MOLECULAR CHARACTERIZATION OF VARIOUS NEWCASTLE DISEASE VIRUS IMMUNOGENIC PROTEINS.

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ABSTRACT: Newcastle Disease (ND) is a viral, fatal and contagious disease that causes severe economic losses to poultry industry. It causes respiratory, neurologic and enteric problems in chicken. The current study was intentioned to isolate, identify and characterize Newcastle Disease Virus from 50 samples taken from a number of poultry farms of Punjab. The NDV was isolated by inoculating 9-11 days old embryonated eggs with the suspected samples and was confirmed through Heamagglutination Assay (HA). Out of 50 samples, 40 were positive and then subjected to PCR using cDNA for isolating the Fusion (F) gene and the Haemagglitinin-neuraminidase (HN) gene. Nine positive samples were sent for gene sequencing and then phylogenetic analysis using NCBI BLAST was carried out, which provided relationship among other previously characterized Newcastle disease virus isolates especially the isolates of Genotype VII that is endemic in Pakistan, India China, Indonesia and Egypt. It was concluded that while HN gene showed close relationship with previously detected isolates, F gene isolates although having wide difference among each other yet showed close relationship with previously detected isolates.

Key words: Newcastle disease, Fusion protein, HN protein, Molecular characterization.

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INTRODUCTION

Poultry is one of the important and vibrant sectors in Pakistan's agriculture based economy (Sadiq, 2004), contributing 1.4% in GDP during 2016-2017 (Economic Survey of Pakistan, 2016-2017). But the development of this organized sector is hampered by various infectious and non-infectious diseases that cause substantial economic losses. One of the chief risks to poultry industry is NDV. Newcastle Disease (ND) is an extremely contagious, an acute viral disease of the birds causing up to 100 per cent mortality in the chickens that are significant natural host of an agent. NDV is an avian paramyxovirus-1 (APVM-1), the member of genus *Avulavirus*, the family *Paramyxoviridae* (Mayo, 2002). This disease is distinguished by presence of lesions in the digestive, nervous and respiratory system (Nanthakumar, 2000) depending upon the virus, disease varies from mild to severe forms. Early signs of ND are anorexia, ruffled feathers, inactiveness, conjunctivitis and swelling. Gradually, the diseased birds develop greenish diarrhea, difficult breathing and inflammation of regions of head and neck. Neurological signs also start to appear gradually, including tremors, spasms, paralysis, torticollis and circular movement. (Okoye *et al*., 2000; Usman and Diarra, 2008 and Barman *et al*., 2010). In Pakistan ND outbreak causes huge economic losses. It not only causes severe losses to poultry but also non- poultry birds. Some virulent forms of NDV that were frequently observed in poultry were also transmitted to wild birds. (Ayala *et al.*, 2016). Also there are also some specific genotypes of virulent NDV that are well maintained in wild birds like cormorants and pigeons etc. (Sabra *et al.*, 2017; He *et al.*, 2018).It has been reported that throughout 2012, outbreak of ND happened in the Wildlife Park, Lahore, which instigated mortality of 190 peacocks approximately (Munir *et al*, 2012).

NDV is a single stranded, negative sense, enveloped RNA virus (Murphy *et al*, 1995), with genome of 15,186 nucleotides long (15.2kb) (de Leeuw, 1999; Zhang *et al.*, 2012) having six genes encoding six proteins; two of these are interactive glycoproteins including the Fusion (F) and the Haemagglutinin-neuraminidase (HN) proteins. F and the HN proteins are specifically involved in the attachment of virus to a cell surface and fusion with cell membrane. Other proteins include nucleocapsid protein (NP), matrix protein (M), phosphoprotein (P), and large (L) polymerase protein which is RNA-dependent (Millar *et al.*, 1988). The attachment and fusion of this virus with the host cell surface is the root of pathogenicity and there should be

ample amount of research on F and HN at molecular level (Boynukara *et al.*, 2013).

Therefore, present study was designed for the isolation and identification of ND virus from different samples of infected birds of poultry Farms of the Punjab. The samples were subjected to HA and HI and PCR using cDNA, to characterize genes of F and HN proteins of NDV. In the end sequencing and phylogenetic analysis of the results was done to identify the relationship status of NDV with other virus isolates.

