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Molecular Characterization of Class II Newcastle Disease Virus from Field Outbreaks in Mizoram, India

Shamana Kochiganti[1], Tridib Kumar Rajkhowa[1], J. Kiran[1], Doris Zodinpui[1], Farhin Aktar Choudhury[1]

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ABSTRACT

Background: Newcastle disease (ND) is a highly contagious and economically important disease affecting poultry of all ages. The causative agent, class II NDV strains are frequently virulent and are classified into at least 21 distinct genotypes with several sub-genotypes. The circulating strains of NDV from mainland India are identified as genotype XIII, whereas emergence of a new genotype XXII has been reported recently from North East Region of India.

Methods: A total of 25 poultry farms comprising a total population of 2165 birds were studied for field outbreaks of ND. Detailed post-mortem examination was conducted on a total of 121 dead birds and field outbreaks were confirmed by the detection of the F gene in tissue samples by reverse transcription PCR. Deduced amino acid sequence analysis of fusion proteins and phylogenetic analysis based on complete F gene were carried out to understand the molecular epidemiology of the circulating NDV strains.

Result: This study has confirmed severe NDV outbreaks in the vaccinated flock, from Mizoram state of India. The three isolates from the outbreaks revealed the presence of the multi-basic amino acid residues at the fusion protein cleavage site (112RRQKRF117) identified as characteristic of velogenic strain. The phylogenetic analysis based on the complete F gene has characterized the isolates belonging to newly identified genotype XXII and subgroup XXII.2. The evolutionary evidence of this new genotype of NDV in the unique ecosystem of NER, India, warrants detailed studies for better understanding of the variant.

Key words: Chicken, F-gene, Genotype XXII, NDV, Phylogenetic analysis.

INTRODUCTION

Newcastle disease is one of the highly contagious infectious diseases of poultry because of its worldwide distribution and the potential for devastating economic losses. It occurs in at least six of the seven continents of the world and is enzootic in many countries including India. The disease is caused by *Avian Paramyxovirus I* (APMV-1) or Newcastle disease viruses (NDV) under the genus *orthoavulavirus* subfamily *Avulavirinae* of the family *Paramyxoviridae* (ICTV, 2019). The virus is a negative sense, non-segmented and enveloped with a single-stranded RNA genome of approximately 15.2 kb (Cao *et al*., 2013; Zhang *et al*., 2012). The NDV genome is composed of six genes that encode their corresponding six structural proteins: Nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and the RNA polymerase (L) (Chambers *et al*., 1986). RNA editing of the P protein produces additional non-structural proteins V and possibly W (Chambers and Samson, 1982; Collins *et al*., 1982; Locke *et al*., 2000; Mebatsion *et al*., 2001; Steward *et al*., 1993). The F glycoprotein which is responsible for the viral particles to fuse with the host cellular membrane is postulated as the main determinant of the viral pathogenicity (Gotoh *et al*., 1992; Ogasawara *et al*., 1992). The World Organisation for Animal Health (OIE) has defined the virulent strains of NDV that have an intracerebral pathogenicity index of 0.7 or higher (2.0 is maximum) or a fusion cleavage site starting from amino acid position 112 with multiple basic amino acids and phenylalanine at position 117 (OIE, 2012).

Based on the consensus amino acid sequences at F gene, NDV is divided into class I NDV and class II NDV strains. The class I NDV strains are grouped into a single genotype and 3 sub-genotypes and class II NDV strains, divided into at least 21 distinct genotypes (I-XXI) made up of several sub-genotypes (Dimitrov *et al*., 2019). NDV viruses of class I are mainly composed of avirulent strains, usually circulating among wild birds, whereas viruses of class II are more frequently virulent (Miller *et al*., 2010) and mainly isolated from poultry.

Class II NDV are also categorised into five pathotypes based on clinical signs in infected chickens and are

[1]Department of Veterinary Pathology, College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl-796 014, Mizoram, India.

Corresponding Author: Tridib Kumar Rajkhowa, Department of Veterinary Pathology, College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl-796 014, Mizoram, India.
Email: tridibraj09@gmail.com

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designated: a) viscerotropic velogenic (VVND), b) neurotropic velogenic (NVND), c) mesogenic, d) lentogenic or respiratory and e) aymptomatic. Keeping in view the divergence of the circulating viral strains and their transboundary emergence, molecular characterization studies are essential in order to keep track of the evolution and genetic diversity of the viral strains. In the present study, we have recorded severe field outbreaks of NDV, isolated the circulating strain and characterized them based on complete F gene analysis.

