Research Article

Molecular Detection Of Newcastle Disease Virus In Clinically Suspected Broiler Chickens From Kirkuk Province, Iraq: Paired Comparison Of Tracheal Swabs And Blood Samples

Huda Lateef Mohammad[1*], Nihad Abdulhussain Jafar[2]

[1]Department of Veterinary Microbiology, College of Veterinary Medicine, University of Tikrit, Tikrit, Iraq, drhudalateef94@st.tu.edu.iq
[2]Department of Veterinary Microbiology, College of Veterinary Medicine, University of Tikrit, Tikrit, Iraq, nihadabid73@tu.edu.iq
Corresponding Author: Huda Lateef Mohammad
ORCID iD: 0009-0004-5664-6545

Abstract

NDV is still considered a major limiting factor on poultry farming in endemic areas. In this field study, we report the presence of NDV RNA in clinically affected broilers in Kirkuk Province, Iraq, comparing paired tracheal swabs and blood samples for conventional RT-PCR detection. A total of 96 broiler chickens with clinical symptoms of NDV infection at the age of 3-6 weeks old from four commercial farms in Kirkuk were selected. One tracheal swab and one blood sample were collected from each chicken and subjected to conventional RT-PCR using specific primers for NDV, which targets the F gene with expected amplification of 387 bp. NDV RNA was detected in 19 out of 96 (19.8%; 95% confidence interval [CI]: 13.1-28.9%). NDV RNA was found in 18 chickens in tracheal swabs (18.8%), while six were positive in blood samples (6.3%). Five chickens were positive in both sample types, thirteen in tracheal swabs, and one in blood. The detection rate in tracheal swabs was significantly higher than that in blood samples (McNemar's exact test, P=0.0018). While the clinical and post-mortem lesions were consistent with NDV infection, it was not enough to classify the virus genotype, pathotype, or virulence.

Keywords: Newcastle disease virus; conventional RT-PCR; broiler chickens; tracheal swabs; blood samples; Iraq.

1. Introduction

Newcastle disease (ND) is among the important infectious diseases that affect poultry production throughout the world and is still endemic in most of the developing countries. ND is caused by Newcastle disease virus (NDV), which has recently been reclassified as avian orthoavulavirus 1 and belongs to the Paramyxoviridae family. NDV is an enveloped virus with a single-stranded non-segmented negative-sense RNA genome encoding the viral proteins responsible for viral replication, attachment, fusion and assembly. Of importance is the fusion (F) protein, which has been shown to be important for determining the pathogenicity of NDV through the amino acids of the F-protein cleavage site (Czeglédi et al., 2006; Ganar et al., 2014; Absalón et al., 2019).

Clinical presentation of ND depends on the strain of the virus, the species of host affected, immunity of the host, age, management and vaccination history. Clinical features include respiratory symptoms, digestive disorders, neurological symptoms, and mortality in the outbreaks caused by virulent strains. Proventricular haemorrhages and haemorrhagic caecal tonsils are some of the gross lesions that support the diagnosis of ND but are not conclusive for the pathotype identification. Molecular characterization, including sequencing of the F-protein cleavage site, is the only way to confirm virulence (WOAH, 2021).

Despite the availability of vaccinations, NDV is still being detected in different poultry production systems. Outbreaks and molecular detection have been reported in various countries, and the prevalence has been found to vary due to sampling approach, diagnostic technique, poultry production system and vaccination (Abozaid & Abdel-Moneim, 2022; Mngumi et al., 2022; Zhang et al., 2022). In Iraq, NDV has been detected in commercial and backyard poultry and sequenced-based studies have identified genetically diverse strains in various governorates (Alazawy & Al Ajeeli, 2020; Faraj et al., 2025).

Conventional RT-PCR is a useful molecular tool for detection of NDV RNA in resource-constrained laboratories. However, the detection yield can vary with the type and quality of the clinical specimen. Respiratory specimens, including tracheal and oropharyngeal swabs, are commonly recommended for direct detection of NDV in live or recently euthanised birds. Blood samples may be useful in some settings but may provide lower molecular detection yield because viraemia can be transient and influenced by the stage of infection (Wise et al., 2004; WOAH, 2021).

