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Occurrence of infectious bronchitis in layer birds in Plateau state, north central Nigeria

Ismaila Shittu[1],*, Dorcas A. Gado[1], Clement A. Meseko[1], Davou C. Nyam[1], Kayode A. Olawuyi[1], Gyang D. Moses[2], Chinonoyerem N. Chinyere[1] and Tony M. Joannis[1]

[1]*Regional Laboratory for Animal Influenza and other TADs, National Veterinary Research Institute, Vom, Nigeria*
[2]*Central Diagnostic Division, National Veterinary Research Institute, Vom, Nigeria*

Abstract
A flock of 54 wk-old layer birds exhibiting signs of respiratory distress, greenish diarrhea, and drop in egg production was investigated. A marked drop in egg production (55%) was recorded with eggs appearing white and soft-shelled. Mortality was in the range of 1%–2% with post-mortem lesions revealing cloudy air sacs, frothy, and congested lungs. Viral RNA was extracted from pooled tissue samples (trachea, lungs, spleen, and liver) and tested for Avian influenza virus (AIV), Newcastle disease virus (NDV), and infectious bronchitis virus (IBV) by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, virus isolation was attempted in 9–11 day-old embryonating chicken eggs (ECE). In order to determine the prevalence of IBV serotype(s) in the flock, serum samples were screened by hemagglutination-inhibition (HI) test using IBV antigens and antisera (Arkansas, Connecticut, and Massachusetts). Neither AIV nor NDV but IBV was detected in the tissue samples by RT-PCR. In addition, virus isolate obtained after four serial passages in ECE produced dwarfed, stunted, and hemorrhagic embryos, and the isolate was confirmed by RT-PCR to be IBV. The serum samples were 100% seropositive for three serotypes with HI titres ranging from 5 to 12 Log_2. In this study, IBV was confirmed as the causative agent of the observed respiratory distress and drop in egg production. Also, the evidence of co-circulation of multiple IBV serotypes was established, this to the best of our knowledge is the first of such report in Nigeria. We recommend extensive molecular and sero-epidemiology of circulating IBV genotypes and serotypes in Nigeria with the aim of developing better control strategies, including vaccination.
Keywords: Drop in egg production, Infectious bronchitis, Respiratory distress, Serotypes, Virus isolation.

Introduction

Globally, respiratory infections are common occurrences in poultry production with associated complexity in terms of causative pathogens involving bacterial and viral agents. Diseases caused by aetiologic agents of respiratory distress, stunted growth, and drop in egg production cause great economic losses to poultry producers worldwide. Individually, they can cause diseases that are exercerbated when co-infections with other viral and bacterial agents are involved. Notable infectious agents that have been implicated in respiratory infections of poultry, include avian influenza virus (AIV), Newcastle disease virus (NDV), *Mycoplasma* spp., avian metapneumovirus, infectious laryngotracheitis virus, and infectious bronchitis virus (IBV) (Villegas, 1998).

Infectious bronchitis (IB) is an acute, viral disease of poultry which has been implicated in severe economic losses mainly due to reduced performance and complication with secondary bacterial and viral co-infection that can result in high mortality (Cavanagh, 2007; Liu, 2005; Sid *et al*., 2015). The virus does not only infect the respiratory tract of chickens with clinical presentations of respiratory distress but also replicate in other sites, including kidney, reproductive, and gastrointestinal systems where it produces pathological lesions with varying severity among different breeds of poultry (Cavanagh, 2007; OIE, 2008; Villegas, 1998).

The aetiologic agent of IB is an enveloped, positive-sense, single-stranded RNA virus with the longest genome length, 27.6 kb, among the RNA viruses. The virus is classified in the genus *gammacoronavirus,* sub-family, *Coronavirinae*, family *Coronaviridae*, order Nidovirales (https://talk.ictvonline.org/taxonomy/). The virus may give rise to different serotypes through recombination among different strains of the virus or by spontaneous mutations in the genes. Several circulating serotypes of the virus which may not confer cross protection on each other have been identified in poultry worldwide. It is, therefore, imperative to identify serotypes circulating in different localities for the choice of effective vaccine (Bijlenga *et al*., 2004; Thor *et al*., 2011). The most prevalent serotype worldwide being the Massachusetts-type (Mass) commonly used as a vaccine candidate (Gaba *et al*., 2010; Li *et al*., 2010). In spite of the widely used vaccine(s), IB remains the

***Corresponding Author:** Ismaila Shittu. Regional Laboratory for Animal Influenza and other TADs, National Veterinary Research Institute, Vom, Nigeria. Email: *ismaila.shittu@gmail.com*

most commonly reported respiratory disease of poultry globally (Thor *et al*., 2011).

