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Research Article

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2025.56.4.666-670

Molecular surveillance of Newcastle disease virus in apparently healthy chicken and ducks reared at domestic-wild bird interface regions of Kerala#

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Citation: Kumar, P.K.S., Ravishankar, C., Kumar, N., Rajasekhar, R., Sumod, K., Palekkodan, H., Singh, A., Jayakrishna, B., Nehru, B.S. and Kailas, P. 2025. Molecular surveillance of Newcastle disease virus in apparently healthy chicken and ducks reared at domestic-wild bird interface regions of Kerala.
J. Vet. Anim. Sci. **56** (4): 666-670

Received: 11.09.2025 Accepted: 24.09.2025 Published: 31.12.2025

Abstract

Newcastle disease (ND) is a highly pathogenic infectious disease of poultry, causing significant economic losses globally. Wild birds act as natural reservoir of Newcastle disease virus (NDV) and spread the virus to domestic poultry. Frequent exposure of backyard poultry to wild birds and/or their excretions increases the risk of acquiring infection from wild birds. In the present study, tracheal and/or cloacal swabs were collected from a total of 242 apparently healthy chicken and ducks from the backyards. The samples were tested for the presence of NDV using matrix (M) gene based quantitative real time polymerase chain reaction (RT-qPCR), where all the samples were found negative. Virus isolation was attempted using specific pathogen free- embryonated chicken eggs (SPF-ECEs). Out of 242 samples, 22 samples (9.09%) showed haemagglutination (HA) across different passages. Genome of NDV could not be amplified in any of these HA-positive samples by using M gene-based RT-qPCR, and L- and F- genes based conventional reverse transcription polymerase chain reaction (RT-PCR). This indicates the presence of haemagglutinating agent other than NDV in the samples. Further studies are needed to identify the haemagglutinating agent detected in this study. All the samples in the present study were negative for NDV which might be due to absence of active infection during sample collection and vaccination status of the birds.

Keywords: *Newcastle disease virus, backyard poultry, ducks, domestic-wild bird interface*

Newcastle disease (ND) is a highly contagious infection known to affect at least 200 avian species worldwide (Zeng *et al*., 2024). It is caused by *Orthoavulavirus javaense*, which belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae*. The genome of NDV consists of non-segmented, single stranded, antisense RNA that contains a 55-nucleotide leader sequence at 3′ end and 114-nucleotide trailer sequence at 5′ end (Gao *et al*., 2019). The NDV genome encodes six structural proteins that includes M (matrix), P (phosphoprotein), NP (nucleoprotein), HN (haemagglutinin-

#Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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neuraminidase), F (fusion) and L (RNA-dependent RNA polymerase) proteins arranged in the order of 3′-NP-P-M-F-HN-L-5′. Non-structural proteins, V and W arise from P protein due to a specific RNA editing that occurs during transcription (Lu *et al*., 2024). Based on their pathogenicity in chickens, NDV strains are classified into lentogenic, mesogenic and velogenic pathotypes. Virulent strains of NDV contains polybasic amino acid motif at the FPCS which can be cleaved by wide range of proteases resulting in systemic spread of infection whereas avirulent strains possess monobasic amino acid motif (Jakhesara *et al*., 2016). NDV strains are categorised into class I and class II based on complete fusion gene sequences and genome size. The complete genome of NDV occurs in three different lengths of either 15,186 bp, 15,198 bp or 15,192 bp. The NDV strains with genome length of 15,198 nucleotides are generally avirulent belonging to class I and are commonly detected in wild waterfowl (Ren *et al*., 2016). The transmission of NDV can occur through inhalation, consumption of contaminated material or direct contact (Rashi *et al*., 2021). Backyard poultry are at a higher risk of acquiring NDV because of their frequent interactions with wild birds (Brown and Bevins, 2017). Lentogenic strains of NDV are frequently maintained in poultry populations without causing overt clinical signs. These apparently healthy birds serve as carriers, facilitating the maintenance and spread of virus within poultry populations (Rahmahani *et al*., 2020). Lentogenic strains of NDV circulating in wild waterfowl have the potential to mutate into virulent NDV and cause outbreaks in chickens (Tsunekuni *et al*., 2010). A study was conducted to detect the presence of lentogenic NDV in backyard poultry and ducks reared in domestic-wild bird interface regions of Kerala as data on this aspect is lacking in Kerala.

