https://dx.doi.org/10.4314/dujopas.v11i1c.19
ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 11 No. 1c March 2025*

Isolation and Molecular Detection of Newcastle Virus in a Private Poultry Farm, Rano Local Government Area, Kano state, Nigeria

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Abstract
Newcastle disease is a highly contagious disease among poultry birds and a major constraint in poultry production. Despite frequent vaccination, the virus keeps circulating among vaccinated and unvaccinated birds. This study determined the prevalence of Newcastle Disease Virus in poultry chicken and carried out molecular confirmation of the isolates obtained from poultry farms in Rano, Rano Local Government Area, Kano state. In this study, Cloacal samples were collected from 120 diseased poultry chickens for isolation of NDV in 9-13-day old specific pathogen free fertile hens' eggs, and 83 samples were positive for the virus. NDV was further detected in positive samples with molecular techniques using F-gene. The virus was isolated from some samples with the prevalence rate of 73.33%. This study revealed circulation of NDV in poultry birds in Kano. This highlights the need for stringent NDV control programs to reduce or eliminate the circulation of the virus in this province.

Keywords: F gene, Kano state, Newcastle disease Virus, Prevalence, Poultry chicken

INTRODUCTION
NDV belongs to the family Paramyxoviridae, genus Avulavirus formerly known as avian paramyxovirus1 (APMV-1), (ICTV, 2020). It infects the respiratory, gastrointestinal, and nervous systems of birds, leading to symptoms ranging from mild respiratory distress to severe neurological disorders, with mortality rates reaching up to 100% in susceptible poultry populations (Hu *et al.,* 2022). NDV possesses an enveloped negative sense single stranded

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RNA genome. The envelope of the virus is made up of 2 glycoproteins, - the haemagglutinin-neuraminidase (HN) and the "F" protein known as the fusion protein (Acheson, 2011).

The genome of NDV is categorized into six structural proteins which are NP, P, M, F, HN, L. and two nonstructural protein V and W. NDV isolates are genetically grouped into 2 classes: class I and class II. The class I isolate has only one genotype whereas the class II is further classified into genotypes I-XVIII which are all virulent in chicken with exception of genotype I, II, and X (Dimtrov *et al.,* 2016).

Newcastle disease virus is a highly contagious pathogen that affects birds especially poultry birds such as chicken, turkey, Quail and wild birds (Kuhn *et al.,* 2019; Abah *et al.,* 2020). NDV was first reported in Nigeria in 1952, after that, different cases have been reported in all agrogeological areas in the commercial, rural, free living and wild range birds making it enzootic across the whole country (Okwor and Eze, 2010; Shittu *et al.,* 2016). In 2008, about 78,526 outbreaks of the disease were reported across Nigeria resulting into about 8.9-billion-naira financial losses (Akambi *et al*., 2020). Newcastle disease virus is commonly cultivated and amplified in embryonated chicken eggs where it replicates in the allantoic fluid, and can also be propagated in Cell lines such as Vero (African Green Monkey cells), CEF (Chicken embryonic fibroblasts), DF-1 (A continuous cell line derived from CEF) and a few others (Santry *et al*., 2018).

Poultry production in Nigeria is the most lucrative among all agricultural businesses contributing to the main source of income in Nigeria. This as well acts as a way of generating food in the country and also creating employment for aspiring farmers. Unfortunately, endemic avian diseases such as Newcastle disease has crippled the economic status in the poultry industry resulting in loss of poultry flocks which jettisons the financial buoyancy of farmers and poor rural occupant who depend on poultry farming as a source of livelihood (Amoia *et al*., 2021; Ekiri *et al*., 2021).

Despite the frequent vaccination practiced by farmers, NDV continues to circulate among vaccinated and unvaccinated birds suggesting continuous mutation of the virus which might possibly result in new genetic variant (Garner *et al.,* 2014). The high rate of NDV infection is not only associated with inadequate immunization, immmunosuppression, it may possibly be due to viral mutation that might disrupt the genomic sequence and biological characterization (Chinyere *et al.,* 2023).

