus*[8,9]. Rhino-orbital-cerebral, pulmonary, cutaneous, gastrointestinal, and disseminated forms are the main clinical manifestations of *R. microsporus* infection[10]. Rhino-orbital-cerebral, pulmonary, and disseminated infections are the most prevalent types of *R. microsporus* infections and have been linked to significant death rates ranging from 78% to 100% in humans, especially the immunocompromised patients[10]. Clinical signs and the isolation of the causative agent in appropriate mycological media can be used to identify *R. microsporus* infections[11-13]. The internal transcribed spacer (ITS) sections of the ribosomal RNA (rRNA) gene were identified macroscopically and microscopically, as well as by polymerase chain reaction (PCR) and sequencing for molecular diagnosis of *R. microsposrus*[14].

Gram-negative, facultatively anaerobic *Klebsiella pneumoniae* (*K. pneumoniae*) is a frequently encountered opportunistic bacterium[15]. *Klebsiella pneumoniae* has been established to be a minor intestinal commensal, which rarely causes enteric disease[16]. However, *K. pneumoniae* has been established to cause pneumonia and urinary tract infections in dogs[16]. *Klebsiella pneumoniae* can be diagnosed based on morphological characteristics on bacteriological media such as treptose sugar agar, blood agar, and McConkey agar[15], and lesions presented in affected organs, such as congestion and interstitial pneumonia[17,18].

Aflatoxin B1 is a potent hepatotoxic mycotoxin produced by fungal growth (*Aspergillus spp*.) in cereals and legumes used in preparing dry dog feeds[19,20]. Clinical cases of canine aflatoxicosis associated with liver damage have been reported[21]. Dog feeds contaminated with aflatoxin are sold in the dog feed market in Ibadan, Nigeria[19]. Case studies of aflatoxin-intoxicated dogs are available in Ibadan, Nigeria, causing toxic hepatopathy with fibrosis and ascites[22,23]. This highlights the local incidence of canine aflatoxicosis, which has been previously reported in other parts of the world[21,24-26]. The exposure to aflatoxin weakens the dogs' immune system and increases the susceptibility to opportunistic infections[27,28].

Reports of concurrent aflatoxicosis with systemic *R. microsporus* and *K. pneumoniae* co-infections are rare. Therefore, the present study aimed to describe a rare case of aflatoxicosis with systemic *R. microsporus* and *K. pneumoniae* co-infections, including its clinical signs, pathological findings, and laboratory diagnostic methods.

2. Case report

2.1. History and clinical presentation

A 4-month-old female Russian Shepherd dog, weighing 16 kg was presented to the Veterinary Teaching Hospital, University of Jos, Jos, Plateau State, Nigeria, on December 22, 2022. The study was conducted after obtaining ethical clearance from the University of Jos Ethical Committee on Animal Care and Use. Main complaints at presentation were haematuria, melena, ascites, and anorexia. Physical examination revealed severe icterus on both ocular and oral mucous membranes, and the bitch lost weight. The dog was initially diagnosed with babesiosis based on a thin blood smear. The dog was treated with 120 mg of Imidocarb (5 mg/kg; CEVA®, France) administered once subcutaneously during the 7-day treatment period. Additionally, 5 mL of ceftriaxone (100 mg/mL; Arya Intraceuticals PVT LTD, India) was given intravenously for four days, 4 mL of furosemide (10 mg/mL; NELB, Nigeria) intravenously for three days, and 100 mL of lactated Ringer's solution (Fidson HealthCare PLC, Nigeria) intravenously with 2 mL of B-complex (Jubaili, Dubai) was administered for two days out of seven. Iron dextran (100 mg/mL; Jubaili, Dubai), 3 mL intramuscularly, was given for three days, and piroxicam (20 mg/mL; A-Nwaeze Pharmaceutical Co., Ltd., China), at 0.3 mg/kg intramuscularly, was injected for three days[29,30]. During the second visit in January 2023, no presence of *Babesia* sp. was detected following a thin blood smear evaluation. However, signs of severe icterus, melena, and ascites persisted. The female dog died on January 28, 2023, and was submitted for a post-mortem examination.

2.2. Sampling

At the second presentation to the Veterinary Teaching Hospital, University of Jos, Jos, Plateau State, Nigeria, the haematological analysis of whole blood, including red blood cells, packed cell volume, hemoglobin, total white blood cells, segmented neutrophils, band neutrophils, lymphocytes, eosinophils, monocytes and basophils from the Russian shepherd bitch was performed using a Sysmex automated haematology analyser (KX-21, Japan). The blood sample was collected according to the method outlined by Shabir et al.[31]. The differential leucocyte counts were performed using a cell counter and viewed under an Olympus light microscope (Shinjuku-ku, Tokyo, Japan) at 1000x magnification with oil immersion after staining the thin blood smear with Giemsa stain (Sigma-Aldrich, Germany).

2.3. Post-mortem examination

Following the physical inspection, the carcass of the Russian shepherd bitch underwent a rigorous post-mortem examination. With timely sample collection, lesions observed on the trachea, lungs, heart, spleen, liver, and intestines were adequately documented. At the Microbiology Laboratory Unit of the Veterinary Teaching Hospital, University of Jos, Jos, Plateau State, Nigeria, samples of the liver and lungs were subjected to microbiological analyses. The samples were preserved in deep freezer at -20^{o}C for 24 hours before culture. The post-mortem examination was done according to the method outlined by Igbokwe[32].

2.3.1. Isolation and characterization of Rhizopus sp.

Swabs from liver tissue samples were inoculated on Sabouraud dextrose agar (SDA; Titan biotech, India) and incubated at 25°C for five days. The fungal isolates were morphologically characterized via the observation of the colour of the front and a rare view of the plate[22]. Isolates were characterized microscopically by staining with Lactophenol cotton blue (Himedia Laboratories, India) and viewed at magnifications of 100x, 400x, and 1000x (using oil immersion) on an Olympus light microscope (Shinjuku-ku, Tokyo, Japan) to assess hyphal and conidial characteristics, according to the method described by Abomughaid[33].

