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ORIGINAL ARTICLE

Pathological and molecular investigation of velogenic viscerotropic Newcastle disease outbreak in a vaccinated chicken flocks

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Abstract Newcastle disease virus (NDV) belongs to genus *Avulavirus* and family Paramyxoviridae. There are thirteen serotypes named APMV-I (Avian Paramyxovirus-I) to APMV-13 of which NDV has been designated as APMV-1. The disease has been reported worldwide affecting both domestic and wild avian species. Morbidity and mortality rates up to 100% have been reported in cases of unvaccinated flocks. Stringent vaccination schedule is practiced in endemic/disease prone areas in order to prevent the disease. Despite this, NDV outbreaks have been reported even in cases of vaccinated populations. In this study we describe detailed pathological and molecular investigation that were undertaken in an organized poultry farm from Bareilly region, Uttar Pradesh, India, involving layer flocks which succumbed to ND outbreak in spite of following strict vaccination protocol. The mortality rate ranged from 76.80 to 84.41% in different flocks with an average mortality of 79.50%. Necropsied birds had gross lesions suggestive of viscerotropic ND including petechial hemorrhages on the proventricular tips, intestinal lumen with necrotic areas covered with hemorrhages, hemorrhagic cecal tonsils, para-tracheal edema and mottling of spleen. The characteristic histopathological lesions were mainly seen in the blood vessels and lymphoid tissues. Vascular changes characterized by congestion, edema, and hemorrhage were found in majority of the organs. Lymphocytolysis in spleen and cecal tonsils was evident. Immunohistochemical studies revealed positive signals mostly in macrophage and lymphocytes. PCR assay was done to confirm the NDV genome, which revealed an amplicon size of 356 bp. The phylogenetic analysis revealed the resemblance of the present isolate (ADS01) with class II genotype NDV XIIIA. The isolate belonged to velogenic NDV as the Minimum Lethal Dose (MLD) and Mean Death Time (MDT) for the present isolate were 10^{-8} and 41 h, respectively. Thus this study clearly demonstrates that in spite of strict vaccination regime and biosecurity procedures, ND continues to be rampant. Hence it is important to effectively administer the present vaccine in addition to strains matching to the field isolates to provide longer and optimal protection against spreading of virus by means of reducing the extent of viral shedding.

Keywords Genotype · Molecular characterization · Pathology · Poultry · Vaccine mismatch · Velogenic viscerotropic Newcastle disease virus

Introduction

There are many infectious diseases causing severe mortality among the birds and in turn causing severe economic losses to the farmers and poultry industry. Owing to its highly infectious nature, Newcastle Disease (ND) is one of the OIE (Office International des epizootics) notifiable diseases amongst avian infectious diseases. Newcastle disease virus (NDV) belongs to the genus *Avulavirus* in the family Paramyxoviridae. There are thirteen serotypes under Paramyxoviridae named APMV-I (Avian Paramyxovirus-I)

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to APMV-13 and Newcastle disease virus (NDV) has been designated as APMV-1. The disease has been reported worldwide and it is currently controlled in some western European countries, the United States and Canada but continues to be a threat in South America, Asia and parts of Africa [1]. Both domestic and wild species are affected but with varying degrees of mortality and morbidity depending upon the host species and also the strain of virus [2]. The duration of incubation period is very short, ranging from 2 to 15 days with an average of 5–6 days. Morbidity and mortality rates up to 100% have been reported in cases of unvaccinated flocks [3]. Direct contact with diseased or carrier birds is the main mode of transmission followed by virus shedding by the infected birds in their feces, thus contaminating the environment. The disease is very contagious in nature as it takes only 2–6 days to affect the entire birds in the susceptible flock.

Virus infectivity is mainly dependent on the cleavage of Fusion (F) protein of the virus, which in virulent strains has been reported to be polybasic in nature, and thus this can be easily cleaved by furin-like proteases appearing in all organs in the host enabling easier and faster viral spread [2].

Phylogenetic analysis of the NDV isolates circulating in India revealed the presence of various genotypes including II, III, IV, VI, XIII, XIIIa and XIIIb. To delineate the genotype involved in the current ND outbreak, the partial genome of ADS01 isolate was sequenced and phylogenetic analysis was done on the basis of partial Fusion gene sequencing. The isolate (ADS01) clustered with other genotype XIIIA isolates, within Class II. ADS01 isolate was categorized as a velogenic NDV strain assessed biologically by the MLD, MDT and polybasic amino acid residues at the F protein cleavage site.

Stringent vaccination schedule are being practiced in endemic/disease prone areas in order to prevent the disease. NDV vaccine strains belonging to genotype I and II are generally being used in vaccines to control the clinical disease [2]. Despite of the stringent vaccination protocol, NDV outbreaks have been reported even in cases of vaccinated populations [4]. In this study, we describe detailed pathological and molecular investigation that were undertaken in an organized poultry farm involving layers of three different age groups belonging to white leghorn breed, which succumbed to ND outbreak in spite of following strict vaccination protocol.

Materials and methods

Farm history, necropsy examination and collection of samples

A disease outbreak among vaccinated layer flocks belonging to White leghorn strain was investigated in an organized poultry farm in Bareilly region, Uttar Pradesh India. The birds belonged to three different age groups of 10–18 weeks (n = 1200), 19–35 weeks (n = 600) and 36–72 weeks (n = 3000). Clinical signs, vaccination history and mortality were recorded. The birds were vaccinated against major viral diseases (Table 1). Necropsy examination of the dead birds (n $\simeq$ 500) was carried out randomly and the gross findings were recorded. Representative tissue samples (n = 25) from all the flocks were collected and stored in 10% formalin for histopathological studies and pooled tissue samples (spleen, trachea and caecal tonsils: n = 10) in − 80 °C for molecular studies.

