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Molecular screening and isolation of Newcastle disease virus from live poultry markets and chickens from commercial poultry farms in Zaria, Kaduna state, Nigeria

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Abstract

Newcastle disease is one of the major economic threats to poultry population because of its high morbidity and mortality varying from 90-100%. It is caused by Avian Paramyxovirus-1 (APMV-1). This research work was carried out to identify Newcastle disease virus (NDV) by using reverse transcription-polymerase chain reaction (RT-PCR) assay and further isolate the virus in embryonated chicken eggs. A total of 127 cloacal swabs were collected from local chickens in live bird market and exotic chickens in commercial poultry farms in Zaria and environs, Nigeria between November, 2014 and January, 2015. Five commercial poultry farms and four live bird markets were purposively sampled. Molecular screening of NDV Matrix-gene (M-gene) was performed on all the samples using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Newcastle disease positive samples were further inoculated into embryonated chicken eggs for isolation of Newcastle disease virus. Isolates were confirmed as Newcastle disease virus by haemagglutination inhibition (HI) test. Newcastle disease virus Matrix-gene was detected in 16 (12.5%) out of 127 cloacal swabs; 13 (10.2%) from live bird markets and 3 (2.3%) from commercial poultry farms. However, only 10 Newcastle disease viruses were isolated in embryonated chicken eggs as confirmed by Haemagglutination inhibition (HI) test. Due to the higher detection rate recorded by reverse transcriptase-polymerase chain reaction (RT-PCR), it is therefore important that molecular technique be made easily accessible so that samples from each suspected outbreaks of NDV be screened so that rapid and confirmatory diagnosis can be achieved.

Keywords: Embryonated chicken eggs, Haemagglutination inhibition test, Newcastle disease virus, RT-PCR

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Introduction

Newcastle Disease Virus (NDV), or avian paramyxovirus type 1 (APMV-1), is an enveloped,

non-segmented, negative-sense, single-stranded RNA virus belonging to the Genus *Avulavirus* of the

Family *Paramyxoviridae* (Mayor, 2002; Alexander and Senne, 2008). The NDV genome is composed of six genes and encodes their corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), RNA polymerase (L) (Chambers *et al.*, 1986). Based on the manifestations of clinical signs in chickens, three forms of the disease were generally classified; viscerotropic velogenic (highly virulent), neurotropic velogenic (highly virulent), and mesogenic (moderately virulent). These pathotypes cause variable respiratory and neurologic signs along with high morbidity (Miller *et al.*, 2010).

Newcastle disease was first reported in Nigeria between December, 1952 and February, 1953 in and around Ibadan (Hill *et al.*, 1953). Since then, the disease has been reported from all parts of the country (Hill *et al.*, 1953; Fatumbi & Adene, 1979; Ezeokoli *et al.*, 1984; Gomwalk *et al.*, 1985; Baba *et al.*, 1995). The disease was also reported to be more common during the dry harmattan season (November-March) (Sa'idu *et al.*, 1994; Halle *et al.*, 1999; Abdu *et al.*, 2005). In Nigeria, the disease has been reported to be endemic in both local and commercial poultry with annual epidemics being recorded in highly susceptible poultry flocks (Adu *et al.*, 1986; Sa'idu *et al.*, 1994; Halle *et al.*, 1999; Orajaka *et al.*, 1999).

Outbreaks of between 200 and 250 of Newcastle disease were reported annually in Nigeria (Okeke and Lamorde, 1988). In Zaria, Newcastle disease was reported to be the most prevalent disease of local and exotic birds (Abdu *et al.*, 1985; Sa'idu *et al.*, 1994; Halle *et al.*, 1999). The disease is reported to be the most important cause of death in chickens in Zaria (Abdu *et al.*, 1992).

