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Molecular Characterization and Pathological Findings of *Syngamus trachea* in Captive White Storks in University of Ibadan Zoological Garden, Nigeria

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ABSTRACT

This case report highlights the clinical presentation, postmortem findings, and diagnostic evaluation of syngamosis and severe dehydration in two white storks (*Ciconia ciconia*). The storks, a male and female, introduced into a zoo four months prior, succumbed to parasitic tracheitis and dehydration. Necropsy and histopathology examinations were conducted while molecular parasite identification was carried out by using species-specific primers. Parasites found were cleaned with isotonic NaCl solution, preserved in 70% ethanol, and examined under microscopes. Intestinal scrapings were used to assess parasite egg burden, and various organ tissue samples were collected and sent for histopathological examination, including the trachea, lung, heart, liver, kidney, proventriculus, ventriculus (gizzard), small and large intestines, and bursa of Fabricius. Histopathological analysis was performed through paraffin embedding and staining with hematoxylin and eosin. Necropsy revealed dehydration, cachexia, parasitic tracheitis, nephrosis, and generalized hemorrhages. Histopathological examination confirmed subacute tracheitis with goblet cell hyperplasia, pulmonary hemorrhages, and renal damage. Molecular analysis identified *Syngamus trachea* via amplification of the 18S rRNA gene. This case report sheds light on the clinical presentation and pathological observation in white storks diagnosed with syngamosis and severe dehydration. The identification of parasitic tracheitis underscores the importance of considering parasitic infections in avian mortality investigations. Dehydration should also be addressed as a significant factor in assessing the health of captive birds.

Keywords: Avian Mortality, Dehydration, Parasitic Tracheitis, Syngamosis, White Stork.

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INTRODUCTION

Syngamosis, a disease caused by the Strongylid nematode *Syngamus trachea* that affects the respiratory tract of various bird species worldwide. Avian orders of all kinds can be negatively impacted by this parasite, with occasional fatal outcomes, particularly among young birds **(Fernando and Barta, 2008).** The transmission of tracheal worms can occur through infectious larvae (L3) in eggs or directly already hatched as free-living L3 by the bird. Indirect transmission takes place through intermediary hosts like earthworms, snails, slugs, and insects. Infected birds house adult worms in their tracheas, where male and female worms engage in perpetual copulation **(Deplazes *et al*., 2021).**

Clinical symptoms in infected birds can vary, with severe cases manifesting as respiratory distress, including gasping for air, coughing, sneezing, and anemia. Factors such as the bird's size, age, and parasite load influence the severity of clinical symptoms **(Fernando and Barta, 2008).**

MATERIALS AND METHODS

History

A pair of adult storks were introduced into the zoo on January, 2023, and after a quarantine period and deworming, they initially appeared healthy. However, the male stork was discovered dead on April 10, 2023 while the female died on the 17th of April, 2023.

Clinical Examination

Necropsy and histopathology examinations were conducted by the Necropsy unit of the Department of Veterinary Pathology, University of Ibadan, while parasite identification was carried out by the Department of Veterinary Parasitology, University of Ibadan. Parasites found were cleaned with isotonic NaCl solution, preserved in 70% ethanol, and examined under microscopes. Intestinal scrapings were used to assess parasite egg burden, and various organ tissue samples were collected and sent for histopathological examination, including the trachea, lung, heart, liver, kidney, proventriculus, ventriculus (gizzard), small and large intestines, and bursa of Fabricius. Histopathological analysis was performed through paraffin embedding and staining with Haematoxylin and Eosin (H&E) **(Robers, 2004).**

DNA extraction, PCR and sequencing

Rectal content was collected from the storks at post-mortem examination. 100 g of rectal content were homogenized, and total DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The extracted DNA was stored at -20°C prior to PCR amplification.

A 1400 bp segment of the 18S rRNA protein gene of nematodes was amplified using a pair of primers, 18S-F (ATTCCGATAACGAACGAGACT), and 81R (TTCCTCCGCTAAATGATATGCTTAA) D2AR primers, that amplify a 1400bp segment of the 18S rRNA protein gene of nematodes **(Carta and li, 2018).** The PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs mix, 1 µl of 10 pmol each of 18S-F & 81R D2AR primers, and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 32 µl with sterile distilled water to which 20 µl of DNA template was added. The PCR was carried out in a 50 µl total reaction volume consisting of 10 µl of 5x GoTaq colorless reaction, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs mix, and 1 µl of 10 pmol of each primer for the GeneAmp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) was used to conduct the PCR. The PCR conditions included an initial cycle of initial denaturation at 94ºC for 5 min, followed by 40 cycles of each cycle consisting of 30 secs of denaturation at 94ºC, 30 secs of primer annealing at 50ºC, 60 secs of extension at 72ºC, and a final extension for 7 min at 72ºC **(Matsubayashi *et al*., 2021).**