The first case of IB was reported in the US in 1930 by Schalk and Hawn (1931) which subsequently spread to other parts of the world (Fabricant, 1998). In Nigeria, however, the first documented evidence of suspected IB disease was by Adene and Ojo (1976). Since the first report in Nigeria, there seems to have been a progressive increase in the prevalence of IB in the country without much attention compared with other prevailing poultry diseases like Newcastle, Gumboro, and avian influenza. Komolafe *et al.* (1990) reported a seroprevalence of 3.3% from the south-eastern part of Nigeria. Thereafter, other reports have established high (15.3%–89%) seroprevalence of IB (Emikpe *et al*., 2010; Mungadi *et al*., 2015; Oyejide *et al*., 1988; Owoade *et al*., 2006) but none of these studies have identified the different serotypes in circulation nor attempt to establish a causal relationship with marked drop in egg production. In another report, Ducatez *et al.* (2009) identified a novel genotype of IBV from birds with inapparent signs of infection in South West Nigeria.

Unlike Newcastle disease (ND) which is relatively well studied in Nigeria and has been known to be enzootic (Adu *et al*., 1986; Nwanta *et al*., 2008; Shittu *et al*., 2015; Solomon *et al*., 2012), IB is less studied and understood with only a few reports available in the public domain on the epidemiology and control in the country. Based on the previous serological reports, the incidence of IB seems to be on the increase across the country (Emikpe *et al*., 2010). In this investigation, we sought to identify the causative agents of reported case of respiratory disease and drop in egg production in Plateau State, Nigeria.

Recently, however, reports of rampant occurrences of respiratory diseases and drop in egg productions have risen in Plateau state, Nigeria (personal communication: Moses GD). This study, therefore, investigates one of such occurrences with the view of identifying the aetiologic agent(s) and determine the seroprevalence of three IBV serotypes in the flock.

Materials and Methods

Case history, sample collection, and processing

In June 2015, during the course of investigating avian influenza outbreaks in Plateau state, Nigeria, a commercial farm (N 9.76466°; E 8.85699°) housing 1,200 laying birds (54 wk-old) reported drop in egg production and respiratory distress with low (1%–2%) mortality. The birds were sourced from a farm that raised birds for point-of-lay sales at the age of 14 wk. According to the farmer's record, all necessary vaccines (Mareks disease, ND, infectious bursal disease, Fowl typhoid, and Fowl pox) had been administered including a trivalent (ND, IB, and egg drop syndrome) killed oil-emulsion vaccine. There was no record of initial priming with live attenuated IBV vaccine. Birds came into lay at the age of about 22 wk and production never reached 80%. At the time of this investigation, the flock size stood at 1,062 laying chickens. During the course of production, many birds were lost without confirmation of the cause of death. Dead birds were collected and subjected to post-mortem examination.

At necropsy, lungs, trachea, liver, and spleen were harvested for virological examination by reverse transcriptase-polymerase chain reaction (RT-PCR) and isolation in embryonating chicken eggs (ECE). Homogenate (20%) of pooled tissue (lungs, trachea, liver, and spleen) was made by grinding in chilled mortar and pestle with addition of phosphate buffered saline (pH 7.2) containing Penicillin (2,000 units/ml), Streptomycin (2 mg/ml), Gentamycin (50 µg/ml), and Amphotericin B (5 mg/ml) as contained in the OIE Manual of Diagnostics (2008). The homogenate was clarified by centrifugation at 3,000 rpm for 10 min and supernatant stored at −80°C until used.

