B-4443
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RESEARCH ARTICLE Indian Journal of Animal Research

Isolation and Molecular Characterization of Newcastle Disease Virus in Layers

Smita Bordoloi, Anju Nayak, A.P. Singh[1], R.V. Singh[2], Kajal Jadav, Amita Dubey[3], Joycee Jogi, Poonam Shakya, K. Himani[4]

10.18805/IJAR.B-4443

ABSTRACT

Background: Newcastle disease (ND) in spite of the availability of vaccines remains a constant threat to poultry producers worldwide. It is prevalent in Indian subcontinent and leads to economic losses. The present study was aimed with isolate and identify virulent Newcastle disease virus (NDV) in layer poultry from field outbreaks.

Methods: Total 47 samples consisting of nasal (05), oropharyngeal (13) and cloacal swabs (11) and tissue samples consisting of trachea (07), lungs (06), larynx (05) were collected from layer birds. For isolation of NDV swab and tissue samples were inoculated in 9-11 days old embryonated eggs via allantoic cavity route. After preparing the viral inoculum, 47 suspected samples (29 swab and 18 tissue samples) were inoculated in 141 embryonated eggs to isolate the virus.

Result: Out of 47 samples 10 (21.27%) samples were positive for HA activity. All the 10 isolates showing HA activity subjected to Reverse-Transcriptase PCR of F gene and 6 were found positive in RT-PCR for F1 gene. The PCR amplified product showed amplicon at 356 bp and 254 bp positive for F1 and F2 gene, respectively. On basis of F gene, 06 (50%) isolates were considered as virulent Newcastle Disease Virus. One isolate sequence was submitted at NCBI with accession MT890653 On phylogenetic analysis MT890653 designated as Class II/ genotype II/ virulent strain and had the motif ^{112}R-R-R-K-R-F^{117} at the cleavage site of the fusion protein.

Key words: Cleavage site, F gene, Layer, NDV, Phylogenetic analysis, Virulent.

INTRODUCTION

Newcastle disease (ND) or Ranikhet disease is a highly contagious viral disease that affects over 250 species of birds of all age groups. It is caused by Newcastle disease virus (NDV) which is a linear, non-segmented single stranded, enveloped, negative sense RNA virus belonging to the order Mononegavirales, family Paramyxoviridae and genus Avulavirus are contained in one serotype and are also known as avian paramyxovirus serotype-1 (APMV-1). RNA genome predicted to have three genome lengths 15,186; 15,192 and 15,198 nucleotides and encodes six genes in the order of 32 -NP-P-M-F-HN-L-52 coding for the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), an attachment protein, the haemagglutinin-neuraminidase (HN) and a large polymerase protein (L) (Kapczynski *et al.,* 2013). NDV differs in virulence and has been grouped into 5 pathotypes: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric. ND is mostly (Alexander, 2003). In India according to OIE (2015) a total of 6,93,840 cases with 198 outbreaks were recorded. While, higher seroprevalence of ND in Kuroilers that is 81.4% (Ghosh *et al.,*2017) was reported in India. The most important component of controlling ND is the need to monitor flock immune response after vaccination. Consequently, the World Organisation for Animal Health (OIE) has included it among the list of diseases that require immediate notification upon recognition (OIE, 2012). Present study aimed on isolate and identify the virulent Newcastle disease virus from layer birds. A total of 47 samples of layers from field outbreaks, consisting of 29 swab samples *viz*. nasal, oropharyngeal and cloacal swabs and 18 tissue samples *viz*. lung pieces, trachea and larynxfro Jabalpur district of Madhya Pradesh were incorporated in this study.

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur.
[1]Animal Biotechnology Centre, Jabalpur,College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur.
[2]Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur
[3]Department of Veterinary Pathology, College of Veterinary science and Animal Husbandry, NDVSU, Jabalpur.
[4]Indian Veterinary Research Institute, Bareilly- 243 122, Uttar Pradesh, India.

Corresponding Author: Smita Bordoloi, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur-482 001, Madhya Pradesh, India.
Email: smita.smitabordoloi.bordoloi@gmail.com

How to cite this article: Bordoloi, S., Nayak, A., Singh, A.P., Singh, R.V., Jadav, K., Dubey, A., Jogi, J., Shakya, P. and Himani, K. (2021). Isolation and Molecular Characterization of Newcastle Disease Virus in Layers. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4443.

Submitted: 08-03-2021 **Accepted:** 24-04-2021 **Online:** 27-05-2021

MATERIALS AND METHODS

Location of work

The work was conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh.

Duration of work

The study was conducted for 2 years from June 2018 to June 2020.