Despite the frequent vaccination of poultry yearly, outbreak of NDV is still reported on some farms in Nigeria. Poultry Chicken (broilers) on a private farm in Rano Local government were observed with symptoms such as nasal discharge, diarrhoea, reduced feed intake, weight loss, twisting of the neck (torticollis), and death which are typical signs of NDV Infection. Therefore, there is need to detect, isolate and confirm NDV among those birds. This research therefore studied the presence of NDV infection as well as the isolation and confirmation of the virus by molecular techniques.

MATERIALS AND METHODS

Study Area

The farm is located in Rano Local Government, Kano State, Nigeria. The state is situated in NorthWestern Nigeria on Latitude 12° 0' 0.0000'' N and longitude 8° 31' 0.0012'' E with an altitude of 484m above sea level. The state has a population of totaling 9,383,628 (NPC, 2006).

The total population of Rano L.G.A. as at 2022 was 243,900 while Kano state had 15,462,200. The inhabitants are majorly Hausa and Fulani, with few settlers from other parts of the country who are there for business activities. The state shares border with other states such as Katsina state, Jigawa state (Dawaki *et al.*, 2017). The major occupation of the people is farming and other agricultural practices such as poultry farming. It experiences average precipitation of about 690mm per year, between the period of June to September. The state is known for its extreme hotness throughout the year, sometimes cold during mid-November to February. The annual temperature range is between 20 - 42^{o} C, but the mean monthly values ranges between 21^{O}C and 23^{O}C in the coolest months. Kano State is a major producer of hides and skins, and also the major producer of sesame, soybean, cotton, garlic, gum Arabic and chili pepper (Sajo *et al.*, 2023).

Ethical consideration

This research was carried out in accordance to Helsinki declaration of the use of animals for research and experiment.

Sample collection

Cloacal samples were collected from 120 poultry birds after proper restraint of birds, with the help of a Veterinary Doctor. A sterile cotton swabs stick was gently inserted into the cloaca of the birds and swirled 5 times and placed in 1ml of viral transport medium containing penicillin (10,000 units/ml), streptomycin (10,000 mg/ml), gentamicin (5000 mg/ml), and Amphotericin B (50 mg/ml). The samples were immediately placed on an ice pack and transported to the laboratory and stored at -80^{o}C until processed.

Preparation of eggs for isolation of NDV

Eggs were candled (9-13 days old specific pathogen free embryonated hens' eggs) and disinfected and arranged in a roll of four eggs per inoculum. Fertile eggs containing active embryos were prepared for inoculation by marking the air sac and labeling them accordingly. Cloacal samples were eluted in a PSGA solution, pooled into groups of four samples per group and labelled by sample codes.

The allantoic cavity was marked and punched for inoculation. About 0.1-0.2mls of the pooled samples was inoculated and sealed with glue. The eggs were incubated at 37^{o}C under humid condition and observed daily to check for viability or mortality for five days, and results recorded. Dead eggs were chilled at +4^{o}C for 1 hour and the allantoic fluid was harvested. It was tested with 10% chicken red blood cells on a tile to observe for haemagglutination. Sterility check using blood agar was carried out on samples that agglutinated. Negative samples at first blind passage were harvested and passed into another set of eggs for a second blind passage. The clean positive samples were placed in aliquots and nucleic acid extracted for Polymerase chain reaction.

The harvested allantoic fluid was tested with 10% SPF chicken red blood cells on tile and rocked for agglutination NDV RNAs from allantoic fluids were extracted using QIAmp RNA mini kit (Qiagen, Germany) for purification of viral RNA according to the manufacturers protocol. The fusion gene was amplified using One Step RT-PCR Kit (Qiagen, Germany).

