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DETECTION OF NEWCASTLE DISEASE VIRUS (NDV) IN LAUGHING DOVES AND THE RISK OF SPREAD TO BACKYARD POULTRY

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ABSTRACT

Newcastle disease (ND) is a highly infectious viral disease of birds caused by the Newcastle disease virus (NDV) and doves have been incriminated in previous outbreaks of the disease that have discouraged backyard poultry productions. This survey was done to detect and characterize the NDV from 184 swabs from the cloacae and pharynxes of 67 trapped laughing doves and 25 backyard poultry birds. The study utilized haemagglutination assay (HA) followed by haemagglutination inhibition (HI) tests on HA positive samples to screen field samples. Conventional reverse transcriptase polymerase chain reaction (RT-PCR) was conducted on the HI positives to characterize the NDV. This study revealed that of 134 dove samples screened, 88 (65.7 %) were HA positive. Of these HA positives subjected to HI testing, 37 (42.1 %) were HI positive. Interestingly, 21 (56.8 %) of the HI positives were also RT-PCR positive: 8 lentogenic, 12 velogenic, while one had both lentogenic and velogenic NDV. Comparatively, of the 50 chicken samples screened, 23 (46 %) were HA positive; and of these, HA positives subjected to HI testing, 16 (69.6 %) were HI positive. Only 4 (25 %) of the HI positives were RT-PCR positive: 3 lentogenic and a velogenic NDV. From this study it was concluded that laughing doves were demonstrated to be infected with either lentogenic or velogenic NDV or both. The use of red blood adsorption-de-adsorption concentration of NDV enhanced the RT-PCR detection using the fusion gene primers NDV-F 4829 and NDV-R 5031. The detection of not only lentogenic but velogenic NDV in laughing doves poses a great risk to backyard poultry production.

Key words: backyard poultry; laughing doves; molecular pathotyping; Newcastle disease virus

INTRODUCTION:

Laughing doves belong to the Kingdom—Animalia, Phylum—*Chordata*, Class—*Aves*, Order—*Columbiformes*, Family—*Columbidae*, Genus—*Streptopelia*, Species—*senegalensis* and subspecies—*senegalensis*. They are small Af-

rican pigeons so named because of their distinct cooing sounds which they make especially in the mornings that mimic human laughter [12]. Laughing doves are commonly called Senegal dove, laughing turtle dove, or little brown dove, depending upon the region in which they are found. Laughing doves are widely distributed throughout most of tropical West Africa, Asia and Arabia. They inhabit desert scrub land from the guinea savannah to the rain forests. Laughing doves usually move in pairs, although monogamous pairs may form large groups of up to 20 to 50 birds in a particular home range. Laughing doves are seldom migratory and may be resident in a particular home range for up to seventeen years.

Adult doves measure about 25 cm long and have an average wing span of 45 cm. Their purplish brown plumage is unique with a pinkish brown breast and a black chequered neck band, purple pink legs and a black bill. The bird is flighty although they may be found nesting very close to human dwellings particularly in garden trees, flower hedges and window ledges. In Nigeria the laughing dove is freely trapped or hunted with catapults and sold as pets or meat to customers from live bird markets. The bird is prolific in the wild and unlike pigeons, do not breed in captivity. Laughing doves (*Streptopelia senegalensis*) in foraging for food, visit households to pick up grains, flour, chaff, and chicken feed spilled on the ground around backyard poultry houses, especially when feeding their nestlings (squabs) and when grains are scarce in the fields [15]. Laughing doves also invade human dwellings in search of drinking water during the dry season [18]. These increase the chances of dove droppings contaminating the premises of households in backyard poultry operations. The soles of footwear of poultry farmers may get contaminated with these wild bird droppings and be transferred into the backyard poultry houses [18]. Also, with broken chicken wire fencing of poultry houses, doves may actually invade such pens and contaminate poultry feed and water with oro-nasal discharges and faeces. Dove droppings on rooftops during the rainy season may be washed down with runoff water into drums and tanks which backyard poultry farmers use to water birds during periods of water scarcity.

Newcastle disease (ND) caused by avian paramyxovirus serotype 1 (APMV-1) viruses can be diagnosed in specimens from both live and dead birds. Most commonly in live birds, swabs of the pharyngeal area and/or cloacae (or faeces) may be tested at appropriate diagnostic laboratories using virus isolation [5, 10]. During seasonal outbreaks of ND, laughing doves and pigeons are usually knocked down and may be found dead under trees or in gutters [17]. These wild doves may act as sentinel animals for the monitoring of ND in an area known for the production of poultry by backyard and large scale poultry farms [10]. The trapping of these doves routinely and sampling for Newcastle disease virus (NDV) may assist avian disease monitoring and surveillance teams predict the eventual outbreak of ND in poultry farms in a region [5, 10]. The "gold standard" for the identification of NDV involves isolation and cultivation in embryonated chicken eggs followed by hemagglutination test, hemagglutination inhibition test and pathotyping of the virus [13]. Pathogenicity is determined by the intracerebral pathogenicity index [13]. These assays are labour intensive and time-consuming, requiring up to ten days to complete. This hinders the authorities in undertaking adequate measures in a timely manner to limit the spread and eradicate the infection [10]. The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) is a test that satisfies the requirements for high sensitivity and specificity, coupled with a short turnaround time [4, 6, 7, 11, 17].