MATERIALS AND METHODS

Study areas and sample collection

The outbreak of ND was investigated in both backyard and commercial poultry farms located in Aizawl districts of Mizoram during the period of 2021 to 2022. A total of 25 poultry farms comprising a total population of 2165 birds with complaints of mortality associated with respiratory disease, severe depression and greenish-to-whitish diarrhoea were studied. The affected birds were clinically examined at the farm premises. A detailed post-mortem examination was conducted on a total of 121 dead birds from affected farms and the gross lesions were recorded. Representative tissue samples comprising of trachea, lungs, proventriculus, spleen and caecal tonsils were collected in 10% buffered formalin for histopathological examination and also preserved at -80°c for molecular diagnosis. Formalin-fixed tissues were subjected to histopathological processing and staining following standard method (Bancroft and Gamble, 2008). Hematoxylin and Eosin-stained individual sections were microscopically examined and the histopathological changes were recorded. The entire work was carried out in the Department of Veterinary Pathology, C. V. Sc. and A. H., CAU(I), Selesih, Aizawl, Mizoram.

Isolation of newcastle disease virus

The RT-PCR positive tissue samples were used to prepare the inoculums for isolation of the NDV field strains. The prepared inoculums (0.2 ml/egg) were inoculated in 9-10 days-old embryonated eggs through allantoic route (Alexander and Senne, 2008) and incubated at 37°C and regularly candled for mortality of the embryos. The embryos that showed mortality within 36-48 hours post inoculation were kept for brief freezing at 4°C for 2 hrs and the allantoic fluid was harvested. The propagation of the NDV was confirmed by the haemagglutination inhibition test (HA-HI) and also by detection of F gene of NDV (Qin *et al*., 2008).

RNA extraction from clinical samples and molecular detection of NDV by reverse transcription-PCR (RT-PCR)

The total RNA was extracted from tissue samples (trachea, lungs, proventriculus, spleen and caecal tonsils) by using Trizol method (Chomczynski and Sacchi, 2006). Reverse transcription of RNA into cDNA was carried out by using cDNA synthesis kit (Fermentas Life Sciences, Canada) according to the manufacturer's protocols. All the tissue samples were tested by RT-PCR for detection of the complete F gene of NDV by using the forward primer 5′-ATGGGCTCCAAACCTTCTAC-3′ and reverse primer 5′-TTGTAGTGGCTCTCATC-3′ (Qin *et al*., 2008). The general conditions for PCR were 95°C for 5 min, 32 cycles of 95°C for 1 min (denaturation), 52°C for 1 min (annealing) and 72°C for 2 min (extension), followed by 72°C for 10 min (final extension). PCR products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide. To rule out any co-infections with other respiratory diseases viruses, all the samples were also tested for infectious bronchitis virus (IBV) (Ji *et al*., 2011) and infectious laryngotracheitis (ILT) (Kirkpatrick *et al*., 2006).

Nucleotide sequencing and analysis

The 1662 bp products of complete F gene of Class II NDV amplified by RT-PCR were purified by using Thermo Scientific GeneJet Gel Extraction kit and cloned into pTZ57R/T vector using InsT/Aclone™ PCR product cloning kit (Fermentas Life Sciences, Canada). The recombinant plasmids containing the F gene were sequenced in DNA sequencing facility at Delhi University, South campus.

Phylogenetic analysis of complete fusion glycoprotein

The phylogenetic analyses of nucleotide sequences from complete F gene of three field isolates were performed as recommended by the recently updated unified phylogenetic classification system for NDV (Dimitrov *et al*., 2019). The dataset comprised 142 reference sequences (Table 1) spanning all 21 distinct genotypes of class II NDV (Dimitrov *et al*., 2019; Rajkhowa *et al*., 2023). The maximum-likelihood and Bayesian methods were used to construct the phylogenetic tree. The sequences were aligned using Muscle (MEGA7) and TrimAl (PhyloSuite v1.2.2) to remove spurious sequences or poorly aligned regions from a multiple sequence alignment (Zhang *et al*., 2020). The phylogenetic tree was inferred by Maximum-likelihood trees based on general time-reversible (GTR) model Tavaré (1986), were constructed by using RaxML version 8.2.11 (Stamatakis, 2014) with 1000 bootstrap replicates. Trees were visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Deduced amino acid sequence analysis of fusion proteins of the three field isolates were compared with three vaccine strains Mukteshwar, LaSota and B1 using MEGA7 (Kumar *et al*., 2016). Amino acid residues in hyper-variable region, fusion protein cleavage site, neutralizing epitopes, heptad repeat regions (HRb, HRc) and transmembrane domains of F protein sequences were compared.

RESULTS AND DISCUSSION

Three distinct field outbreaks of NDV were confirmed from different commercial broiler farms located at Selesih, Zemabawk and Melthum areas in Aizawl district of Mizoram, during the study period from December 2021 to December 2022. In all three farms' birds were vaccinated with LaSota strain (primary dose) and R2B strain of NDV (booster dose).