MATERIAL AND METHODS

Collection and preservation samples: A total of 50 samples from Islamabad and different farms of Punjab were collected from infected birds and transported to the lab. Autopsy of birds was also accompanied and different tissues including caecal tonsils (n= 10), spleen (n= 10), intestine, trachea along with cloacal swabs and blood samples were collected (caecal tonsils (n= 10), spleen (n= 10), trachea (n= 10), intestine (n= 10), cloacal swabs (n= 7) and blood samples (n= 3). Blood samples were collected in EDTA contained vacutainer, while the organ samples were placed in separate sterile, tightly sealed labeled container having Phosphate Buffer Saline(PBS)/Glycerol viral transport medium (VTM). Cloacal swabs were collected by using sterile sampling swabs. Samples were stored at -20°C for advanced processing.

Virus isolation in embryonated eggs: Virus isolation was carried out in 9-11 days old embryonated eggs. After disinfecting them with 10% formalin these eggs were incubated at 37°C with the relative humidity of 60%. The eggs were candled post incubation. The virus was injected in the eggs by chorioallantoic sac route. Each egg was inoculated with 0.1 ml of the inoculum and re-incubated at 37°C.

The eggs were subjected to candling twice daily. Embryos were chilled to 4°C for overnight. After 72 hours, allantoic fluid from embryonated eggs was harvested and tested for the occurrence of virus by HA using 1% red blood cells of chicken and PCR.

Detection of virus by haemagglutination assy (HA): Fifty µl of Phosphate Buffer Saline Solution was added in each well of round bottom immunoassay plate.50µl of Allantoic fluid (AF) was added in first well of the each row of the plate. With micropipette the AF was serially diluted by transferring 50µl to the next well on right. 50µl from the last well was discarded. The last well served as negative control with no virus. After that 50µl of 1%RBCs were added to the each well and mixed it gently. At room temperature, plate was incubated almost for 30 minutes and after that results were recorded.

RNA extraction and PCR: Extraction of viral RNA was done from the AF of the samples that showed positive HA results, using TRIzol LS Reagent kit as per instruction of the manufacturer. The quantity of RNA was determined using nanodrop and was stored at -70°C until further processing. The nano-drop quantification of the samples was performed using a Nano-drop quantifier (Quawell® UV-vis Spectrophotometer Q5000).

The PCR using cDNA was carried out by the use of Maxime RT PreMix Kit. Template RNA 1µg and distilled water to a total volume of 20µL was added into the Maxime RT PreMix tubes. The cDNA synthesis reaction mixture was first heated at 45ºC for sixty minutes and then will be heated at 95ºC for five minutes. The cDNA samples were immediately used for PCR and remaining cDNAs were stored at -20ºC.

PCR for F gene: Synthesized cDNA was subjected to the PCR using primers which were F gene specific and these were previously used by (Nanthakumar et al., 2000).

Following primers were used; For primer: 5'-GCAGCTGCAGGGATTGTGGT- 3' Rev primer: 5'-TCTTTGAGCAGGAGGATGTTG-3'.PCR product size of fusion gene segment was 356 bp using these primers. The PCR amplification was carried out by using following conditions: one cycle at 94ºC for 2.5 minutes for initial denaturation, 35 cycles at 94ºC for 45 seconds, 58ºC for 45 seconds, 72ºC for 45 seconds and last step was final extension at 72ºC for 5 minutes.

PCR for HN gene: Synthesized cDNA was directed to the PCR process by using HN gene specific primers previously used by (Iram et al., 2014). Primers used for HN gene are as follows: For primer 5'-CATACACAACATCAACATG-3'and Rev primer 5'-GGTAGCCCAGTTAATTTCCA-3'.PCR product size of fusion gene segment was 519 bp using these primers. The PCR amplification was carried out and thermal profile is as follows: One cycle for 5 minutes at 94ºC for initial denaturation, 35 cycles with 94ºC for 30 seconds, 54ºC for shorter time (30 seconds) and 72ºC for 1 minute and last step of final extension at 72ºC for 10 minutes. PCR products were visualized by electrophoresis in the 1.5 % (w/v) agarose gel under ultraviolet light and pictures were taken by using a gel documentation system (UV trans-illuminator-FisherBiotech®).