2.3.2. Isolation and characterization of Klebsiella sp.

The lung samples were inoculated onto McConkey (Sigma-Aldrich, Germany) and 5% sheep blood agar (Himedia, India) by streaking and incubating aerobically for 24 hours at 37°C. Isolate was characterized macroscopically using parameters such as colour, colony size, consistency/texture, margin, and elevation[34]. Microscopy of the isolate was performed using Gram staining and observation with an oil immersion objective (1000x) to examine cellular characteristics, including Gram reaction, shape, and cell arrangement. Based on colonial morphology and microscopic features, the isolate was subjected to selected biochemical tests, including catalase, oxidase, indole, citrate, and triple sugar iron (TSI) tests, for confirmation according to the method described by Rawy et al.[34].

2.4. Polymerase chain reaction evaluation for Rhizopus microsporus

The fungal isolate's DNA was extracted using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Both negative and positive controls were included in the study, of which the negative control was distilled water, while the positive control was a known *R. microsporus* strain P2 sample. The extract was then amplified using the PCR technique with the following primer pair, including ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') Forward[35] and ITS-4 (5' TCCTCCGCTTATTGATATGC-3') reverse[35], which targeted the ITS rDNA. The cycling conditions was carried out according to the Woo et al.[35] with slight modifications. The conditions included an initial denaturation at 95^{o}C for two minutes, followed by 35 cycles of denaturation at 95^{o}C for one minute, annealing at 55^{o}C for one minute and extension at 72^{o}C for two minutes. This was followed by a final extension at 72^{o}C for 10 minutes, after which the reaction was held at 4^{o}C until terminated. The amplicon was further resolved on a 1.5% agarose gel stained with ethidium bromide and visualized using the Chemi Genius gel imaging system (Syngene, USA).

2.5. Polymerase chain reaction evaluation for Klebsiella pneumoniae

The DNA of the bacterial isolate obtained from the lung samples was also extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for conventional PCR. Both negative and positive controls were included in the study. The negative control was distilled water, and the positive control was a known positive *Klebsiella* sp. sample from previous cultures diagnosed as *Klebsiella* sp. Forward KLEB-F 5'-GTAATGTCTGGGAACTGCC-3' and reverse KLEB-R 3'-CCACCTTCCTCCAGTTTATC-5' primers[36] of 16s *Klebsiella* species were used for the PCR amplification. The cycling conditions were as reported by Arenas et al.[36] with some modifications. The initial denaturation was for one minute at 94°C, then 40 cycles of denaturation at 94°C for one minute, annealing at 56°C for one minute, and extension at 72°C for one minute. This was followed by a final extension at 72°C for three minutes, after which the reaction was held at 4°C until termination. The amplicons were also resolved on a 1% agarose gel stained with ethidium bromide and visualized using a Chemi Genius gel imaging system (Syngene, USA).

2.6. Sequencing Rhizopus microsporus and Klebsiella pneumoniae

The positive amplicons of *R. microsporus* and *K. pneumoniae* were shipped to LGC Genomics Germany (GmbH) for Sanger sequencing using the respective primers. The sequence data were later edited using BioEdit software[37] and identified using the BLASTn tool. Reference sequences were downloaded from GenBank and used together with the sequences from this study for the respective phylogenetic analyses. Specifically, the CLUSTAL W program of MEGA version 11 software[38] was used for multiple alignment of the *R. microsporus* and *K. pneumoniae* sequences, respectively, at default settings. The respective phylogenetic trees were then constructed using the

maximum likelihood method of MEGA 11 and Kimura-2 parameter model[39] with 1000 bootstrap replicates.

2.7. Determination of aflatoxin B1 concentration

Aflatoxin B1 determination via the aflatoxin M1 metabolite by enzyme-linked immunosorbent assay (ELISA) was carried out on the urine sample obtained from the bladder during post-mortem examination. Urine aflatoxin M1 level was analysed using the Helica® aflatoxin M1 ELISA kit (Helica® Santa Ana, CA, USA) on STAT FAX 4200 ELISA reader (Awareness Technology Inc., USA) according to the manufacturer's instructions as follows.

The urine samples were diluted 1:20 with distilled water. Before use, all reagents were kept at room temperature. The PBS-T powder packet was reconstituted to prepare the wash buffer by washing out its contents with a gentle stream of distilled water into a 1L container. For each standard and sample tested, a single mixing well was used. Each reagent was mixed by swirling the reagent bottle before use. 200 µL of the conjugate was dispensed into each dilution well. Using a fresh pipette tip for each, 100 µL aliquots of each standard were dispensed and diluted with the urine sample to the appropriate dilution well containing conjugate. The standard, urine sample, and the conjugate were mixed by priming the pipettor at least three times. The location of each standard and sample was recorded throughout the test. Using a new pipette tip for each transfer, 100 µL from each dilution well was transferred to the corresponding antibody-coated microtiter well. The antibody-coated microtiter wells, each diluted 1:100 in dilution buffer, were incubated at room temperature for 15 minutes. Contents from the microwells were poured into a discard basin. Each microwell was filled with PBS-T wash buffer, and the contents were decanted into a discard basin. The wash was repeated a total of five times. The microwells were tapped face down onto a layer of absorbent towels to eliminate any residual buffer. The required amount of substrate reagent (120 µL /strip/well) was measured and dispensed into a separate container. To each microwell, 100 µL of the substrate reagent was added, and the mixture was incubated at room temperature for five minutes, with the lid covered to prevent exposure to direct light. Next, 100 µL of stop solution was introduced in the same order as the substrate solution. The optical density (OD) of each microwell was measured using a microtiter plate reader at 450 nm, and the aflatoxin B1 concentration was determined from the calibration curve of the standard solutions.