Haemagglutination Test and determination of 4HA unit virus for HI test

0.025 ml of PBS was dispensed into each well of a plastic V-bottomed microtitre plate. 0.025 ml of the allantoic fluid was placed in the first well. Two fold dilutions of 0.025 ml volumes of the virus suspension were made across the plate. A further 0.025 ml of PBS was dispensed to each well. 0.025 ml of 1% (v/v) chicken RBCs was dispensed to each well. The solution was mixed by tapping the plate gently. The RBCs were allowed to settle for about 40 minutes at room temperature. HA was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs.

Haemagglutination Inhibition (HI) Test (OIE, 2012)

0.025ml of PBS was dispensed into each well of a plastic V-bottomed microtitre plate. 0.025 ml of serum was placed into the first well of the plate. Two fold dilutions of 0.025 ml volumes of the serum was done across the plate. 4 HAU virus/ antigen in 0.025 ml were added to each well and the plate was left for a minimum of 30 minutes at room temperature. 0.025 ml of 1% (v/v) chicken RBCs was added to each well and after gentle mixing, the RBCs was allowed to settle for about 40 minutes at room temperature. The HI was the highest dilution of serum causing complete inhibition of 4 HAU of antigen.

Isolation of Newcastle Disease Virus in embryonated eggs (OIE, 2012 and Alexander and Senne, 2008)

For isolation of virus suspected samples were processed to prepare viral inoculums treated with antibiotics and lastly inoculums were filtered with 0.22µm filter before inoculation in embryo. A volume of 0.2 ml of supernatant was injected into the nine day old embryonated chicken eggs in triplicate by allantoic cavity route and was incubated at 37ºC for 7 days. Candling was performed daily and observed the mortality for 7 days. Allantoic fluid was harvested and subjected to HA test for presence of virus. Determination of thermostable property of Newcastle disease virus was done by exposing infected allantoic fluid to 56°C in water bath for 10, 15, 30 and 60 min. The titre and viability of the virus was detected by HA test. The organs (lungs, intestine, spleen, caecal tonsils and proventriculus) were collected at the time of post mortem examination. The inoculated embryos were examined for any gross abnormal changes.

RNA extraction and cDNA synthesis

The total RNA extraction from allantoic fluid, swab or tissue samples was done by using TRIZOL reagent (Sambrook and Russell, 2001) with some minor modification and quantified in Nanodrop OD at 260. The cDNA synthesis was done using PrimeScript™1st strand cDNAsynthesis kit (Takara) as per manufacturer's instructions.

Reverse transcriptase polymerase chain reaction (RTPCR)

RT-PCR was carried out using NDV genome-specific primers for Fusion protein gene using two (F1 and F2) primer (F5 'GCAGCTCGAGGGATTGTGGT3' & R5'TCTTTGAGCAGG AGGATGTTG3' for F1 (F5'CCTTGGTGACTCTATCCGCAG 3' & R5'CTGCCACTGCTAGTTGTGATAATCC 3' for F2); (Mohamed *et al*., 2005 and Guan *et al*., 2001).

Amplification was performed usingDreamTaq™ Green PCR Master mix 12.5 µl; 2 µl each of primers (10 pmol), 2 µl cDNA (2 µg/20 µl) and nuclease free water up to 25 µl in PCR tubes.

Cycling conditions were set as shown in Table 01. The amplified products were checked for the presence of the desired bands on 1.2% agarose gel.

Sequencing of PCR product

The RT-PCR products of one isolate (L/JBP/OS-3) was subjected to nucleotide sequencing through outsourcing at Eurofins Genomics India Pvt Ltd., Bangalore by Sanger sequencing method using forward and reverse primers for F1 gene.

Phylogenetic analysis

The sequence of F1 genes of Newcastle disease virus was analyzed for evolutionary lineages among themselves and

Table 1: Reaction protocol of PCR cycle for F Gene.

Step	For F1 Gene		For F2 Gene	
	Temperature	Time	Temperature	Time
Initial Denaturation	94°C	4 min	94°C	3 min
Denaturation	94°C	2 min	94°C	1 min
Primer annealing	60°C	2 min	57°C	1 min
Extension	72°C	2 min	72°C	1 min
Final Extension	72°C	5 min	72°C	Hold

The reaction was carried out for 35 cycles.

with various Indian and World isolates of NDV. The sequences were analyzed using BLAST (Basic Local Alignment Search Tool) and the Clustal-W (CLUSTAL 2.1 multiple sequence alignment) and Molecular Evolutionary Genetic Analysis (MEGA X) version was used for construction of phylogenetic tree.

Analysis of cleavage site of F gene

The sequence data were compiled with the EditSeq Module of Molecular Evolutionary Genetic Analysis (MEGA X) software. Nucleotide and deduced amino acid sequences of the F gene corresponding to the N terminus of the fusion protein (amino acid residues1-118) of NDV were aligned using the Clustal-W (CLUSTAL 2.1 multiple sequence alignment) of MEGA X software. The nucleotide sequence data of the fusion protein gene used in this study were obtained from GenBank database (Gen Bank, 2004).