This study was designed to by-pass the isolation and cultivation of the Newcastle disease virus in embryonated chicken eggs which are time consuming, by utilizing the HA and HI tests to screen field samples and to detect the virus from the oropharyngeal and cloacal swab samples of laughing doves and then confirm the pathotype by RT-PCR and gel-electrophoresis [16, 17, 18].

MATERIALS AND METHODS

Study area

The study areas fell within latitude 11°10'38"N, longitude 7°37'43"E and latitude 11°8'18"N, longitude 7°041'3"E, occupying a land area of 26.59 km^2. The residential areas used for this study were selected by convenience and comprised of the residential clusters in Zaria, especially the staff quarters of A. B. U. Samaru, Zaria, Kaduna State, Nigeria in West Africa (Fig. 1).

Study design

A survey for NDV was carried out on trapped doves and backyard poultry birds from the premises of backyard

poultry farmers in Areas BZ, E, G and C (R1), and areas H, Silver Jubilee of A. B. U. Staff quarters, and Palladan (R2), and from the live bird market in Samaru (R3). These areas are located in Zaria, Kaduna State, Nigeria, West Africa (Fig. 1).

Sampling

Laughing doves are not an endangered species in Nigeria and the birds are usually trapped or shot with catapults by hunters for sale as food or pets. No special ethical clearance was needed therefore in this study, since the birds were treated humanely and were released back into their natural habitats after the sample collections. A total of sixty-seven laughing doves were randomly trapped and sampled along with twenty-five chickens sampled from the backyard poultry from the residential areas based on convenience. This was subject to the number of doves that were trapped during the six month survey period from September 2014 to February 2015. Thus, a sum total of 184 oropharyngeal and cloacal swabs were collected and tested. We purposed to sample at least ten doves and five chickens from each residential area.