2.8. Histopathological evaluation

The lung, heart, liver, spleen, and kidney samples were fixed in 10% neutral-buffered formalin (BDH Chemicals Ltd., Poole, England). The samples were thereafter processed by dehydration in graded methanol (70%, 80%, 95% and 100%), cleared using xylene, impregnated in bakers that contained paraffin wax incubated in vaccum air oven at 60°C, embedded in plastic embedding rings, sectioned at 5 µm using a microtone, deparaffinized with xylene, rehydrated in graded concentrations of alcohol (100%, 95%, 80% and 70%) stained with haematoxylin and eosin (H&E; BDH Chemicals LTD Poole, England) and examined using a light microscope according to the method outlined by Baker et al.[40].

3. Results

The mixed infection in the present study was initially diagnosed as babesiosis and septicaemia, warranting treatment with medicines against *Babesia* and bacterial infections. There was no parasitaemia, and the erythrogram did not indicate anaemia. The icterus was not due to an acute haemolytic disease, but associated with the patient's hepatic condition. When the dog died after treatment failure, and *K. pneumoniae* was isolated from the post-mortem sample, it implied the bacterium was resistant to the antibiotic used. Both infections continued to invade the tissues until several organs were affected, and death occurred.

3.1. Haematology

The haematological evaluations revealed a normal erythrogram; however, the leucogram showed a mild leucopenia (Table 1).

3.2. Gross pathology

At post-mortem, physical examination revealed severe emaciation and icterus of the ocular and oral mucous membranes. Upon opening the carcass, generalized icterus was observed. There was severe congestion of both lungs with marked frothy exudates within the trachea (Figure 1). Severe cardiomegaly (Figure 2) and moderate splenomegaly (Figure 3) were observed. Severe generalized nodular lesions were observed on the liver (Figure 4). The intestines were ballooned with severe serosal and mucosal haemorrhages, and severe ascites (1.5 Litres) of yellowish fluid was recovered from the abdominal cavity (Figure 5).

Table 1. Haematological parameters of a Russian shepherd bitch diagnosed with mixed infections of *Rhizopus microsporus* and *Klebsiella pneumoniae*

Haematological parameter	Patient value	Reference value
Red blood cell (× 10^{12}/L)	6.9	4.95-7.87
Packed cell volume (%)	45	35-57
Hemoglobin (g/dL)	12.3	11.9-18.9
Total white blood cell count (× 10^9/L)	4.400	5.0-14.1
Segmented neutrophils (× 10^9/L)	3.476	2.9-12.0
Band neutrophils (× 10^9/L)	0.132	0-0.45
Lymphocytes (× 10^9/L)	0.704	0.4-2.9
Eosinophils (× 10^9/L)	0.044	0-1.3
Monocytes (× 10^9/L)	0.044	0-1.4
Basophils (× 10^9/L)	-	0-0.14

Reference values are according to the MSD Veterinary Manual[41]

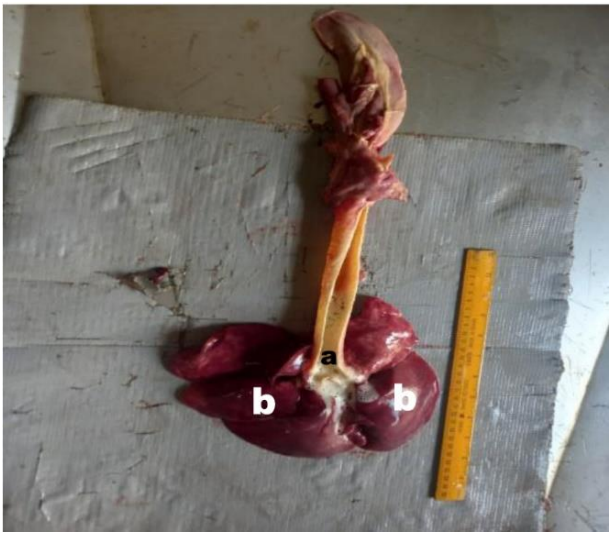

Figure 1. Tongue, trachea, and lungs of a 4-month-old Russian shepherd bitch diagnosed with mixed infection of *Rhizopus microsporus* and *Klebsiella pneumoniae.* a: Marked frothy exudates within the trachea, b: Severe congestion in both lungs.

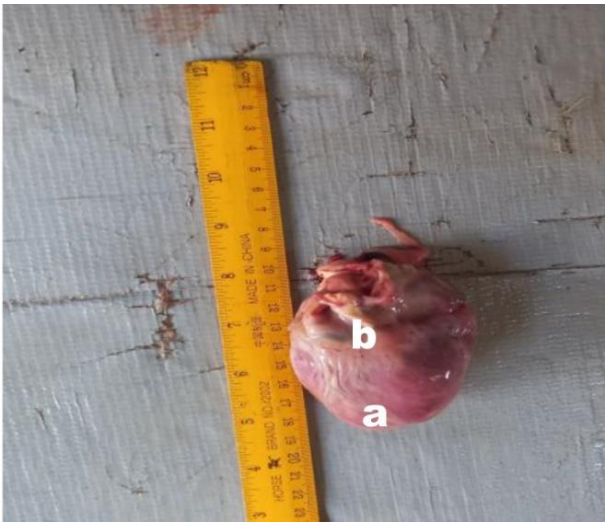

Figure 2. The heart of a 4-month-old Russian shepherd bitch diagnosed with mixed infections of *Rhizopus microspor*us and *Klebsiella pneumoniae.* a: Severe cardiomegaly, b: Marked serous atrophy of fat.