Despite the economic threat posed by ND in Nigeria and well known importance of rapid and effective diagnosis of the disease, traditional methods of diagnosis based on clinical signs and post-mortem findings couple with some serology-based diagnostic methods like haemagglutination inhibition (HI), virus neutralization (VN) and agar gel immunodiffusion (AGID) tests are often used in veterinary hospitals and some veterinary institutions. But these are generally time consuming when compared with single method of genome detection of the virus. The reverse transcription-polymerase chain reaction (RT-PCR) has now being considered best method of diagnosis over other confirmatory test methods in the field of diagnosis of ND (Singh *et al.*, 2005; Krzysztof *et al.*, 2006). Therefore, the aim of this work is to use RT-PCR technic to screen for Newcastle disease viruses and further isolate them in embryonated chickens eggs; to see whether all

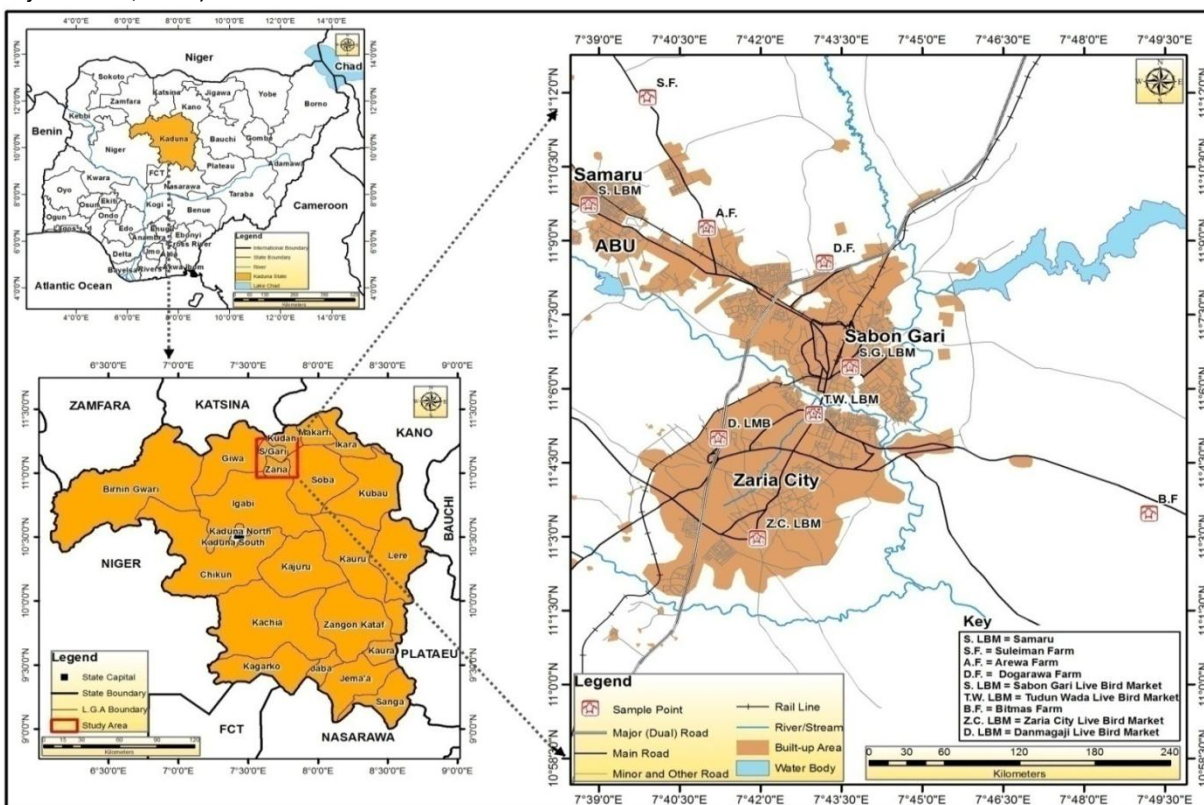


Figure 1: Map of Zaria and environs showing sampled locations

Newcastle disease viruses detected by RT-PCR can be recovered by isolation.