The master mix for 25µl total volume PCR was prepared from reagents containing RNAse free water, PCR buffer 5x, deoxynucleotide triphosphates (dNTPs), 10µL of forward and reverse primers targeting the F-gene F5296F: 5'-ATTGGTAGCGGCTTGATCACTG-3', 0.5µL RNAse inhibitor and 5.0µL RNA (Qiengen kits) (Table 1). The Mastermix was prepared in a 25µL

reaction mix consisting of 2µL of total viral RNA/extracted RNA template from Anionic Linear Polyacrylamide Facilitators (ALFs). The reaction mixture was loaded into the thermocycler with the following cycling conditions according to the manufacturer's instruction. 50^{o}C at 30mins, 94^{o}C for 15mins, 40 cycles of 94^{o}C for 30sec, 55^{o}C for 1min and 68^{o}C for 2mins and a final extension at 68^{o}C for 10mins.

All PCR products were visualized with 500 bp DNA Ladder (Qiangen Germany) on 1.5% agarose gel containing ethidium bromide.

Preparations and Casting of gel for Electrophoresis
The gel was weighed and added to the Tris-Borate Ethylene diamine tetra-acetic Acid (TBE) buffer. The agarose solution was dissolved under a low heat and cooled.

The comb was placed in the casting tray to form wells, the agarose solution was poured into the casting tray and allow to solidify at room temperature, the gel in the electrophoresis is covered with buffer.

Table 1. MASTER MIX COMPOSITION

REAGENTS	**1X (µL)**	
RNase.free water	11.0	
PCR Buffer 5X	5.0	
dNTPs	1.0	
NDV-F4217F (10µM)	1.0	
NDV-F5457R(10µM)	1.0	
RNase Inhibitor	0.5	
One Step RT-PCR Enzyme Mix	0.5	
RNA	5.0	
FINAL VOLUME	25.0	

Electrophoresis
The DNA samples were mixed with ethidium bromide loading dye and loaded with the DNA ladder into the wells. One kbp plus DNA ladder was used as band marker and each lane of the wells having positive and negative controls (Figure 1). The electrophoresis voltage was set at 120V for 35 minutes until dye has migrated. Fusion (F) gene PRIMER used for NDV -F5296F: 5'-ATTGGTAGCGGCTTGATCACTG-3'

Visualization of gene bands
The bands were visualized with an ultraviolet trans illuminator Gel Documentation system (ENDURO GDS Labnet, aplagen).

Statistical analysis
Prevalence was determined using descriptive analysis Balachandran *et al*. (2014).

RESULTS
Egg mortality of cloacal samples from poultry chickens in Rano, Rano Local Government Area Kano State
During the first passage in 24 hours, embryo mortality was observed in 8 groups. In 48 hours, embryo mortality was observed in 7 groups. At 72 hours, egg mortality was recorded in 6 groups. Only one group (51-54) was observed for egg mortality at 98 hours (Table 2). Prevalence rate was 60%.

For the second passage, two groups showed egg morality within 24 hours. In 48 hours, egg mortality was observed in two groups, while no egg morality was reported in 72 hours (Table 3). Therefore, overall prevalence = 12 + 72 eggs mortality= 84/120 x 100 = 70%.

Spot Haemagglutination and agar sterility test result

The results showed that, out of the 93 eggs spotted, 15 were positive for haemagglutination. with a prevalence of 63.0 % (Table 4). No bacterial or fungal growth was observed in all sample during sterility test.