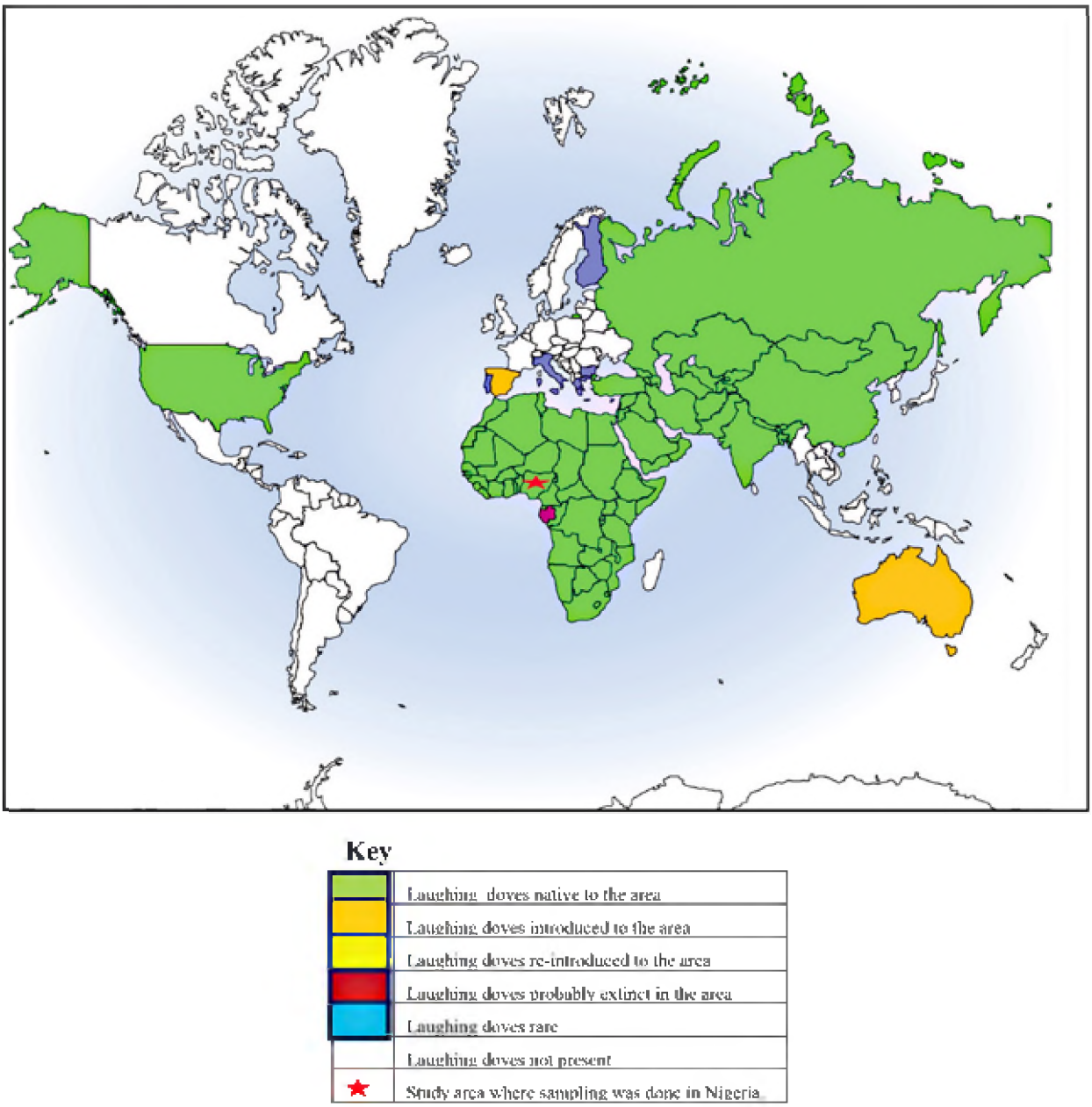

Fig. 1. Worldwide distribution of the Laughing dove [oiseaux.net/maps/laughingdove.html]

Eligibility criteria of participants

All residents with backyard poultry farms who had raised broilers, layers or local chickens for commercial or domestic purposes in the last three years or more were considered as backyard poultry farmers. Farmers with up to 300 birds stocking capacity were considered to have standard backyard poultry farms.

Trapping of laughing doves

Three locally constructed bamboo cages with apparently healthy laughing doves and grains of guinea corn (sorghum) and millet, placed around backyard poultry farms, were used to lure and trap laughing doves into the study areas. The best trapping successes were obtained between 10 a. m.—12 noon and 4 p. m.—6 p. m.; times at which laughing doves foraged from the ground. Between 12 noon—4 p. m. the doves were either watering themselves or resting high up in the trees.

Each dove trapped and sampled was tagged by clasping a non-corroding metallic colour-coded bracelets and rubber bungs on their shanks. Each residential area had a different colour code: Area BZ-green, Area E/G-blue, Area C-golden, Silver Jubilee and Area H-red/pink, and Palladan-silver; while subject doves from the Samaru live bird market were marked on their shanks with black indelible ink. This was to prevent double sampling of subjects and in a follow up future study assess whether laughing doves migrated from one residential area to another area or were limited to the areas in which they were trapped initially (Fig. 2).

Sample collection

Sixty-seven pairs of oropharyngeal and cloacal swabs were collected from randomly trapped laughing doves close to backyard poultry farms. Each dove trapped, ringed and sampled was released back into the wild but was not sampled again even if re-trapped. Fourteen, twenty-eight and twenty-five doves were sampled from clusters 1, 2 and the Samaru live bird market respectively. A similar collection was done on five chickens conveniently selected from three out of four residential areas in cluster 1 and two out of three residential areas in cluster 2, making a total of twenty-five pairs of samples. Oropharyngeal and cloacal swabs from doves trapped close to backyard poultry farms and the Samaru live bird market were eluted in 2.0 ml of viral transport medium in screw cap tubes, and frozen at –20 °C until analysis.

Fig. 2. Trapping (top) and tagging (below) of laughing doves

Preparation of washed chicken red blood cells

Whole blood was collected into EDTA-coated tubes from apparently healthy local chickens with no known history of Newcastle disease vaccination or clinical disease. The whole blood was washed with dextrose gelatine veronal (DGV) solution (pH 7.4), and 10 %, 1 % and 0.1 % suspensions of the washed chicken red blood cells were prepared and stored at 4 °C until needed [16].

Antigen

Newcastle disease virus antigen was obtained by reconstituting commercially sold Newcastle disease La Sota vaccine and using it fresh for the HI test [13, 15, 18]. The antigen had a titre of 1:256 with a 4 HAU of 64. Sterile PBS was prepared with a pH of 7.4 to serve as a diluent.

Positive control serum

Hyper-immune serum with antibodies to Newcastle disease was obtained by raising ten broilers to 8 weeks

of age, and vaccinating them at week 1, 3, 6 and 8 with La Sota vaccine. Ten millilitres (10 ml) of whole blood was collected from these ten broilers at 10 weeks of age, pooled together, kept to stand, and allowed to coagulate to obtain the serum. The serum was decanted into a screw cap sterile container and stored frozen at –20 °C [16]. The HI titre was determined to be 1:64. Sterile PBS with pH 7.4 served as a diluent for the negative control which was a blank of negative serum.

Laboratory analysis for detection of Newcastle disease virus

Haemagglutination Assay and Haemagglutination Inhibition assay (HA & HI):

Each oropharyngeal and cloacal swab was subjected to HA and HI tests in U-shaped micro-well titre plates. To each drop (0.02 ml) of test sample placed in a well, one drop of one percent (1 %) washed chicken red blood cells was added. The sides of the plate were tapped lightly, and then the plate covered with a piece of paper, and left to stand for 30—45 minutes. The result was read as positive for haemagglutination if a diffuse mat of red blood cells was observed in the well and negative if a button of clumped red blood cells settled at the base of the well. These were compared with the reactions of the negative (RBC and PBS only) and positive (RBC, La Sota NDV and PBS) control wells. Then 0.02 ml of the positive control serum was added to each haemagglutination positive well and the positive control wells to observe for the inhibition of the haemagglutination. HI positive samples transformed from a diffuse mat of red blood cells to a button of clumped cells at the bottom of such a well, while HI negative samples remained as diffuse mats of red blood cells [16]. This was compared with the positive and negative control wells.