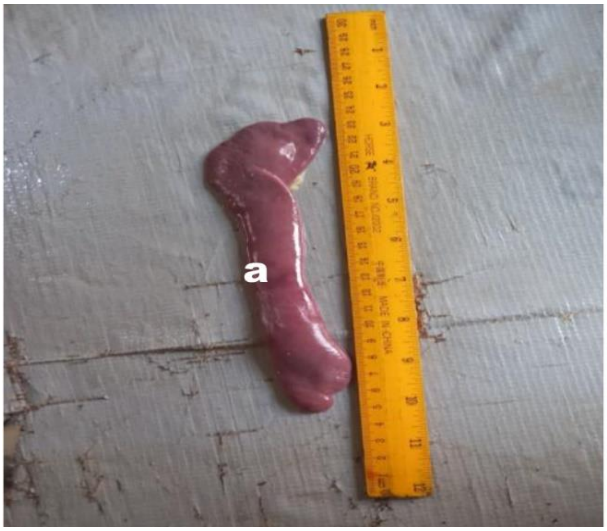

Figure 3. The spleen of a 4-month-old Russian shepherd bitch diagnosed with mixed infection of *Rhizopus microsporus* and *Klebsiella pneumoniae*. a: Moderate splenomegaly.

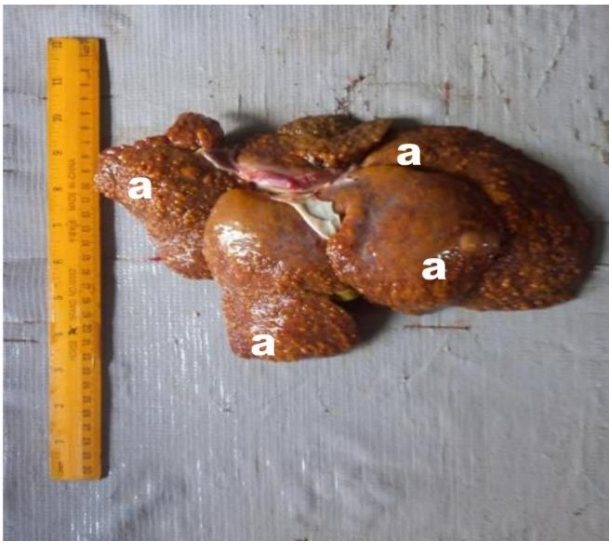

Figure 4. The liver of a 4-month-old Russian shepherd bitch diagnosed with mixed infections of *Rhizopus microsporus* and *Klebsiella pneumoniae.* a: Severe icteric and generalized nodules.

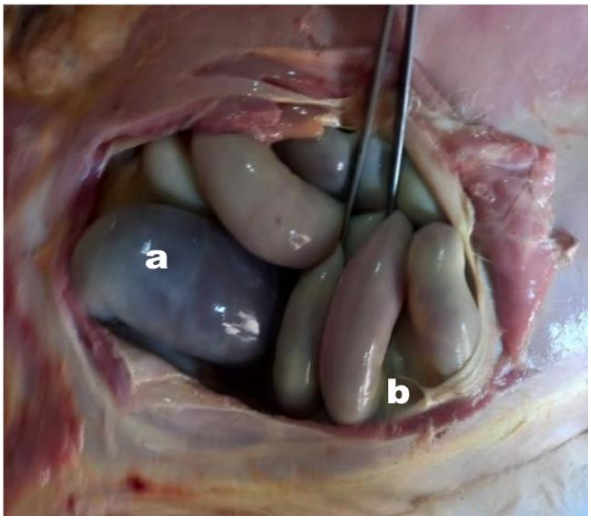

Figure 5. Intestines of a 4-month-old Russian shepherd bitch diagnosed with mixed infections of *Rhizopus microsporus* and *Klebsiella pneumoniae*. a: Moderate ballooned intestines with severe serosal haemorrhage, b: Marked severe yellowish ascitic fluid.

3.3. Cultural and molecular identification of Rhizopus sp.

Culture plate of SDA inoculated with liver sample of the Russian shepherd bitch showing light-gray growth after incubation at room temperature for 7 days (Figure 6). Sporangiophore with sporangiospores, columella with branching hyphae without cross walls and rhizoid were observed microscopically in the morphology of the *R. microsporus* (Figure 7). The gel electrophoresis results for *R. microsporus* in the present study, using ITS primers, showed a positive band at 750 bp for the positive sample (Figure 8). The phylogenetic tree obtained from the sequencing of *R. microsporus* in the present study (accession number PQ208715) is presented in Figure 9.

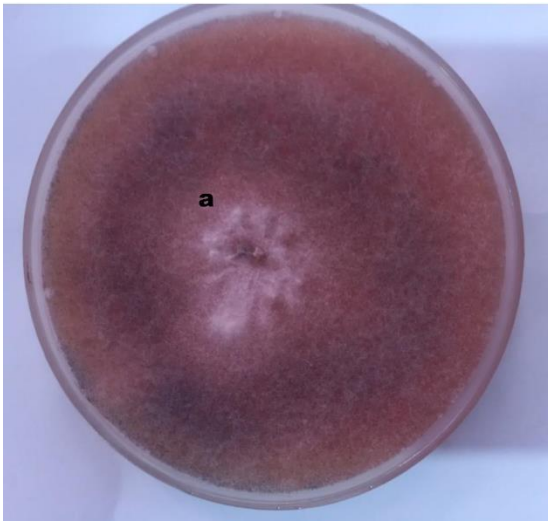

Figure 6. Culture plate inoculated with the liver sample of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microsporus* and *Klebsiella pneumoniae.* a: Light-grey color on Sabouraud dextrose agar after 7 days, identified as *Rhizopus microsporus.*

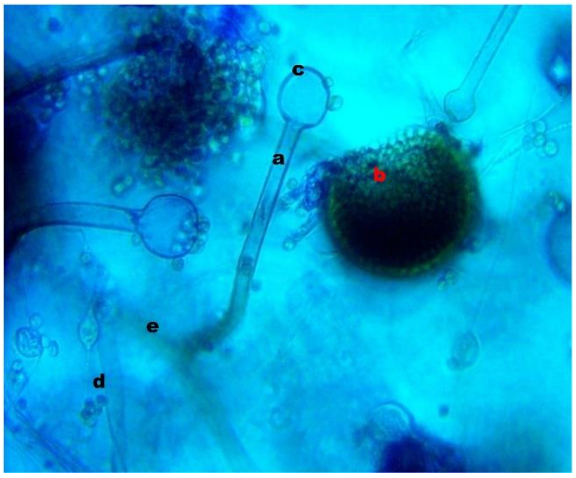

Figure 7. Morphology of *R. microsporus* isolated from the liver sample of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microspores* and *Klebsiella pneumoniae*. a: Sporangiophore, b: Sporangiospores, c: Columella, d: Branching hyphae devoid of cross walls, and e: Rhizoid. After culture on SDA for 7 days Lactophenol cotton blue stain, 1000x.