Molecular detection of NDV by PCR and Electrophoresis

The electropherogram of amplicon from extracted RNA of some samples showed bands (kbp) corresponding to F gene of NDV (Figure). The F-gene has a molecular weight of 502 kbp corresponding to that of the ladder

TABLE 2: Egg mortality of cloacal samples from poultry chickens in Rano, Rano Local Government Area Kano State

SN/	Sample ID	24h	48h	72h	98h	120h	No of samples with mortality
1.	1-4	0/3	0/3	0/3	0/3	0/3	0
2.	5-8	0/3	0/3	0/3	0/3	0/3	0
3.	9-12	1/3	0/2	0/2	0/2	0/2	4
4.	13-16	0/3	0/3	0/3	0/3	0/3	0
5.	17-20	3/3	0/0	0/0	0/0	0/0	4
6.	21-24	0/3	0/3	1/3	0/2	0/2	4
7.	25-28	0/3	0/3	0/3	0/3	0/3	0
8.	29-32	0/3	0/3	0/3	0/3	0/3	0
9.	33-36	0/3	0/3	0/3	0/3	0/3	4
10.	41-44	1/3	0/2	2/2	0/0	0/0	4
11.	45-48	2/3	0/1	1/1	0/0	0/0	4
12.	49-52	0/3	0/3	0/3	0/3	0/3	-
13.	51-54	0/3	1/3	0/2	2/2	0/0	4
14.	55-58	0/3	2/3	0/1	0/1	0/1	4
15.	59-62	0/3	0/3	0/3	0/3	0/3	0
16.	63-66	0/3	3/3	0/0	0/0	0/0	4
17.	67-70	0/3	0/3	0/3	0/3	0/3	4
18.	71-74	0/3	0/3	0/3	0/3	0/3	0
19.	75-78	0/3	0/3	0/3	0/3	0/3	0
20.	79-82	0/3	0/3	0/3	0/3	0/3	0
21.	83-86	0/3	0/3	0/3	0/3	0/3	0
22.	87-90	0/3	0/3	0/3	0/3	0/3	4
23.	91-94	0/3	0/3	3/3	0/0	0/0	4
24.	95-98	1/3	0/2	2/2	0/0	0/0	4
25.	99-102	3/3	0/0	0/0	0/0	0/0	4
26.	103-106	1/3	1/2	0/1	0/1	0/1	4
27.	107-110	0/3	3/3	0/0	0/0	0/0	4
28.	111-114	2/3	1/1	0/0	0/0	0/0	4
29.	115-118	0/3	1/3	0/2	0/2	0/2	4
30.	119-120	0/3	0/3	2/3	0/1	0/1	0
31.	Controls	0/3	0/3	0/3	0/3	0/3	
Totals = 120		14	12	11	2	0	72

Prevalence rate = $^{72}/_{120}$ X 100 = 60%

Table 3. Egg mortality of cloacal samples from poultry chickens in Rano local government Area Kano State during the second passage of samples (P2)

Sample ID	24 HOURS	48HOURS	72 HOURS	NO OF SAMPLES
1. 1-4	1/3	0/2	0/2	4
2. 5-8	0/3	0/3	0/3	0
3. 13-16	0/3	0/3	0/3	0
4. 25-28	0/3	1/3	0/2	4
5. 29-32	0/3	0/3	0/3	0
6. 49-52	0/3	0/3	0/3	0
7. 59-62	0/3	0/3	0/3	0
8. 71-74	0/3	0/3	0/3	0
9. 75-78	0/3	0/3	0/3	0
10. 79-82	0/3	2/3	0/1	4
11. 83-86	3/3	0/0	0/0	0
12. 87-90	0/3	0/3	0/3	0
13. controls	0/3	0/3	0/3	0
Total =120	09	06	01	12

