Molecular Characterization of Avian Influenza H9N2 and H5N1 from Outbreak in a Backyard Layer Farm in Kaduna State, Nigeria.

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ABSTRACT
Avian influenza viruses (AIVs) have been reported in Nigeria since 2006, and the co-circulation of high-pathogenic avian influenza H5N1 and low-pathogenic avian influenza H9N2 subtypes in poultry has been reported. These Avian Influenza virus subtypes could lead to severe economic losses to the poultry farmers and may force the farmers out of the poultry production leading to unemployment for the staff of the farm. The co-infection with both highly pathogenic (H5N1) and low pathogenic (H9N2) could lead to possible re-assortment and the production of novel strain that can be highly pathogenic to chicken and may have the capacity to infect humans and be transmitted from human to humans which may lead to a pandemic of influenza. The clinical signs observed were somnolence, marginal cyanosis of the combs, ruffled feathers, matted vent and whitish/yellowish diarrhoea; while the post mortem examinations revealed dehydrated carcass, multiple foci of petechial haemorrhages on the abdominal fats, congested and necrotic-friable liver, edematous and congested lungs, congested spleen with multiple foci of necrosis, hemorrhagic and misshaped ovarian follicles, egg yolk peritonitis, swollen and hemorrhagic kidneys, erosion of the proventricular mucosa, congested trachea with mucoid exudates, and sinusitis. The disease was diagnosed based on the clinical signs, post mortem findings, competitive Enzyme-Linked Immunosorbent Assays (ELISAs) and clade-specific RT-qPCR, for the detection of co-circulating highly pathogenic avian influenza virus H5N1 and low pathogenic H9N2. The result of all sera (5/5) tested for Antibodies against Avian influenza Nucleoprotein produced positive results while 80 % of sera (4/5) tested positive for the H9 subtype of AIV. Antibodies against the H5 and H7 subtypes were not detected. The results of RRT-PCR and RRT-PCR/H5 were positive for both tracheal and cloacal samples. RRT-PCR/ H7 & H9 yielded negative results.

Keywords: Avian Influenza; Layer farm; ELISA; H5N1; H9N2
INTRODUCTION
Avian influenza (AI) is a highly contagious viral disease of domestic and wild birds occurring worldwide. The aetiologic agent of the disease, avian influenza viruses (AIV) are segmented, single-stranded, negative sensed ribonucleic acid (-ssRNA) viruses belonging to the genus Influenza A viruses in the family Orthomyxoviridae [1]. They are categorized into 16 haemagglutinin (H) and nine neuraminidase (N) subtypes [2]. In poultry, AIV are generally grouped based on the severity as low pathogenic avian influenza viruses (LPAI) such as H9N2 subtypes; and the highly pathogenic avian influenza viruses (HPAI) such as H5N1 and H5N8 strains [3]. Outbreaks of HPAI H5N1 instigated disastrous economic effects with alarming public health concerns in the world owing to the ability of the virus to cause up to 55% and 100% mortality rates in both poultry and humans, respectively [4]. While birds infected with the LPAI H9N2 are usually asymptomatic or show mild clinical signs, however, fatal outbreaks have equally been documented [5, 6]. In addition, co-circulation of HPAI H5N1 and LPAI H9N2 has been reported in poultry [7, 17] which presented possible assortment scenarios to generate novel influenza viruses capable of causing fatal infections in both animals and humans, particularly in an epidemiological niche like Africa where multiple species co-socialize [8, 9]. In Nigeria, the first outbreak of HPAI H5N1 was reported in a poultry farm in Kaduna state and it spread to other states of the federation incurring severe economic loss especially in Northern Nigeria [10, 11, 12, 13].

MATERIALS AND METHODS
Case Report
One sick and three dead 40-weeks-old layer (chickens) from the flock of 1,500 commercial layers were presented to the avian and aquatic clinic of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nigeria on 23rd January 2021, with the complaint of increasing mortality on daily basis despite treatment. The condition started 3 days before the presentation. The mortality patterns were 6, 50, 300+, 700+ during the preceding four days. There were 2 sets of layers on the farm, the younger ones kept on a deep litter system and the olders on battery cage, the disease started with the younger flock before it spread to the olders on the fourth day of the outbreak. Routine vaccination using NDV Lasota was conducted 3 weeks before the outbreak. The production was 65% and the chickens were treated with Diclazuril and cocktails of Neomycin, Chloramphenicol, Oxytetracycline.