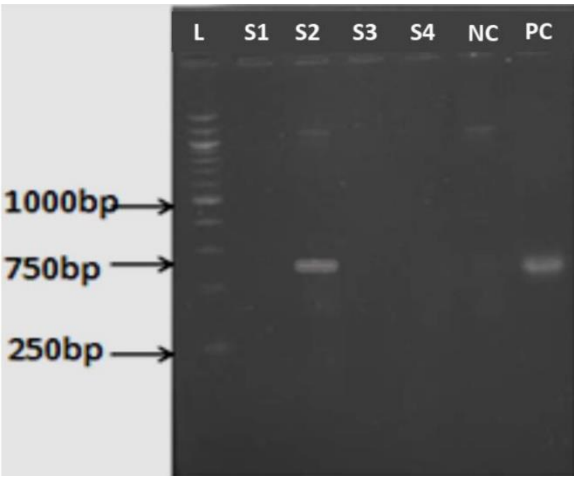

Figure 8. Gel image showing the resolution of *Rhizopus microsporus* DNA amplified using ITS primers. Sample 2 (S2): positive at 750 bp, S1, S3, S4: Negative. L: Ladder, NC: Negative control, PC: Positive control.

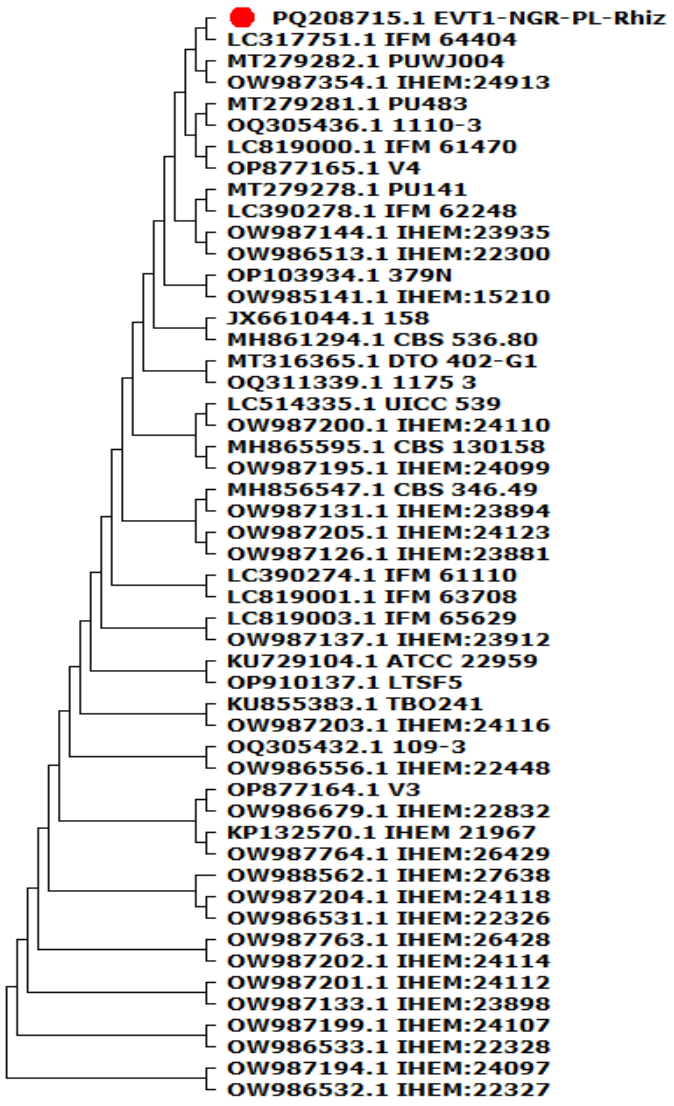

Figure 9. Phylogenetic analysis using the sequence *Rhizopus microsporus* PQ208715. Maximum likelihood phylogenetic tree showing the relationship between the *Rhizopus microsporus* isolate found in the present study and the GenBank reference strains based on the nucleotide sequences of the 750 bp-ITS region of their rRNA gene. The isolate reported in the present study is indicated with a solid red circle.

3.4. Cultural and molecular identification of Klebsiella sp.

The *K. pneumoniae,* after culture from the lung sample of a 4-month-old Russian shepherd bitch, showed creamy growth on a blood agar culture plate without any hemolysis (Figure 10) and numerous Gram-negative bacilli that were sampled from the lung, as shown in Figure 11. The PCR result for the *K. pneumoniae* isolate indicated a positive result at 1070 bp on the base pair ladder (Figure 12). The phylogenetic tree for *K. pneumoniae* in the present study, with accession number PQ152238, is presented in Figure 13.

3.5. Aflatoxin B1 concentration

The aflatoxin B1 level was assessed using the aflatoxin M1 metabolite through ELISA on a urine sample obtained from the bladder of the Russian shepherd bitch at post-mortem examination. The result obtained was 10,000 ng/mL.

Figure 10. Growth of *Klebsiella pneumoniae* on blood agar inoculated with lung sample of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microsporus* and *Klebsiella pneumoniae.* Mucoid colony of *K. pnuemoniae* with elevated margins after incubation at 37°C for 24 hours was observed.