Viral RNA extraction

Viral RNA was extracted from the homogenate and ALF samples using QIAmp viral RNA mini kit (Qiagen, Germany). In addition, NDV Ulster 2C (IZSVe, Padua, Italy), IBV M-41 strain (Charles River Laboratories, CT), and AIV H5N2 (IZSVe, Padua, Italy) were extracted as positive controls, while nuclease-free water was used as a negative control. The RNA extracts were eluted in 60-µl elution buffer according to the manufacturer's instruction and stored at −80°C for further analysis.

RT-PCR and gel electrophoresis for AIV and NDV, IBV

As shown in Table 1, primers targeting the matrix (M) gene were used to amplify AIV and NDV, while the 5′-untranslated region was amplified in IBV.

To amplify the M gene of NDV, the One-Step RT-PCR kit (Qiagen, Germany) was used in a 25-µl reaction mixture containing 5.0 µl of 5× PCR buffer, 1.0 µl dNTP mix (10 mM each), 1.0 µl of each primer (10 µM), 0.5 µl RNase Inhibitor (40 U/µl, Promega), 0.5 µl RT-PCR Enzyme Mix, 5.0 µl of RNA template, and nuclease-free water was added to make up to the final volume. The reaction was performed on GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, CA) under the following cycling conditions: 50°C for 30 min, 94°C for 15 min; 40 cycles of 94°C for 30 sec, 55°C for 1 min and 68°C for 2 min, and a final extension at 68°C for 10 min.

Using the primers in Table 1 as described by Callison *et al.* (2006), IBV was amplified with a One-Step RT-PCR protocol (Qiagen, Germany) in a 25-µl reaction mixture containing 5.0 µl of 5× PCR buffer, 1.0 µl dNTP mix (10 mM each), 0.5 µl of each primer (10 µM), 0.5 µl RNase Inhibitor (40 U/µl, Promega), 0.5 µl RT-PCR Enzyme Mix, 5.0 µl of RNA template and nuclease-free water to make up to the final volume. The following cycling conditions were used: 50°C at 30 min, 94°C for 15 min; 40 cycles of 94°C for 30 sec,

Table 1. Primer sequences used for the amplification of AIV, NDV and IBV.

Primer ID	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Product size (bp)	Reference
AIV	CTTCTAACCGAGG TCGAAACG	AGGGCATTTTGGACAAAG/ TCGTCTA	240	Fouchier *et al.*, 2000
NDV	AAGGAGCCTTGATCTATC TGTCGG	TGTGCCCCTTCTCCAG CTTAGTA	280	Unpublished
IBV	GCTTTTGAGCCTAGCGTT	GCCATGTTGTCACTG TCTATTG	143	Callison *et al.*, 2006

52°C for 30 sec, 68°C for 30 sec, and a final extension at 68°C for 7 min.

AIV was amplified using GeneAmp® Gold RNA PCR kit (Applied Biosystems, CA) as previously described (Fouchier *et al*., 2000). All the PCR products alongside 50 bp DNA molecular weight marker (Fermentas) were analyzed by gel electrophoresis using 1.5% agarose stained with Ethidium bromide (0.5 µg/ml) and visualized in a Biostep dark hood DH-40/50 imaging analysis system (Biostep, Germany).

Virus isolation

To isolate the aetiologic agent, 0.2 µl of the supernatant was inoculated via the allantoic cavity route into five 10-day-old specific antibody negative ECE. Allantoic fluids (ALFs) were harvested after 5 d of incubation at 37°C and blind passaged for additional four times in fresh ECE (OIE, 2008). At each passage, embryos were observed for IB lesions which include stunted growth, haemorrhagic, and curled embryo. Harvested ALFs were stored at −80°C for further analysis.

Seroprevalence

To determine the prevalence of IBV serotype(s) in the flock, 32 blood samples were obtained via the brachial vein of the chickens. Briefly, 3–5 ml of blood was collected from each bird in syringes. The samples were left to clot in a slanted position and separated sera were decanted into sterile tubes afterward. The sera were transported in a cool box to the laboratory and stored at −20°C until tested. To determine the serotypes of IBV in the flock, the sera were screened using a panel of IBV antigens and antisera [Arkansas (Ark), Connecticut (Conn) and Mass (Charles Rivers Laboratories, CT)] in a hemagglutination-inhibition (HI) test according to the standard methods (OIE, 2008).