Histopathology

The tissue samples were fixed in 10% neutral buffered formalin and processed by routine paraffin-embedding technique. Briefly, 4–5 µm thick sections were taken followed by Haematoxylin and Eosin (H&E) staining for detailed microscopic studies [5].

Immunohistochemistry

Immunohistochemical staining was performed on the tissues selected from tissue blocks based on the screening of H&E stained sections. Immunohistochemical staining was conducted using an avidin–biotin complex immunoperoxidase method following antigen retrieval by microwave based method [6]. Anti-NDV polyclonal antibody (ABCAM, USA) raised in poultry was used as the primary antibody. Briefly, duplicate sections of each tissue sections were stained with NDV primary antibody at a dilution of 1:100. After washing the sections thrice with PBS-T (Phosphate Buffered Saline-Tween 20), these were treated with biotylated anti-chicken antibodies raised in rabbit (1:100, Sigma Aldrich, USA) for 1 h, followed by three washings with PBS-T. Finally, the sections were subjected

Table 1 Vaccination regime followed in the farm

S. No	Day	Vaccine	Route
1.	2	MD	S/C
2.	6	IB + LaSota	Occular
4.	15	IBD intermediate	Occular
5.	24	IBD intermediate	Occular
6.	30	Fowl pox	Wingweb
8.	8th week	IB + LaSota	Occular
10.	11th week	R2B	I/M
12.	16th week	IB + LoSota	Occular
		ND killed	S/C
14.	Once in 4–6 weeks	ND LaSota	Occular

to 1:100 dilution of HRP-conjugated avidin (Sigma Aldrich, USA) for 1 h. Post-washing with PBS-T, the enzyme reactions were developed in AEC staining kit (Sigma Aldrich, USA) to produce a dark red precipitate and the sections were counterstained with Mayer's hematoxylin. Tissue sections were processed for negative control as above by omitting primary antibody in the procedure.

Genome detection studies (RNA isolation, cDNA synthesis, RT-PCR and nested PCR)

The tissue samples stored in − 80 °C were subjected to total RNA extraction with TRIzol reagent (Invitrogen, USA) as per the manufacturer's instructions. The extracted RNA was used to synthesize cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following manufacturer's instructions. RT-PCR was performed with the primers targeting the *fusion* gene of NDV (Forward primer: GCAGCTGCAGGGATTGTGGT; reverse primer TCTTTGAGCAGGAGGATGTTG) [7]. The RT-PCR was performed in a 25 µl reaction mixture containing 3.0 µl cDNA (5 ng/µl), 12.5 µl DreamTaq PCR Master mix 2× (Thermo Scientific, USA), 1.0 µl forward primer (10 p mol/µl), 1.0 µl reverse primer (10 p mol/µl) and 7.5 µl nuclease free water. The cyclic conditions for primary amplification were initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s, 72 °C for 45 s and final extension at 72 °C for 10 min.

Nucleotide sequencing and phylogenetic analysis

Partial *fusion* protein gene of 535 bp size was amplified [8]. The PCR products were purified using GeneJET PCR purification kit (Thermo Fisher Scientific, USA) and then sequenced in both directions using commercial sequencing services (Eurofins, Bangalore). Forward and reverse nucleotide sequences were curated and aligned using MEGA 7.0 software followed by BLAST analysis (http://blast.ncbi.nlm.nih.gov/) to find the most related sequences. The evolutionary history of the isolate ADS01 isolate of NDV was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [9]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [10]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ G, parameter = 1.7867)). The rate variation model allowed for some sites to be evolutionarily invariable ([+ I], 22.4665% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 59 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 523 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [11]. The sequence analysis of the predicted amino acids of the amplicon of the present ADS01 isolate was carried out to determine the presence of virulent amino acid motif in the F protein cleavage site [12]. Lentogenic strains of NDV have been shown to depict the FPCS cleavage site motif ^{112}G/E-K/R-Q-G/E-R-L^{117} with few basic amino acid residues at carboxyl terminus of the F2 protein while the virulent strains (mesogenic and velogenic) possess ^{112}R/K-R-Q-R/K-R-F^{117} motif with two pairs of basic amino acids and a Phenylalanine in the N terminus of the F1 (residue 117). In ADS01 strain the sequence of the FPCS motif is ^{112}R-R-Q-K-R-F^{117}.

GenBank accession numbers

The sequenced data was submitted to GenBank and the accession number obtained was MG013984.

Virus isolation and identification

Virus propagation was carried out in 9 days old embryonated eggs procured from Hatchery Unit of ICAR-CARI, Izatnagar. The pooled samples from the trachea, brain and spleen stored in − 80 °C were triturated, filtered using 45 µm syringe filter and the filtrates were used to inoculate the embryonated eggs for virus isolation via allantoic route following standard protocol [1]. The presence of NDV in the allantoic fluid (AF) was identified by means of Haemagglutination (HA) and Haemagglutination inhibition (HI) test. The tests were performed in V-bottom/shaped microtiter plates as described by earlier workers [1, 13]. RT-PCR was carried following cDNA synthesis from the AF to confirm the presence of NDV [14].

Calculation of minimum lethal dose (MLD) and mean death time (MDT)

MLD was performed in 9 days old embryonated eggs. Serial tenfold dilution of the harvested allantoic fluid was prepared and 0.1 ml of the dilutions (10^{-6}, 10^{-7}, 10^{-8} and

10^{-9}) was inoculated into the allantoic cavity (5 eggs/dilution). The MLD corresponds to the highest virus dilution that causes all the embryos inoculated to die. Thereafter, mean death time (MDT) was calculated based on the mean time in hours for the minimum lethal dose to kill all the inoculated embryos [13].