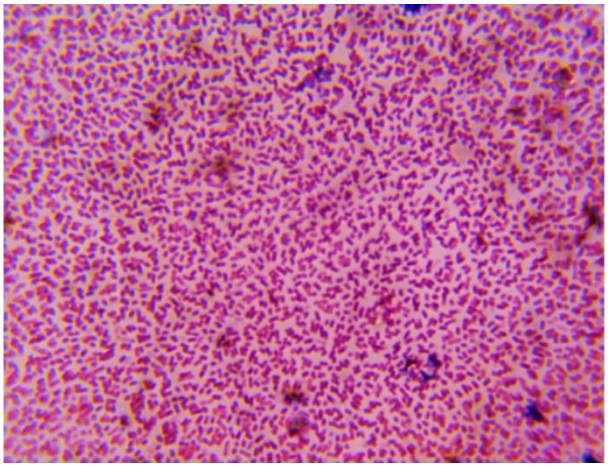

Figure 11. Microscopic morphology of *Klebsiella pneumoniae* from the lung sample of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microsporus* and *Klebsiella pneumoniae* cultured on blood agar for 24 hours, showing Gram-negative rod-shaped bacilli (1000×, Gram stain).

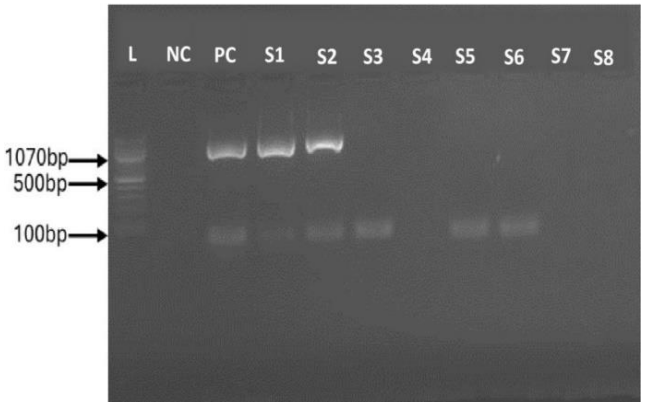

Figure 12. Gel image showing the resolution of *Klebsiella pneumoniae* DNA amplified using *16S* primers. Samples 1 and 2 (S1 and 2): Positive at 1070 bp. Negative samples: S3, S4, S5, S6, S7 and S8. L: Ladder, NC: Negative control, PC: Positive control, S1-S8: Samples 1 to 8.

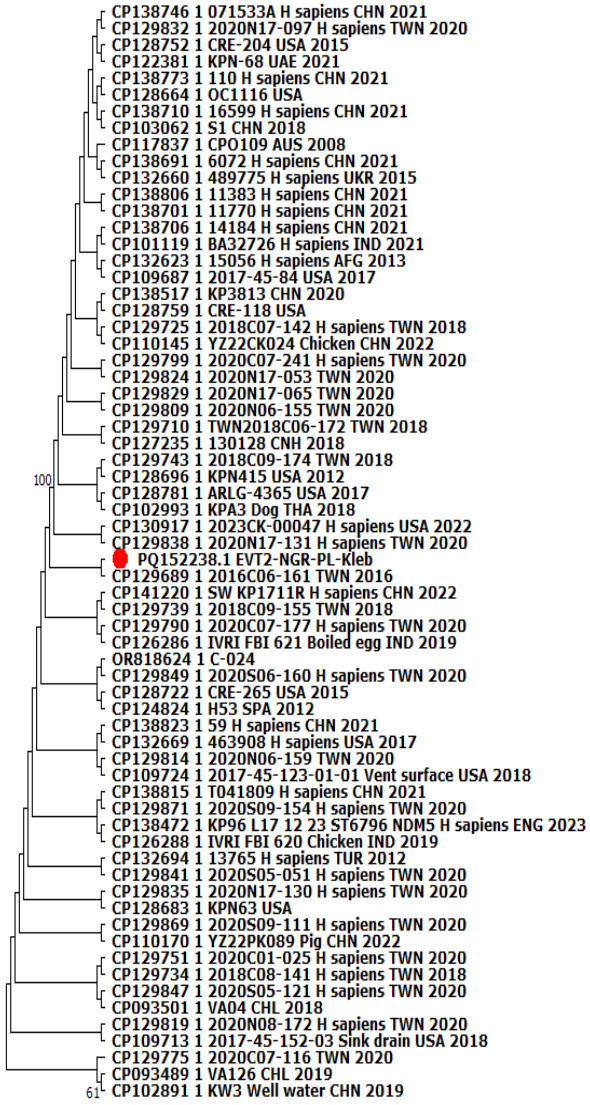

Figure 13. Phylogenetic analysis using the 16S sequence of *Klebsiella pneumoniae* PQ152238. Maximum likelihood phylogenetic tree showing the relationship between the *Klebsiella pneumoniae* isolate found in the present study and the GenBank reference strains based on the nucleotide sequences of the 1070 bp segment of their rRNA gene. The isolate reported in the present study is indicated with a solid red circle.

3.6. Histopathological findings

There was severe vascular congestion and severely thickened interalveolar septa in the lungs (Figure 14). The heart revealed markedly wavy cardiac myofibers, accompanied by severe cardiac myofiber necrosis and fragmentation, with vascular congestion (Figure 15). Severe vacuolar degeneration and vascular congestion with bridging fibrosis were observed in the liver of the affected dog (Figure 16). Figure 17 shows a section of the spleen with moderate lymphocytic depletion, while severe glomerular and renal tubular necrosis were recorded in the kidneys of the affected dog (Figure 18).

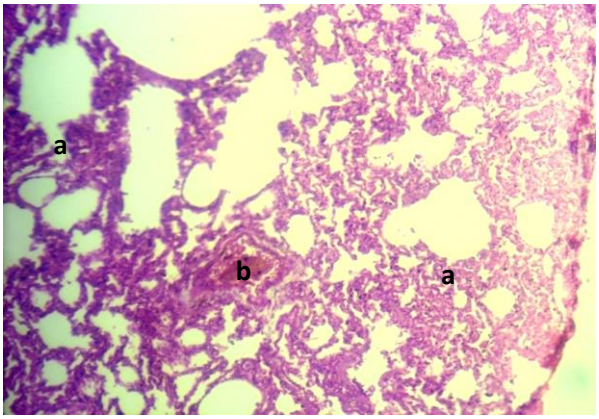

Figure 14. Lungs of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microspores* and *Klebsiella pneumoniae.* a: Severe vascular congestion, b: Severely thickened inter-alveolar septa. 100x, H&E.