Materials and Methods

Study area

Zaria is located between 7° 44' E and latitude 11 o 07 11° 7' N within the Sudan savannah zone (www.distancesto.com). Zaria is found on the high plains of northern Nigeria, in sub-Saharan Africa (Ubogu *et al.*, 2011). The sampled locations for this study were distributed within Zaria and environs (Figure 1).

Inclusion criteria

Commercial poultry farms that reported a suspected case of Newcastle disease to Ahmadu Bello University Veterinary Teaching Hospital, Zaria, were selected for this study. In addition, live bird markets with chickens showing obvious clinical signs of Newcastle disease were also considered for this study.

Category of birds sampled

Local chickens were sampled from live bird markets and only exotic chickens were sampled from commercial poultry farms.

Sampling of birds

A total of 127 birds were purposively sampled in both commercial poultry farms and live bird markets in Zaria and environs. Samples were collected in four commercial poultry farms and five live bird markets; 69 samples from commercial farms and 58 samples from live bird markets (Table 1 and 2).

Sample collection

A cloacal swab was collected from each bird between November, 2014 and January 2015 and

transported in virus transport media (Hanks' transport medium) containing 2000 U/ml of penicillin, 2 mg/ml of streptomycin, 0.05 mg/ml of gentamycin and 100 U/ml of mycostatin in cold flask. The samples were stored in a refrigerator at -20 °C in the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, prior to transporting them in ice at 4 °C to Avian Influenza Unit of Virology Laboratory, NVRI Vom, Plateau state, where they were stored at -20 °C until Newcastle disease virus detection.

Molecular detection

Viral RNA was extracted directly from the swab samples. Viral extraction was performed in the Avian Influenza Unit of Virology laboratory, National Veterinary Research Institute Vom, Plateau state Nigeria, using QIAgen RNeasy Mini Kit (QIAgen, Hilden, Germany) according to the manufactures protocol.

One-step RT-PCR, using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) was performed for the amplification of 280 base pair fragment for the Matrix gene using the following primers;

APMV-1 M4079F 5'-

AAGGAGCCTTGATCTATCTGTCGG-3' Forward

APMV-1 M4337R 5'-TGTGCCCTTCTCCAGCTTAGTA-3' Reverse.

Using 25 µL reaction volume, reaction mixtures containing 11.5 µL Nuclease free water, 5.0 µL 5x PCR buffer, 0.5 µL dNTPs, 0.5 µL Avian Myeloblastosis Reverse transcriptase enzyme, 0.5 µL Rnase Inhibitor, 5.0 µL of RNA template and 1.0 µL,

Table 1: Distribution of Newcastle disease virus in chickens amongst farms/markets sampled in Zaria and environs, Nigeria

S/no	Name of poultry farms/markets	No. of chickens sampled	Number of positive samples from each farm (Based on M-gene screening test)
1	Dogarawa farm	17	0
2	Suleiman farm	22	2
3	Arewa farm	20	1
4	Bitmas farm	10	0
5	Danmagaji market	12	5
6	Zaria city market	11	0
7	Sabon Gari market	10	4
8	Tudun Wada market	13	4
9	Samaru market	12	0
Total		127	16

Table 2: Results of inoculating Newcastle disease positive samples in to embryonated chicken eggs

Samples I.D	Embryonic mortality	H.I result	Comment
01NGZCK015	+	+	NDV isolated
02NGZCK015	+	+	NDV isolated
03NGZCK015	+	+	NDV isolated
04NGZCK015	+	+	NDV isolated
05NGZCK015	+	+	NDV isolated
06NGZCK015	+	+	NDV isolated
07NGZCK015	+	+	NDV isolated
08NGZCK015	+	+	NDV isolated
09NGZCK015	+	+	NDV isolated
10NGZCK015	+	+	NDV isolated
11NGZCK015	+	-	NDV not isolated
12NGZCK015	+	-	NDV not isolated
13NGZCK015	+	-	NDV not isolated
14NGZCK015	+	-	NDV not isolated
15NGZCK015	+	-	NDV not isolated
16NGZCK015	+	-	NDV not isolated

