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Rapid Communication

Sequence and phylogenetic analysis of virulent Newcastle disease virus isolates from Pakistan during 2009–2013 reveals circulation of new sub genotype

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ABSTRACT

Despite observing the standard bio-security measures at commercial poultry farms and extensive use of Newcastle disease vaccines, a new genotype VII-f of Newcastle disease virus (NDV) got introduced in Pakistan during 2011. In this regard 300 ND outbreaks recorded so far have resulted into huge losses of approximately USD 200 million during 2011–2013. A total of 33 NDV isolates recovered during 2009–2013 throughout Pakistan were characterized biologically and phylogenetically. The phylogenetic analysis revealed a new velogenic sub genotype VII-f circulating in commercial and domestic poultry along with the earlier reported sub genotype VII-b. Partial sequencing of Fusion gene revealed two types of cleavage site motifs; lentogenic ^{112}GRQGRL^{117} and velogenic ^{112}RRQKRF^{117} along with some point mutations indicative of genetic diversity. We report here a new sub genotype of virulent NDV circulating in commercial and backyard poultry in Pakistan and provide evidence for the possible genetic diversity which may be causing new NDV out breaks.

Introduction

Newcastle disease (ND) is a highly contagious viral disease of birds and has been regarded as one of the most important diseases of poultry and other birds throughout the world (Aldous and Alexander, 2001) in which infection with the highly virulent viruses may result in high mortality, with varied clinical signs depending upon the virus strain, host immune status, co-infection and environmental conditions. Outbreaks of ND frequently result in severe economic losses in poultry. It is caused by virulent ND virus (NDV). This enveloped virus has a negative-sense single-stranded RNA genome of approximately 15 kb, which code for six major proteins, including nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin-neuraminidase (HN) protein and RNA polymerase (L) (Lamb and Kolakofsky, 1996). Two of the interactive surface glycoproteins, the fusion (F) and the hemagglutinin neuraminidase (HN) proteins, are involved in cell surface attachment and cell membrane fusion. Commonly, the F-protein cleavage site sequence is considered as the primary molecular determinant of NDV virulence (Glickman et al., 1988). However, previous work has also demonstrated that NDV strains which carry exactly the same F-protein cleavage site had significant differences in their virulence (Diel et al., 2012; Kim et al., 2007). Furthermore, in addition to F-protein, the HN-protein also contributes to virulence (de Leeuw et al., 2005). Based on genome size, the NDVs are grouped into class I (I genotype) and class II (XV genotypes). However, a new genotype XVI has been recently identified to be included in class II (Courtney et al., 2013). Despite routine vaccinations and strict biosecurity measures, sporadic outbreaks of virulent form of ND occur occasionally in different parts of the Pakistan, primarily affecting backyard poultry. However, the number of outbreaks involving commercial poultry dramatically increased during 2011–2012. Most of the damages were recorded in younger flocks, especially the commercial broiler flocks were hit badly. No change in vaccine type or vaccination strategy was helpful in controlling this disease. Extensive use of live and/or killed ND vaccines under different vaccination programs was attempted within short life span of broiler chicken, but no significant respite could be provided to the farmers in this regard. The intensity of outbreaks decreased significantly during early 2012, however, a new wave of ND outbreaks has appeared again since the winter of 2012. As a matter of fact it was found that the maximum antibody titers obtained after repeated shots at the age of 3 weeks of chicks were around 4.5 $\log_2$, which were not sufficient to provide protection upon subsequent NDV exposure. However, flocks having HI antibody titers of more than

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Table 1
NDV Isolate IDs and genbank accession numbers.