Results

Clinical signs and mortality

Clinical signs appeared suddenly with marked depression, anorexia, sharp drop in egg production, respiratory rales, greenish diarrhea, edematous swellings of the head, cyanosis of the combs, and conjunctivitis with ocular discharge. Prostration was observed in many birds followed by death within a span of 10 days. The mortality rate recorded in different age groups were 84.41, 83.00 and 76.80%, respectively, with an average mortality of 79.50% (Table 2).

Gross findings

Necropsied birds had facial swelling with mucoid to catarrhal exudate from the nares. Hemorrhages in the lower eyelid conjunctiva were evident in majority of the cases. Gross lesions were prominent in the mucosa of the proventriculus, small and large intestine. Proventriculus showed range of lesions from hemorrhagic line at the junction between esophagus and proventriculus, petechial hemorrhages on the proventricular tips (Fig. 1a), ecchymotic to suffusions covering the mucosa. Mottling of spleen was evident characterized by numerous multifocal pale areas on the surface (Fig. 1b). Grossly, the intestines revealed discolored areas of necrosis from the serosal surface. On opening the intestines, the lumen contained numerous necrotic areas covered with hemorrhages and mucoid content (Fig. 1c, e), and on washing the necrotic areas they revealed raised button type of ulcers which were yellowish in color. These lesions were seen either diffusely throughout the intestine or seen spread in multifocal areas. Cecal tonsils were swollen, prominent and hemorrhagic in all the cases examined (Fig. 1d). In few cases there were pale necrotic foci in the pancreas. Respiratory tract lesions were predominantly of mucosal hemorrhage and marked congestion of the trachea and para-tracheal edema. Airsacculitis was present in majority of the cases, and thickening of the air sacs with catarrhal or caseous exudates was observed. Layer chickens had free egg yolk in the abdominal cavity. The ovarian follicles were often flaccid, highly congested, with petechiae on their surfaces and appeared degenerative (Fig. 1f). The bursa of Fabricius was edematous.

Histopathological findings

The characteristic histopathological lesions were mainly seen in the blood vessels and lymphoid tissues. Vascular changes characterized by congestion, edema, and hemorrhages were found in majority of the organs. The tunica media of the arterioles were hyalinized, endothelial cells of the blood vessels were swollen and necrosed. Few blood vessels contained thrombosis in the lumen. Depletion of lymphoid tissue was the prominent finding in majority of the lymphoid organs including spleen, bursa, intestinal lymphoid aggregates like Peyer's patches and cecal tonsils. In upper respiratory tract, the lesions were seen throughout the length of the trachea. Cilia were lost. In the mucosa of the upper respiratory tract, congestion, edema, and dense cellular infiltration of lymphocytes and macrophages were seen (Fig. 2a). Marked lesions of the lung were seen with severe congestion and edema of the parabronchi. Air sacs revealed mild to moderate edema with mixed cell type infiltration. Sections from proventriculus revealed necrotic and denuded surface epithelium with severe hemorrhages (Fig. 2b). Pancreas revealed multifocal areas of necrosis. Multi focal necrotic areas were found throughout the spleen (Fig. 2c). Focal vacuolation and destruction of lymphocytes was seen in the cortical areas and germinal centers of the spleen. Inclusion bodies were observed in the spleen. Marked degeneration of lymphocytes in the medullary region was seen in the bursa of Fabricius. Spleen also contained severe reticular cell hyperplasia. Pancreas exhibited multi-focal areas of necrosis and interstitial edema (Fig. 2d). Intestinal mucosal epithelium was denuded completely and the structure of the villi was lost. The mucosa was necrotic with severe inflammatory reaction with mixed cell populations in the lamina propria (Fig. 2e). Bacterial colonies were found adhered to the necrotic debri attached to the ulcerated area. In case of cecal tonsils, there was severe lymphocytolysis, loss of lymphocytes severe

Table 2 Flock details of the investigated farm

Flocks	Age group (weeks)	Number of birds	Number of birds dead	Mortality rate (%)
Layers shed I	10–18	1200	1013	84.41
Layers shed II	19–35	600	498	83.00
Layers shed III	36–72	3000	2305	76.80