Clinical Signs
Upon farm visit the clinical signs observed were somnolence, marginal cyanosis of the combs, ruffled feathers, depression, and whitish/yellowish diarrhoea, white shelled eggs, soft-shelled egg, matted vent and death.

Post Mortem Findings
Post mortem examinations were conducted on the dead chickens submitted to the clinic. The lesions recorded were multiple foci of petechial haemorrhages on the abdominal fat, congested and necrotic-friable liver, edematous and congested lungs (Plate D), congested spleen with multiple foci of necrosis, hemorrhagic and misshaped ovarian follicles, egg yolk peritonitis, swollen and hemorrhagic kidneys, erosion of the proventricular mucosa, congested trachea with mucoid exudates (Plate C) and sinusitis.
Plate I: (A) Showing ruffled feathers, cyanotic comb and wattles; (B) Showing depression and Somnolence.

Plate II: (C) Mucoid and haemorrhagic trachea; (D) Showing congested lungs, heart and Haemorrhagic ovarian follicles.

**Clinical Diagnosis**

Based on the history, clinical signs and post mortem findings the following differential diagnosis were suggested Avian influenza, Newcastle disease and Fowl cholera and a tentative diagnosis of NCD was decided.

**Treatment**

After samples were collected and sent for laboratory analysis. Gentamycin injection at 0.3ml/chicken intramuscularly for 3 days and Vitamino-trace at 1gm/5l of drinking water for 7 days were recommended.
**Laboratory Analysis**

**Antigen/Antibody Detection by ELISA**

Oropharyngeal swabs, cloacal swabs and blood samples collected from 5 birds were received for avian influenza screening. Sera were separated from blood samples by spinning blood tubes at 3000 g for 15 minutes. Harvested sera were tested by an Antibody capture Indirect ELISA for Avian Influenza nucleoprotein. Positive samples were further tested by H5, H7 and H9 competitive ELISA (ID.Vet, Grabels, France) according to the manufacturer's instruction. Results were read using an ELISA machine (BioTek ELX 800). BioTek Instruments, Inc., Vermont, USA.

Antigen capture ELISA for the detection of Influenza A virus Nucleoprotein (Biocheck, Fokkerstraat, Reeuwijk, Netherlands) was used to test for group-specific viral nucleoprotein antigen in pooled tracheal and cloacal swabs using BioTek ELX 800 absorbent microplate reader (BioTek Instruments, Inc., Vermont, USA.) following manufacturers recommendation. Positive swabs were kept frozen at -20°C and immediately shipped to the Regional laboratory for Animal Influenza and other Transboundary Animal Diseases, National Veterinary Research Institute, Vom, Plateau State, Nigeria for molecular detection and characterization of the Avian Influenza virus.

**Nucleic acid extraction and Real-Time Polymerase Chain Reaction**

Total RNA was extracted from pooled oropharyngeal and cloacal swabs using Qiagen RNeasy Mini Kit, (Qiagen, Hilden, Germany) according to the manufacturer instructions. Eluted RNA was stored at -80°C. Real-time RT – PCR (RRT – PCR) test for detection of Influenza A matrix gene on a Rotor G (QIAGEN Co, CA) was carried out [15]. Positive reactions were further subtyped by RRT – PCR H5, H7 and H9 [16]. Primers and probe oligonucleotide sequences used are