The affected birds showed respiratory disease characterized by severe depression, cyanosis of combs and wattles, conjunctivitis and nasal discharges, coughing and respiratory rales accompanied by greenish diarrhoea. Detailed post-mortem examination of dead birds revealed petechial haemorrhages at the tip of proventricular glands, haemorrhagic ulceration on the caecal tonsils, severely congested or haemorrhagic trachea and lungs and enlarged, mottled spleen (Fig 1a to 1c). Microscopical examination consistently showed haemorrhagic tracheitis with sloughed-off mucosa, pneumonia characterized by severe congestion, oedema, haemorrhages and mononuclear infiltration in the parabronciolar parenchyma; lymphoid depletion with haemorrhages in spleen and caecal tonsils and haemorrhagic proventriculitis (Fig 1e and 1f). The outbreaks were confirmed by detection of the complete F gene of class II NDV in representative tissue samples (Spleen, Caecal tonsils, lungs and trachea).

Three field strains of the NDV were successfully isolated in 9-10 days old embryonated eggs and the NDV propagation was confirmed by the RT-PCR and HA-HI test. The embryos in the inoculated eggs died between 36-48 hours and showed dwarfing, curling and haemorrhages throughout the body surfaces (Fig 1d).

The amplified 1662 bp product encompassing the complete F gene of NDV was sequenced, analyzed, submitted to GenBank and obtained the accession no. OQ427364, OQ427365 and OQ427366 (Table 1). The deduced amino acid analysis revealed presence of multi-basic amino acid residues at the fusion protein cleavage

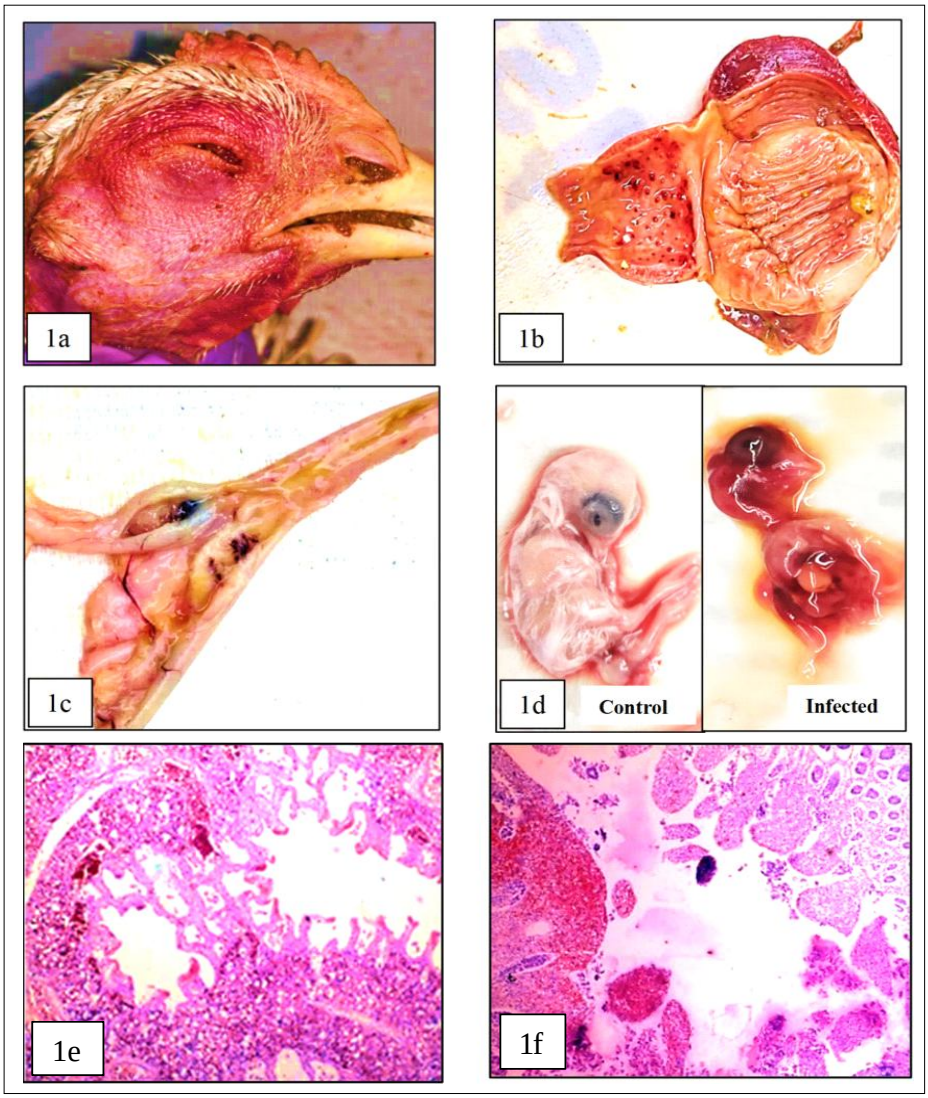

Fig 1: 1a) Conjunctivitis and congestion of comb and wattle, 1b) Patecheal haemorrhages at the tip of proventricular glands. 1c) Haemorrhagic and ulcerative caecal tonsils, 1d) Curling, subcutaneous oedema and haemorrhages in infected chicken embryo. 1e) Congestion, haemorrhages and oedema in parenchyma of parabronchi (H and E, 100X), 1f) Necrosis and haemorrhages in the lamina propria of caecal tonsil (H and E, 100X).