The amplified product was run on an agarose gel to verify successful amplification. In order to validate amplification, the integrity of the amplified 1400 bp gene fragment was examined on a 1.5% agarose gel. 1.5% agarose gel was then made using the prepared buffer (1X TAE buffer). For five minutes, the suspension was microwaved to a boil. The molten agarose was dyed with 3 l of 0.5 g/ml ethidium bromide after being allowed to cool to 60°C. The casting tray's slots were fitted with combs, and molten agarose was then poured into the combs. The wells were created by giving the gel 20 minutes to solidify. The gel tank was filled with the 1XTAE buffer to just barely cover the gel. After loading the 100 bp DNA ladder into well 1 of each PCR product, two microliters (2 l) of 10X blue gel loading dye were added to the 4 l of each PCR product to make it easier to load the samples into the wells and monitor the gel's development. The gel was electrophoresed at 120V for 45 minutes before being imaged under UV transillumination. By comparing the mobility of a 100 bp molecular weight ladder with experimental samples run alongside it in the gel, the sizes of the PCR products were calculated.

The PCR reagents were eliminated from the amplicon using the ethanol purification protocol. Briefly, in a new, sterile 1.5-l Eppendorf tube, each 40 µl PCR amplified product received 7.6 l of Na acetate 3 M and 240 l of 95% ethanol. The mixtures were properly mixed by vortexing, and the tubes were then kept at -20°C for at least 30 minutes. The pellet was washed by adding 150 l of 70% ethanol and mixing, then centrifuged for 15 min at 7500 g and 4°C after removing the supernatant for 10 min at 13000 g and 4°C (inverted tube on trash once). Then, resuspend the tube with 10 l of sterile distilled water and store it at -20°C before sequencing. Remove the supernatant once more (invert the tube in the trash), invert the tube on a paper towel, and let the tube dry in the fume hood at ambient temperature for 10-15 minutes. The purified fragment was quantified using a Nanodrop of model 2000 from Thermo Scientific and tested on a 1.5% agarose gel run at 110V for about an hour as before to confirm presence of purified product **(Matsubayashi *et al*., 2021).**

The amplified fragments were sequenced in two directions using a Genetic Analyzer 3130xl sequencer from Applied Biosystems following the manufacturers' manual, while the sequencing kit used was that of the BigDye terminator v3.1 cycle sequencing kit. Chromas 2.6.6 (http://technelysium.com.au/Chromaslite.html) was used to view and edit the DNA sequences.

The NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the sequences and retrieve similar sequences in the GenBank. The phylogenetic tree was constructed via multiple sequence alignments of the nucleotide sequence of the 18s rRNA gene and sequences retrieved

from the GenBank using MEGA version 11.0.10 **(Tamura *et al.*, 2021).**

RESULTS

The carcass displayed dry, glossy shanks with tightly adhered skin to underlying muscle tissue in 2/2. Severe atrophy of the breast muscle was evident, with visible keel bones in 2/2. Approximately 20 adult worms were observed along the trachea's mucosal surface in 1/2. The tracheal mucosa was diffusely hyperemic from the anterior portion to the tracheobronchial junction in 1/2. The kidneys appeared pale and somewhat swollen. There were moderate focal areas of ecchymotic haemorrhages on the epicardium, pericardium and thigh muscle in 1/2. The proventricular tips, proventricular junction and mucosal surface of the gizzard had moderate petechial and ecchymotic haemorrhages in 1/2. These findings indicate that parasite infection, dehydration, and associated organ damage and suspected immune suppression significantly impacted the stork's health and eventual demise.

Summary of Gross Findings:

Severe dehydration 2/2, cachexia 2/2, severe parasitic tracheitis 1/2, acute catarrhal tracheitis 2/2, marked nephrosis 1/2, Generalized haemorrhages: moderate, petechial and ecchymotic (pericardium, epicardium, gizzard, proventriculus and thigh muscle) in 1/2.