RESULTS AND DISCUSSION

Isolation of virus in embryonated chicken eggs from swab and tissue samples of layers

Overall from live bird 04 HA positive sample, 03 samples HA titre was equal to or higher than 2^4 while 01 samplesshowed 2^3 titre. All the nasal swabs samples of layer birds showed no HA activity. 3 HA positive oropharyngeal swab samples showed higher HA titre, equal to or higher than 2^4 that is 1:16 or above. Similarly, from one HA positive cloacal swabs samples of layer birds HA titre was 2^6 as shown in Table 2 Fig 1.

A total of 18 tissue samples *viz*. 06 lung, 07 trachea, 05 larynx were collected at the time of necropsy from layer birds, which were clinically showing drowsiness and respiratory signs and at necropsy haemorrhage in proventriculus, congestion in larynx, trachea and lung were suspected for Newcastle disease virus infection. All the 18 samples were inoculated in 54 embryonated eggs in triplicates *via* allantoic cavity route. Embryonated eggs that showed mortality after 24 hours of incubation and presence of haemorrhagic lesion were selected for harvesting of allantoic fluid and HA test. All HA positive (03) NDV suspected samples were having HA titre equal to or higher than 2^3. While, from 05 lyranx sample mortality was recorded in 07 embryonated eggs and 02 sample allontoic fluids showed HA activity. From both HA positive larynx samples of layer birds the HA titre was equal to or higher than 2^4. From 07 trachea sample mortality was recorded in 06 embryonated eggs and 01 samples allontoic fluid showed HA activity. Whereas, from 02 NDV suspected trachea samples of layer, embryonic mortality was recorded but the HA activity was not determined. From tissues overall NDV isolation rate in layer birds was 33.33% (6/18). Individually from tissues, larynx 40% (2/5), trachea 14.28% (1/7) and lungs 50% (3/6) samples were suspected for Newcastle disease virus as shown in Table 02. A total of 47 suspected samples (29 swab and 18 tissue samples) were inoculated in 141 embryonated eggs to isolate the virus. The overall isolation of virus from layer poultry was 10/47 (21.27%).

Gowthaman *et al.,* 2013 isolated NDV form all the pooled samples from natural outbreak of Newcastle disease in turkeys, Japanese quails and chicken in a multi–species poultry farm in northern India and they found mean HA titer varied between 4 (2^2) to 8(2^3) in chickens, whereas it remained high in turkeys and quails and varied between 32(2^5) to 1024 (2^{10}). Balachandran *et al*. (2014) isolated and characterized Newcastle disease virus from vaccinated commercial layer chicken and the incidence of ND was most commonly noticed in 20-50 week of age and between the months of September to November. Kianizadeh *et al*. (1999) recovered 12 isolates of NDV from different outbreaks in Iran and found ICPI varying from 1.7 to 1.96 and described them as characteristic velogenic NDV. Leow *et al*. (2011) reported outbreaks during extreme hot months of April to June in layer flocks. In the present study the occurrence was seen in winter months also.

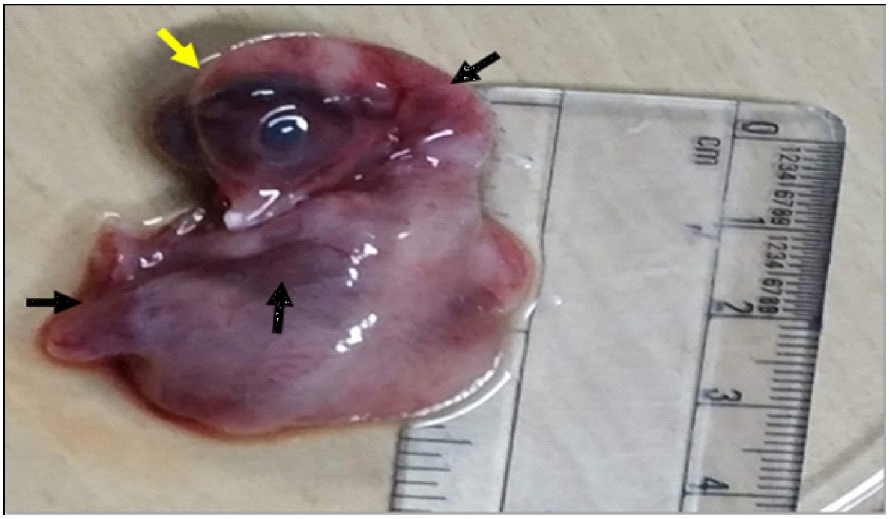

Fig 1: Lesions on embryo inoculated with L/JBP/OS-3 isolate of NDV showing diffuse haemorrhages around head, leg and body (black arrow) along with edema of head (yellow arrow).

Table 2: Isolation of Newcastle disease virus in embryonated chicken eggs from swab and tissue samples in layer.