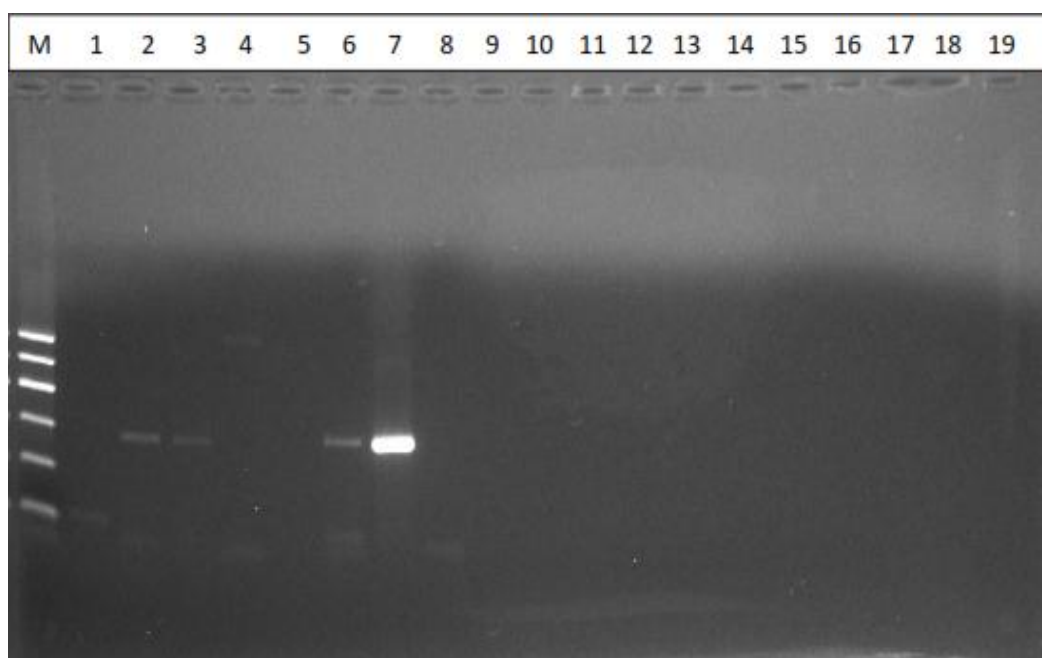


Figure 2: RT-PCR product bands (lanes 2, 3 and 6) of partially amplified Newcastle disease virus M-gene from cloacal swabs of chickens in commercial poultry farms. The expected amplicon size is 280 bp. Lane 7 and 8 are positive and negative controls, respectively. M represents 100 bp ladder

each of the primers were first cycled in a 96-well thermo cycler (Applied Biosystems) at 50 °C for 30 minutes for reverse transcription. This was followed by initial denaturation at a temperature of 94 °C for 2 minutes. A 35 cycles of denaturation, annealing and elongation of the templates were achieved at the cycling conditions of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute respectively. Final extension of the templates was achieved at 72°C for 7 minutes.

The products were electrophoresed at 120V for 30 minutes in 2% agarose gel stained with ethidium bromide and bands were visualized using gel electrophoresis.

Virus isolation

Newcastle disease virus positive samples (by RT-PCR) contained in Hanks viral transport media were vortexed to ensure thorough mixing. 0.2 ml cloacal