Table 1: The field strains (highlighted in bold) along with the reference NDV strains used for this study.

Isolate	Year	Country	Host	Accession number	Genotypes (Dimitrov *et al.*, 2019)
NDV/MZ01/IND/23	2023	Mizoram, India	Chicken	OQ427364	XXII. 2
NDV/MZ02/IND/23	2023	Mizoram, India	Chicken	OQ427365	XXII. 2
NDV/MZ04/IND/23	2023	Mizoram, India	Chicken	OQ427366	XXII. 2
2_1334	2002	Australia	Chicken	AY935490	I.1.1
99_868_hi	1999	Australia	Chicken	AY935495	I.1.1
Queensland	1966	Australia	Chicken	M24693	I.1.1
NJ_A_101_1383	2001	USA	Redknot	EF564816	I.1.2.1
BHG/Sweden/94	1994	Sweden	Black_headed_gull	GQ918280	I.1.2.1
Tyva_14	2014	Russia	Gull	KX352834	I.1.2.1
Ishi	1962	Japan	Chicken	AB465607	I.1.2.2
AK_44500_136	2009	USA	Northern_pintail	KC503476	I.1.2.2
Nikita_530_FFNK2	2008	Russia	Redpoll	KC503479	I.1.2.2
FarEast_2713	2001	Russia	Duck	AY965079	I.2
NIE08_121	2008	Nigeria	Spur_winged_goose	HG326605	I.2
AK_44493_716	2009	USA	American_green_winged_teal	KC503453	I.2
Lasota	1946	USA	Chicken	AF077761	II
TX_GB	1948	USA	Chicken	GU978777	II
Hitchner_B1	1947	USA	Chicken	JN872151	II
Mukteswar	1940	India	Avian	EF201805	III
SPVC_Karachi_1	1974	Pakistan	Chicken	GU182327	III
Novo_Selo_1161	1995	Bulgaria	Pigeon	MH996904	III
Herts	1933	UK	Fowl	AY741404	IV
Plovdiv_1153	1959	Bulgaria	Pullet	MH996900	IV
Coast_8278	1982	USA	Parrot	JN872189	V.1
498109_15	2007	Honduras	Chicken	JN872194	V.1
95066_9	2001	Nicaragua	Fighting_cock	JN942027	V.1
Distrito_Federal_462	2004	Mexico	Dove	EU518682	V.2
Estado_de_Mexico_466	2006	Mexico	Chicken	EU518684	V.2
NC04_635	2010	Mexico	Chicken	JQ697744	V.2
PA_810	2008	USA	Pigeon	JX901367	VI.2.1.1.1
NJ_721	2007	USA	Pigeon	JX901351	VI.2.1.1.1
TX_1185_kidney_26981_3_A	2015	USA	ECDO	MG018211	VI.2.1.1.1
sms12	2012	China	Pigeon	JX094510	VI.2.1.1.2.1
248_	1998	Belgium	Pigeon	JX901110	VI.2.1.1.2.1
LHLJ_110813	2011	China	Pigeon	JX486553	VI.2.1.1.2.1
SH_167	2013	China	Pigeon	KT163262	VI.2.1.1.2.2
11_09620	2011	Belgium	Pigeon	JX901124	VI.2.1.1.2.2
Ningxia_2068	2016	China	Pigeon	MG840654.1	VI.2.1.1.2.2
GB1168	1984	Great_Britain	Domestic_fowl	AF109885	VI.1
NY	1984	USA	Pigeon	FJ410145	VI.1
S_1	2002	China	Pigeon	FJ865434	VI.1
PG_JS_1	2005	China	Pigeon	FJ480825	VI.2.2.2
100	2008	China	Pigeon	JX244794	VI.2.2.2
LJS_1	2004	China	Pigeon	KJ607163	VI.2.2.2
TX_209682	2002	USA	Waterfowl	JN872180	VI.2.2.1
12339	1998	USA	Pigeon	JN872182	VI.2.2.1
101	2001	USA	Pigeon	JX901312	VI.2.2.1
NIE09_1898	2009	Nigeria	Pigeon	HG326604	VI.2.1.2
B2_Isiolo	2012	Kenya	Laughing_dove	JX518532	VI.2.1.2

Table 1: Continue....

Table 1: Continue....