Gene sequencing and phylogenetic analysis: Positive PCR products with required sized DNA were sent for sequencing to Macrogen, Korea after PCR cleanup. The sequence data along with other related genes were analyzed using NCBI-BLAST software. The cDNA sequences of F and the HN gene were then aligned by using Clustal W method using Seaview® software. The pairwise distances were computed using MEGA7®. A phylogenetic tree was constructed using MEGA7®

software. Previously reported genotypes around the world, as well as, in Pakistan were analyzed.

RESULTS

A total of 50 samples were collected. For isolation of NDV virus the samples were inoculated in 9-11 days old embyonated eggs and after 3 days of incubation they were tested with Haemagglutination Assay (HA). Mean Death Time (MDT) of 10 samples was determined to evaluate the pathogenic potential of the virus (Table 1). The 40 positive samples were then used for RNA isolation and molecular characterization.

Table 1: Mean Death Time of Embryos inoculated with NDV.

Chicken embryos (n= 10)	Time to Death (Hrs)		
	24	48	72-90
Died	1/10	7/10	2/10
Percentage	10%	70%	20%

Ten percent of the embryos died within the 24 hours showing the velogenic strain while ninety percent of the embryos were dead after 48-72 hrs, showing the mesogenic and lentogenic strains.

Confirmation of NDV by PCR: Suspected samples gene fragments were detected by RT-PCR using F and HN gene specific primers. For HN gene 519 bp gene fragments (Fig. 1) and for F gene 356 bp gene fragments (Fig. 2-3) were observed when samples were allowed to run on 1.5 percent agarose gel. The primers showed equal sensitivity and particularity with extracted RNA from suspected samples by way of genomic RNA of standard NDV.

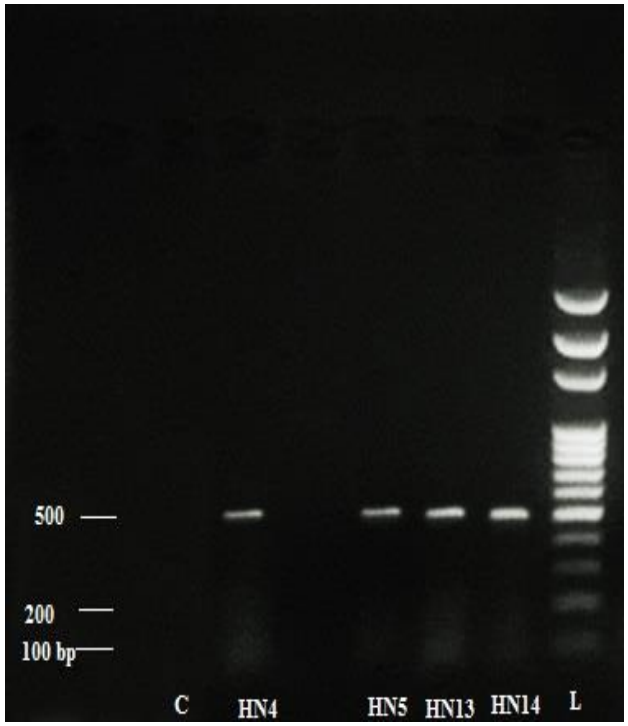

Figure 1: PCR products of 519bp for HN gene on 1.5% agarose gel with 100bp ladder (L: 100bp Ladder, C: Control, Sample names: HN4, HN5, HN13, HN14)