To compute the geometric mean titre (GMT), the individual serum sample titres from the same serotype were added up and averaged. The values obtained were then reported as the GMT after cross-checking against GMT values given in the Brugh's table (Villegas, 2008).

Results and Discussion

In the infected birds, clinical signs observed include white, soft-shelled eggs, greenish diarrhoea, and respiratory distress (coughing, sneezing, and rales). As at the time of sampling, egg production had dropped by approximately 55%. Mortality was observed before the birds came into lay and continued during lay with a range of 1%–2%. At post-mortem, lesions observed include cloudy air sacs, frothy and congested lungs, whitish, cheesy materials on the serosal surface of the intestine, white nodules on the surface of the ventricles, proventriculus and intestines, and presence of ascitic fluid in the abdomen. These signs and lesions are consistent with findings from previous reports (Awad *et al*., 2014b; Ballal *et al*., 2005). Due to the similarities in clinical and pathological lesions presented by infections involving respiratory viruses, clinician that based their diagnosis on these non-pathognomonic signs may miss the aetiological agents. Though the mortality rate in the affected flock was low (1%–2%), it is consistent with the report by Awad *et al.* (2014a). Generally, single infections with IBV result in low mortality. However, exacerbation by concurrent infection with other pathogens of viral or bacterial origin have been reported (Jackwood, 2012).

As shown in Figure 1, the tissue homogenate was positive for IBV by RT-PCR and negative for AIV and NDV. Upon inoculation of ECEs with the tissue homogenate, no noticeable changes were observed in the embryos in the first few passages. However, at passage four, embryo death with characteristic IB lesions, including curling, dwarfing, and hemorrhages on the embryos (13 d of age) were conspicuously discernible (Fig. 2). Allantoic fluids harvested from the eggs of both dead and live embryos did not cause agglutination of chicken red blood cells in spot hemagglutination test (data not shown) and this confirms the absence of hemagglutinating agent. In this study, we have shown that IBV which is less described and often given less attention and not NDV or AIV was the causative agent of infection in the 54-wk-old laying birds showing respiratory signs and severe drop in egg production. Although ND was first suspected by the consulting clinician due to its enzootic status in Nigeria. In a limited study, the prevalence of IB was found to equal that of ND confirming the increasing important enzootic status of IB in Nigeria poultry (Shittu unpublished data). In this study, successful isolation of IBV in embryonating eggs was accomplished after four blind passages with the embryos developing lesions characteristic of IB such as stunting and dwarfing (Fig. 2). For IBV isolation, ECE and tracheal organ cultures (TOC) are substrates of choice although TOC has an edge over ECE in that stasis of the tracheal cilia could