Limited molecular data are available for clinically suspected broiler chickens in Kirkuk Province, Iraq. Moreover, few field studies from the region have directly compared paired tracheal swabs and blood samples collected from the same birds. Therefore, the present study aimed to detect NDV RNA by conventional RT-PCR and compare the detection yield of paired tracheal swabs and blood samples from clinically suspected broiler chickens under field conditions.

2. Materials and methods

2.1. Study design and sample collection

A field-based cross-sectional study was conducted in Kirkuk Province, Iraq, from September 2025 to February 2026. A total of 96 clinically suspected broiler chickens aged 3-6 weeks were sampled from four commercial broiler farms located in Kirkuk City, Daquq, and Laylan. Twenty-four birds were examined from each farm. Birds were selected on the basis of respiratory and/or neurological signs suggestive of Newcastle disease.

From each bird, one tracheal swab and one whole-blood sample were collected, resulting in 192 paired specimens included in the comparative analysis. Tracheal swabs were placed in 1 mL of TRIzol reagent. For blood samples, 0.25 mL of whole blood was mixed with 0.75 mL of TRIzol reagent. No cloacal or faecal samples were tested by RT-PCR in the final analysis. Samples were transported under cold conditions and stored at -80 °C until RNA extraction.

According to information provided by farm managers, the investigated flocks followed routine ND vaccination programmes involving an inactivated oil-emulsion ND vaccine administered at 1 day of age, with subsequent doses at 7 and 14 days of age. Complete individual bird-level vaccination records were not available; therefore, vaccination history was treated as farm-reported background information and was not analysed as an independent variable. LaSota strain material was used in the laboratory only as a positive amplification control for the RT-PCR assay.

Because this study included only clinically suspected birds and did not include healthy control birds, the observed positivity rate should be interpreted as the detection rate among clinically suspected broilers rather than the true prevalence of NDV in the wider broiler population.

2.2. RNA extraction

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA; Cat. No. Z3100) with a modified TRIzol-chloroform phase separation step. Specimens stored in TRIzol reagent were mixed with 200 µL of chloroform, incubated for 10 min at room temperature, and centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase was transferred to a clean RNase-free tube, mixed with an equal volume of 70% ethanol, and applied to a spin column.

The column was centrifuged at 14,000 × g for 1 min, followed by two washing steps using 600 µL of Wash Solution 1 and Wash Solution 2. DNase treatment (40 µL) was applied for 15 min. RNA was eluted in 50 µL of nuclease-free water at 60 °C. RNA concentration and purity were assessed using a NanoDrop spectrophotometer, and A260/A280 ratios were recorded before downstream reverse transcription (Promega Corporation, 2025).

2.3. Primers

A conventional RT-PCR primer pair targeting the NDV fusion (F) gene was used for amplification. The primer set was commercially synthesised by Macrogen and was based on a published conventional PCR assay for NDV detection (Majed et al., 2013). The forward primer NDV/Fa was 5'-TCAAACATATACACCTCATCCCAGACAGG-3' and the reverse primer NDV/Ra was 5'-CTGCCACTGCTAGTTGGGATAAATCC-3'. The expected amplicon size was 387 bp. Primers were supplied at a working concentration of 10 µM.

LaSota strain material was included as a positive amplification control to verify that the RT-PCR run was functioning under the laboratory conditions. This control was used to validate amplification and was not used to determine the genotype, pathotype, or virulence of the field-positive samples. Sterile nuclease-free water was included as the negative control.

2.4. cDNA synthesis and conventional RT-PCR

Reverse transcription and conventional PCR amplification were performed as a two-step procedure. For cDNA synthesis, each 20 µL reaction contained 4 µL of 5× master mix, 10 µL of extracted RNA, and 6 µL of nuclease-free water. Reverse transcription was performed at 25 °C for 10 min, followed by 42 °C for 15 min and enzyme inactivation at 85 °C for 5 s.

Each PCR reaction was prepared in a final volume of 25 µL containing 12.5 µL of 2× PCR master mix, 1 µL of each primer (10 µM), 2 µL of cDNA, and 8.5 µL of nuclease-free water. Thermal cycling consisted of initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 minAnalysis of PCR products (10 µL) was done using electrophoresis in 1.5% agarose gels stained with ethidium bromide followed by visualization under UV light and photography. All samples with a positive amplification band at the 387 bp site were termed positive. Quantitation of the viral load was not done since this was endpoint RT-PCR.