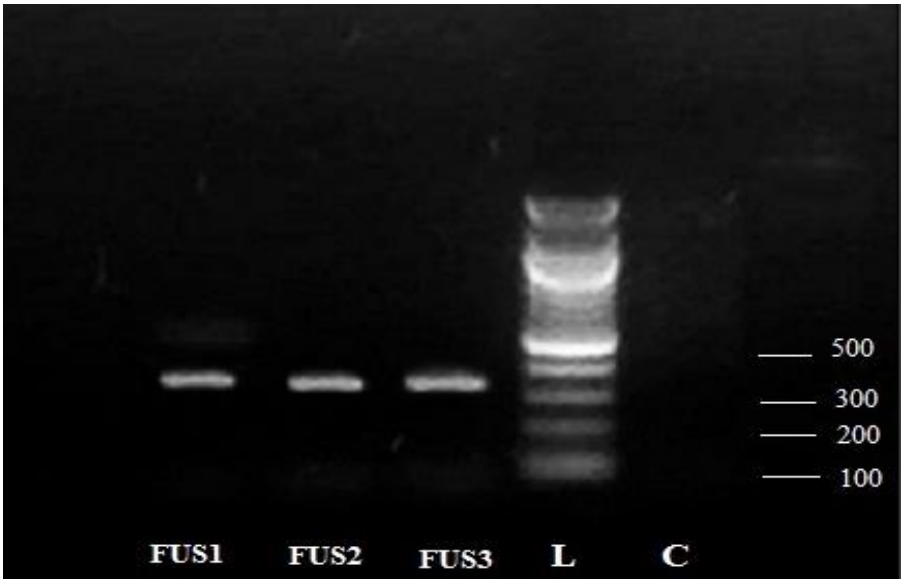

Figure2: PCR products of 356 bp for F gene on 1.5% agarose gel with 100bp ladder. (L: 100bp Ladder, C: Control, Sample names: FUS1,FUS2, FUS3).

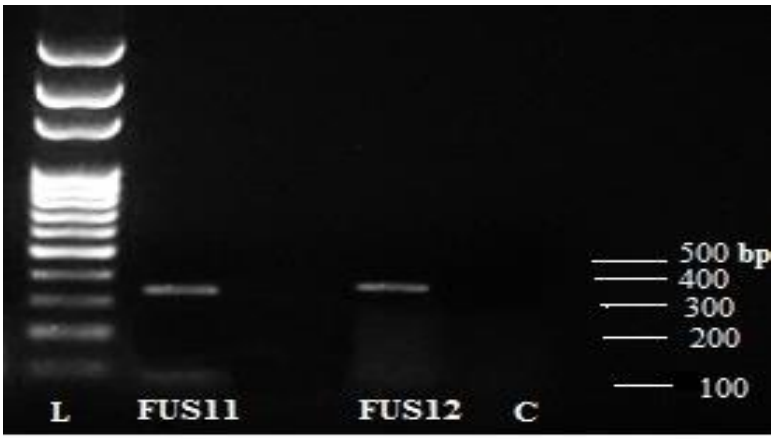

Figure 3: PCR products of 356 bp for F gene on 1.5% agarose gel with 100bp ladder. (L: 100 bp Ladder, C: Control, Sample names: FUS11, FUS 12).

Phylogenetic analysis of NDV strain: The selected positive isolates were subjected to nucleotide sequencing. The analysis of nucleotide sequence was done by using BLAST software. The sequences were aligned by Clustal W using MEGA7 program. Phylogenetic analysis of F gene sequences showed that although our sequences were placed in separate cluster they were closely related to other previously identified velogenic strains mostly found in India, China and Egypt (Fig 4). On the other hand the phylogenetic study of the HN gene sequences exposed that the isolates showed homology with previously isolated NDV samples of pigeon from Egypt, Lahore (Pakistan), Ukraine and distantly related to the samples isolated from China, Nigeria, Japan and Brazil (Fig 5). The Phylogenetic trees were made by Neighbor- joining method using MEGA7 software.

To determine pairwise distance or amount of substitutions among the gene sequences MEGA7 software was used. The diversity values estimate the Evolutionary Divergence between the provided sequences (Table 2 and 3).