NIE13_92	2013	Nigeria	Pigeon	HG424627	VI.2.1.2
98_Guizhou	1998	China	Pheasant	EF589133	VII.1.1
Shandong_Pyan	2004	China	Chicken	EF579733	VII.1.1
Ibaraki_SG106	1999	Japan	Chicken	AB853927	VII.1.1
Liaoning_1_2009	2009	China	Chicken	KC542905	VII.1.1
Behshahr	2015	Iran	Chicken	KX268351	VII.1.1
A7	1996	China	Fowl	AY028995	VII.1.2
18	2003	China	Pigeon	GQ338309	VII.1.2
Jiangsu_JS02	1999	China	Goose	DQ227246	VII.1.2
RBWW_3	2013	South_Africa	Chicken	MF622047	VII.2
Karachi_AW_1	2014	Pakistan	Parakeet	KU862293	VII.2
Banjarmasin_10	2010	Indonesia	Chicken	HQ697254	VII.2
5620	2016	Namibia	Chicken	KY747479	VII.2
152608_ancestral	1993	Netherlands	Chicken	JN986837	VII.2
Trenque_Lauquen	1970	Argentina	Chicken	AY734534	VIII
QH1	1979	China	Chicken	FJ751918	VIII
AF2240	1960	Malaysia		JX012096	VIII
FJ_1	1985	China	Chicken	AF458009	IX
ZJ_1	1986	China	Chicken	FJ436303	IX
F48E8	1948	China	Chicken	FJ436302	IX
TX_130	2011	USA	Mottled_duck	FJ705468	X
ndv42_AI09_4117	2009	USA	Redhead	KX857716	X
99_376	1999		Mallard	FJ705466	X
MN_AI10_3434	2010	USA	Mallard	KX857721	X
MG_725	2008	Madagascar	Chicken	HQ266602	XI
MGMNJ	2009	Madagascar	Chicken	JX518882	XI
MGS1595T	2011	Madagascar	Chicken	JX518884	XI
Apurimac_50009	2005	Peru	Chicken	KU594615	XII.1
Lurin_40871	2004	Peru	Gamecock	KU594616	XII.1
Arequipa_VFAR_81	2015	Peru	Chicken	KU594618	XII.1
GD_12	2011	China	Goose	JN627504	XII.2
GD_1003	2010	China	Goose	JN627507	XII.2
FS_SS_292	2013	China	Goose	MF278927	XII.2
45445_3	1995	South_Africa	Ostrich	JN942034	XIII.1.1
47385_11	2010	Tanzania	roller	JN942043	XIII.1.1
Chiwoko	2015	Zambia	Chicken	MF409241	XIII.1.1
SPVC_Karachi_43	2008	Pakistan	Chicken	GU182323	XIII.2.1
SPVC_Karachi_33_	2007	Pakistan	Chicken	GU182331	XIII.2.1
University_Diagnostic_Lab_12	2010	Pakistan	Chicken	KF113338	XIII.2.1
ndv42_gopalpura_4	2013	India	Chicken	KM056349	XIII.2.2
Polashbari	2014	India	Chicken	KT734767	XIII.2.2
Nagpur_3	2011	India	Chicken	KX372707	XIII.2.2
EMM_7	2011	Iran	Chicken	JQ267579	XIII.1.2
EMM_2	2008	Iran	Chicken	JQ267584	XIII.1.2
EMM_1	2008	Iran	Chicken	JQ267585	XIII.1.2
NIE09_2071	2009	Nigeria	Turkey	HF969205	XIV.1
VIR_1377_7	2006	Niger	Chicken	JN872165	XIV.1
VRD08_36	2008	Nigeria	Chicken	JQ039386	XIV.1
NIE08_453	2008	Nigeria	Chicken	HF969187	XIV.2
NIE10_139	2011	Nigeria	Chicken	HF969210	XIV.2
KD_TW_03T_N45_720	2009	Nigeria	Chicken	KY171990	XIV.2

Table 1: Continue....

Table 1: Continue....