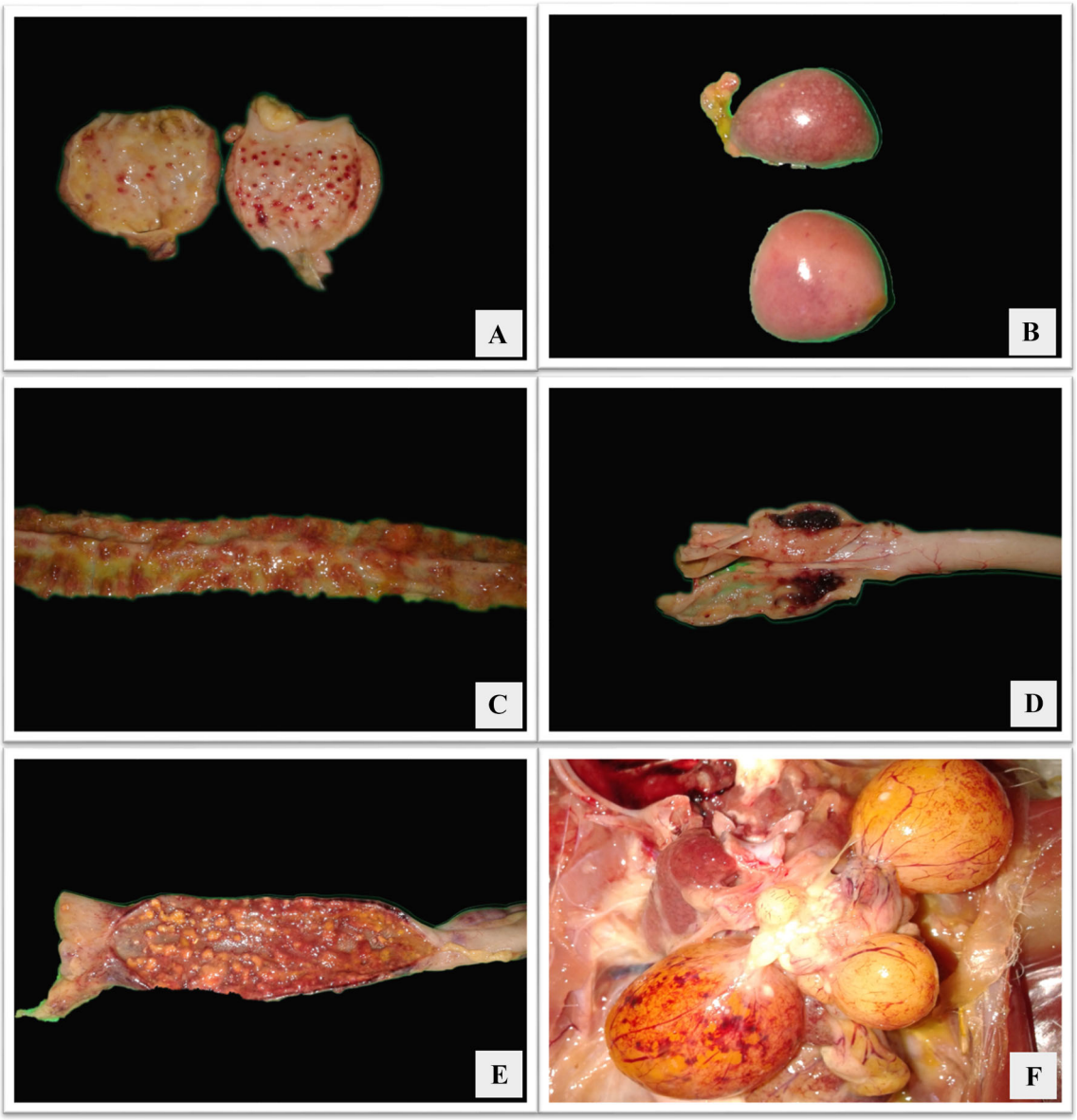

Fig. 1 **a** Proventriculus showing hemorrhages in the glandular tips; **b** Spleen with numerous multifocal pale areas giving a mottling appearance; **c** Mucosa of duodenum showing multifocal to coalescing raised hemorrhagic to necrotizing ulcerated areas; **d** Caecal tonsils with hemorrhages; **e** Mucosa of rectum showing multifocal to coalescing raised necrotic ulcerated areas; **f** Congested ovarian follicles with petechial to ecchymotic hemorrhages

necrosis and hemorrhages. Necrotic debri was observed in the lumen of the caecum (Fig. 2f).

Immunohistochemistry

Tracheal epithelium showed strong positive signals, sub-mucosal macrophages showed positive signals and the lymphocytes showed the same results (Fig. 3a). Epithelial cells and cells in lamina propria of proventriculus papillae also showed positive signals (Fig. 3b). In spleen, positive signals were present diffusely and mostly in macrophages and lymphocytes surrounding the central arteriole (Fig. 3c). Pancreas showed strong positive signals from the necrotic areas (Fig. 3d). Intestinal lymphoid aggregates and macrophages in sub mucosal layer and in lamina propria showed intense positive signals (Fig. 3e, f). The denuded and necrotic contents in the lumen revealed strong positive signals.

Genome detection studies

An amplicon of size 356 bp on 1.5% agarose gel was visualized, indicating the presence of NDV genome in the samples (Fig. 4).