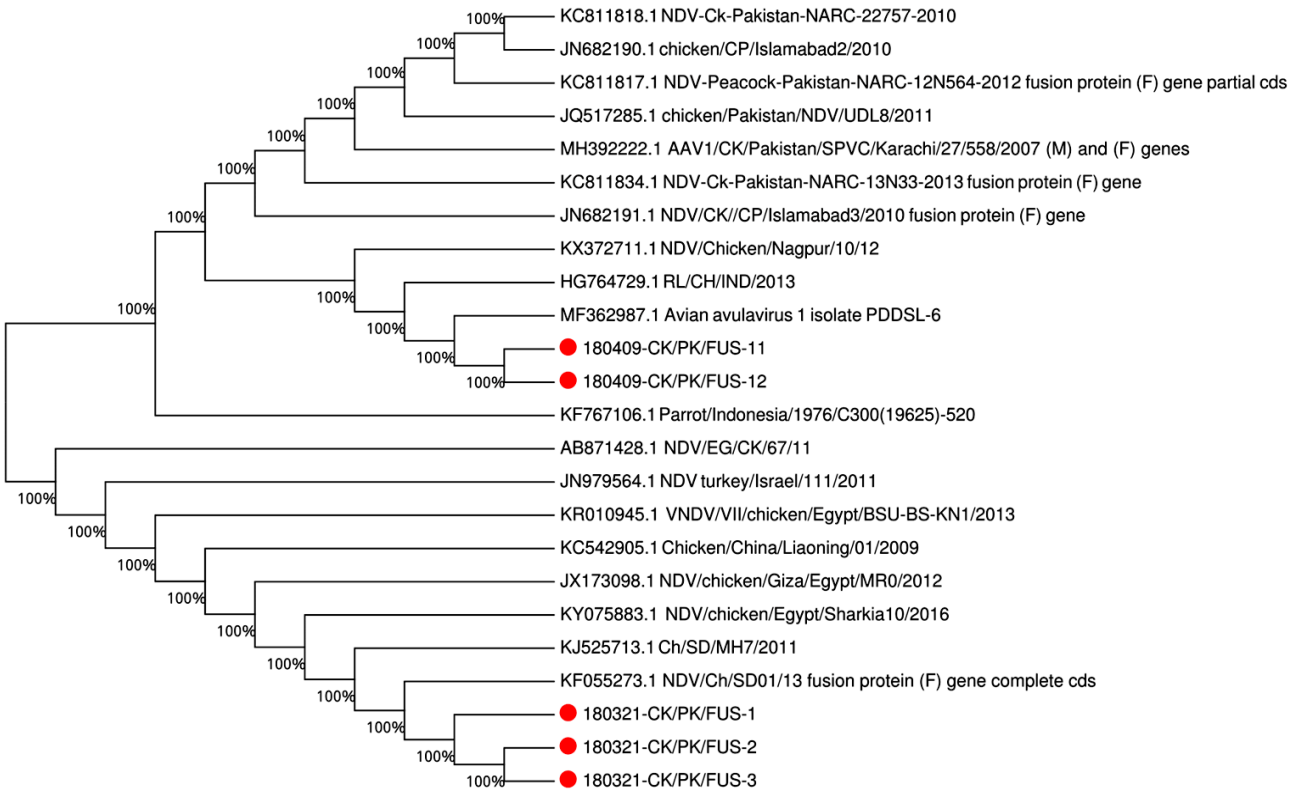

Figure Error! No text of specified style in document.**: Figure showing phylogenetic tree for Fusion gene. The red dot (●) shows our samples**