Tentative Postmortem Diagnosis

Syngamosis with severe dehydration in 2/2

Fig.1: Dead white stork

Fig.2: Necropsy of a white stork nestling (WS3) showing the opened trachea with *Syngamus trachea* forming the typical Y-shape

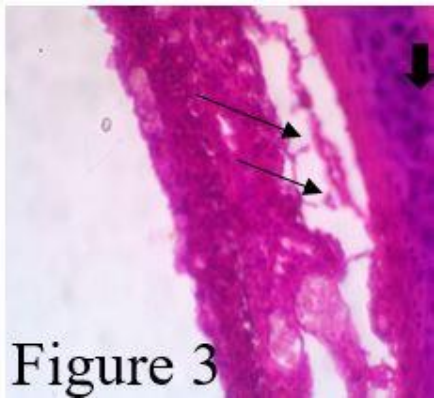


Fig.3: Focal-extensive erosion and ulceration of the tracheal mucosa (long arrows) with thickening of the lamina propria (arrowheads) due to a mixed cell tracheitis and focal granuloma formation in animal

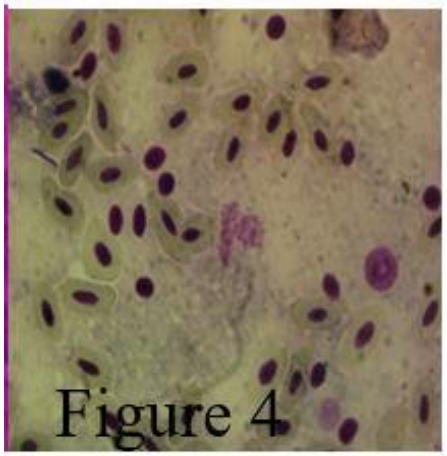


Figure 4: Eggs of *Syngamus trachea*

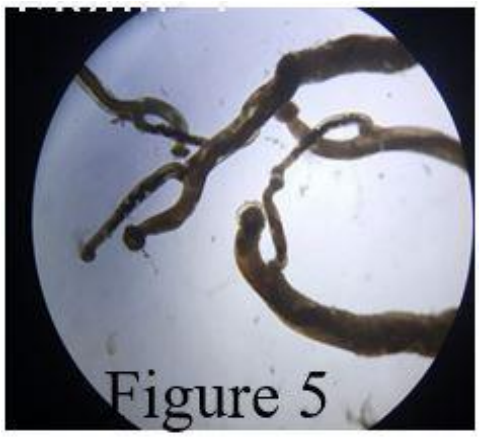


Fig. 5: Stereo microscope image of a *Syngamus trachea* pair locked in permanent copulation.

Laboratory Investigation

The trachea was characterized by tracheitis characterized by a mixed inflammatory response, which was severe in 1/2 and mild in 1/2. The inflammatory cell population was predominantly heterophils with some

lymphocytes and macrophages. There is also a moderate goblet cell hyperplasia with loss of ciliated epithelium in 2/2. The lungs are showing predominantly extravasated RBCs in alveoli and around the parabronchus. The summary includes: Trachea: Tracheitis, sub-acute with goblet cell hyperplasia.

Lungs: Pulmonary hemorrhage

Coprological Examination and Worm Identification

Various diagnostic techniques, including fecal sedimentation and floatation, were employed to examine intestinal contents. Magnifications of 100x and 200x-400x were used for microscopy examination. However, the samples submitted were positive for *Syngamus trachea* eggs.

Polymerase Chain Reaction and Sequence analyses results

The agarose gel electrophoresis **(Fig. 6)** shows that the 1400 bp segment of the 18s rRNA protein gene of nematodes was successfully amplified in samples from selected nematodes.