Sequence ID	Accession number	ICPI	MDT	Fusion gene cleavage site
NDV-CK-Pakistan- NARC-12N-607–2012	KC811804	Not done	Not done	^{112}RRQKRF^{117}
NDV-BYP-Pakistan- NARC-12N-558–2012	KC811805	1.85	36	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-28865-2011	KC811806	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-28863-2011	KC811807	1.80	33	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-28826-2011	KC811808	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-28859-2011	KC811809	1.712	49	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-28789-2012	KC811810	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-12N126-2012	KC811811	1.7	42	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-28991-2012	KC811812	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-12N80-2012	KC811813	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-12N148-2012	KC811814	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-19225-2010	KC811815	1.5	58	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-12N153-2012	KC811816	Not done	Not done	^{112}RRQKRF^{117}
NDV-Peacock-Pakistan- NARC-12N564-2012	KC811817	1.6	45	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-22757-2010	KC811818	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-22452-2010	KC811819	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-11432-2009	KC811820	1.65	50	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-15193-2009	KC811821	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-15986-2009	KC811822	1.7	42	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-12N149-2012	KC811823	0.35	106	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-12N171-2012	KC811824	Not done	Not done	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-24653-2011	KC811825	Not done	Not done	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-22003-2010	KC811826	0.375	108	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-12N596-2012	KC811827	Not done	Not done	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-19887-2010	KC811828	Not done	Not done	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-12N202-2012	KC811829	Not done	Not done	^{112}GRQGRL^{117}
NDV-CK-Pakistan- NARC-12N152-2012	KC811830	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan-NARC-13N94-2013	KC811831	1.85	36	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-13N81-2013	KC811832	Not done	Not done	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-13N39-2013	KC811833	1.9	38	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-13N33-2013	KC811834	1.72	53	^{112}RRQKRF^{117}
NDV-CK-Pakistan- NARC-13N20-2013	KC811835	1.75	52	^{112}RRQKRF^{117}
NDV-BYP-Pakistan- NARC-13N07-2013	KC811836	1.737	46	^{112}RRQKRF^{117}

MDT: mean death time, ICPI: in**tracerebral pathogenicity index.**

6 $\log_2$ were reported to better withstand the virus exposure (data not shown). This prompted us to investigate the mutation in new isolates which could have increased viral pathogenicity among the evolving genotypes. In this regard 33 NDV viruses were selectively sequenced to determine the extent of mutation among these isolates which could possibly explain current increase in their pathogenicity. The partial fusion gene sequences were submitted to Genbank and were assigned accession numbers accordingly [KC811804–KC811836] (Table 1).

Results and discussion

In-vivo pathotyping of 33 NDV isolates earlier recovered from the ND positive flocks during 2009–2013 led to the categorizing of 26 NDV isolates into velogenic type with ICPI and MDT values ranging between 1.5 to 1.9 and 38 to 58 h, respectively whereas 7 NDV isolates belonged to lentogenic type (Table 1). In addition to this 4 isolates from backyard poultry and domestic birds were also found to be of velogenic type, indicating the role of backyard poultry in disease epizootology under the current situation. F-protein gene sequence analysis showed that 26 isolates, including those recovered from backyard poultry and domestic birds had cleavage site ^{112}RRQKRF^{117} which is characteristic of velogenic strains, supporting the in-vivo Pathotyping results. Additionally, 11 isolates had serine at 107 position belonging to virulent sub genotype VII-b while 15 isolates had S107A mutation belonging to the new virulent sub genotype VII-f. Furthermore, 7 isolates had cleavage sites motifs ^{112}GRQGRL^{117} confirming their lentogenic nature (Table 1). On the other hand some point mutations were also observed in the partially sequenced F-protein gene of these Pakistani NDVs.

The phylogenetic analysis of these isolates grouped 26 isolates into virulent genotype VII in class II cluster, while the remaining 7 isolates grouped as lentogenic genotype II (Fig. 1). Velogenic genotype VII isolates were further categorized into two sub genotypes, whereas 11 isolates clustered along previously reported Pakistani sub genotype VII-b (Munir et al., 2012). Fifteen isolates clustered tightly (99.8–100% sequence homology) with recently reported new Indonesian sub genotype VII-f (Xiao et al., 2012). It is the first report of new sub genotype VII-f of NDV circulating in commercial and backyard poultry from Pakistan.