RBC adsorption-de-adsorption concentration of NDV

Prior to the RNA extraction and RT-PCR, the NDV was concentrated by adsorption and de-adsorption of the viral particles to and from washed red blood cells respectively [8]. This was done by centrifuging 1 ml of oropharyngeal or cloacal swab HI positive samples in a micro-centrifuge tube for 10 minutes at 12,000 rpm twice. Next, 300 µl of supernatant was transferred into a new centrifuge tube and incubated with 50 µl of washed chicken RBC, then spun at 150 rpm for 30 minutes at room temperature to adsorb the NDV to the RBC (adsorption step). The mix was further spun at 2,110 rpm for 5 minutes at room temperature to concentrate the suspended RBC into a pellet at the base of the tube. The supernatant was decanted. The pellet of RBC with adsorbed NDV was re-suspended in 300 µl of PBS and then 50 µl of 5 mM EDTA and 50 µl of beta mercaptoethanol were added to the mix and incubated at 37 °C for 5 minutes (de-adsorption step). The tube was spun at 2,400 rpm for 5 minutes to separate the NDV from the RBC. Finally, 200 µl of the NDV-rich supernatant was pipetted for the RNA extraction.

RNA extraction

The concentrated 200 µl of the NDV-rich test samples were subjected to the RNA extraction using a BIONEER AccuPrep® Viral RNA extraction kit. To every 200 µl of NDV-rich test sample, 400 µl of a binding buffer (VB) were added, and then transferred into 1.5 ml micro-centrifuge tubes and vortexed for 5 seconds. The tubes were incubated for 10 minutes at room temperature. Then, 100 µl of isopropanol was added to the tube, lightly vortexed for 5 seconds, and then spun for 10 seconds. The binding column was fitted into the 2 ml collection tube. The liquid was transferred into the binding column. The lids were closed carefully and centrifuged for 1 minute at 8,000 rpm. Following the centrifugation, the binding column was transferred to another 2 ml collection tube. Then 500 µl of washing buffer 2 was added and centrifuged for 1 minute at 8,000 rpm. After centrifugation, the binding column was transferred to a 2 ml collection tube. Thereafter, 500 µl of washing buffer 2 was added and centrifuged for 1 minute at 8,000 rpm. The collection tube was spun down once more at 13,000 rpm for 1 minute to remove the ethanol completely. The binding column was transferred to a 1.5 ml collection tube, 50 µl of elution buffer was added, and allowed to stand for 1 minute to allow the buffer to permeate the column. The eluted RNA was retrieved by spinning down at 8,000 rpm for 1 minute and used directly for the RT-PCR.

Reverse transcription—polymerase chain reaction (RT-PCR) test

The RNA extracted from fifty-three of the HI positive samples were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) technique using the primer set NDV-F 4829 (5'-GGTGAGTCTATCCGGARGATACAAG-3') and NDV-R 5031 (5'-TCATTGGTTG-

CRGCAATGCTCT-3'). These primers amplified the Fusion-gene segments of the NDV antigen that coded for lentogenic, mesogenic or velogenic NDV.

The PCR reaction mix per extracted RNA sample contained 13 µl RNase-free water, 50 µl PCR Buffer 2 ($MgSO_4$ 2.4 mM; dNTPs 1.6 mM), 1 µl Primer NDV-F 4829, 1 µl Primer NDV-R 5031, 1 µl Enzyme mix (Taq polymerase enzyme) making a total reagent volume of 45 µl. The mixture was vortexed for a few seconds, then transferred into 0.2 ml PCR tubes. Next, 5 µl of extracted RNA was added making a final volume of 50 µl. The reverse transcription and amplification of cDNA was achieved following opti-