Type of sample	Clinical findings	Post inoculation embryo mortality in days						Embryo mortality	Lesion	HA
		2	3	4	5	6	7	after 24 hrs		titre
Nasal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	0	0/3	0	0
Nasal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	0	0/3	0	0
Nasal swab	Drowsiness and respiratory signs	0	0	0	0	2	0	2/3	present	-
Nasal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	2	0	2/3	present	-
Nasal swab	Drowsiness and respiratory signs	0	0	0	0	1	0	1/3	present	-
Oropharyngeal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	1	2	3/3	present	2^3
Oropharyngeal swab	Drowsiness and respiratory signs	0	0	0	0	0	2	2/3	present	-
Oropharyngeal swab	Drowsiness and respiratory signs	0	0	0	0	0	2	2/3	present	-
Oropharyngeal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	0	0/3	-	-
Oropharyngeal swab	Drowsiness, respiratory signs and diarrhea	0	0	1	0	0	1	2/3	present	-
Oropharyngeal swab	Drowsiness and inappetance	0	0	1	0	0	0	1/3	absent	-
Oropharyngeal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Oropharyngeal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Oropharyngeal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Oropharyngeal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Oropharyngeal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	1	2	3/3	present	2^5
Oropharyngeal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	3	3/3	present	2^4
Oropharyngeal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	0	0/3		
Cloacal swab	Drowsiness, respiratory signs and diarrhea	0	0	1	1	0	1	3/3	present	2^6
Cloacal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3		
Cloacal swab	Drowsiness and respiratory signs	0	0	1	0	0	0	1/3	present	-
Cloacal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3		
Cloacal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	1	1	2/3	present	-
Cloacal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3		
Cloacal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	1	0	1/3	present	-
Cloacal swab	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Cloacal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	2	0	2/3	present	-
Cloacal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	1	1/3	present	-
Cloacal swab	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	0	0/3	-	-
Lungs	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Lungs	Drowsiness and respiratory signs	0	0	0	1	1	1	3/3	present	2^4
Lungs	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Lungs	Drowsiness and inappetance	0	0	0	0	1	2	3/3	present	2^5
Lungs	Drowsiness and respiratory signs	0	0	2	0	0	1	3/3	present	2^3
Lungs	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Trachea	Drowsiness, respiratory signs and diarrhoea	0	0	0	0	0	0	0/3	-	-
Trachea	Drowsiness and respiratory signs	0	0	0	0	0	2	2/3	present	-
Trachea	Drowsiness and respiratory signs	0	0	0	0	0	1	1/3	absent	-
Trachea	Drowsiness, respiratory signs and diarrhoea	0	0	0	0	0	0	0/3	-	-
Trachea	Drowsiness and inappetance	0	0	0	0	0	0	0/3	-	-
Trachea	Drowsiness and inappetance	0	0	0	0	2	1	3/3	present	2^5
Trachea	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Larynx	Drowsiness and respiratory signs	0	0	0	0	0	2	2/3	present	2^5
Larynx	Drowsiness and respiratory signs	0	0	0	0	0	3	3/3	present	2^4
Larynx	Drowsiness and respiratory signs	0	0	0	0	0	0	0/3	-	-
Larynx	Drowsiness, respiratory signs and diarrhea	0	0	0	0	0	0	0/3	-	-
Larynx	Drowsiness and respiratory signs	0	0	0	0	1	1	2/3	present	-

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) of F gene

The F glycoprotein is responsible for fusion between the cellular and viral membranes and subsequent virus genome penetration and infectivity. The F gene of 10 HA positive NDV isolates were amplified by RT-PCR using NDV-F1 and F2 primers with the expected band size of 356 and 254, respectively. Out of 10 isolates 08 isolates were showing positive for molecular identification. For Fusion protein gene of Newcastle disease virus F1, F2, 06 and 04 isolates were positive showing amplification band at 356bp and 254 bp, respectively (Table 3, Fig 2 and 3).

The conserved amino acid sequence "RRQKR" of F gene segment can be amplified by the primer F1 and F2 indicating that the isolates were velogenic NDV (Aldous and

Table 3: Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) of different NDV isolates.