All of these NDV VII-f isolates only showed 87.6–89.7% sequence homology of F-protein gene with previously reported sub genotype VII-b, while 86.0% and 76.4% sequence homology with Mukteswar and vaccinal LaSota strains, respectively. This variation could be one of the possible reasons for altered pathogenicity parameters observed in new sub genotype and subsequent ineffectiveness of currently used NDV vaccines. These findings are also supported by the fact that the new sub genotype VII-f isolates were first time recovered during 2011, which was the time when the frequency of NDV outbreaks increased and peaked in 2012. Though the virulent sub genotype VII-b was also circulating in poultry during 2011–2012, the crucial observation that the sub genotype VII-f was recovered from the poultry flocks with severe respiratory problems, high mortality rates along with different outbreaks pattern than those recorded with sub genotype VII-b cannot be ignored. In conclusion, we first time report the presence of a new NDV sub genotype VII-f circulating in poultry in Pakistan, which has caused enormous losses as compared to other sub genotypes of virulent type VII, earlier prevalent

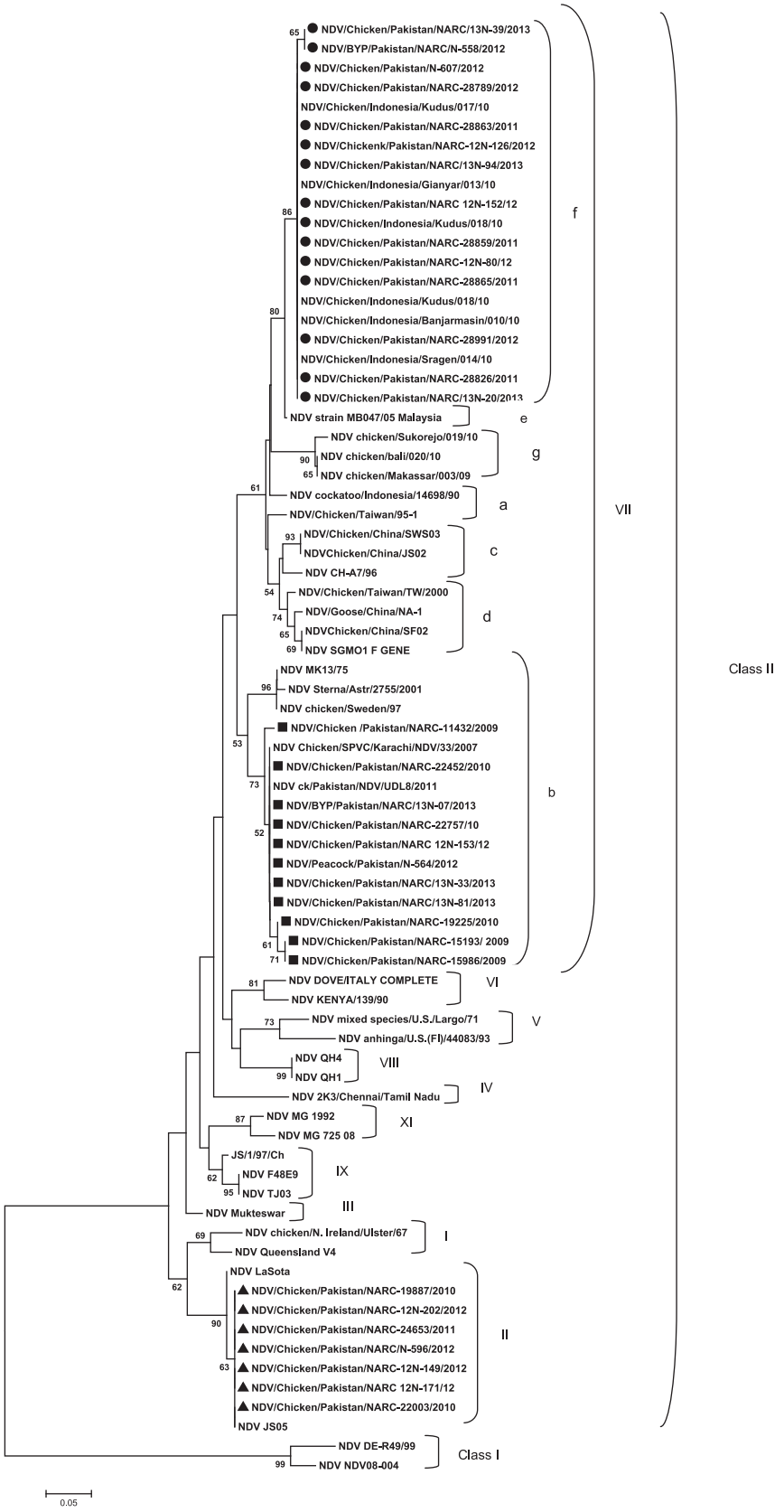

Fig. 1. Phylogenetic tree of NDV genotypes. Phylogenetic tree of the nucleotide sequences of Pakistani NDVs based on the partial sequences of F gene. The nucleotide coding regions tree was generated by neighbor joining method (with maximum composite likelihood) as implemented in MEGA version 4. Numbers at the nodes indicate confidence level of a bootstrap analysis with 1000 replications as a percentage value. Scale bar indicates 0.05 nucleotide substitutions per site. The Pakistani isolates in the current study are marked in dark circles (genotype VII-f), dark squares (genotype VII-b) and dark triangles (genotype II).

in the field since 2009 and caused sporadic outbreak of low intensity. Another important finding of this study is the interaction amongst wild and domestic birds and backyard poultry, therefore serving as a continuous reservoir for evolving of NDVs by recombination and subsequent transmission of the new variants to commercial poultry or backyard poultry itself (Chong et al., 2010). Un-planned and occasional use of ND vaccination in the backyard and domestic poultry appears to be one of the major factors affecting the ND control strategy in commercial poultry in this country. Therefore the strategies for NDV surveillance, disease control, and vaccination need to be re-evaluated for protecting the commercial poultry from ND in the countries considered endemic for the Newcastle disease.