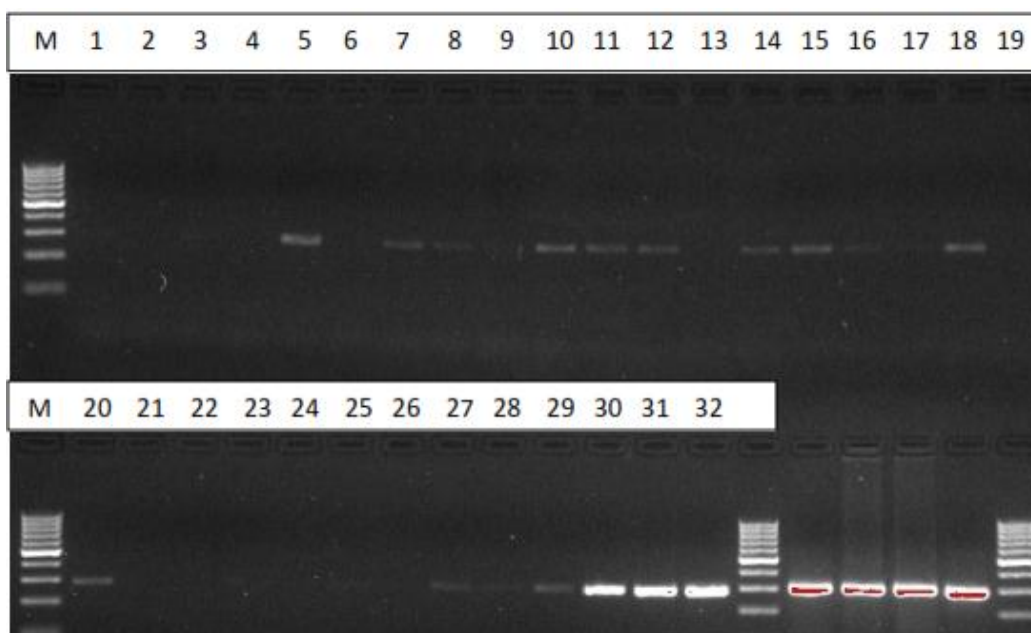


Figure 3: RT-PCR product bands (lanes 5, 7, 8, 10, 11, 12, 14, 15, 16, 18, 20, 27, and 29) of partially amplified Newcastle disease virus M-gene from Cloacal swabs of chickens in live bird markets. The expected amplicon size is 280 bp. Lane 30, 31 and 32 are positive controls. M represents 100 bp Plus ladder

samples was inoculated using 1 ml syringe into each of three 9-day-old embryonated chicken eggs obtained from Poultry farm of National Veterinary Research Institute Vom, Plateau state, Nigeria, via the allantoic route. The eggs were incubated at 37 °C and candled with a 25 voltage bulb twice daily for 4 days. Eggs showing embryonic death at 24 hours and up to day 4 were chilled at 4 °C. Eggs that were viable after 4 days of incubation were also chilled. Allantoic fluid from each of the eggs was harvested and tested for haemagglutination (HA) using 1% chicken red blood cells. The HA-negative samples were passaged once more in to each of two embryonated chicken eggs. The isolates were confirmed using HI and kept at -20 °C.

Results

The M-gene RT-PCR test was carried out on each of the 127 cloacal swab samples, targeting 280 base pair DNA fragment. However, only M-gene positive samples were inoculated in to embryonated eggs for virus isolation. The RT-PCR protocol detected the presence of APMV-1 RNA in 16 out of 127 clinical samples included in this research; three from commercial poultry farms and thirteen from live bird markets (Figure 2 and 3). Ten of the samples positive by RT-PCR were also positive by virus isolation. Six samples; 3 from commercial farms and 3 from live bird markets were positive by RT-PCR but gave a negative result by virus isolation.

The result of isolation shows that 10 samples were positive for Newcastle disease virus (Table 2). In all the isolates, embryos died within 48 to 72 hours post inoculation. Mortality of the embryo was observed with some isolates within 24-hour period. This, according to OIE, (2012) should be subjected to further passage (s).

Consequently, the four HA-negative samples from first inoculation were subjected to first passage and the result showed that all the four samples were positive for Newcastle disease virus as confirmed by HI. These samples were previously confirmed by RT-PCR as Newcastle disease virus positives.