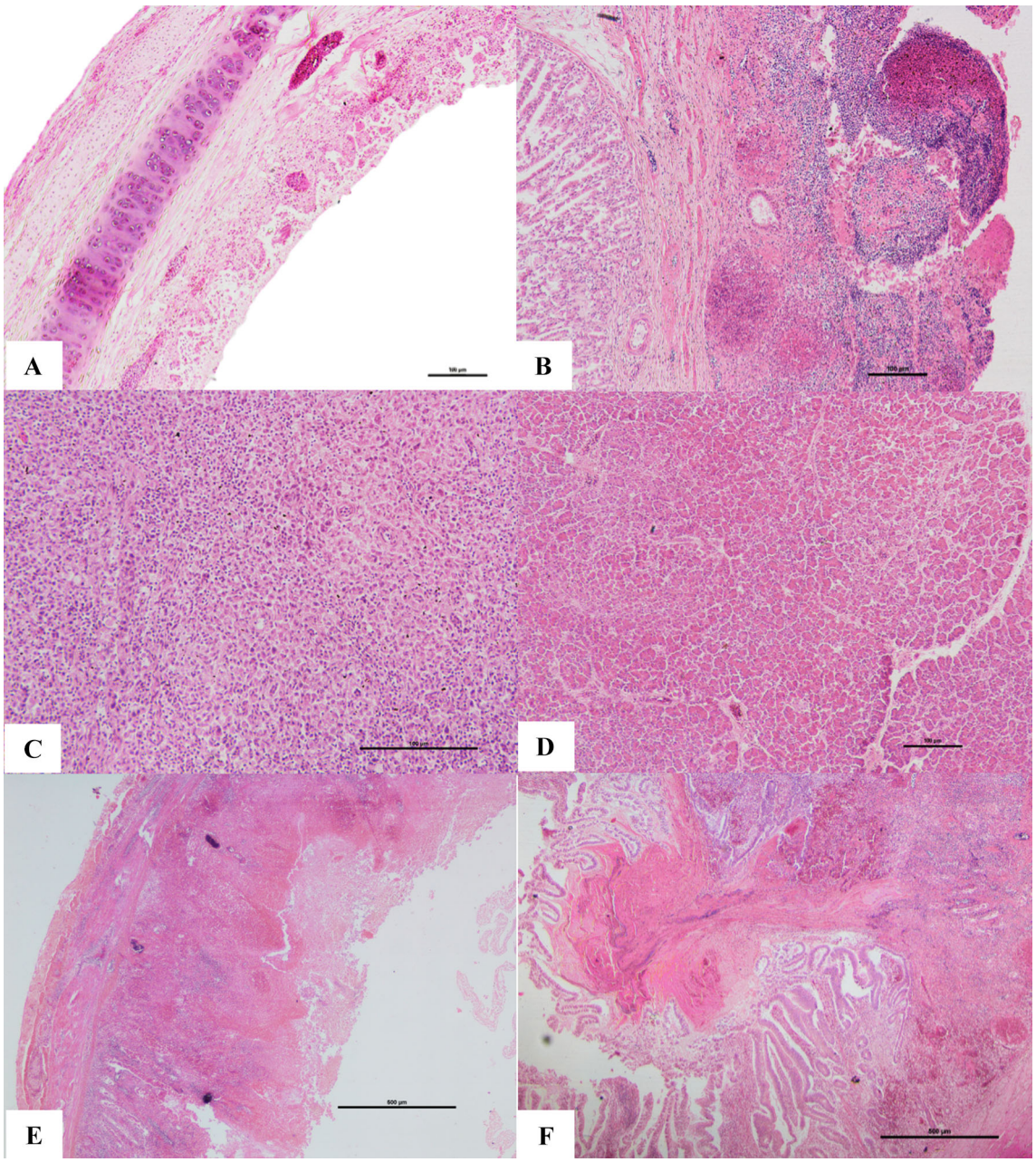

Fig. 2 **a** Trachea with necrotic and denuded surface epithelium with edematous submucosa and mononuclear cell infiltration. H&E 10×; **b** Proventriculus showing necrotic and denuded surface epithelium with severe hemorrhages H&E 10×; **c** Spleen showing focal to coalescing lymphoid depletion with associated reticular cell hyperplasia H&E 20×; **d** Pancreas with multi-focal areas of necrotic foci and interstitial edema. H&E 10×; **e** Duodenum with fusion of villi and the surface epithelium showing necrotic changes and sub-mucosa reveal severe infiltration with mononuclear cells and hemorrhage. H&E 4×; **f** Caecal tonsil showing severe lymphocytolysis, necrotic epithelium and eosinophilic exudates in the lumen admixed with fibrin and inflammatory cells. H&E 4×

Sequencing and phylogenetic analysis

Partial *fusion* protein gene of size 535 bp was amplified and sequencing of the PCR amplicon was done followed by phylogenetic tree construction. The phylogenetic analysis revealed the resemblance of ADS01 isolate with genotype NDV XIIIA (Fig. 5). The 'F' protein cleavage site amino acid motif for ADS01 isolate was ^{111}G-R-R-Q-K-R-F^{117} which is a virulent amino acid motif and thus the isolate was classified as velogenic.