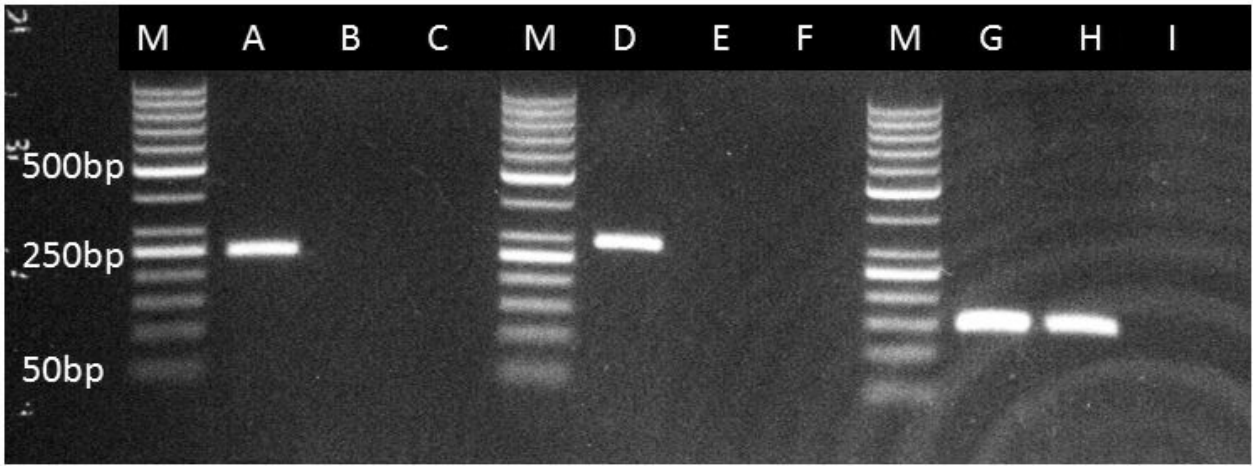

Fig. 1. Gel electrophoresis of RT-PCR products for AIV, NDV, and IBV. (M): 50 bp DNA molecular weight marker; (A, D, and G): positive controls for AIV H5N2, NDV Ulster 2C, and IBV M-41 strain at 240, 280, and 143 bp, respectively; (B, E, and H): test samples from the suspected flock investigated for AIV, NDV, and IBV, respectively; (C, F, and I): negative control for each assay conducted in the investigation.

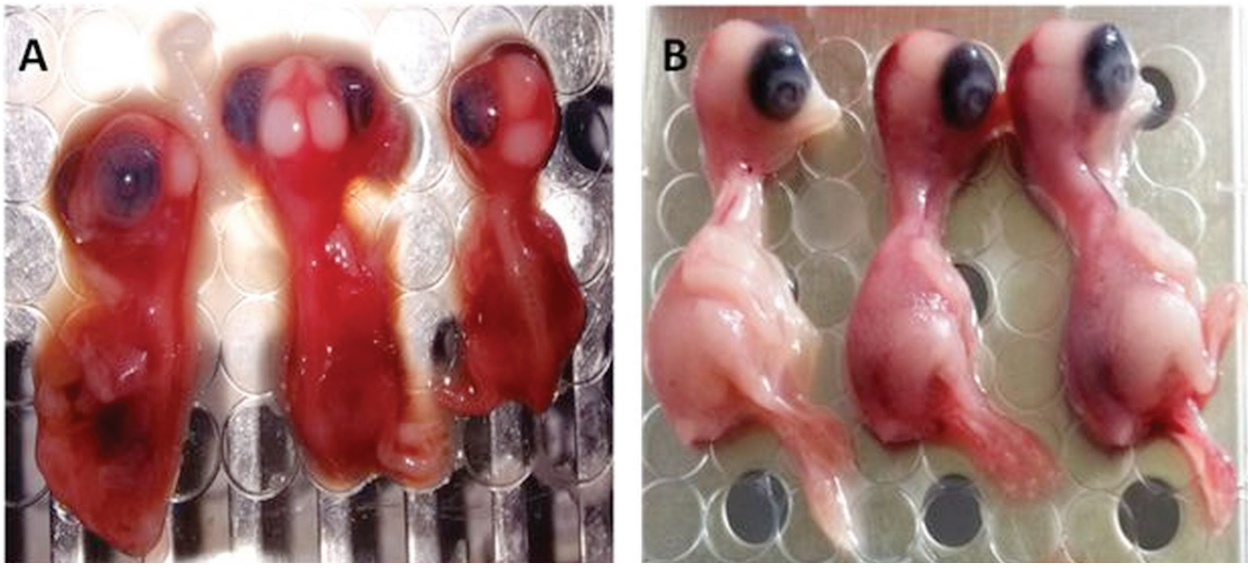

Fig. 2. IBV-infected and uninfected 13-day-old embryos. (A): Infected embryos with characteristic IB lesions of stunted growth, curling, and cutaneous hemorrhage. (B): Uninfected embryos.

be observed in the former upon primary inoculation (OIE, 2008). In this study, ECE isolation technique was found to be equally useful.