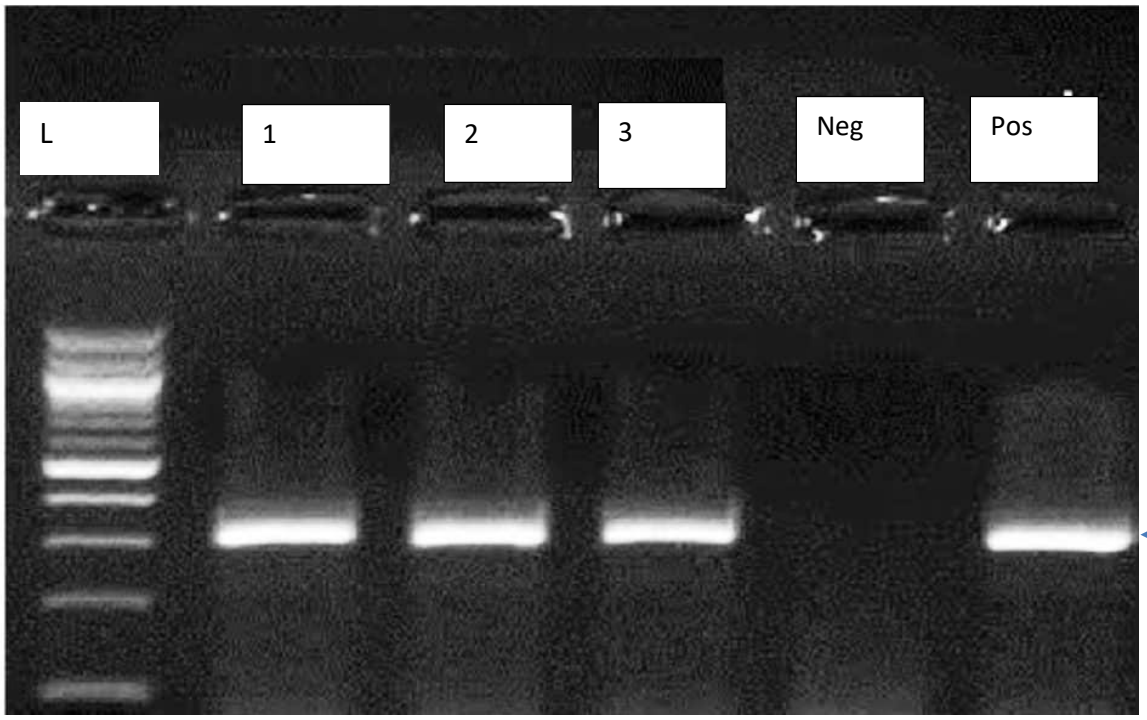

Figure 1: Newcastle disease virus PCR results of cloacal swaps from poultry chickens in Kano. Pooled samples 1, 2, and 3 were positive for cDNA bands, well 5 and 6 are negative and positive controls respectively. L represent the 100 bp DNA ladder

500bp

Table 4. Spot Haemagglutination and sterility of allantoic fluid of cloacal samples of poultry chickens from Rano, Rano Local Government Area Kano State

Sample ID	No of eggs spotted	No Negative	Growth on BA	No of samples
1-4	3	3	No growth	
5-8	3	3	No growth	
9-12	3	-	No growth	**4**
13-16	3	3	No growth	
17-20	3	3	No growth	
21-24	3	3	No growth	
25-32	3	3	No growth	
29-32	3	3	No growth	
33-36	3	3	No growth	
37-40	3	3	No growth	
41-44	3	-	No growth	**4**
45-48	3	3	No growth	
49-52	3	3	No growth	
51-54	3	-	No growth	
55-58	3	3	No growth	
59-62	3	3	No growth	
63-66	3	-	No growth	**4**
67-70	3	3	No growth	
71-74	3	3	No growth	
75-78	3	3	No growth	
79-82	3	3	No growth	
83-86	3	3	No growth	
87-90	3	3	No growth	
91-94	3	-	No growth	**4**
95-98	3	3	No growth	
99-102	3	3	No growth	
103-106	3	-	No growth	**4**
107-110	3	3	No growth	
111-114	3	3	No growth	
115-118	3	3	No growth	
119-120	3	3	No growth	
Total =120	93	75		20

DISCUSSION

In Africa, ND is enzootic, causing huge economic losses to farmers, and in Nigeria, an estimated 78,526 outbreaks of ND are reported annually, with an estimated financial burden of $24.72 million (8.9 billion naira), and there is the lack of characteristic clinical signs in many bird species, infected with NDV isolates, which poses a serious challenge for the rapid identification and diagnosis of the infection by this virus (Akanbi *et al*., 2020). This study determined the prevalence of Newcastle Disease Virus in poultry Chickens and also carried out molecular confirmation of the isolates obtained from poultry farms in Rano, Rano Local Government Area, Kano state.