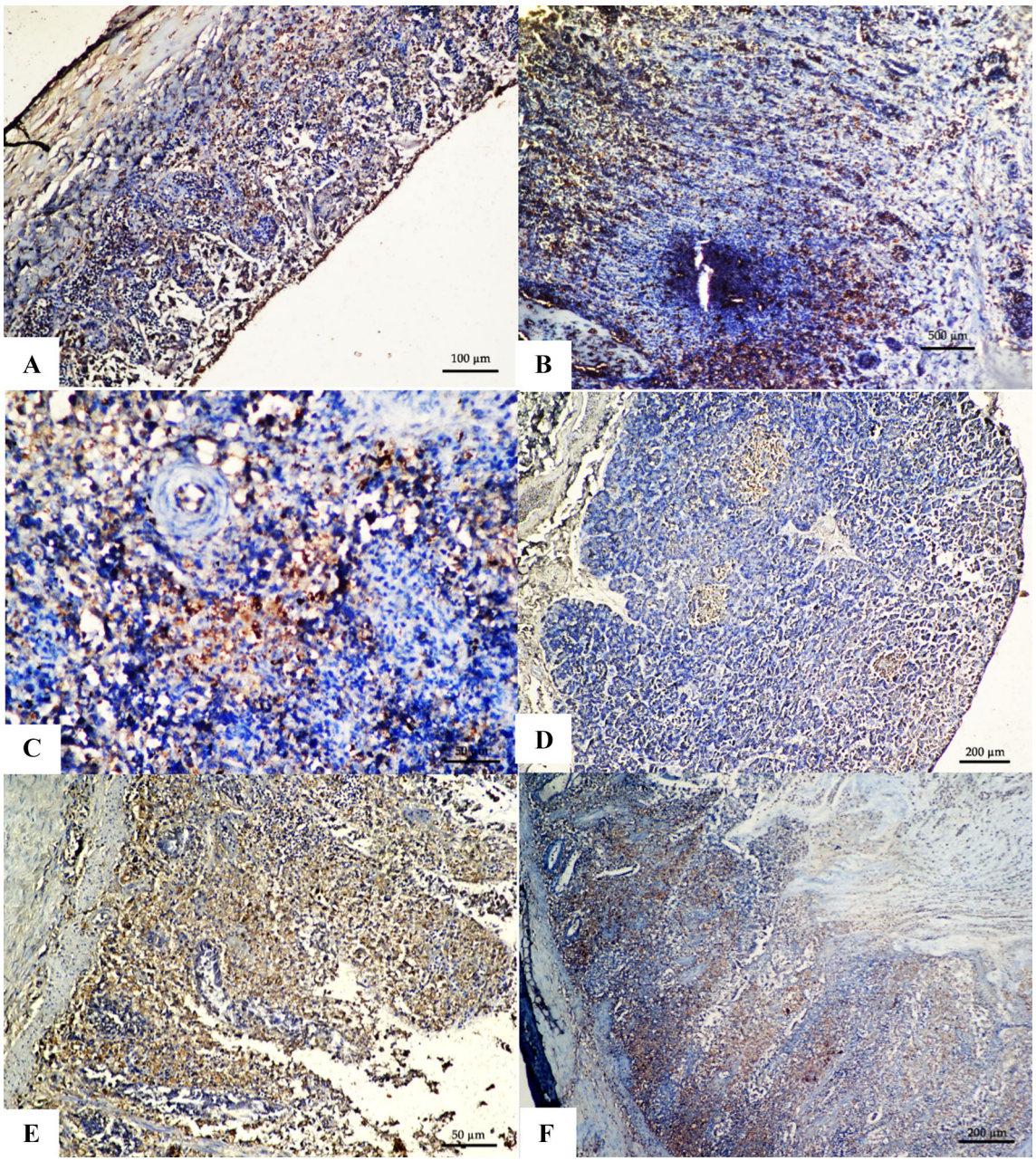

Fig. 3 **a** Trachea with strong positive signals from the necrotic mucosa, sub-mucosa and inflammatory cells. IHC 20×; **b** Proventriculus showing strong positive signals from the necrotic mucosa and inflammatory cells IHC 40×; **c** Spleen showing strong positive signals in the RE cells and macrophages from the areas surrounding the central arteriole in the PALS area IHC 40×; **d** Pancreas with strong positive signals from the necrotic areas. IHC 10×; **e** Duodenum showing strong positive signals from the inflammatory cells in the lamina propria and sub-mucosa IHC 40×; **f** Caecal tonsil with strong positive signals from lymphocytes and other inflammatory cells in the sub-mucosa. H&E 20×

Isolation and identification

The chicken embryos that died before 36 h post-inoculation with tissue triturate were discarded and the allantioc fluid (AF) was found negative for HA, HI and RT-PCR. Most of the embryos died between 36 to 48 h post inoculation and their AF were found positive for HA, HI and RT-PCR testing. Grossly, the virus infected embryos were edematous, had typical hemorrhage in the occipital area of the embryos along with hemorrhages throughout the body surface (Fig. 6a, b). The pooled AF showed HA titre of 1:512 and HI titre of 2^{10} RT-PCR revealed an amplicon size of 350 bp indicating the presence of NDV in the harvested AF.

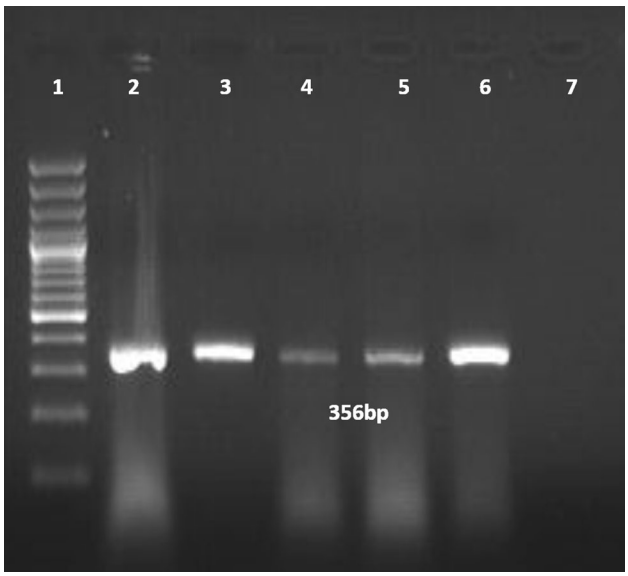

Fig. 4 Agarose gel electrophoretic picture showing 356 bp PCR amplified product of fusion protein gene (Lane 1–DNA ladder; lane 2–positive control; lanes 3 to 6–field test samples; 7–non template control

Calculation of minimum lethal dose (MLD) and mean death time (MDT)

The MLD and MDT for the present isolate (ADS01) were found to be 10^{-8} and 41 h respectively.

Discussion

Newcastle disease exhibits a range of pathogenicity in chickens depending on its virulence that varies from per-acute disease with nearly 100% mortality to subclinical disease with no outward lesions. The MDT is a measure of viral pathogenicity where it determines the virulence of an isolate by the duration between inoculation of virus and time of death in embryonated chicken eggs. Velogenic strains cause the chicken embryos to die in less than 60 h, lentogenic strain fails to kill the embryos even after 90 h, and the strains that cause embryo mortality between 60 and 90 h are mesogenic [3]. In the present study, the MDT for the virus isolate (ADS01) was found to be 41 h, thus the isolate is pathogenic and falls in velogenic category.