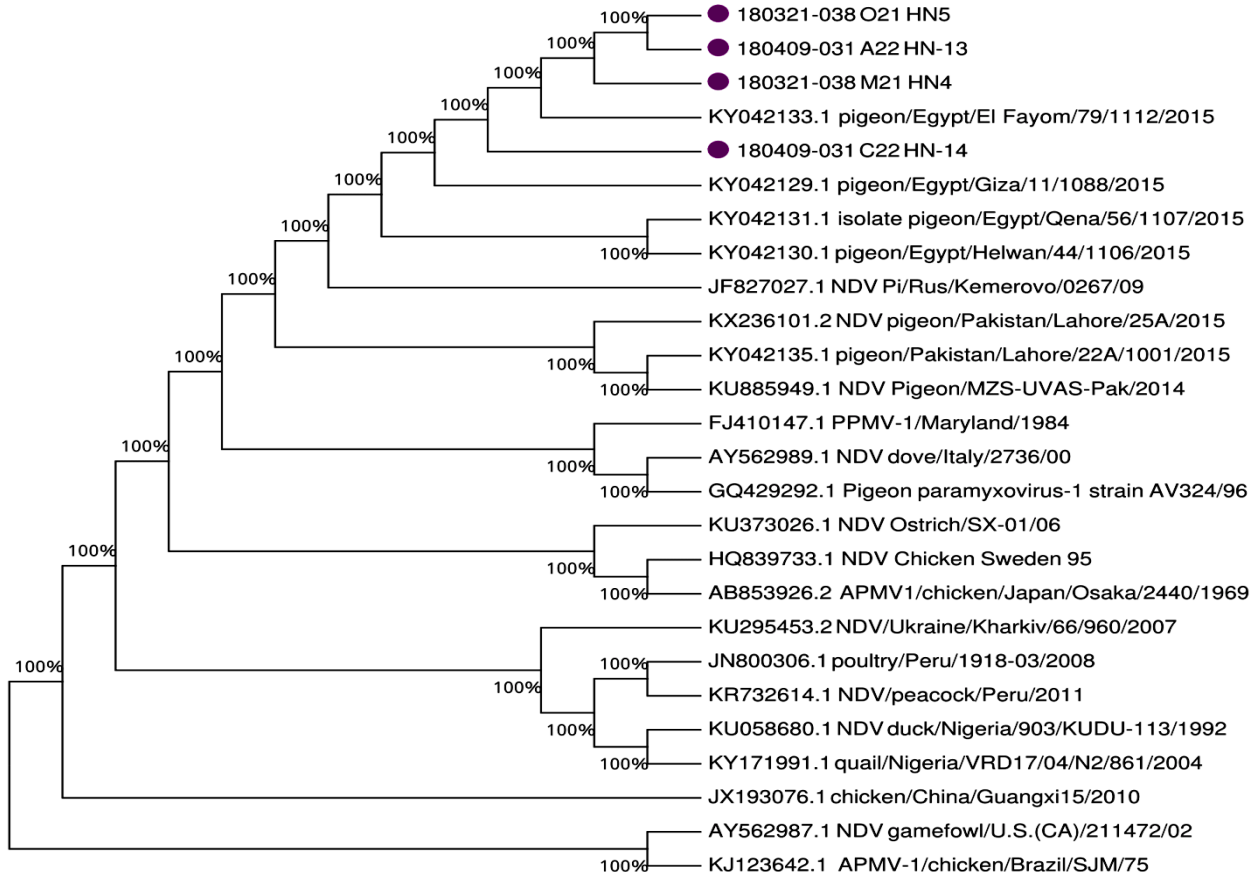

Figure 5: Figure showing phylogenetic tree for HN gene. The purple dot (●) shows our samples.

Table 2: Table showing pairwise mean diversity among F gene sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	2
1. 180409-CK/PK/FUS-11																				
2. 180409-CK/PK/FUS-12	0.000																			
3. 180321-CK/PK/FUS-1	0.171	0.171																		
4. 180321-CK/PK/FUS-2	0.171	0.171	0.000																	
5. 180321-CK/PK/FUS-3	0.171	0.171	0.000	0.000																
6. KX372711.1 NDV/Chicken/Nagpur/10/12	0.000	0.000	0.171	0.171	0.171															
7. KF767106.1 Parrot/Indonesia/1976/C300(19625)-520	0.027	0.027	0.152	0.152	0.152	0.027														
8. KC811818.1 NDV-Ck-Pakistan-NARC-22757-2010	0.034	0.034	0.180	0.180	0.180	0.034	0.049													
9. JQ517285.1 chicken/Pakistan/NDV/UDL8/2011	0.034	0.034	0.180	0.180	0.180	0.034	0.049	0.000												
10. JN682190.1 chicken/CP/Islamabad2/2010	0.041	0.041	0.190	0.190	0.190	0.041	0.056	0.007	0.007											
11. MH392222.1 AAV1/CK/Pakistan/SPVC/Karachi/27/558/2007 (M) and (F) genes	0.034	0.034	0.180	0.180	0.180	0.034	0.049	0.000	0.000	0.007										
12. KC811834.1 NDV-Ck-Pakistan-NARC-13N33-2013 fusion protein (F) gene	0.034	0.034	0.180	0.180	0.180	0.034	0.049	0.000	0.000	0.007	0.000									
13. JN979564.1 NDV turkey/Israel/111/2011	0.144	0.144	0.020	0.020	0.020	0.144	0.126	0.153	0.153	0.162	0.153	0.153								
14. AB871427.1 NDV/EG/CK/101/12	0.153	0.153	0.013	0.013	0.013	0.153	0.134	0.162	0.162	0.171	0.162	0.162	0.007							
15. KJ567597.1 chicken/China/SDZB11/2013	0.171	0.171	0.013	0.013	0.013	0.171	0.152	0.180	0.180	0.190	0.180	0.180	0.020	0.027						
16. KC542905.1 Chicken/China/Liaoning/01/2009	0.144	0.144	0.020	0.020	0.020	0.144	0.126	0.153	0.153	0.162	0.153	0.153	0.000	0.007	0.020					
17. KF055273.1 NDV/Ch/SD01/13 fusion protein (F) gene complete cds	0.171	0.171	0.000	0.000	0.000	0.171	0.152	0.180	0.180	0.190	0.180	0.180	0.020	0.013	0.013	0.020				
18. KY075883.1 NDV/chicken/Egypt/Sharkia10/2016	0.153	0.153	0.027	0.027	0.027	0.153	0.134	0.162	0.162	0.171	0.162	0.162	0.007	0.013	0.027	0.007	0.027			
19. JN682191.1 NDV/CK//CP/Islamabad3/2010 fusion protein (F) gene	0.048	0.048	0.180	0.180	0.180	0.048	0.063	0.013	0.013	0.020	0.013	0.013	0.153	0.162	0.180	0.153	0.180	0.162		
20. KC811817.1 NDV-Peacock-Pakistan-NARC-12N564-2012 fusion protein (F) gene partial cds	0.041	0.041	0.189	0.189	0.189	0.041	0.056	0.007	0.007	0.013	0.007	0.007	0.162	0.171	0.189	0.162	0.189	0.171	0.020	