2.5. Clinical assessment and post-mortem examination

Prior to post-mortem examinations, the birds were clinically examined and presence or absence of clinical signs such as those relating to the respiratory, gastrointestinal and nervous systems was noted. The clinical signs recorded included breathing difficulties such as gasping, nasal discharge, coughing, depression/lethargy, green diarrhea, tremors, torticollis and paralysis of the legs or wings.

All 96 birds that were euthanized using cervical dislocation (the approved animal study protocol) underwent post-mortem examination. Focus was placed on gross lesions consistent with Newcastle Disease, such as petechial/ecchymotic hemorrhages in the proventricular mucosa and enlarged, edematous or hemorrhaged caecal tonsils. Photographs were taken of representative gross and clinical features.

2.6. Ethical approval

The study protocol was approved by the College of Veterinary Medicine, University of Tikrit, Iraq, under approval number CVT/2025/042. Sampling, handling, euthanasia, and post-mortem examination were conducted in accordance with internationally recognised animal welfare guidelines and the conditions of the approved protocol. Consent for sampling was obtained from farm owners or authorised farm managers before sample collection.

2.7. Statistical analysis

SPSS version 26.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The individual bird was considered the primary unit of analysis. Categorical variables were summarised as frequencies and percentages. A bird was considered NDV-positive when at least one of its paired samples was positive by conventional RT-PCR. Detection proportions were reported with 95% confidence intervals calculated by the Wilson score method.

The detection yields of paired tracheal swabs and blood samples collected from the same birds were compared using the exact McNemar test. A P value <0.05 was considered statistically significant. Farm-level positivity rates were summarised descriptively because the study included only four farms and was not designed to evaluate farm-level risk factors. A formal sample size calculation was not performed; the sample size was determined by field accessibility and the availability of clinically suspected birds during the study period.

3. Results

3.1. Clinical signs

Clinical signs compatible with Newcastle disease were recorded in the 96 clinically suspected broiler chickens included in the study. Respiratory signs were the most frequently observed findings. Gasping or difficulty in breathing was recorded in 78 birds (81.3%), nasal discharge in 65 birds (67.7%), and coughing in 58 birds (60.4%). Depression or lethargy was observed in 52 birds (54.2%), while greenish diarrhoea was recorded in 48 birds (50.0%). Neurological signs included tremors in 31 birds (32.3%), torticollis in 22 birds (22.9%), and paralysis of the legs or wings in 15 birds (15.6%).

3.2. Gross pathological findings

The gross post-mortem examination showed lesions indicative of Newcastle Disease. Proventricular hemorrhages were present in 18 birds (18.8%). The lesions included petechial and ecchymotic hemorrhages in the proventriculus lining. Hemorrhagic and swollen caecal tonsils were seen in 15 birds (15.6%) and varied in degree of swelling and surface hemorrhages. Torticollis was noted in 22 birds (22.9%). These lesions were compatible with NDV infection, however, were not enough to classify the pathotype and virulence of the virus.

3.3. RNA quality

The RNA was isolated from the matching tracheal swab and blood specimens obtained from the 96 bird subjects. The results of spectrophotometric evaluation indicated the presence of good-quality RNA in both the samples. The average A260/A280 ratio was 1.94 ±0.06 in tracheal swab and 1.89±0.09 in the blood specimen. No faecal/cloacal samples were used for RT-PCR analysis.

3.4. NDV detection by conventional RT-PCR

NDV RNA was detected by conventional RT-PCR in 19 of 96 birds, giving an overall bird-level positivity rate of 19.8% (95% CI: 13.1-28.9%). A total of 192 paired samples were examined, including 96 tracheal swabs and 96 blood samples. Tracheal swabs were positive in 18 of 96 birds (18.8%; 95% CI: 12.2-27.7%), whereas blood samples were positive in 6 of 96 birds (6.3%; 95% CI: 2.9-13.0%).

Among the 19 positive birds, 5 were positive in both sample types, 13 were positive only in tracheal swabs, and 1 was positive only in blood. The detection yield of tracheal swabs was significantly higher than that of paired blood samples according to exact McNemar's test (P=0.0018). Amplification products of the expected 387 bp size were observed in positive samples and in the LaSota positive control, whereas no amplification was detected in the negative control.