Among velogenic category there are two forms based on visceral or nervous signs viz., viscerotropic (VVNDV) and neurotropic (VNNDV). Grossly, viscerotropic form is characterized by the presence of necrosis, ulceration of the lymphoid tissues in the intestines, splenomegaly, necrosis in the spleen, hemorrhages and necrosis of caecal tonsils [15–18]. Microscopically, in the viscerotropic form, the lymphoid tissues seen scattered throughout the body from intestines to lymphoid organs reveal severe necrosis [17, 18]. Immunohistochemistry has been used to study the distribution of virus in the tissues of NDV infected birds. By immunohistochemistry, VVNDV could be detected throughout the body in all the primary and secondary lymphoid tissues including thymus, bursa, spleen, and cecal tonsils. In the spleen, macrophages surrounding the penicilliary arteries showed intense positive signals, while in other lymphoid organs the positive signals emanated in the middle of the lymphoid follicles containing macrophages and lymphocytes. In the cecal tonsil, NDV signals were noticed from macrophages within the lamina propria. Thus, the gross, microscopical and immunohistochemical findings recorded in the present study were in corroboration with findings noticed in viscerotropic NDV category.

Phylogenetic analysis of NDV isolates reported during 1989–2013 has revealed the presence of various genotypes circulating in India including Genotype XIII genotype XIIIa, genotype VI, genotype III and genotype II in cranes and chicken [12]. Apart from various genotypes reported, pathotypic and sequence characterization of NDV from vaccinated chickens revealed circulation of genotype II, IV and XIII in the country [19]. Genotype XIII (XIIIb) was isolated in commercial vaccinated layer farms [20] and from Emu [21]. In the present study, partial sequencing of fusion protein gene followed by phylogenetic analysis revealed the present isolate (ADS01) that was of class II genotype NDV XIII and the finding was subsequently supported by deduced amino acid sequence analysis of F protein cleavage site which revealed a virulent amino acid motif.

The mortality in the investigated layer flock was 79.50%. Mortality rate varies among the affected flocks depending upon the strain of the virus, vaccination status, genetic resistance and species of the birds [2]. ND Outbreaks in fully susceptible chickens are generally devastating with mortality up to 100% [22]. But in the present study, there was a substantial mortality among the vaccinated birds. Severe mortality due to ND among vaccinated birds have also been reported earlier [23, 24]. The one reason may be attributed to the virus shedding of the healthy birds post-vaccination. It has been reported that the vaccination against ND helps in protecting the poultry against mortality and clinical outcome, and it's quite imperative that these vaccines could not stop shedding of viral particles which may act as a prime source of infection in well vaccinated flocks [4, 25, 26]. It is well established that the amount of viral shedding depends on host's immunity, affected host species, the dose and infectivity of the challenge virus, the type and dose of ND vaccine used and the duration between vaccination and challenge [27, 28]. Usage of vaccines that match the field genotype significantly have been reported to reduce viral shedding compared to mismatched vaccines [29, 30]. The other