From Table 2 in this study embryonic mortality/egg death was observed under 24 hours in samples from five groups. This result is consistent with the observation of Angeliya et al. (2022) who observed embryonic mortality from 48 hours. Embryos inoculated with NDVs that have a virulent pathotype die in less than 60 h and show clinical signs such as bleeding, hairlessness, and stunting, which is consistent with the molecular characterization of the six virulent NDVs, while embryos inoculated with non-virulent NDV will not normally die until the 5th day of observation (Angeliya et al., 2022). Embryos that died within 24 hours post-

infection (PI) are not considered to be due to virus activity, are in some cases excluded and regarded as death due to non-specific causes (Alazawy and Al Ajeeli, 2020). This means that the embryos that died within 24 hours post-infection did not die as a result of virus infection. Egg mortality may occur as a result inappropriate handling of eggs during inoculations. From Table 2, it was also observed that some embryos died 48 hours' post inoculation. This observation is in agreement with the result reported by Hamisu et al. (2016) who reported embryonic mortality within 48-72 hours in a similar study on molecular screening and isolation of Newcastle disease virus from live poultry markets and chickens from commercial poultry farms in Zaria, Kaduna state.

Egg mortality was observed in 6 groups and one group in 72 and 98 hours respectively. Amer et al. (2018) in a similar study on Isolation and Identification of H9N2 Avian Influenza and Newcastle disease viruses co-infections in chicken, carried out their incubation for 48h, although, their samples were those that showed partial M segment RT-PCR positive reaction In this study, no embryo mortality was reported after 120 hours during first passage, this does not agree with the observations of Sharmin et al (2013) were he reported that mortality of embryos started within 120 post infection.

From this study, the results obtained (Table 2) showed that the overall prevalence of NDV on the farm studied was 46.66%. the prevalence rate in this study did not agree with the prevalence rate of 17% by Abraham *et al.* (2014) in a similar study to determine the Prevalence of Newcastle disease antibodies in local chicken in Federal Capital Territory, Abuja, Nigeria. This may be attributed to variations in seasons, with high occurrence in the months of March and October which coincide with onset of the rainy season and dry season, respectively. The high wind movement could aid in spread of infection from one poultry farm to another (Abraham *et al.,* 2014). The prevalence of 46.66% obtained in this study is comparatively higher than the 15.40% prevalence obtained by Muhammed and colleagues in the same study area (Kano) and higher than the prevalence of 29% reported in Zaria.

From table 3, During the second passage egg mortality was observed in groups 1-4, 52-54, 99-102, 24 hours post inoculation. This was in disagreement with that described by Sharmin *et al.* (2013) were egg mortality started 48 hours post infection. This results did not corroborates with the observation reported by Balanchandran *et al.* (2014) who reported The isolation in 66.67% of samples were positive in the first passage, 20.83 % in the 2 passage and 12.50 % in the 3 passage. in a similar study Isolation and characterization of Newcastle disease virus from vaccinated commercial layer chicken. A better viral isolation is usually achieved when samples are subjected to three passages in embryonated eggs. Therefore, about 85 % of the positivity in the 1 passage, and 10% needed a second blind passage and in exceptional cases, three blind passages were needed. Hence variation may be due to the nature and virus titer of samples used which serve a crucial role in the virus isolation (Balachandran *et al.,* 2015).