Materials and methods

Tracheal and/or cloacal swabs were collected from a total of 242 apparently healthy domestic chicken (n= 142) and ducks (n = 100) that are reared in domestic-wild bird interface regions of Kerala. The sampling was carried out from March 2025 to July 2025. The samples were collected from seven districts of Kerala, *viz*., Alappuzha (n = 38), Ernakulam (n = 23), Kannur (n = 45), Kozhikode (n = 41), Malappuram (n = 30), Thrissur (n = 40) and Wayanad (n = 25), focusing on the locations around water bodies and paddy fields that are frequently visited by wild and migratory birds. The swabs were collected in sterile phosphate buffered saline (PBS), containing antibiotic-antimycotic solution (Penicillin, 2000 units/ml, Streptomycin, 2 mg/ml, Gentamicin, 50 µg/ml and Amphotericin, 0.5 µg/ml) and one per cent bovine serum albumin (BSA) with a pH ranging between 7.0-7.4. All the samples were transported to laboratory maintaining the cold chain and stored at -80 ºC until further processing.

All the tracheal and/or cloacal swabs were processed for virus isolation in specific pathogen free embryonated chicken eggs (SPF-ECEs) and molecular detection of the NDV inside the BSL-3 facility of the Indian Council of Agricultural Research - National Institute of High Security Animal Diseases (ICAR-NIHSAD), Bhopal. Virus isolation was attempted from all the samples by inoculating in triplicate into 10-day-old SPF-ECEs through allantoic route. Inoculated eggs were incubated at 37 ºC in a humidified incubator (65% relative humidity) for 5 days or till death of the embryo. After overnight chilling at 4 ºC, allantoic fluids were harvested aseptically. Three serial passages of the samples were carried out in SPF-ECE. The collected allantoic fluids were subjected to Haemagglutination (HA) test using freshly prepared one per cent (v/v) chicken RBC to detect the presence of any haemagglutinating agent (WOAH, 2021).

Extraction of RNA was carried out from all the clinical samples and allantoic fluid samples that showed haemagglutination using QIAamp Viral RNA Mini Kit (QIAGEN, Germany). The extracted RNA was screened for the presence of NDV genome using matrix gene-based quantitative real time polymerase chain reaction (RT-qPCR). The primer pair, forward primer (AGTGATGTGCTCGGACCTTC) and reverse primer (CCTGAGGAGAGGCATTTGCTA), and probe ([FAM] TTCTCTAGCAGTGGGACAGCCTGC [BHQ-1]) were used to detect the matrix gene of NDV (Wise *et al*., 2004). The cycling conditions were 50 °C for 30 min (reverse transcription), 95 °C for 5 min (initial denaturation) followed by 45 cycles of 95°C for 15 sec (denaturation), 60 °C for 45 sec (annealing) and 72 °C for 30 sec (extension).

The complementary DNA (cDNA) was synthesised from the RNA of HA positive allantoic fluid samples and their respective clinical samples using First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturers protocol and stored at -20 °C for further use.