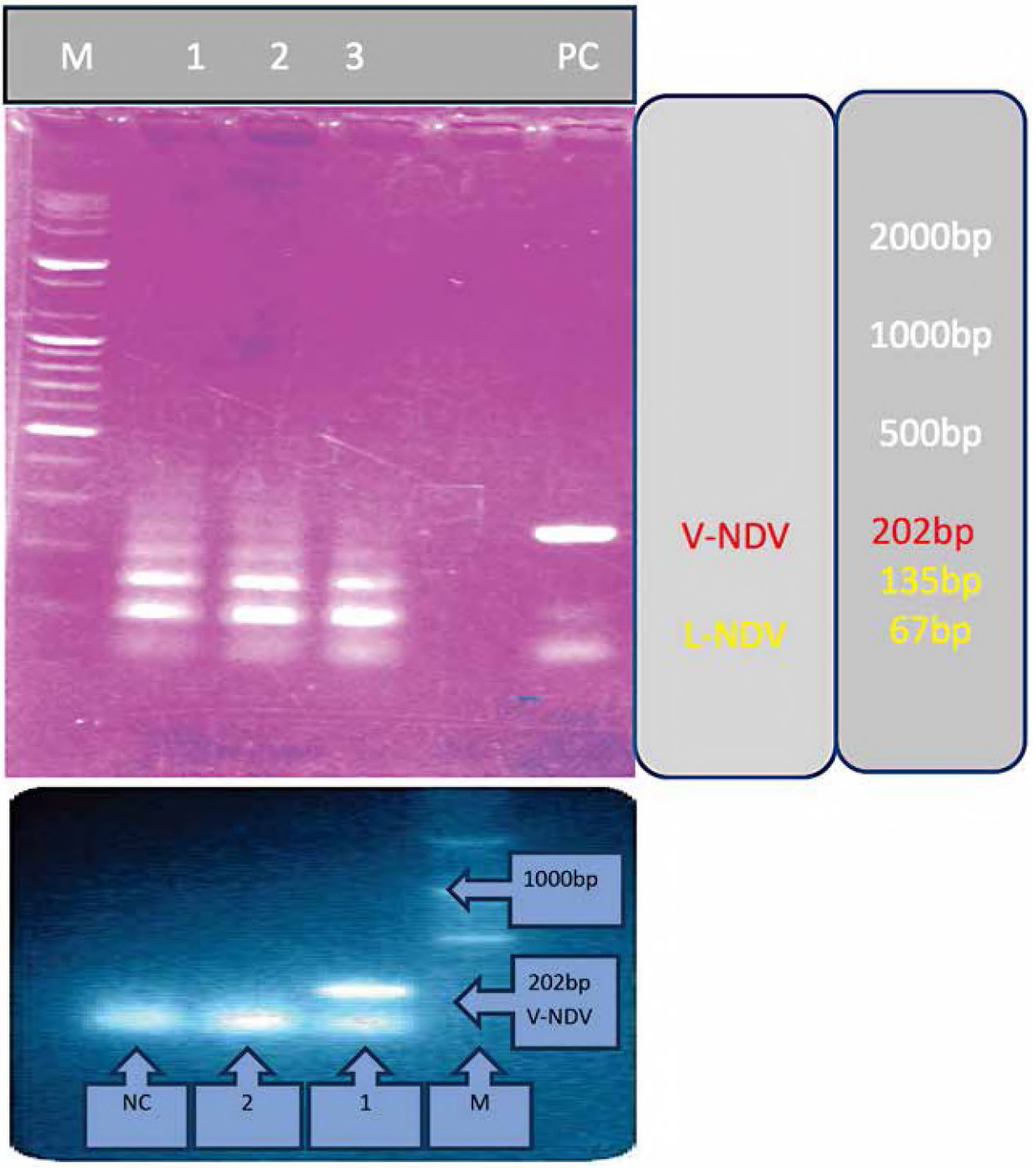

Fig. 3. Gel electrophoretogram of RT-PCR products from dove and chicken samples
Lane M—marker; Lanes 1, 2 and 3—NDV samples from doves in cluster 1, 2 and the Samaru live bird market respectively; PC—positive control from optimized velogenic NDV compared with lentogenic NDV from La Sota vaccine; NC—negative control; bp—base pair; V-NDV—velogenic Newcastle disease virus; L-NDV—lentogenic Newcastle disease virus. Swabs 1 and 3 were oropharyngeal swabs while swab 2 was a cloacal swab

mization of the cycling conditions using the NDV RNA extracted from the La Sota vaccine which also served as a positive control. Placing the reaction mix in the thermocycler, cDNA was produced by heating to 42 °C for 60 minutes, followed by raising the temperature to 94 °C for 5 minutes to inactivate the enzymes. cDNA was amplified in 40 cycles, consisting of 95 °C for 15 seconds, 49 °C for 30 seconds, and then 72 °C for 30 seconds. After that, the 40 cycles enzymes were inactivated by maintaining the temperature at 72 °C for 7 minutes. Amplicons were stored at 4 °C until use.

Agarose gel electrophoresis

Amplicons were transferred into the wells of a 2 % agarose gel for gel electrophoresis. The results were visualized after gel electrophoresis as follows: Lentogenic NDV appeared as 2 bands of approximately 135 bp and 67 bp, while mesogenic or velogenic NDV appeared as a single band of approximately 202 bp, respectively (Fig. 3).

Data analysis

The percent positives and frequency tables were calculated and drawn for the detection rates obtained by HA, HI and RT-PCR tests on oropharyngeal and cloacal swabs obtained from the doves and chickens from the field survey.

RESULTS

Detection of Newcastle disease virus

Out of 134 oropharyngeal and cloacal swab samples from the laughing doves examined, 88 (65.7 %) were HA positive. Of the 88 HA positives, 37 (42.1 %) were HI positive. On subjection of the 37 samples that were both HA and HI positive to conventional RT-PCR, 21 (56.8 %) of them were positive. The detection rate was higher in the cloacal (45.7 %) than the oropharyngeal (38.1 %) samples (Table 1).

Meanwhile, of the 50 samples from chickens subjected to HA test, 23 (46 %) showed haemagglutination and sixteen (69.6 %) of the 23 HA positives were HI positive. In order to determine the pathogenicity of the NDV, the sixteen samples that showed HA that were inhibited by the positive control hyper-immune serum were subjected to conventional RT-PCR. Only four (25 %) of such HA and HI positive samples were positive by RT-PCR. The detection rate was higher in the oropharyngeal (75.0 %) than the cloacal (63.6 %) samples (Table 2).