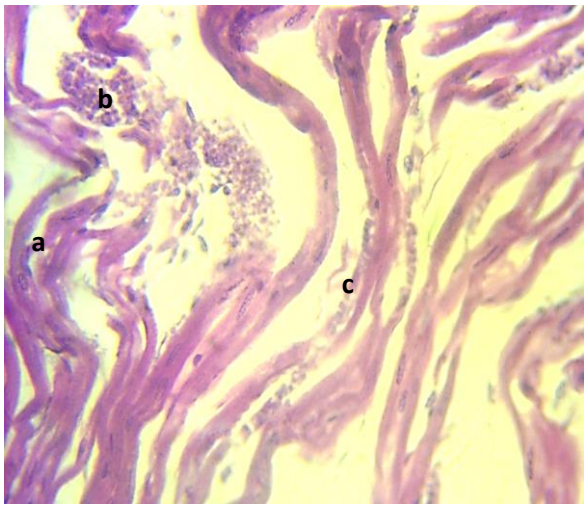

Figure 15. Heart of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microspor*us and *Klebsiella pneumoniae.* a: Severely wavy cardiac myofibers, b: severe vascular congestion c: Severe cardiac myofiber necrosis and fragmentation. 400x, H&E.

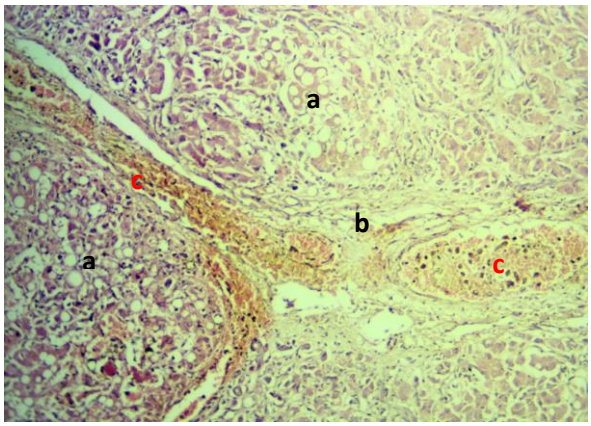

Figure 16. Liver of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microsporus* and *Klebsiella pneumoniae*. a: Severe vacuolar degeneration, b: Bridging fibrosis, c: Severe vascular congestion. 100x, H&E.

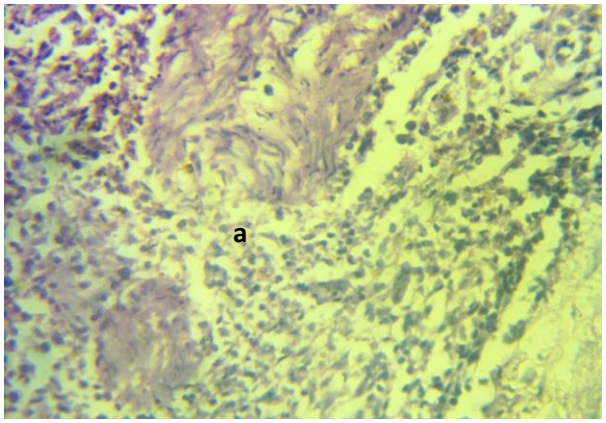

Figure 17. Spleen of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microsporus* and *Klebsiella pneumoniae*. a: Moderate lymphocytic depletion. 400x, H&E.

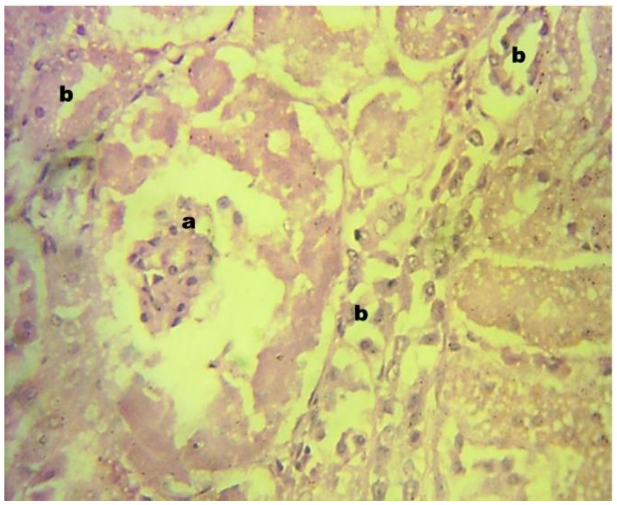

Figure 18. kidney of a 4-month-old Russian shepherd bitch diagnosed with mixed microbial infections of *Rhizopus microsporus* and *Klebsiella pneumoniae*. a: Severe glomerular epithelial necrosis, b: Tubular epithelial necrosis. 400x, H&E.

4. Discussion

Aflatoxicosis as indicated by the elevated level of aflatoxin B1 in the urine sample at 10,000 ng/mL, was observed as the main factor causing toxic hepatopathy. In the present study, the hepatic damage was characterized by the presence of nodular lesions and microscopically by vacuolar degeneration, bridging fibrosis, and vascular congestion when observed under a light microscope. These findings are consistent with previous reports in dogs affected by aflatoxicosis resulting from the ingestion of contaminated commercial feed[19]. The reduced synthetic capacity of the liver would lead to hypoproteinaemia and decreased oncotic pressure, with transudation of oedematous fluid into the abdominal cavity, producing ascites. Aflatoxins may have contributed to the renal tubular and glomerular necrosis documented in this study, similar to the findings of Alaka et al.[22]; however, renal dysfunction was not consistently observed in canine aflatoxicosis[25]. Aflatoxin has been reported in dry dog feeds in Nigeria[19] and contaminated feed could be a source of the toxin in canine aflatoxicosis[21,22,42]. Ingesting aflatoxin could have disrupted

intestinal immunity and mucosal integrity[43], predisposing the dog to diseases caused by opportunistic pathogens such as *K. pneumoniae* and *R. microsporus*, findings similar to those of the present study, which attributed systemic dissemination from the intestinal microbiota. These organisms contaminate the environment and could have been associated with the dog feeds and water as sources of infection.