The synthesised cDNA was subjected to conventional reverse transcription polymerase chain reaction (RT-PCR) targeting the L gene of NDV. For the amplification of L gene, forward primer (CTCAGCAGGCAATGGAAGAA) and reverse primer (GCTCAGGAGTGATAAAGACTTG) were used as described by Kim *et al*. (2008). The cycling conditions were 95 °C for 5 min (initial denaturation), 40 cycles of 95 °C for 45 sec (denaturation), 54 °C for 45 sec (annealing) and 72 °C for 45 sec (extension) followed by a single cycle at 72 °C for 5 min (final extension). The cDNA was also screened using the primers, APMV F (ACGGGTAGAAGATTCTGGATCC) and APMV R (CCARGTAGGTGGCACGCATATT) targeting the F gene of NDV (Rajasekhar *et al*., 2015). The cycling conditions for amplification of F gene of NDV were 95 °C for 5 min (initial denaturation), 40 cycles of 95 °C for 1 min (denaturation), 53 °C for 45 sec (annealing) and 72 °C for 1 min 25 sec (extension) followed by a single cycle at 72 °C for 5 min (final extension). The PCR products were

subjected to agarose gel electrophoresis using submarine gel electrophoresis system (Thermo Orion, USA). After electrophoresis, the gel was observed under long range UV light and photograph was taken by using Gel Doc (Azure biosystems) system.

Results and discussion

In the present study, wild birds such as lesser whistling ducks, Asian open billed storks, Asian woolly necked storks, black headed ibis, kites, herons, egrets, cormorants and house crows were most frequently observed in the immediate vicinity of backyard poultry during sample collection. Ceñidoza *et al*. (2019) and Karamendin and Kydyrmanov (2021) stated that cormorants and egrets are considered as potential reservoirs of NDV, as they maintain close associations with migratory birds and frequently interact with poultry populations around food and water sources.

All the 242 samples turned out to be negative for NDV when tested by matrix gene-based RT-qPCR. Marks *et al*. (2014) conducted a similar study on backyard poultry reared in proximity to migratory bird wintering sites and reported that all the samples tested negative for NDV using RT-qPCR. However, after passaging all these samples in SPF-ECEs, twelve samples showed HA activity in first passage with titre ranging from 2^2 to 2^8 (Fig. 1). All the samples were subjected to second passage, where three samples retained hemagglutination ability in second passage with HA titre between 2^3 and 2^7. Out of the samples that were HA negative in first passage, 10 samples showed hemagglutination in second passage with titre ranging from 2^2 to 2^5. Samples that were HA positive in second passage were inoculated for third passage where four samples retained hemagglutination ability after third passage with HA titres of 2^3 and 2^4. A total of 22 samples (9.09%) showed haemagglutination out of 242 samples. across different passages.

Fig. 1. Haemagglutination test result after first passage of clinical samples in specific pathogen free embryonated chicken eggs (Rows A – H: Allantoic fluid from eggs inoculated with the clinical samples as indicated; Columns 1 – 12: Two-fold serial dilutions of allantoic fluid)

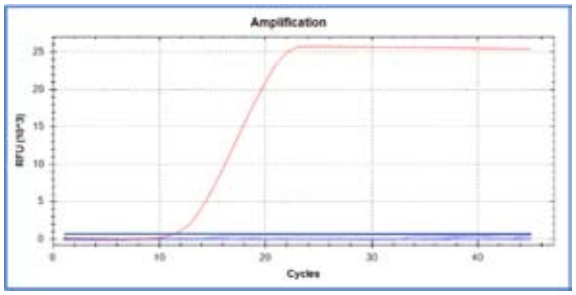

Fig. 2. Amplification plot of clinical samples (blue lines) and positive control (red line) tested for NDV genome by M gene-based RT-qPCR