Cluster 1 (Areas BZ, C, E, and G) had 23 (82.1 %) of the 28 samples from the doves trapped close to backyard poultry houses, being HA positive. Nine (39.1 %) of the 23 HA positives were also HI positive. However, four (44.4 %) of the nine samples that were both HA and HI positive were RT-PCR positive. While cluster 2 (Areas H, Silver Jubilee and Palladan) from which 56 samples from doves were examined, 41 (73.2 %) were HA positive. Twelve (29.3 %) of the HA positives were HI positive. Four (33.3 %) of the twelve HA and HI positives were RT-PCR positive. Cluster 3 (Samaru live bird market) had 50 swabs from the doves and 24 (48 %) were HA positive. Of the 24 HA positives 16 (66.7 %) were HI positive also. Interestingly, 13 (81.3 %) of the 16 HA and HI positives were detectable by conventional RT-PCR (Table 3).

For comparison with the dove swabs, fifty swabs were obtained and examined from chickens from backyard poultry farms in clusters 1 and 2. Thirty swabs obtained from chickens in cluster 1 (Areas BZ, C, E and G) were subjected to HA tests and fifteen (50 %) were HA positive. Nine (60 %) of the fifteen HA positives were also HI positive. However, only three (33.3 %) of the HA and HI positives were detectable by the conventional RT-PCR. Of the twenty swabs from chickens in cluster 2 (Areas H, Silver Jubilee and Palladan), eight (40 %) were HA positive. Seven (87.5 %) of the HA positives were also HI positive. Upon subjection of these seven HA and HI positives to RT-PCR, only one (14.3 %) was positive (Table 4).

Table 5 summarizes the pathogenicity of the detectable NDV from HA and HI positive swabs by RT-PCR from doves and chickens from the three sampling clusters (R1, R2 and R3). Of the four swabs from fourteen doves trapped, sampled and examined from cluster 1 (Areas BZ, C, E and G) that were HA, HI and RT-PCR positive, three were velogenic while one was lentogenic NDV. Of the four swabs from twenty-eight doves from cluster 2 (Areas H, Silver Jubilee and Palladan) that were HA, HI and RT-PCR positive, two were velogenic, one was lentogenic and one was a mixture of both velogenic and lentogenic NDV. Following the trend, thirteen swabs from the twenty-five doves in cluster 3 (Samaru live bird market) were HA, HI and RT-PCR positive. Of these thirteen positives, seven were velogenic, while six were lentogenic NDV. In compar-

Table 1. Detection of Newcastle disease antigen in Laughing doves trapped around backyard poultry farms and the live bird market sampled in Samaru, Zaria, Nigeria

Subject	Swab type	No. tested	HA positive (%)	HI positive (%)	PCR positive (%)
Dove	Oropharyngeal	67	42 (62.7)	16 (38.1)	11 (68.8)
Dove	Cloacal	67	46 (68.7)	21 (45.7)	10 (47.6)
Total		**134**	**88 (65.7)**	**37 (42.1)**	**21 (56.8)**

Table 2. Detection of Newcastle disease antigen in chickens from backyard poultry farms sampled in Samaru, Zaria, Nigeria

Subject	Swab type	No. tested	HA positive (%)	HI positive (%)	RT-PCR positive (%)
Chicken	Oropharyngeal	25	12 (48.0)	9 (75.0)	2 (22.2)
Chicken	Cloacal	25	11 (44.0)	7 (63.6)	2 (28.6)
Total	**–**	**50**	**23 (46.0)**	**16 (69.6)**	**4 (25.0)**

Table 3. Detection of NDV in laughing doves using HA, HI and RT-PCR tests according to sample area sub-units, in Samaru, Zaria, Nigeria

Subject	Area	Sample type	No. tested	HA positive (%)	HI positive (%)	PCR positive (%)
Dove	Cluster 1	OPS/CS	28	23 (82.1)	9 (39.1)	4 (44.4)
Dove	Cluster 2	OPS/CS	56	41 (73.2)	12 (29.3)	4 (33.3)
Dove	Cluster 3	OPS/CS	50	24 (48.0)	16 (66.7)	13 (81.3)
Total	**–**	**–**	**134**	**88 (65.7)**	**37 (42.0)**	**21 (56.7)**

OPS/CS—Oropharyngeal swab and cloacal swab; Cluster 1—Areas BZ, C, E and G
Cluster 2—Areas H, Silver Jubilee and Palladan; Cluster 3—Samaru live bird market

Table 4. Detection of NDV in chickens from backyard poultry farms using HA, HI and RT-PCR tests according to sample area sub-units, in Samaru, Zaria, Nigeria

Subject	Area	Sample type	Number tested	HA positive (%)	HI positive (%)	PCR positive (%)
Chicken	Cluster 1	OPS/CS	30	15 (50.0)	9 (60.0)	3 (33.3)
Chicken	Cluster 2	OPS/CS	20	8 (40.0)	7 (87.5)	1 (14.3)
Total			**50**	**23 (46.0)**	**16 (69.6)**	**4 (25.0)**