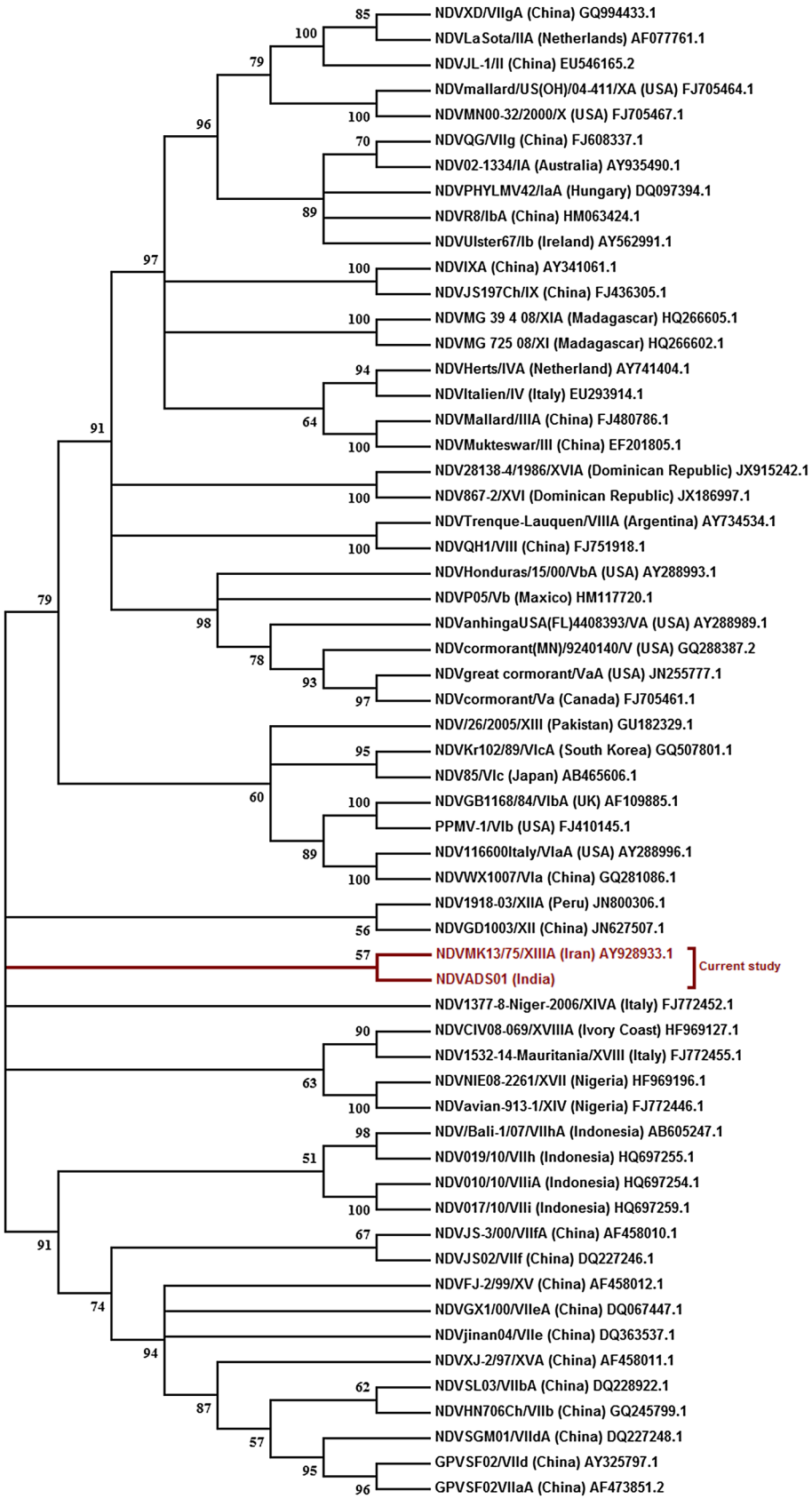

Fig. 5 The phylogenetic analysis of the sequenced partial Fusion protein gene showing the resemblance of ADS01 isolate with genotype NDV XIIIA

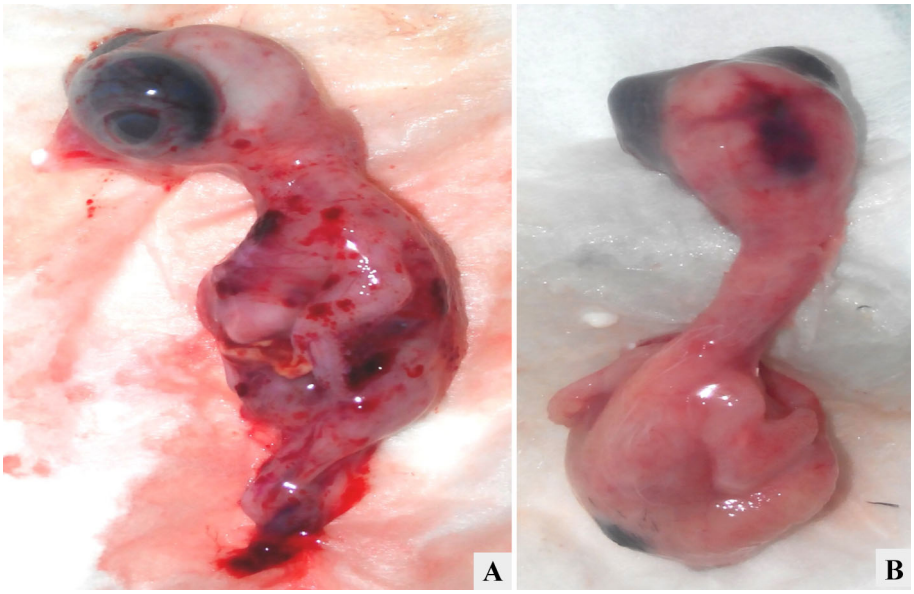

Fig. 6 **a** Embryos from embryonated eggs infected with tissue triturate showing petechial to ecchymotic hemorrhages throughout the body surface; **b** Typical hemorrhage in the occipital area of the infected embryo

reason for disease outbreak in vaccinated flock may be due to inadequate protection conferred by the present vaccine strain to the vaccinated flocks, and few researchers also described that it is need of the hour for newer vaccination strategies against ND [4, 31, 32]. Hu et al. [29] and Miller et al. [30] claimed that matched vaccines generated from field isolates provide enhanced protection to the chickens and reduced the viral shedding in turn reducing the transmission to the uninfected flock. LaSota vaccinated commercial broilers showed varying protection levels against two strains viz., AF2240-I (genotype VIII) and IBS002 (genotype VII) during challenge studies. But, the inactivated genotype VII NDV recombinant vaccine conferred full protection against both NDV isolates along with reduction in virus shedding [33], proving the effectiveness of matched genotype vaccines in conferring full protection.

On contrary to the above findings, Bwala et al. [34] and Liu et al. [35] showed that genotype II vaccine (LaSota) conferred 100% cross-protection against heterologous viruses of genotypes V, VIb, VIg, VIId and IX, thus excluding the role of genotype-matched vaccines in ND outbreak. Dortmans et al. [36] proved that administration of LaSota strain protected chickens when challenged with a velogenic virus (NL/93, genotype VII) completely without any clinical disease and absence of viral shedding. The adapted vaccine from NL/93 strain yielded similar results compared to the LaSota based vaccine. The authors concluded that the currently practiced routes for vaccine application like aerosol (spraying) or oral administration via drinking water do not guarantee that every bird in the flock receive an optimum vaccine dose, which is the reason behind vaccination failure. Susta et al. [37] reported that LaSota vaccine strain successfully protected chickens against the genotype VII and XVII NDV strain with non-significant virus shedding. Efficacy of vaccines was not affected even after repeated challenge with virulent NDV [38].

Thus it is very clear that apart from vaccination, other electrifying factors that predispose for vaccine failure include live vaccine virus being neutralized by the maternal antibodies, vaccines being inappropriately handled and administered thus causing inactivation of the live vaccine virus, and infection with other agents like infectious bursal disease virus, inclusion body hepatitis, Marek's disease and mycotoxins [14, 39].

As reported, vaccine failure may be because of inadequate application procedures of vaccines and vaccine mismatch. Thus, as India poultry population is endemic to ND, it will be very appropriate to incorporate local virus strain along with including genotype II vaccine in the vaccination regime and by following appropriate application procedures, which would enhance post-vaccinal immunity, antibody specificity, prevention of clinical disease and mortality, and reduce viral shedding from vaccinated birds. By following such matched vaccine practices and adopting strict bio-security measures, several deadly infectious poultry diseases can be contained in a better way thus preventing morbidity and mortality in the poultry flocks and in turn enhancing the productivity of the birds.

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