28138_4	1986	Dominican_Republic	Chicken	JX915242	XVI
Queretaro_452_1947	1947	Mexico	Chicken	JX915243	XVI
867	2008	Dominican_Republic	Chicken	JX186997	XVI
NIE10_310	2011	Nigeria	Chicken	HF969176	XVII
NIE08_2042	2009	Nigeria	Chicken	HF969191	XVII
NIE08_2199	2009	Nigeria	Chicken	HF969194	XVII
1532_14	2006	Mauritania		FJ772455	XVIII.1
ML038	2007	Mali	Guinea_fowl	JF966389	XVIII.1
ML57051T	2010	Mali	Chicken	JX518885	XVIII.1
CIV08_42	2007	Ivory_Coast	Chicken	HF969218	XVIII.2
CIV08_32	2006	Ivory_Coast	Village_weaver	HG326600	XVIII.2
ML57072T	2010	Mali	Chicken	JX518886	XVIII.2
MN_92_40140	1992	USA	Cormorant	FJ705456	XIX
WI_272409	2003	USA	Cormorant	JN942024	XIX
FL_41105	2012	USA	Cormorant	KC433530	XIX
Ibaraki_SM87	1987	Japan	Chicken	AB853928	XX
ZhJ_2	1986	China	Chicken	AF458016	XX
88_M	1988	Korea	Quail	KY042142	XX
ETHMG1C	2011	Ethiopia	Chicken	KC205479	XXI
11RS98_102VIR	2011	Italy	Dove	JN638234	XXI.2
10VIR7155	2010	Italy	Turtle_dove	KU377533	XXI.2
12VIR1876_1	2012	Italy	Turtle_dove	KU377535	XXI.2
Lahore_AW_2	2015	Pakistan	Pigeon	KU862298	XXI.1.2
22A	2015	Pakistan	Pigeon	KY042135	XXI.1.2
Jallo_Lahore_221B	2016	Pakistan	Pigeon	KY042141	XXI.1.2
Vladimir_687	2005	Russia	Pigeon	JF824032	XXI.1.1
Lahore_125	2015	Pakistan	Pigeon	KY042136	XXI.1.1
73_OP_G29	2015	Egypt	Pigeon	KY042132	XXI.1.1
M4	2012	Bangladesh	Chicken	MK934291	XXII. 1
BD_C161	2010	Bangladesh	Chicken	MK934289	XXII. 1
F-16	2012	Bangladesh	Chicken	MK934292	XXII. 1
G3	2012	Bangladesh	Chicken	MK934293	XXII. 1
Sak008	2017	Bangladesh	Chicken	MK934295	XXII. 1
Sak084	2017	Bangladesh	Chicken	MK934296	XXII. 1
ND/MZ/IND/1	2016	Mizoram, India	Chicken	KY828155	XXII. 1
ND/MZ/IND/2	2016	Mizoram, India	Chicken	KY828156	XXII. 1
ND/MZ/IND/3	2016	Mizoram, India	Chicken	KY828157	XXII. 1
ND/MZ/IND/4	2016	Mizoram, India	Chicken	KY828158	XXII.2
ND/MZ/IND/5	2016	Mizoram, India	Chicken	KY828159	XXII.2
ND/MZ/IND/6	2016	Mizoram, India	Chicken	KY828160	XXII.2
ND/MZ/IND/7	2016	Mizoram, India	Chicken	KY828161	XXII.2
ND/SKM/IND/8	2019	Sikkim, India	Chicken	OK149201	XIII.2.2
ND/SKM/IND/9	2019	Sikkim, India	Chicken	OK149202	XXII. 1
ND/MZ/IND/10	2020	Mizoram, India	Chicken	OK149203	XXII. 1

site between 112 and 116 and phenylalanine at position 117 (^{112}RRQKRF^{117}) were observed in all three field isolates confirming the isolates as velogenic strain (Table 2) (OIE, 2012). The comparison of amino acid sequences of fusion proteins of the three isolates with the vaccine strains Mukteshwar, LaSota and B1 showed 91.29% to 91.65%, 89.11% to 89.47%, 88.93% to 89.29% sequence identity respectively. Also, a comparison with genotype XIII strains that are recorded as circulating strains in mainland India, showed 89.87% to 93.67% sequence homology with the three field isolates. Although all three isolates showed similarity with the newly identified XXII genotype strains earlier identified from NER, India, two mutations at the positions N9V and P10S were observed in the

Table 2: Comparison of F protein amino acid changes at the hyper-variable region, fusion protein cleavage site and neutralizing epitopes.