Isolates	F1 gene	F2 gene
L/JBP/OS-1	+	-
L/JBP/OS-2	+	-
L/JBP/OS-3	+	-
L/JBP/CS-4	+	+
L/JBP/LP-5	-	+
L/JBP/LP-6	+	+
L/JBP/TP-7	-	+
L/JBP/LP-8	+	-
L/JBP/LP-9	-	-
L/JBP/LP-10	-	-
Percent positive	**06/10 (60%)**	**04/10 (40%)**

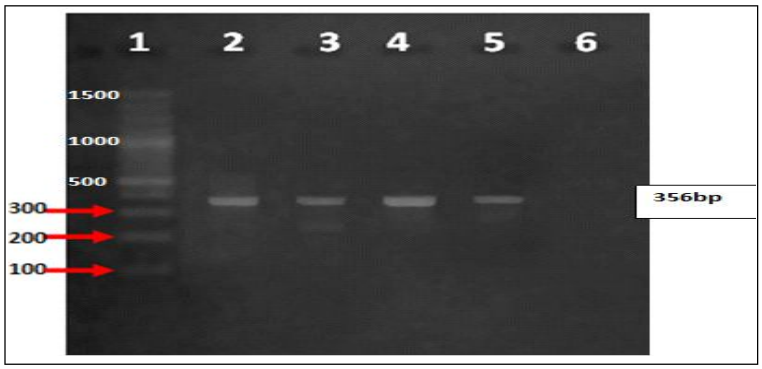

Fig 2: Agarose gel electrophoresis of F1 gene showing PCR amplification product of 356 base pair

Lane 1: 100bp ladder

Lane 2, 3, 4 and 5 Positive Sample

Lane 6: Negative Control

Table 4: Results of nucleotide blast search for MT890653 sequence (L/JBP/OS-3) similarity in the GenBank.

Isolates	GenBank Accession No	Country of origin	% Identity with MT890653
chicken/NDV/India/IVRI/Bareilly/08/2018	MK544897	India	99.35
chicken/NDV/India/IVRI/Ambala/05/2017	MK544895	India	99.35
chicken/NDV/India/NVC/Nagpur/03/2017	MK544892	India	99.35
Amar	MT362718	India	99.35
AAvV-1	MT036311	India	99.35
NDV/Pelican/Telineelapuram/2018	MN901912	India	99.35
chicken/NDV/India/NVC/Nagpur/04/2017	MK544891	India	99.02
NDV/F/17/RSP-1	MH577763	India	99.02
PDDSL-7	MF362988	India	99.02
410/16A,	MF422129	India	99.02
KKD	MT362717	India	99.02
Eluru	MT362714	India	99.02
DV_E/PUNE/MH/IND/2018/F	MK213740	India	98.69
248-13A2	MF422128	India	98.69
248-13A2	MF422128	India	98.69
Hyd	MT362719	India	98.69
VPP5	MT362715	India	98.69
AAvV-1	MT178234	India	98.69
96-15	MF422125	India	98.05
D2/Hyd/2018	MT740246	India	98.04

Alexander, 2001) whereas LaSota being a lentogenic strain having an amino acid sequence as "GRQGRL" (Nanthakumar *et al*., 2000) could not be amplified with this primer pair.

Guan *et al*. (2001) subjected field isolate to RT-PCR of the fusion (F) gene amplified by gene specific primers into 254 bp product except in case of LaSota vaccine. The F glycoprotein is responsible for fusion between the cellular and viral membranes and subsequent virus genome penetration and infectivity. The primer F1 and F2 could amplify the conserved amino acid sequence "^{112} RRQKR ^{116}" of F gene segment in viral genome indicating that the isolates were velogenic NDV (Aldous and Alexander, 2001) whereas, LaSota being a lentogenic strain having an amino acid sequence as "GRQGRL" (Nanthakumar *et al*., 2000b) could not be amplified with this primer pair. Mohammed *et al*. (2013) isolated viral RNA from 34 field samples and 26 HA positive allantoic fluid for the detection of NDV genome by RTPCR using NDV specific primers and amplified F gene at 387 bp.

Nucleotide sequencing and blast search

The RT-PCR products of two isolates of layers (L/JBP/OS-3) was subjected to nucleotide sequencing through outsourcing at Eurofins Genomics India Pvt Ltd., Bangalore by Sanger sequencing method using forward and reverse primers for F gene. The sequence (MT 890653) were subjected to NCBI blast search.

The results of the blast search revealed that the isolate L/JBP/OS-3 had close similarity with the isolates from India had 99.35%, 99.02%, 98.69%, 98.05% and 98.04% identity (Table 4).

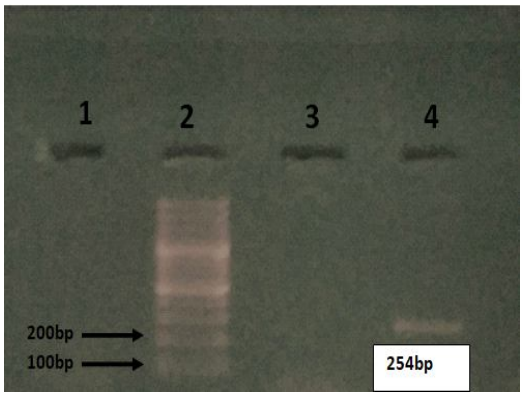

Fig 3: Agarose gel electrophoresis of F2 gene showing PCR amplification product of 254 base pair