**Table 1. RRT-PCR primer and probe oligonucleotide sequences**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer/Probe</th>
<th>Sequence (5’ – 3’) *</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza A</td>
<td>Forward primer (M+25)</td>
<td>AGATGAGTCTTCTAACCAGGTCG</td>
<td></td>
</tr>
<tr>
<td>M Gene</td>
<td>Reverse primer (M-124)</td>
<td>TGCAAAAAACATCTTCAAGTCTCTG</td>
<td></td>
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<tr>
<td></td>
<td>Probe (M+64)</td>
<td>TCAGGGCCCCCTCAAGGCCCAG</td>
<td></td>
</tr>
<tr>
<td>AI Virus</td>
<td>Forward primer H5 – F</td>
<td>TTTATCAACAGTGAGCGAG</td>
<td></td>
</tr>
<tr>
<td>Subtype H5</td>
<td>Reverse primer H5NE – R</td>
<td>CCAG(T)AAAGATAGACCAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CCCTAGCAGTGCGGCAATCATG</td>
<td></td>
</tr>
<tr>
<td>AI Virus</td>
<td>Forward primer H7 – F</td>
<td>TTTGGTTTACGGTCCGG</td>
<td></td>
</tr>
<tr>
<td>Subtype H7</td>
<td>Reverse primer H7 - Deg R</td>
<td>GAAGAA(C)AAGGCC(T)CATTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CATCATGGTTCATCTTCTTGCCCAT</td>
<td></td>
</tr>
<tr>
<td>AI Virus</td>
<td>Forward primer H9F</td>
<td>ATGGGGTTTGCTGCC</td>
<td></td>
</tr>
<tr>
<td>Subtype H9</td>
<td>Reverse primer H9R</td>
<td>TTATACAAATGTTGCAC(T)CTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TTCTGGGATCATGCAATGG</td>
<td></td>
</tr>
</tbody>
</table>

* Parentheses show alternative nucleotides in degenerate primers/probes.
LABORATORY RESULTS

ELISA and PCR
All sera (5/5) tested for antibodies against Avian influenza nucleoprotein produced positive results while 80% of sera (4/5) tested positive for the H9 subtype of AIV. Antibodies against the H5 and H7 subtypes were not detected. The results of RRT-PCR and RRT-PCR/H5 were positive for both tracheal and cloacal samples. RRT-PCR/H7 & H9 yielded negative results.

Microbial Culture and Sensitivity Test
The microbiology result of the liver showed *Escherichia coli*, the bacterial isolate was resistant to Streptomycin, Neomycin, Thiamphenicol, Doxycycline and Penicillin but sensitive to Gentamycin, Florfenicol, Colistin, and Enrofloxacin.

DISCUSSION

In the present case, Newcastle disease was considered because of the presenting signs of high mortality, white shelled eggs and soft-shelled eggs including the haemorrhages seen in the trachea and ovarian follicles which are similar to the signs reported by previous works [19]. [17] reported white shelled eggs, low mortality (7%) and a drop in egg production which are different from the mortalities (100%) recorded in this case. The sera were positive for H9 AIV antibodies while all samples were negative for H5 and H7 AIV viruses. This is so because H5 and H7 cause highly pathogenic Avian Influenza which usually kills all the chickens in the flock within a very short period, therefore most or all the chickens will die from the peracute phase of the disease. On the other hand, the LPAI has very low morbidity and mortality and may last for weeks in a flock [7].

All the swabs were positive for H5 Avian Influenza virus and negative for H7 and H9 Avian Influenza virus. This is not surprising because the clinical signs and high mortality recorded are pointing towards Highly Pathogenic Avian Influenza which is currently ravaging poultry in Nigeria.

On the 2nd of February, 2021 the Department of Veterinary and Pest Control Services of the Federal Ministry of Agriculture and Rural Development issued a circular VSD/276/S4/C4/1/24 notifying all the Directors of Veterinary Services of all the states in Nigeria of a resurgence of HPAI in Nigeria after 2 years of no reported outbreak in the country. The report further stated that there were 3 outbreaks in Kano State and one in Plateau State. The presence of the 2 Influenza viruses can lead to the assortment and production of novel offspring that can be more deadly to chickens and may have the capacity to infect humans and may have the capacity for inter-human transmission.

Conclusion

In conclusion, this is a case of HPAI (H5N1) complicated with LPAI (H9N2) which can have a lot of implications for the poultry industry in Nigeria and also Public Health Significance. Hence biosecurity is very important in the prevention of the disease.

Acknowledgement

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