Material and methods

The tissue samples obtained from the suspected morbid birds were processed and inoculated into 9-day embryonated specific-pathogen-free (SPF) eggs for the recovery of causal viruses using standard procedures (Alexander, 1989). The initial characterization of the isolates was performed by using the hemagglutination inhibition test with NDV-specific antisera (Alexander, 1989). The intracerebral pathogenicity index (ICPI) and mean death time (MDT) were subsequently determined as described previously (World Organization for Animal Health, 2004). Viral RNA was extracted using QIAamp viral RNA mini kit according to manufacturer's instructions (QIAGEN). One step RT-PCR was performed on Veriti 96-well thermal cycler (Applied Biosystems) using Superscript ™ One step RT-PCR with Platinum Taq kit (Invitrogen) with previously described degenerative primers specific to fusion (F) protein gene, encompassing proteolytic cleavage site of F0 protein (Panda et al., 2004). Amplicons of the appropriate sizes were subsequently excised and purified using gel extraction kit (QIAquick Gel Extraction Kit, QIAGEN). The purified PCR products were directly used for cycle sequencing reactions using BigDye® Terminator v3.1 kit, ABI. The products of the sequencing reactions were cleaned using PERFORMA® V3 96-Well short plate (Edge Bio) and sequenced in a 4 capillary Applied Biosystems 3130 Genetic Analyzer. The consensus sequence of the nucleotide sequences was generated using DNA Star, Lasergene (Lasergene, V.8.0.2 DNA Star, Madison WI) software. Sequence and phylogenetic analysis were carried out using MEGA-4 software and sequences were submitted to genbank (Table 1).

Author's contribution

NS and KN designed and conceived the research and provided consultation. NS and KN wrote the manuscript. NS and AG edited the manuscript and analyzed the data. NS, FR, SR and AR performed the experiments. MAA and AAM provided consultation, reagents and materials. All authors read and approved the final manuscript.

Competing interests

Authors do not have any competing interests.

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