Lane 1: 100bp ladder
Lane 2: Negative Sample
Lane 3: Negative Control
Lane 4, 5 Positive Sample

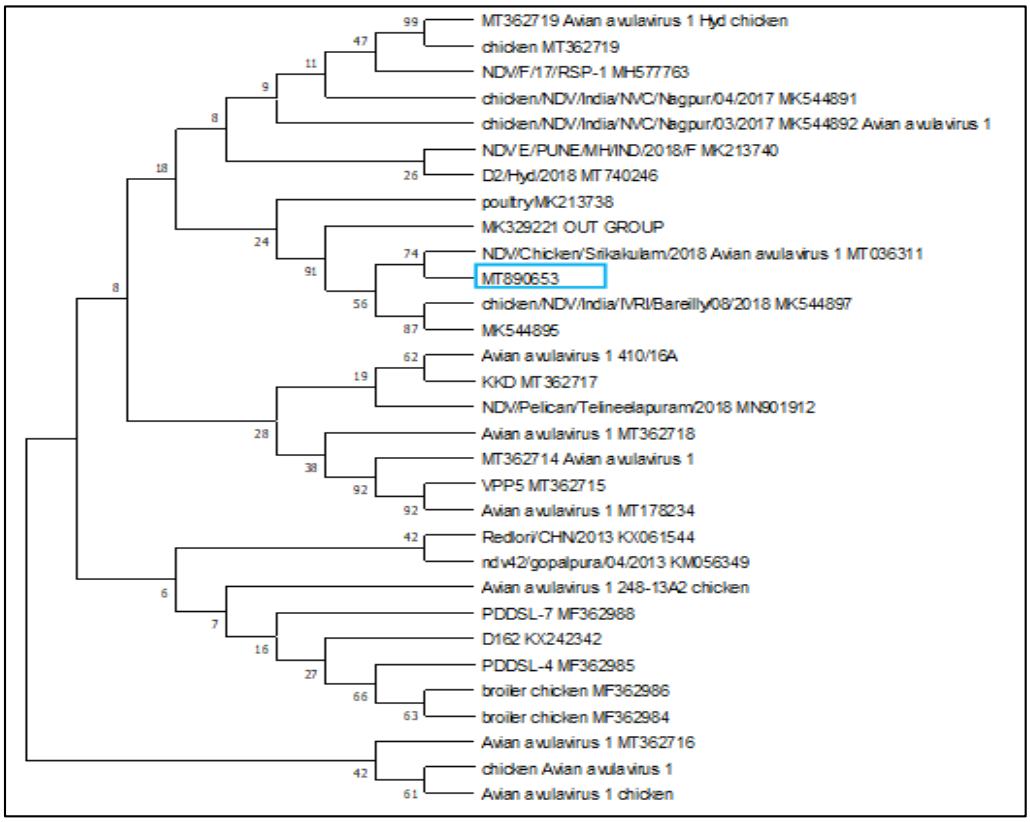

Fig 4: Phylogenetic tree of NDV isolate (L/JBP/OS-3) with other NDV strains was constructed using neighbour joining method with bootstrap values calculated for 1000 replicates.

Multiple alignment of nucleotide sequences and phylogenetic analysis

The coding region of F gene of (L/JBP/OS-3) was aligned with the corresponding region of the F gene of different NDV strains belonging to 18 different genotypes from worldwide, downloaded from the GenBank. A phylogenetic tree was constructed (Fig 4). In the phylogenetic tree, L/JBP/OS-3 isolate clustered with the velogenic strain (Texas GB) under genotype II.

The phylogenetic tree based on the similarity of isolate L/JBP/OS-3 with different isolates worldwide respectively. Sequence analysis of the F protein of isolate L/JBP/OS-3 is 99.11% amino acid identity with that of strain BC (Table 4). Phylogenetic analysis showed that L/JBP/OS-3 isolate closely related with the virulent NDV strain Texas GB and Beaudette C in genotype II of class II viruses, respectively (Fig 5). The findings of present study are resembled with the findings of (Mohamed *et al*., 2011). The F protein cleavage site sequence is a well-characterized determinant of NDV pathogenicity in chickens (Panda *et al*., 2004). Virulent NDV strains typically contain a polybasic cleavage site (R-X-K/R-R↓F), which is recognized by intracellular proteases present in most cell types. The cleavage site of all Egyptian strains contained four basic amino acids at positions 112-116 (^{112}R-R-Q-K-R↓F-I$^{118)}$. The presence of the phenylalanine (F) residue at position 117 also a possible contributor to the neurological effects (Lamb and Parks, 2007).

Analysis of the fusion protein cleavage site

The amino acid sequences of the fusion protein cleavage site (amino acid residue 112 to 118) of different strains belonging to different genotypes are shown in Table 5. The isolate (L/JBP/OS-3) possessed the amino acid sequences ^{112}R-R-R-K-R-F^{117}at F0 cleavage site, which was identical to the motif of velogenic type of NDV; presence of multiple basic amino acid sequence indicates the virulent strain.