After 48 hours and 72 hours mortality was recorded in three groups and one group respectively. These results agreed with that reported by Mihreteab *et al.* (2017), when he observed that selected samples for virus isolation were found to be positive for virus isolation in embryonated chicken eggs, and all inoculated embryos died between 55 to 60 h post inoculations, in an outbreak investigation of Newcastle disease virus from vaccinated chickens in Eritrea.

In a similar studies involving many passages, Mebatsion *et al*. (2001) recorded a prevalence of 32% after about 5 passages. He reported that extra egg passages were required for NDV-P1 to

be detected using the HA test, suggesting that some form mutants which replicate slowly when inoculated into the allantoic cavity of 9 to 11-day-old embryonated SPF chicken eggs. In another similar study, Balachandran *et al*. (2014) reported that those samples which were found negative for NDV at 3 passage were further passaged twice before being discarded as negative. This is thus one of the limitations of this study as it was terminated at 2 passages. Further passages could have enhanced the prevalence of the disease in the flock studied since the entire flock was displaying symptoms of the disease and some of the virus isolates may be slow in replicating. Velogenic, mesogenic and lentogenic strains of NDV killed the embryo in about 60 hrs, 60 to 90 hrs and more than 100 hrs respectively (Balachandran *et al*., 2014). Samples with a low titer of NDV showed negative results in the first isolation and require passage in the embryonated chicken eggs more than two times for positive results, and several isolates have to be passaged repeatedly to obtain a uniform titer of 2^6, which are used in the treatment of macroscopic and microscopic observations of embryonic lesions after NDV inoculation (Angeliya *et al*., 2022).

From Table 4, results showed that the allantoic fluids of some samples were negative while others were positive for spot haemaglutination assay. A prevalent of 16.66% was recorded. In a similar study by Alazawy and Al Ajeeli (2020), field samples with suspected and isolated NDV propagated in the allantoic cavities of 10-day-old fertile SPF chicks were also positive in the rapid HA tests within a few seconds.

Sterility test on blood agar showed no growth of bacteria. In a similar study, Kasozi *et al.* (2014) carried out HA activity detected in bacteriologically sterile fluids harvested from inoculated eggs.

From Figure 1 Molecular confirmation of representative samples were positive for F-gene. This study agrees with the findings of Shabirr *et al.* (2018) where F-gene was used to detect NDV. Analysis of F protein cleavage site of NDV is a favourable method to distinguish virulent and avirulent NDVs. However, since both velogenic and mesogenic NDVs share similar virulent type of F cleavage site motifs, this method cannot be used to separate velogenic and mesogenic NDVs (Balachandran *et al*., 2014), thus, MDT and ICPI tests which are carried out to distinguish the virulence of the isolated strains. Also in a similar study by Alazawy and Al Ajeeli (2020) on Isolation and molecular identification of wild Newcastle disease virus isolated from broiler farms of Diyala Province, Iraq, a representative sample (i.e. three birds) were randomly selected from the NDV-infected group for molecular detection using RT-PCR, and two samples were positive in the APMV-1 M-gene procedure. At present, RT-PCR-based techniques for the detection and typing (pathotyping and genotyping) of APMV-1 RNA in the allantoic fluid of inoculated fowl eggs are becoming increasingly common in diagnostic laboratories, but the genetic variability of APMV-1 isolates should be considered carefully as potential cause for false negative results of genetic-based laboratory tests (Kasozi *et al*., 2014).

CONCLUSION

This analysis demonstrates the existence of NDV in the poultry chickens, and indicated the continual movement and exchange of viruses amongst poultry chickens in Kano. Egg inoculation confirmed the presence of NDV in some cloacal samples, which was further confirmed by spot haemagglutination assay of the positive samples. molecular detection by PCR and electrophoresis also revealed the presence of F-gene which is one of the typical genes of NDV. Therefore, the disease occurring in these chickens was ND caused by NDV.

CONFLICT OF INTEREST

The authors declare that there was no conflict of interest

FUNDING

The study was not in receipt of any funding issues involving human and animal studies

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