OPS/CS—Oropharyngeal swab and cloacal swab; Cluster 1—Areas BZ, C, E and G; Cluster 2—Areas H, Silver Jubilee and Palladan

Table 5. Pathotype of NDV detected in subjects based on RT-PCR and gel electrophoretyping in Samaru, Zaria, Nigeria

Subject	Area	Sample	PCR positive (%)	Lentogenic (%)	Velogenic (%)	Mixed Pathotype (%)
Dove	Cluster 1	OPS/CS	4 (44.4)	1 (25.0)	3 (75.0)	0
Dove	Cluster 2	OPS/CS	4 (33.3)	1 (25.0)	2 (50.0)	1 (25.0)
Dove	Cluster 3	OPS/CS	13 (81.3)	6 (46.2)	7 (53.8)	0
Chicken	Cluster 1	OPS/CS	3 (33.3)	2 (66.7)	1 (33.3)	0
Chicken	Cluster 2	OPS/CS	1 (14.3)	1 (100)	0	0
Total			**25 (47.2)**	**11 (44)**	**13 (52)**	**1 (4)**

OPS/CS—Oropharyngeal swab and cloacal swab; Cluster 1—Areas BZ, C, E and G
Cluster 2—Areas H, Silver Jubilee and Palladan; Cluster 3—Samaru live bird market

ison of the three swabs from chickens in cluster 1 that were HA, HI and RT-PCR positive, two were lentogenic while one was velogenic NDV. The single HA, HI and RT-PCR positive swab from chickens in cluster 2 was lentogenic NDV (Table 5).

DISCUSSION

NDV detection and characterization

This study was able to demonstrate that there were high levels of the NDV circulating in wild laughing doves and backyard chickens. Chantal et al. [2] had reported that chickens were more often infected by the NDV than other birds. They also reported that chickens from live bird markets were significantly more often positive for the NDV than birds at commercial or backyard farms which contributed to the enzootic circulation of the NDV. This is in agreement with the findings from this study in which the detection of the NDV in doves was significantly higher in birds from the Samaru live-bird market than trapped wild doves from the residential areas in clusters 1 and 2. This could be attributed to the poor biosecurity measures at the live-bird market. Doves trapped from within and outside Zaria were kept with chickens and other birds in small cages at the live bird market. This increased the possibility of infection between susceptible and infected birds shedding the virus due to the close proximity of birds in such small cages, poor hygiene, poor nutrition, transportation stress and overstocking. Residents from the two clusters 1 and 2 who buy live doves or chickens from the live-bird market and return home with them may help in the enzootic circulation of NDV in Samaru-Zaria. Chantal et al. [2] explained that chickens in live-bird markets in Nigeria were sourced from both vaccinated (commercial or backyard poultry) and unvaccinated (free-range local chickens) flocks. Vaccination likely reduced the expression of clinical signs of Newcastle disease. This did not necessarily suppress viral shedding from asymptomatic chickens. The relatively high detection of the NDV from apparently healthy backyard chickens from the two clusters 1 and 2 in our study was not unexpected as farmers vaccinated their chickens as often as once every month. Chantal et al. [2] had also reported that outbreaks of Newcastle disease in vaccinated flocks have been increasingly reported from Nigeria suggesting a suboptimal protection by vaccination.

In this study, our detection of NDV from oropharyngeal swabs was higher than from cloacal swabs of chickens. This agreed with Haque et al. [7] who reported that the viral isolation rate from clinical samples was found highest in the tracheal swabs (90 %) compared to cloacal swabs (85 %) and serum (65 %). However, in our own study, the reverse was the case for samples from doves with detection higher in cloacal swabs than oropharyngeal swabs.

Also, it was discovered that the NDV from doves were slightly more of the mesogenic or velogenic [3] than the lentogenic pathotypes. This agrees with the findings from Kerri et al. [9] who reported that Pigeon Paramyxovirus-1 (PPMV-1) from doves were typically mesogenic by intra-cerebral pathogenicity index (ICPI) in chickens. It has also been reported that PPMV-1 isolates from doves increased their virulence in chickens after passage and therefore represented a genuine threat to poultry production [12]. The isolation of a virulent strain of the NDV from field samples required reporting to the Office International des Epizooties (OIE) [13]. With the detection of virulent or mesogenic NDV from laughing doves in Zaria in this study there is a need for reporting to the OIE by the Veterinary Agencies of the Nigerian Government following standard reporting protocol.