Strain	Hypervariable region																				Fusion protein cleavage site						Neutralizing epitope		
Positions	3	4	5	8	9	10	11	13	14	16	17	19	20	22	24	25	26	27	28	29	30	112	113	114	115	116	117	72 to 79	157 to 171	**343**
B1	S	R	P	K	N	P	A	M	M	T	I	V	A	V	S	C	I	C	P	A	N	G	R	Q	G	R	L	D K E A C A K A	S I A A T N E A V H E V T D G	**L**
Lasota	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	- - - - - - - -	- - - - - - - - - - - - - - -	-
Mukteshwar	P	-	S	R	I	-	V	L	-	-	-	I	T	A	-	Y	V	R	L	T	S	R	-	-	R	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
ND MZ IND 1	-	K	-	R	I	-	I	L	L	I	-	-	M	I	-	-	V	R	Q	T	S	R	-	-	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
ND MZ IND 2	-	K	-	R	I	-	I	L	L	I	-	-	M	I	G	-	V	R	Q	T	S	R	-	-	K	-	F	- - - - - - - T	- - - - - - - - - - - - - - N	-
ND MZ IND 3	-	K	-	R	I	-	I	L	L	I	-	-	M	I	G	-	V	R	Q	T	S	R	-	-	K	-	F	- - - - - - - T	- - - - - - - - - - - - - - -	-
ND MZ IND 4	-	K	-	R	I	-	I	L	-	I	T	-	M	I	G	-	V	-	Q	T	S	R	-	-	K	-	F	- - - - - - - T	- - - - - - - - - - - - - - -	-
ND MZ IND 5	-	K	-	R	I	-	I	L	-	I	T	-	M	I	G	-	V	-	Q	T	S	R	-	-	K	-	F	- - - - - - - T	- - - - - - - - - - - - - - -	-
ND MZ IND 6	-	K	-	R	I	-	I	L	-	I	T	-	M	I	G	-	V	-	Q	T	S	R	-	-	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
ND MZ IND 7	-	K	-	R	I	-	I	L	R	I	T	-	M	I	-	-	V	-	Q	T	S	R	-	-	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - N	-
ND SKM IND 8	-	K	-	R	I	-	I	L	L	I	T	-	M	I	-	-	-	-	L	T	-	R	-	R	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
ND SKM IND 9	-	K	-	R	I	-	I	L	L	I	-	-	M	I	-	-	V	R	Q	T	S	R	-	-	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
ND SKM IND 10	-	K	-	R	I	-	I	L	L	I	-	-	M	I	-	-	V	R	Q	T	S	R	-	-	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
NDV MZ01 IND 23	-	K	-	R	V	S	I	L	L	I	-	-	M	I	-	-	V	R	Q	T	S	R	-	-	K	-	F	- - - - - - - -	- - - - - - - - - - - - - - -	-
NDV MZ02 IND 23	-	K	-	R	V	S	I	L	L	I	-	-	M	I	-	-	V	R	Q	T	S	R	-	-	K	-	F			-
NDV MZ04 IND 23	-	K	-	R	V	S	I	L	L	I	-	-	M	I	-	-	V	R	Q	T	S	R	-	-	K	-	F			-

Amino acids matching with that of Mukteshwar strain are highlighted with blue and amino acids variations with the genotype XXII variant strains are highlighted with yellow.