Table 3: Table showing pairwise mean diversity among HN gene sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. KY042135.1 pigeon/Pakistan/Lahore/22A/1001/2015																				
2. KU373026.1 NDV Ostrich/SX-01/06	0.260																			
3. JN800306.1 poultry/Peru/1918-03/2008	0.428	0.321																		
4. AY562987.1 NDV gamefowl/U.S.(CA)/211472/02	0.590	0.502	0.599																	
5. KX236101.2 NDV pigeon/Pakistan/Lahore/25A/2015	0.032	0.293	0.433	0.553																
6. KU885949.1 NDV Pigeon/MZS-UVAS-Pak/2014	0.022	0.291	0.445	0.611	0.051															
7. HQ839733.1 NDV Chicken Sweden 95	0.291	0.158	0.288	0.512	0.324	0.316														
8. KU058680.1 NDV duck/Nigeria/903/KUDU-113/1992	0.372	0.296	0.163	0.469	0.392	0.388	0.244													
9. KY042134.1 pigeon/Egypt/El Fayom/84/1113/2015	0.203	0.260	0.368	0.575	0.233	0.231	0.251	0.381												
10. AB853926.2 APMV1/chicken/Japan/Osaka/2440/1969	0.248	0.140	0.255	0.496	0.279	0.264	0.096	0.236	0.229											
11. AY562989.1 NDV dove/Italy/2736/00	0.298	0.263	0.405	0.575	0.332	0.298	0.236	0.368	0.291	0.214										
12. KJ123642.1 APMV-1/chicken/Brazil/SJM/75	0.442	0.391	0.416	0.236	0.430	0.485	0.354	0.323	0.425	0.285	0.435									
13. Y17341.1 NDV HN gene (strain 3/91)	0.286	0.177	0.333	0.473	0.292	0.318	0.169	0.280	0.266	0.118	0.269	0.334								
14. 180321-038 M21 HN4	0.203	0.260	0.368	0.575	0.233	0.231	0.251	0.381	0.000	0.229	0.291	0.425	0.266							
15. 180321-038 O21 HN5	0.203	0.260	0.368	0.575	0.233	0.231	0.251	0.381	0.000	0.229	0.291	0.425	0.266	0.000						
16. 180409-031 A22 HN-13	0.203	0.260	0.368	0.575	0.233	0.231	0.251	0.381	0.000	0.229	0.291	0.425	0.266	0.000	0.000					
17. 180409-031 C22 HN-14	0.203	0.260	0.368	0.575	0.233	0.231	0.251	0.381	0.000	0.229	0.291	0.425	0.266	0.000	0.000	0.000				
18. JX193076.1 chicken/China/Guangxi15/2010	0.421	0.356	0.422	0.497	0.394	0.444	0.329	0.308	0.420	0.271	0.423	0.341	0.348	0.420	0.420	0.420	0.420			
19. KR732614.1 NDV/peacock/Peru/2011	0.455	0.329	0.013	0.613	0.459	0.472	0.296	0.181	0.377	0.262	0.430	0.426	0.341	0.377	0.377	0.377	0.377	0.432		
20. KY171991.1 quail/Nigeria/VRD17/04/N2/861/2004	0.453	0.343	0.219	0.532	0.466	0.470	0.308	0.086	0.426	0.298	0.411	0.390	0.348	0.426	0.426	0.426	0.426	0.349	0.238	
21. GQ429292.1 Pigeon paramyxovirus-1 strain AV324/96	0.269	0.212	0.348	0.573	0.302	0.286	0.174	0.313	0.250	0.155	0.065	0.384	0.230	0.250	0.250	0.250	0.250	0.388	0.371	0.384