In the present study, leucopenia, splenomegaly, and splenic lymphocytic depletion were markers of immunosuppression and may have promoted the pathogenicity of *R. microsporus* infection in the dog. *Rhizopus microsporus* infection is rare in dogs, and this appears to be the first report of a canine case associated with fatality in Nigeria. One human case of *Rhizopus spp.* was reported in Abuja, Nigeria, although the patient was brought into the country from Togo in West Africa[44]. No previous case of *R. microsporus* infection involving the liver in a dog has been reported in the literature. However, a dog having nasal mycosis was reported to have *R. oryzae* infection causing epistaxis and mucopurulent discharge[45]; and two dogs were reported to suffer from *R. arrhizus* infection with systemic and gastro-hepatic lesions characterized by pyogranulomatous inflammation, cavitated hepatic nodules, vasculitis, and thrombosis[46].

In the present study, minimal cellular inflammatory exudates were observed in the liver and kidney, whereas the lung showed interstitial pneumonia, likely toxigenic due to bacterial toxins, aflatoxin, and rhizonin. The rhizonin A (mycotoxin from *R. microsporus*) was reported to cause cellular degeneration and necrosis in hepatic and renal tissues[9], but both rhizonin A and B were reported to be highly toxic to tissues[11]. Inflammatory responses in the liver and kidneys could have been ameliorated by reduced immunity due to neutropenia and impaired function, and by the occurrence of coagulative necrosis. There is evidence that defective phagocytic function of neutrophils is needed for the occurrence of *R. microsporus* infection[47]. Chronic, high levels of aflatoxin exposure may suppress pro-inflammatory cytokine expression, reduce inflammatory cell populations and migration, and reduce cellular infiltration in affected tissues[28].

In dogs, *K. pneumoniae* (enterobacteria) can cause necrotizing fibrinous enteritis with intestinal haemorrhages and septicemia[16]. Exposure to aflatoxin would impair intestinal immunity, thereby creating an environment in which K. pneumoniae could cause intestinal pathology and invade the bloodstream[43]. The bacteria in the intestine are expected to reach the liver via the enterohepatic circulation and from the liver to the lungs. The isolation of *K. pneumoniae* from lung tissue might suggest that the bacterium caused pulmonary congestion and thickening of the interalveolar interstitium, with partial collapse of the alveolar spaces, which corresponded to the histological appearance of an interstitial pneumonia[18]. The outer membrane vesicles of *K. pneumoniae* release active molecules that induce pro-inflammatory effects, causing lesions of interstitial pneumonia in mice[17], suggesting that *Klebsiella* septicaemia was the cause of the lung lesions in the present case.

This case report is unique because the causative agents associated with this fatal disease in a dog have not been previously reported in Jos, Nigeria. Exposure to aflatoxin through dog feeding is an important issue for regulatory control of feeds in the marketplace and for raising awareness among dog owners who feed their dogs homemade rations prepared from locally procured offal and/or grains from markets. Feed and water hygiene are mandatory in kennels to reduce environmental contamination and the risk of exposure to opportunistic pathogens, such as those isolated in the present case. *Klebsiella pneumoniae* can colonize the intestinal microbiota and create epidemiological risks as a zoonotic pathogen, especially when it has multiple drug resistance[48]. Although the zoonotic and public health significance of *R. microsporus* may not be apparent, it is important to avoid mouldy feeds in dogs that may expose them to mycotoxicosis and mycosis.

5. Conclusion

In the present study, mixed microbial infections with *R. microsporus* and *K. pneumoniae* in an aflatoxin-exposed dog caused degenerative, necrotic, and/or inflammatory lesions in the liver, kidney, heart, lung, spleen, and intestine, leading to organ failures and death. The use of laboratory investigations using microbial culture and identification, polymerase chain reaction, and sequencing were important in achieving a definitive diagnosis. These findings suggest the need to monitor dog feeds for mycosis and dog feeding practices in the locality (Jos, Nigeria), as well as investigate the incidence of aflatoxicosis and other mycotoxicosis, along with the occurrence of drug-resistant *Klebsiella* among the cohort of dogs visiting the Jos University Veterinary Teaching Hospital.

Declarations

Ethical considerations

Prior to publishing the present study, the authors reviewed all ethical challenges, including plagiarism, consent to publish, and data fabrication, falsification, and no artificial intelligence was used in conducting the present study.

Competing interests

The authors have declared no conflict of interest.

Authors' contributions

Emmanuel Vandi Tizhe, Pam Dachung Luka, Benshak John Audu, James Satvil Dalis, Lucius Chidiebere Imoh, Anvou Rachel Dalyop, Victoria Isioma Ifende, and Arthur Obinna Oragwa have conceptualized, collected data, performed PCR and sequencing, and written the manuscript. Asinamai Athliamai Bitrus, Ussa Delia Tizhe, Polycarp Tanko, Deborah Maigawu Buba, George Yilzem Gurumyen, Chidiebere Uchendu, and Idris Ayodeji Azeez

were responsible for data collection, analysis, case management, and literature review. Charibu Hurdison Dishon and Peace Ochai helped in diagnosis, data collection, and literature review. Ikechukwu Onyebuchi Igbokwe was responsible for conceptualization, data collection, and mentorship. All authors have contributed and confirmed the last edition of the manuscript.

Availability of data and materials

All results presented here were generated by the authors and presented for publication.

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