3.5. Farm-level distribution of NDV-positive birds

NDV-positive birds were detected in all four examined farms. Farm A had the highest observed bird-level positivity rate, with 9 of 24 birds testing positive (37.5%). Farm C had 5 positive birds (20.8%), Farm B had 3 positive birds (12.5%), and Farm D had 2 positive birds (8.3%). Overall, 19 of the 96 examined birds were positive for NDV RNA (19.8%). Farm-level differences were summarised descriptively because the study was not designed to evaluate farm-level risk factors.

4. Discussion

The present study detected NDV RNA in clinically suspected broiler chickens from Kirkuk Province, Iraq, and directly compared paired tracheal swabs and blood samples for conventional RT-PCR detection. The RNA of NDV was found in 19.8% of the tested birds. Positive birds could be found in all four farms, which means that molecular detection did not happen at one sampling point alone. The observed rate needs to be considered as a detection rate from clinically suspected birds, not as prevalence of NDV in poultry overall, as control birds that were healthy have not been included.

The main finding was the higher detection rate of tracheal swabs compared to blood samples. 18 out of 96 birds had a positive test with tracheal swabs, while only 6 birds were positive with the blood samples. Out of the discordant pairs, 13 birds were positive in tracheal swabs only, while just one bird was positive in blood only. This finding proves the suitability of using respiratory samples for the molecular detection of NDV in clinically suspected broilers. The finding is consistent with the respiratory involvement typical for NDV infection and recommendations for the priority use of respiratory samples for detection (Wise et al., 2004; WOAH, 2021).

Low positivity in blood samples doesn't mean that blood samples cannot be used for NDV detection. Rather, under the field conditions and testing approach used in this study, blood samples provided a lower detection yield than paired tracheal swabs. Detection in blood may be influenced by the timing of sample collection, transient viraemia, stage of infection, and the amount of viral RNA present. Because serial sampling and real-time quantitative RT-PCR were not performed, the present study cannot determine whether differences between specimen types reflect viral load, infection stage, or tissue distribution.

Clinical and gross pathological findings supported the suspicion of ND but were not used as independent evidence of viral pathotype or virulence. Torticollis, tremors, paralysis, proventricular haemorrhages, and haemorrhagic caecal tonsils are compatible with NDV infection; however, such findings are not sufficient to classify the virus as velogenic, neurotropic, or viscerotropic. Accurate pathotype determination requires sequencing of the F-gene cleavage site and/or validated pathogenicity testing, which were not performed in the present study.

The farm managers reported routine use of an inactivated oil-emulsion ND vaccine at 1 day, 7 days, and 14 days of age. However, complete bird-level vaccination records were unavailable; therefore, the study could not evaluate vaccine efficacy or relate vaccination history to individual RT-PCR results. LaSota strain material was used only as a positive control to verify amplification during RT-PCR. This control confirms that the assay run was functional but

does not distinguish vaccine-related RNA from circulating field strains, and it does not establish virulence. Because sequencing was not performed, the relationship between the detected NDV RNA and vaccine or field strains could not be determined.

Farm-level positivity varied from 8.3% to 37.5%. These differences were presented descriptively because the study included only four farms and was not designed or powered to identify farm-level risk factors. Therefore, the higher positivity observed in Farm A should not be interpreted as proof of higher outbreak severity, vaccine failure, or management-related risk without additional epidemiological data.

The study has several limitations. Sequencing and phylogenetic analyses were not performed; therefore, genotype, molecular epidemiology, and virulence could not be determined. No independent reference standard or healthy control group was included; therefore, the comparison between sample types represents detection yield rather than diagnostic sensitivity or specificity. Real-time quantitative RT-PCR was not performed, so viral loads could not be measured. Complete bird-level vaccination records were unavailable, and the number of farms was limited. Despite these limitations, the within-bird paired design provides practical evidence that tracheal swabs yield more positive conventional RT-PCR results than paired blood samples in clinically suspected broilers.