All the HA positive allantoic fluid samples were found to be negative for NDV based on M gene-based RT-qPCR and L- and F- genes based conventional RT-PCR (Figs. 2, 3 and 4). To further confirm the result, the respective clinical samples were also tested by L- and F-genes based conventional RT-PCR and the results were negative. The attempted amplification of NDV genome targeting different genes rule out the possibility of false-negative results in the present study. Similar findings were reported in Switzerland (Schelling *et al*., 1999) and Iran (Rezaeianzadeh *et al*., 2011), where NDV genome could not be detected in any of the tracheal and cloacal swabs collected from apparently healthy backyard poultry. Hence the haemagglutination may have been due to some agent other than NDV. The absence of NDV genome in the samples collected during present study may be due to absence of active infection in birds at the time of sample collection. Rezaeianzadeh *et al*. (2011) stated that the absence of NDV in samples might be due to lack of active infection during sample collection. Also, birds infected with low-pathogenic NDV strains excrete the virus for a shorter duration than those infected with virulent strains (Dai *et al*., 2013). The lack of infection in backyard poultry could also

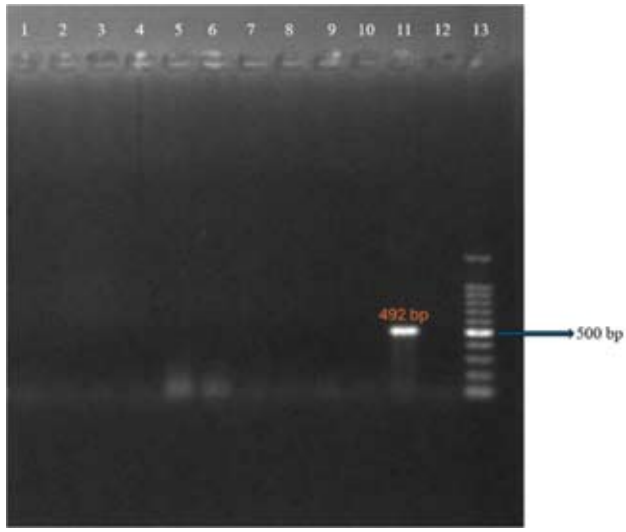

Fig. 3. Agarose gel electrophoresis of RT-PCR amplicons of L gene of NDV (Lanes 1 – 10: Clinical samples, Lane 11: Lasota vaccine [positive control], Lane 12: No temple control, Lane 13: 100 bp marker)

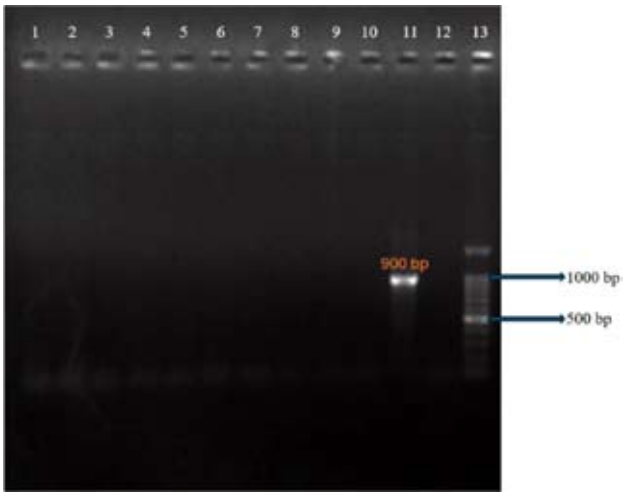

Fig. 4. Agarose gel electrophoresis of RT-PCR amplicons of F gene of NDV (Lanes 1 – 10: Clinical samples, Lane 11: Lasota vaccine [positive control], Lane 12: No temple control, Lane 13: 100 bp marker)

be attributed to the vaccination practices implemented and it has been reported that administration of vaccine against NDV causes 100-fold reduction in viral shedding (Puro and Sen, 2022).

Conclusion

The present study revealed the absence of NDV circulation in apparently healthy chicken and ducks that are reared near domestic-wild bird interface regions of Kerala. The detection of virus depends on various factors such as stage of infection, virus shedding and vaccination status of the birds. Further studies need to be carried out to identify the haemagglutinating agent detected in this study.

Acknowledgements

The authors thank the Director, ICAR-NIHSAD, Bhopal, and the Dean, College of Veterinary and Animal Sciences, Pookode for providing facilities for conduct of the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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