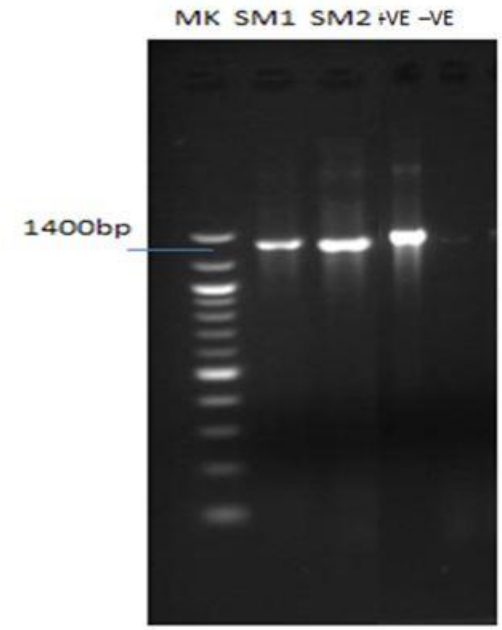
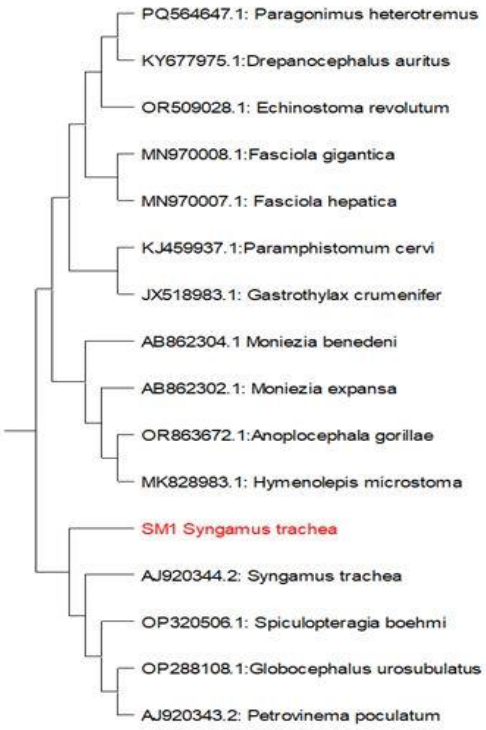


Fig 6: Agarose gel electrophoresis of 1400 bp portion of suspected 18s rRNA protein gene of nematodes. Lane 1: Molecular marker, lane 2: Stork 1 (SM 1), lane 3: Stork 2 (SM 2), lane 4: Positive Control (+VE), lane 5: Negative Control (-VE).

The phylogenetic analysis revealed the 1400 bp sequences from this diagnosis cluster with *Syngamus trachea* sequences from the GenBank **(Fig. 7).**

Fig 7:



Phylogenetic analysis of *Syngamus trachea* sequences from this study with other nematode sequences from the GenBank. The phylogenetic tree was constructed via multiple sequence alignments of the nucleotide sequence of the nematode 18s rRNA gene and sequences retrieved from the GenBank. The tree was analysed by the maximum likelihood method with bootstrapping (1000). *Syngamus trachea* clusters are labelled red.

DISCUSSION

Syngamosis, caused by *Syngamus trachea*, is a recognized respiratory parasite affecting a broad spectrum of avian species. The presence of tracheal worms and associated tracheitis confirmed the diagnosis in this stork. The histopathology lesions in the respiratory system with a mixed cell tracheitis or sub-acute tracheitis align with expectations for this condition and the report produced by **Meister *et al.*, *(2022)*** who recorded a mixed cell tracheitis, pulmonary hemorrhage and granuloma formation with multinucleated giant cells. Severe dehydration likely contributed to cachexia and overall poor body condition. It was unclear how old the dead white storks were. At the time of their deaths, all affected white storks were nestlings lacking the capacity to fly. Therefore, we presume that the birds contracted the infection either by eating tainted food or from paratenic hosts that the parents had regurgitated to feed their young. Two white storks under investigation in a different study from 2020 **(Michalczyk *et al.*, 2020)** had adult stages of S. *trachea*. The white stork under examination may not have a completely developed immune system, which raises the possibility of both a parasite infection and a fatal infection-related

consequence. It has been suggested that a key risk factor for several diseases in white storks is immunological immaturity during the first three weeks following hatching **(Olias *et al*., 2011; Jovani and Tella, 2004).**

The majority of Syngamosis cases occur in the spring to summer, coinciding with the white stork breeding cycles, as previously reported **(Nevarez *et al*., 2002; Cole, 2001).** This may possibly be explained by the vulnerability of immature birds. As a result, as birds age, the prevalence of S. *trachea* infection declines significantly in free-living populations, indicating that the majority of natural hosts develop tolerance to the parasite (Fernando and Barta, 2008). Juveniles have a larger chance of infection as well as the development of more severe clinical symptoms, because the adult worms grow in the trachea and produce mucus as a result, smaller and younger birds have narrower airways and are consequently more susceptible to luminal obstruction **(Nevarez *et al*., 2002).** This bird's health may have been weakened by the parasite infection.

CONCLUSION

This case report sheds light on the clinical presentation and pathological observation in white storks diagnosed with syngamosis and severe dehydration. The identification of parasitic tracheitis underscores the importance of considering parasitic infections in avian mortality investigations. Dehydration should also be addressed as a significant factor in assessing the health of captive birds.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest

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