Table 5: NDV isolates analyzed in this study showing fusion protein cleavage site.

Accession No./ NDV isolates of different strains/ Pathotype	Country	Host	Genotype	Cleavage Site
M2469 4/ Ulster/67/L	Northern Ireland	Chicken	I	GKQGRL
M24692 / D26/D26/76/L	Japan	Duck	I	GKQGRL
M24693/ V4/66/L	Australia	Chicken	I	GKQGRL
AF077761/ LaSota/46/L	USA	Chicken	II	GRQGRL
M23407 / Texas GB/ 48/ V	USA	Chicken	II	RRRQKF
X04719/Beaudette C/M	USA	Chicken	II	RRQKRF
AB070382 /Sato/30/V	Japan	Chicken	III	RRQRRF
AF224505 / Mukteswar/V	India	Chicken	III	RRQRRF
M24701/Miyadera/ V	Japan	Chicken	III	RRQRRF
M33855 /Texas/V	USA	Chicken	IV	RRQRRF
M24702/ Herts/33 /V	Great Britain	Chicken	IV	RRQRRF
AF001106/ CA1085/71/ V	USA	Chicken	V	RRQKRF
AF001107/ H-10/72/ V	Hungary	Chicken	V	RRQKRF
AF001133/ A-24/96/V	Australia	Chicken	VI	RRQKRF
AJ880277/ IT-227/82 /V	Hungary	Pigeon	VI	GRQKRF
AF001135 /RI-3/8/V	Indonesia	Chicken	VIIa	RRQKRF
AF001126/ E-1/93/V	Hungary	Chicken	VIIa	RRQKRF
AF109884/AE232/96/V	United Arab Emirates	Partridge	VIIb	KRQRRF
AF136779/MZ-48/95/V	Mozam	Chicken	VIIb	RRQKRF
AF083965/ TW/84C/V	Taiwan	Chicken	VIIc	RRQKRF
AF458013/JS-2/98/V	China	Chicken	VIIc	RRQKRF
AF364835/Ch/98-3/V	China	Chicken	VIId	RRQKRF
AB512616/ TW/08-07	Taiwan	Chicken	VIIe	RRQKRF
AF136762/SG-4H/65/V	South Africa	Chicken	VIII	RRQKRF
AF048763/AF2240/60/V	Malaysia	Chicken	VIII	RRQKRF
AY508514/ IX /AF2240/60/ V	China	Chicken	IX	RRQRRF
AF458009/ IX/ FJ-1/85/V	China	Chicken	IX	RRQRRF
AY372163/ X/ TW-C69-10-36/V	Taiwan	Chicken	X	RRQKRF
DQ097393 /DE-R49/99/A	Germany	Duckling	Class I	ERQERL
JX436341/ V	Pakistan	Chicken	XII	GRQGGL
JN800306 / V	Peru	Chicken	XIII	RPRGRL
HF969179/V	Africa	Chicken	XVIII	APEPRL
FJ772463 /XVI /V	Burkina Faso	Chicken	XVI	-
JF966386 / XIVb / V	France	Chicken	XIVb	RRRKRF
AF458014 /XV /V	China	Chicken	XV	-

There are no changes in the single nucleotides of representative amino acids; hence no mutation occurred (Fig 3). The sequence was submitted at NCBI with accession MT890653. On phylogenetic analysis MT890653 was designated as Class II/ genotype II/ velogenic strain (Fig 6).

The amino acid sequence at fusion protein cleavage site is a major determinant of NDV virulence (Millar *et al*., 1986). Presence of multiple amino acids at neucleotide position from 112 to 117 indicates the velogenic strains (Vegad and Katiyar, 2017). Virulent NDV strains typically contain a polybasic cleavage site (R-X-K/R-R↓F), which is recognized by intracellular proteases present in most cell types. In a study Mohamed *et al*. (2011) found the cleavage site of all Egyptian strains contained four basic amino acids at positions 112-116 (^{112}R-R-Q-K-R↓F-I^{118}). In addition, the presence of the phenylalanine (F) residue at position 117