5. Conclusions

NDV RNA was detected by conventional RT-PCR in clinically suspected broiler chickens from Kirkuk Province, Iraq. Tracheal swabs showed a significantly higher detection yield than paired blood samples and should be prioritised for conventional RT-PCR detection of NDV under comparable field conditions. Clinical and gross pathological findings were compatible with Newcastle disease, but viral genotype, pathotype, and virulence could not be determined without sequencing or pathogenicity testing. Further studies incorporating F-gene sequencing, phylogenetic analysis, real-time RT-PCR, healthy control birds, detailed vaccination records, and broader farm-level sampling are recommended to clarify the molecular epidemiology of NDV in the region.

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Authors' declarations

Publication consent

Both authors approved the final version of the manuscript and consented to its publication.

Data and material availability

The raw data supporting the findings of this study, including bird-level clinical findings, gross pathological findings, and paired RT-PCR results, are available from the corresponding author upon reasonable request.

Conflict of interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

HLM designed the study, collected samples, performed the laboratory work, including RNA extraction and RT-PCR, conducted the statistical analysis, interpreted the data, and prepared the manuscript. NAJ supervised the study, contributed to animal care, performed the post-mortem examinations, and critically revised the manuscript. Both authors read and approved the final manuscript.

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Tables

Table 1. Frequency of selected clinical and gross pathological findings in examined chickens (n=96)

Finding	Positive birds (n)	Positive (%)
Gasping/difficulty in breathing	78	81.3
Nasal discharge	65	67.7
Coughing	58	60.4
Depression/lethargy	52	54.2
Greenish diarrhoea	48	50.0
Tremors	31	32.3
Torticollis	22	22.9
Paralysis of legs or wings	15	15.6
Proventricular haemorrhages	18	18.8
Haemorrhagic/enlarged caecal tonsils	15	15.6

Table 2. Conventional RT-PCR detection of NDV RNA according to sample type

Sample type	Number tested	Positive (n)	Positive (%)
Tracheal swab	96	18	18.8
Blood sample	96	6	6.3
Total specimens	192	24	12.5
Birds positive in at least one specimen	96	19	19.8

Table 3. Bird-level distribution of NDV-positive birds by farm

Farm	Location	Birds examined	Positive birds (n)	Positive (%)
A	Kirkuk City	24	9	37.5
B	Daquq	24	3	12.5
C	Laylan	24	5	20.8
D	Kirkuk City	24	2	8.3
Total	-	**96**	**19**	**19.8**

Figures

Fig. 1. Representative clinical and gross pathological findings in clinically suspected broiler chickens from Kirkuk Province, Iraq. (A) Torticollis. (B) Petechial and ecchymotic haemorrhages in the proventricular mucosa. (C, D) Enlarged and haemorrhagic caecal tonsils. These findings were compatible with NDV infection but were not sufficient to determine viral genotype, pathotype, or virulence.

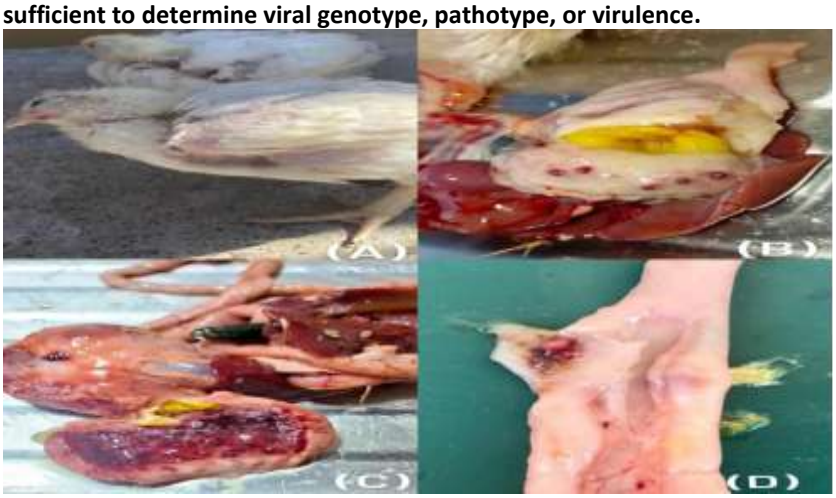

Fig. 2. Agarose gel electrophoresis of conventional RT-PCR products targeting the NDV F gene. The expected amplicon size was 387 bp. M: 100 bp DNA ladder; lanes 1-19: representative NDV-positive samples; PC: LaSota positive control; NC: negative control. The figure demonstrates presence or absence of the expected amplification product only; band intensity was not used for viral-load estimation.