Discussion

As shown in table 2, despite relatively higher number of chickens sampled in commercial poultry farms when compared to chickens sampled in live bird markets in this study, only 3 chickens were positive for Newcastle disease virus in the former as against 13 in the later. This may be attributed to moderate biosecurity measures in all commercial poultry farms sampled. In addition, all the farms kept only exotic birds, and as reported by Abdu *et al.* (2005) outbreaks of Newcastle disease were reported to be more likely in farms that kept exotic birds together with local chickens and other poultry. This finding is contrary to the findings of Ezeokoli *et al.* (1984) who reported that local chickens are less likely to develop clinical Newcastle disease than exotic breed. Halle *et al.* (1999) also reported that

exotic breed of chickens are about twice more likely to be infected with Newcastle disease virus than the local chickens. However, Sa'idu *et al.* (2005) reported that both local and exotic chickens are equally susceptible to Newcastle disease virus.

Among commercial poultry farms visited, there are no much differences in biosecurity measures and source of supply for their day old chicks.

In the live bird markets, sanitation ranges from minimal in Sabon Gari, Tudun Wada and Danmagaji to moderate in Samaru and Zaria city. This may partly explained the distribution of Newcastle disease virus among live bird markets in Zaria (Table 2). Furthermore, birds were over crowded in Sabon Gari, Tudun Wada and Danmagaji live bird markets when compared with chickens in Samaru and Zaria city live bird markets during this study.

The low detection rate (Figures 2 and 3), may be due to the presence of hemagglutinating viruses/ other avian paramyxovirus serotypes (e.g serotype 2, 3, 6, 7) that can cause infection in chickens other than NDV in the samples. However, this finding disagreed with the finding of Hassan *et al.* (2010) that identified 20 samples (58.8%) using RT-PCR out of 34 clinically diagnosed samples from layers and broilers in Sudan. Variation between these results probably occurred because Hassan *et al.* (2010) collected his samples from chickens clinically diagnosed with Newcastle disease on the basis of postmortem lesions, while samples from this study were collected on the basis of apparent clinical signs of the disease and history. The faint bands seen may be due to low template concentration in the samples. Though Waner *et al.* (2007) reported that Brain heart infusion media was superior in maintaining RNA/virus quality than Hanks media which we used in this study, but no reason was given for this difference. Haque *et al.* (2010) also reported that NDV concentration is higher in trachea than cloacal site and samples from this study were collected from cloacal site. They attributed the higher concentration to be due to major rout of the spread of NDV, which is aerosol, and therefore there is high probable chance of the virus concentration in the trachea.

In addition, Waner *et al.* (2007) also reported that when there is low Newcastle disease virus

concentration in samples, the Brain heart infusion and glycerol virus transport media were superior to the Hanks media for the recovery of live Newcastle disease virus during isolation, although no reason was given. This may explained the reason of low number of isolates recovered from this study. Haque *et al.* (2010) reported that virus concentration is low in cloacal swabs when compared with other clinical samples, consequently, isolation rate was usually low in cloacal swabs. Okwor and Eze, (2011) found 58 isolates (3.2%) from a total of 1800 samples. However, unlike samples collected from clinically suspected chickens of this study, they sampled from clinically healthy commercial chickens in Nsukka and its environs, Nigeria.

In conclusion, lack of availability coupled with difficulty in accessing the few rapid and confirmatory diagnostic methods resulted in not only unavoidable confusion between Newcastle disease and other infectious diseases of poultry, but also false negative result due to nonviable viral particle isolated using commonly used embryonated chicken eggs. Consequently, persistent endemicity of ND is not surprising. An important tool in the face of outbreak of ND is the rapid and confirmatory detection of the virus which is a prerequisite for the accurate and effective adaptation of ND control. Although virus isolation followed by identification using most of the serological tests are considered as the standard methods for the confirmation of the disease (OIE, 2012), but these are always considered time consuming and expensive compared to that of genome detection of the virus as a single method. Therefore, genome detection of NDV both from the clinically sick and dead birds is very important for early, reliable and rapid confirmatory diagnosis of ND in poultry during outbreak of the disease compared to any other single or combined conventional methods of diagnosis (Jestin & Jestin, 1991; Pedersen *et al.*, 2000).

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