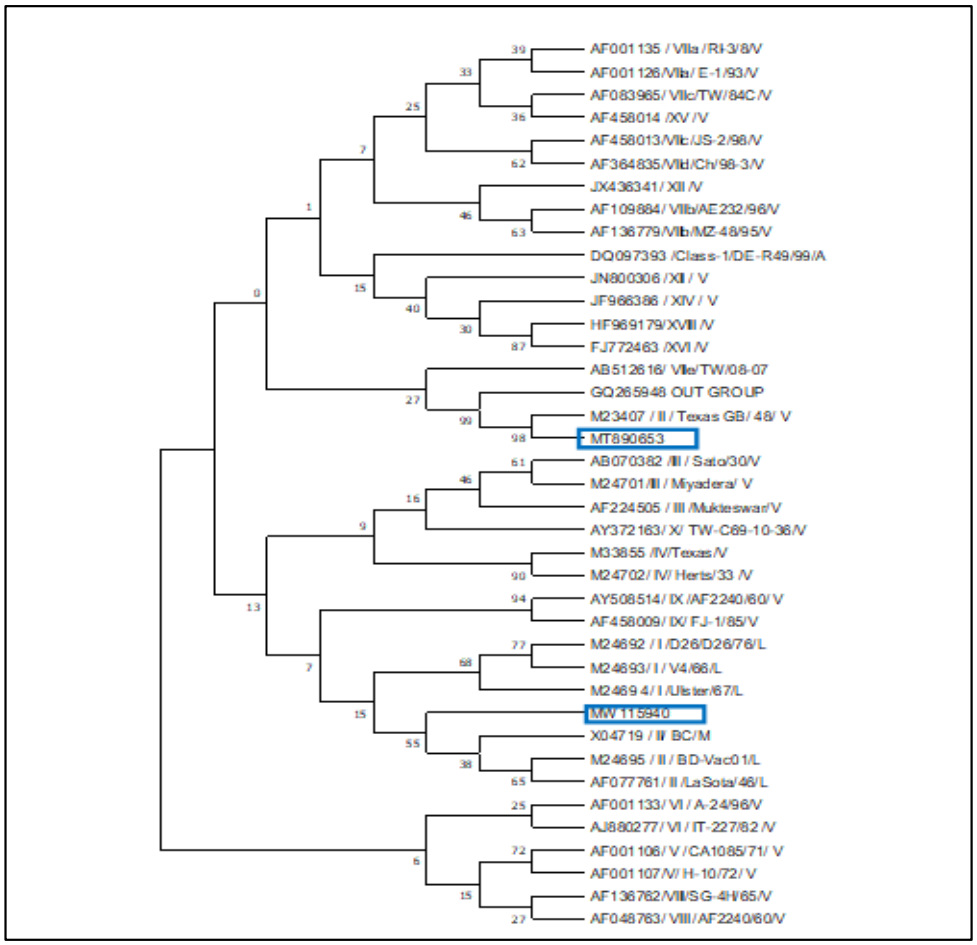

Fig 5: Phylogenetic tree of NDV isolate with 18 NDV genotype 35 strains was constructed using neighbour joining method with bootstrap values calculated for 1000 replicates.

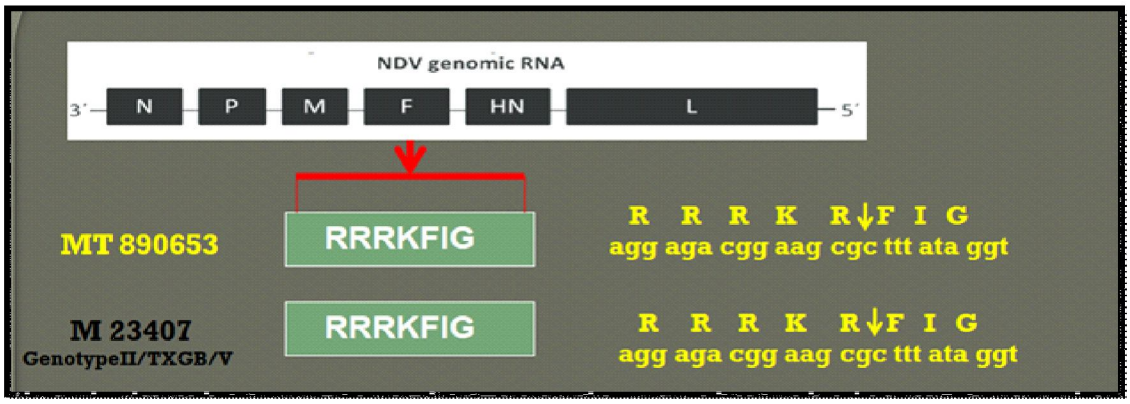

Fig 6: Cleavage site of F gene of NDV isolate with reference strains.

has been described as being a possible contributor to the neurological effects (Lamb and Parks, 2007).

CONCLUSION

The study concluded that conventionally 10/47 (21.27%) were positive for NDV and molecular identification showing 8/10 (80%) positive for F gene. On basis of F gene, 06 (50%) isolates were considered as velogenic Newcastle Disease Virus and 02(20%) isolates may have mixed infection can be velogenic/mesogenic/lentogenic. One isolate sequence is submitted at NCBI with accession MT890653 On phylogenetic analysis MT890653 designated as Class II/ genotype II/ velogenic strain and had the motif ^{112}R-R-R-K-R-F^{117} at the cleavage site of the fusion protein, which was typical of velogenic NDV isolates.

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