DISCUSSION

Newcastle disease (ND) is an extremely contagious and fatal disease of poultry and of wild birds globally that is triggered by infections with lethal strains of APMV-1. (Millar et al., 1988; Wajid et al., 2017)

According to a report of FAO, the mean death time (MDT) value of ND depends upon its pathotype. If the strain is lentogenic the MDT value will be more than 90 hrs. while if the strain is mesogenic the embryo will die within 60-90 hrs. In case of velogenic strain the embryo death occurs in less than 60 hrs.

In a country like Pakistan the tools and approaches used for molecular diagnostic are not up to date and not available equally to all veterinary diagnostic institutes. HI is the mostly used method for the diagnosis of NDV infection that is accessible almost in all the veterinary laboratories. Therefore, NDV recognition is not always being done by using diagnostic techniques like RT–PCR and real time PCR (Abbas et al., 2014).

In the current study PCR procedure was adopted for NDV detection. Fragments of HN gene of 519 bp and F gene of 356 bp were successfully amplified from various NDV isolates obtained from the vicinity of Islamabad and different areas of Punjab. According to Seal et al (1995) although serological identification yet remains the most common choice due to cost effectiveness, yet RT-PCR tends to be a more trustworthy method for swift detection of NDV. PCR results can also be used for genetic analysis using various methods and tools.

It has been observed that out breaks due to NDV genotypes VII and VIII have caused massive economic losses to Pakistan poultry industry. In recent studies, the HN gene from numerous NDV outbreaks in commercial poultry farms revealed a stable nucleotide pattern and essential amino acid replacements in Pakistan's prevalent field viruses.(Abbas et al., 2014; Farooq et al., 2014)

Great efforts had been done globally to combat NDV in the poultry industry including intensive vaccination programs, application of hygienic measures and quarantine measures. Pakistan's transmission and spread of NDV is largely dependent on non-poultry species that kept in captivity in the same geographic region. It is also unclear from outbreaks that each of the outbreaks relates to a particular spill-over event from poultry farms or other unknown reservoirs.

Close distances between poultry farms and pet birds and the backyard poultry points lead to the presence of epidemiological connections. The uninterrupted presence of NDV in species other than poultry recommends the development of further integrated control approaches that would consist of active investigation in pet nurturing sites and or establishes in which exhibition birds and the wild birds are retained in captivity like zoos and parks.

According to Shabbir et al (2013) less biosecurity and un-matched field strain in NDV vaccine are accountable for

the continuous presence and circulation of ND in Pakistan. It has been observed that NDV strains occur as single serotype and vaccination with the one strain gives protection to birds against all the genotypes. But wide-ranging vaccination (three or even four times) in the life of bird of just 5–6 weeks marks in unnecessary stress on the immune status and thus revealing to the clinical infections of NDV. Further studies should focus onpathogenic characterization of isolates on the basis of MDT as well as intracerebral pathogenicity index and antigenic characterization by cross neutralization test of the chicken isolates and isolates of other birds' species.

Conclusion: It was concluded that majority of the samples collected from the vicinity of Islamabad and Rawalpindi were positive for NDV. Deduced gene sequences suggested that no significant genetic change was observed among the genes sequences of F and the HN protein in the isolates. Further studies should focus on the genotypic characterization of NDV from isolates of Islamabad and Rawalpindi region, preparation of DNA vaccines using these gene sequences and determination of effect of F and HN protein on immune system of bird.

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