Newcastle disease virus could be detected by subjecting oropharyngeal swabs (OPS) and or cloacal swabs (CS) from laughing doves and backyard poultry to haemagglutination assay as a screening test since the NDV has haemagglutinating properties [14]. Subjecting positive haemagglutinating samples to haemagglutination inhibition assay using hyper-immune serum allowed us to be able to narrow down the confirmatory detection of NDV cheaply. Running the reverse-transcription polymerase chain reaction on such HI positives we were able to confirm the presence of the Newcastle disease virus RNA, classify the NDV into lentogenic or mesogenic/velogenic pathotypes and reduce the number of samples to be subject to RT-PCR which is quite expensive in developing economies. The sensitivity of RT-PCR could be increased by concentrating the virus in swabs by a red blood cell adsorption-de-adsorption technique [8]. The higher detection of the ND antigen from cloacal swabs than oropharyngeal swabs of laughing doves may be attributed to the fact that birds were primarily exposed to the NDV by the oro-nasal routes. The localization of the virus in the gastrointestinal tract is common in the lentogenic Newcastle disease as the lentogenic

NDV has a monobasic amino acid motif at the F-cleavage site 112 G-R/K-Q-G-R*L 117 which is cleaved extracellularly by trypsin-like proteases found in the respiratory and more so in the intestinal tract [9]. It has been reported that the Newcastle disease virus that was detected in pigeons was mostly the lentogenic strains which could mutate to the mesogenic/velogenic strains after passaging in chickens [12]. The vaccination of chickens with La Sota vaccine produces a mild respiratory form of Newcastle disease [16] and may have influenced the detection pattern, with higher detection rates from the oropharyngeal swabs than from the cloacal swabs of chickens. This study reaffirmed that HA and HI tests can be used to detect NDV from field samples. Also, other haemagglutinating avian pathogens not limited to Avian Influenza (H5N1) virus, infectious bronchitis virus [1] and Egg-drop syndrome ad enovirus, and unlikely agents like *Mycoplasma gallisepticum*, *M. septicum* and Avian Pathogenic *Escherichia coli* with haemagglutinating properties could be diagnosed similarly. In our study, the HA positives that were not HI positive could be due to the avian influenza virus or any of the haemagglutinating pathogens mentioned previously.

Wambura [18] had reported that the NDV had only been demonstrated in Nigerian pigeons by serological evidence and this posed a risk to village chickens [15]. Our study revealed that the NDV could be demonstrated directly from oropharyngeal and cloacal swabs from doves and chickens. The subjection of such swabs to HA tests followed by HI tests demonstrated the presence of the NDV. The conventional RT-PCR using the primer set NDV-F 4829 and NDV-R 5031 was able to pathotype the NDV demonstrated from swabs that were both HA and HI positive, as either lentogenic and or velogenic [19]. The "gold standard" for the diagnosis of the NDV involves isolation and cultivation in embryonated chicken eggs, followed by HA, HI and ICPI [13] which are labour intensive and time consuming. The methods employed in our study used HA to screen field swab samples rapidly. HA positives were then subjected to HI test which was also easily done. And then samples that were both HA and HI positive were subjected to RT-PCR for pathotyping. In order to improve the sensitivity of the RT-PCR, the HA and HI positives were subjected to a red blood cell adsorption-de-adsorption concentration prior to the RT-PCR which mimicked the results reported by Jianzhong and Chengqian [8]. The use of HA, HI and RT-PCR in our study seemed cheaper, faster, easier, convenient and more feasible for backyard poultry outbreak investigations than the gold standard for the NDV detection. There was also no need to sample the birds twice as we would have done if we depended on serology to differentiate between the active infection and previous exposures.

CONCLUSIONS

From our study the following conclusions were made:

About 65.7 % of the oropharyngeal and cloacal swabs from the trapped laughing doves sampled in Samaru-Zaria exhibited haemagglutination, 42.1 % of which was due to Newcastle disease virus. Further, studies should be geared towards screening laughing doves for haemagglutinating avian pathogens particularly: Avian Influenza virus, Egg-drop syndrome adenovirus, and *Mycoplasma gallisepticum*; using HA, HI, and RT-PCR for monitoring and surveillance purposes.

About 38.1 % of the NDV detected in the trapped laughing doves sampled in Samaru-Zaria were demonstrated to be lentogenic, 57.1 % were mesogenic/velogenic while 4.8 % were a mixture of both lentogenic and velogenic NDV. The sequencing and phylogenetic blasts of the isolated samples from doves and chickens would be able to confirm whether the NDV in the doves and chickens were of the same origin.

There may be enzootic circulation of the NDV between chickens and laughing doves. There may be a need to consider the vaccination of laughing doves with thermostable I-2 Newcastle disease vaccine so that velogenic NDV in doves does not wipe off a large number of laughing doves or result in an epizootic of Newcastle disease in wild birds.

It is likely that frequent passaging of lentogenic NDV from doves in chickens resulted in mesogenic or virulent NDV being shed to doves again; hence the detection pattern observed in this study. Further studies should be done on the tagged doves to evaluate the migratory patterns of laughing doves amongst the different residential areas. The veterinary agencies of governments should also encourage more research on the role of laughing doves in the spread of Newcastle disease to poultry.

Running HA-HI on field samples from doves and chickens as a screening test before subjecting positive HA-HI samples to RT-PCR cut down costs that would have

been incurred if all field samples were subject to RT-PCR directly. This modified HA-HI screening method for field samples from wild and domestic birds should be made into a commercial kit that could easily be used by backyard poultry farmers to screen for the NDV in their flocks so as to know when to give booster La Sota NDV vaccines.

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