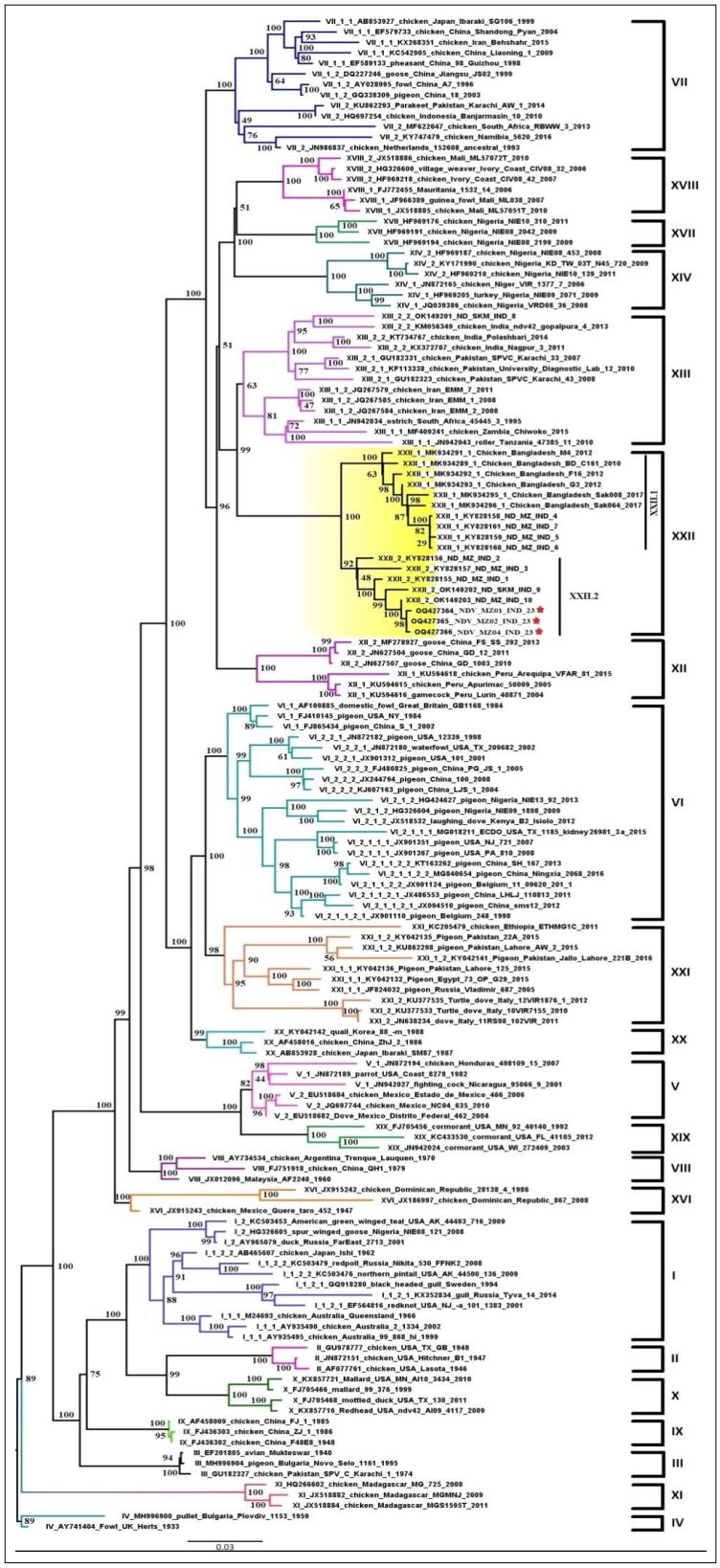

Fig 2: Phylogenetic analysis based on full-length nucleotide sequences of the fusion gene of three field isolates from Mizoram state of India (marked with) with selected isolates representing all class II newcastle disease virus sub/genotypes (n=142).

hypervariable region (Table 2). No distinct variations were observed in the neutralizing epitope, heptad repeat regions HRa, HRb, HRc and transmembrane domains.

The phylogenetic analysis based on the complete F gene was carried out as per the recommendation put forth by the recently updated unified phylogenetic classification system for NDV (Dimitrov *et al*., 2019). The generated phylogenetic tree has depicted close grouping of all the three isolates with newly identified class II NDV genotype XXII and subgroup XXII.2 (Fig 2).

Our study has recorded outbreaks of ND that have resulted 90-100% mortality in the three affected commercial broiler farms. The outbreaks occurred despite the vaccination against the disease with LaSota strain followed by a booster dose with R2B strain. Consistent observation of haemorrhagic proventriculitis, enteritis, ulcerative and haemorrhagic caecal tonsils accompanied with haemorrhagic tracheitis and pneumonia have strongly suggested that the circulating strains are velogenic NDV (vvNDV). The outbreaks were confirmed by detection of complete F gene of NDV by RT-PCR assay (Qin *et al*., 2008). Further, the field strains were also isolated in 9-10 days old embryonated eggs.

The presence of multi-basic amino acid residues at the fusion protein cleavage site between 112 and 116 and phenylalanine at position 117 (^{112}RRQKRF^{117}) (Gowthaman *et al.* 2019; OIE, 2012) in all three field isolates have further confirmed the isolates as velogenic strain complementing our pathological findings (Table 2). The comparison of amino acid sequences of the entire fusion proteins of the three isolates with the vaccine strains Mukteshwar, LaSota and B1 revealed only 88.93% to 91.29% sequence identity suggesting independent evolution of the circulating NDV strains in Mizoram, India. Deduced amino acid analysis of all three isolates showed similarity with the newly identified XXII genotype strains earlier identified from NER, India, with additional two mutations at the positions N9V and P10S in the hypervariable region (Table 2).

The updated unified phylogenetic classification system for NDV (Dimitrov *et al*., 2019) has classified the class II NDV into at least 20 distinct genotypes (I to XXI). Following this classification, the field isolates from NER, India and Bangladesh were classified into a new genotype and designated as XXII with two subgroups XXII.1 and XXII.2 (Rajkhowa *et al*., 2023), while the NDV strains from rest of the mainland India were identified as genotype XIII (sub-genotype XIII.2.2.). Earlier studies (Khorajiya *et al*., 2015; Das and Kumar, 2017; Gowthaman *et al*., 2019; Mariappan *et al*., 2018) have also identified the NDV currently circulating and causing field outbreaks of ND from rest of the mainland India, as the genotype XIII. To understand the relation between the three field isolates, with all the 21 distinct genotypes of class II NDV strains, we have carried out the phylogenetic analysis based on the complete F gene (Dimitrov *et al*., 2019; Rajkhowa *et al*., 2023). The generated phylogenetic tree has clearly characterized the three field isolates into the newly identified genotype XXII and subgroup XXII.2 of class II NDV.

CONCLUSION

NER, India, stands at high risk for transmission of transboundary diseases like ND, as it shares its boundaries with neighbouring countries namely China, Bhutan, Nepal, Myanmar and Bangladesh. The region lies at the junction of the Eastern Himalayas and the Indo-Burma biodiversity hotspot that provides a unique ecosystem for evolution and emergence of pathogens. Therefore, continuous surveillance and monitoring of infectious pathogens of livestock and birds in the region is of paramount interest for the control and prevention strategy of such diseases. The present study on field outbreaks of ND in vaccinated flock of birds has recorded severe clinical disease with high morbidity and mortality. The circulating field strains have been identified as velogenic strains and characterized in the genotype XXII, subgroup XXII.2 of class II NDV. The evolutionary evidence of a new genotype of NDV in the ecosystem of NER, India, warrants detailed studies on the whole genome of the identified genotype as well as animal experimentation to identify the possibility of neutralization escape variants.

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Ethics approval statement

No live animals were harmed during the present study conducted at any point of time.

Conflict of Interest

The authors declare no conflict of interest.

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