According to the farm records, the birds were vaccinated with inactivated oil-emulsion vaccine which contained IBV antigen. However, this seemed not to have protected the birds against morbidity, mortality, and decreased egg production. It has been reported that chickens with low antibody level to IBV serotype could experience severe drop in egg production, whereas those with high antibody level are less affected in terms of egg quality and production possibly as a result of immune protection (Ballal *et al*., 2005). Available literature show that the use of inactivated IB vaccines alone does not confer adequate protection on the birds except where they are first primed with live attenuated IB vaccines during the early stages of production (Cook *et al*., 2012). In addition, IBV serotypes do not cross-protect (Jackwood *et al*., 2010), thus the vaccine must be designed based on circulating serotypes in the locality. In Plateau State, there are no available data on the circulating IBV strains. Although most vaccines being used on the field in Nigeria are predominatly Mass serotype, detection of other serotypes in this investigation is a further indication and support speculation that the vaccine strains being

Table 2. Infectious bronchitis virus serotypes report from a commercial farm exhibiting respiratory distress and drop in egg production.

S/No.	Serotypes	Number of samples	Number of positives	% positive
1.	Massachusetts (Mass)	32	32	100
2.	Connecticut (Conn)	32	32	100
3.	Arkansas (Ark)	32	32	100
4.	Mass & Conn	64	64	100
5.	Mass & Ark	64	64	100
6.	Conn & Ark	64	64	100
7.	Mass, Conn & Ark	96	96	100

Table 3. Distribution of HI titres of positive samples for different IBV serotypes.

No. of samples	Serotypes	Titre									GMT
		1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	
32	Mass	-	-	3	1	10	7	6	2	3	490.5
32	Conn	-	4	8	4	6	2	4	2	2	215.3
32	Ark	-	-	2	1	12	5	4	6	2	534.9

used differ from some of the serotypes in circulation. Furthermore, Ducatez *et al.* (2009) identified a novel IBV serotype "IBADAN" from southwestern Nigeria and no information exists on the ability of the vaccine strains in use to protect birds against this novel strain (de Wit *et al*., 2010). It is, however, not known if this serotype circulates in the northern part of the country.

Antibody prevalence and high GMT titre distribution of the three serotypes of IBV used in the study for Mass, Conn, and Ark are 490.5, 215.3, and 534.9, respectively, as shown in Tables 2 and 3. In all 32 serum samples tested, 100% seropositivity was also observed for Mass, Conn, and Ark serotypes. In addition, concurrent infections with Mass/Conn, Mass/Ark, Conn/Ark, and Mass/Conn/Ark serotypes were observed.

Interestingly, the HI results for the three serotypes (Mass, Conn, and Ark) tested in this study revealed 100% seropositivity (Table 2). This clearly shows that the three IBV serotypes are present in the farm and may be in circulation in Plateau State with the possibility of other hitherto unreported serotypes. As reported by Jackwood (2012), several serotypes and variants of IBV circulate around the world with some having specificity for a particular location, making them indigenous to those places. Such may include the newly described serotype by Ducatez *et al*. (2009) which we could not test for in our samples due to unavailability of strain specific diagnostic reagent. The Mass strain of IBV has been reported to be widespread across the globe possibly due to its use as a vaccine (de Wit *et al*., 2010). In Nigeria, breeder stocks are often vaccinated with live IBV vaccine using Mass-like strains at much younger ages (Ducatez *et al*., 2009). However, in this case, a trivalent killed-adjuvanted vaccine containing IBV was said to have been administered without prior priming with live IBV vaccine. As previously reported (Bijlenga *et al*., 2004; de Wit *et al*., 2010), antibody response to killed-adjuvanted IBV vaccine without priming the birds with live attenuated IB vaccine are usually poor. It can, therefore, be deduced that the high titre of antibodies to the three IBV serotypes detected in this study (Table 3) may not have emanated from vaccination, but could be a result of recent or continuous infection with circulating strains of the virus as also shown by molecular detection and virus isolation. To the best of authors' knowledge, co-circulation of multiple serotypes of IBV as described here is the first documented report from Nigeria.

Conclusion

This report underscores the need to investigate, by laboratory diagnostic methods, all cases presenting with respiratory distress and drop in egg production for IB. In the present case prior to further laboratory, most clinical diagnoses are based on signs and pathological lesions. Future observation and investigation should be designed to investigate the different IBV serotypes and genotypes in circulation across the country with the aim of producing vaccine (s), based on the identified serotypes, for combating the menace of IB in the Nigerian poultry population.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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