

Isolation, Identification and Molecular Characterization of Highly Pathogenic Newcastle Disease Virus From Field Outbreaks

Asma Ashraf¹; Mohammad Slah U Din²; Muhammad Habib¹; Mujahid Hussain²; Shahid Mahboob^{1,3*}; Khalid Al-Ghanim³;

¹Government College University Faisalabad, Faisalabad, Pakistan; ²Nuclear Institute of Agriculture and Biology Faisalabad, Pakistan; ³King Saud University, Department of Zoology, College of Science, P. O. Box 2455, Riyadh, Saudi Arabia.

ABSTRACT

Newcastle disease (ND) is a major infectious disease of the poultry caused by a virulent strain of Avian Paramyxovirus – 1, that is a single strand non-segmented negative sense RNA virus. ND virus is major threat to the poultry industry in many countries of the world. The study was aimed to isolate and identify Newcastle disease virus (NDV) by using a haemagglutination inhibition (HI) test and reverse transcription-polymerase chain reaction (RT-PCR) assay. A total 100 samples of infected and dead birds were collected from different poultry farms. The weight of the birds was ranged 1000-1200g. The birds were divided into 3 groups. Haemagglutination assay (HA) was performed to detect the presence of NDV in suspension of infected homogenized tissues and it was found that HA is not the best method to detect the virus when it is in trace amounts. RT-PCR using NDV specific primers analyzed different clinical and postmortem samples. Reverse transcriptase polymerase chain reaction and specific primers was used for determining the presence of viruses. It was found that the virus was present in most of the infected samples except the serum of infected birds. During multiple sequence alignment (MSA) it was found that, our isolates have high homology (98%) with other reported NDV isolates. Phylogenetic analysis revealed that our isolate was closely related with viscerotropic velogenic types of NDV, which are highly pathogenic Newcastle disease virus.

Key words: Newcastle disease; epidemic; molecular characterization; avian virus; RT-PCR

¹Authors for correspondence: shahidmahboob60@hotmail.com

INTRODUCTION

Newcastle disease (ND) is an important viral disease of poultry and other bird species disregarding of sex and age [1-4]. ND is a major cause of huge economic losses in various parts of the world [5-6]. Newcastle disease virus (NDV) is belong to a group of the avian paramyxovirus - I [7-9]. The disease is characterized by an involvement of digestive, respiratory and nervous systems [10-11]. This disease can vary in nature from mild to severe, depending upon the type of the virus. In non-vaccinated chickens, the morbidity and death rates may be up to 100% each, depending upon the virulent intensity of NDV. In recent years, outbreaks have continuously occurred in Pakistan resulting in huge losses. Infected samples indicated the presence of virus identified by chicken embryonated egg inoculation and haemagglutination assay [2].

NDV has a wide range of hosts range, inclusive of approximately 241 species [12] belongs to 27 orders out of which 50 orders of birds [13]. In various developing countries, ND is an endemic and thus have a high economic impact. Due to this disease, poultry industry is facing losses of billions of Rupees annually in Pakistan [14] and millions of dollars worldwide [15].

ND is a serious threat to the poultry industry [16]. The rate of mortality and morbidity of poultry in unvaccinated flock [2] varies from 90-100%, depending upon the strain of ND virus [10]. The outbreaks of ND are regularly reported from all continents of the world [17]. "An intermittent form of ND present in Pakistan throughout the year, only a limited number of cases are reported annually. A severe outbreak of ND occurred during 2012 at Jallo Wildlife Park in Lahore, Pakistan, caused by APMV- 1 serotype. Within a week, it took the lives of approximately 190 peacocks with a 100% mortality rate and 50% loss of the susceptible birds. Isolation of virus and serological diagnostics, such as HI Test, ELISA and molecular diagnostic tests like real time PCR confirmed the presence of velogenic Newcastle disease virus" [18]. The study was aimed to isolate and identify Newcastle disease virus (NDV) by using a haemagglutination inhibition (HI) test and reverse transcription-polymerase chain reaction (RT-PCR) assay from the dead birds from the poultry farms of Punjab province of Pakistan.

MATERIALS AND METHODS

Collection of infected samples:

The samples were collected during 2013 from the poultry farms of different areas of Punjab province, Pakistan. A total 100 samples of infected and dead birds were collected from different poultry farms. The weight of the birds was ranged 1000-1200g. The birds were divided into 3 groups. Post-mortems were conducted and infected tissues, i.e. intestine, proventriculus, spleen, lungs and trachea were collected from the dead chickens. After collection, the samples were transported on ice packs and stored at -20 °C to -25 °C for further processing.

Virus isolation in embryonated eggs:

"Virus isolation was carried out as described by [19-20]. Eggs from healthy and ND-seronegative chickens were used which were conventionally raised. Tissue was homogenized as a 10% (w/v) suspension in PBS containing antibiotics (streptomycin, penicillin). After centrifugation, 0.1 ml supernatant was inoculated into the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. Allantoic fluid from dead embryos or in live embryos after 72 hours of incubation was collected and NDV was detected by HA test".

Haemagglutination assay (HA):

The titer of the virus in allantoic fluid or tissue homogenate was determined by hemagglutination assay as described by [20-22].

Reverse transcription-polymerase chain reaction:

Allantoic fluid was used for RNA extraction by following the protocol of Favor Prep.™ Viral Nucleic Acid Extraction mini kit. RNA extracted by using 150µl of allantoic fluid, 570µl of VNE Buffer, 570µl of ethanol, 500µl Wash Buffer 1 and 750µl of wash buffer. Each RNA sample was dissolved in 40 µl sterile RNase-free water and stored at -70°C.

The complementary DNA was synthesized using 2µl of the total RNA. 1 µl of random primer hexamers, 9 µl of nuclease free water, 4.0 µl of 5X Reaction Buffer, "1.0 µl of Ribo-Lock RNase Inhibitor (20u/ µl), 2.0 µl of 10 mM dNTP Mixture, 1.0 µl of Revert Aid M-MuL V Reverse Transcriptase (200u/ µl) was added and the mixture

centrifuged and then was incubated for 60 min at 42°C". The reaction terminated after heating it at 70°C for 5 minutes, then after briefly spin the tube before cDNA was used for PCR amplification. RT-PCR was performed as described by [25]. NDV-F /NDV-R primers were selected to amplify a 202 bp fragment of the F gene including the cleavage site. "Primer sequences are shown in Table-1. PCR was carried out in a 50 µl reaction containing 5.0 µl 10X buffer, 2.0 µl 25 mM MgCl₂, 2.0 µl 10 mM dNTP, 0.2 µl Taq, 0.8 µl NDV-F

primer (100 pmol), 0.8 µl NDV-R primer (100 pmol), 1.5 µl cDNA, and 37.7 µl DEPC was added to each tube. The amplification profile started with one cycle at 94°C for 2 min. followed by 35 cycles of 94°C for 15 sec, 48°C for 30 sec and 72°C for 30 sec and final extension of 72°C for 10 min. Sterile RNase free water or tissue samples from animals slaughtered on day 0, were used as negative controls".

Table 1: Specific primers used for RT-PCR

Sr. No.	Gene	Primer	Primer Sequence	PCR Product	Reference
i.	F	NDV-F	5'-GGTGAGTCTATCCGGARGATAACAAG-3'	202bp	Creelan <i>et. al.</i> , (2002)
ii.	F	NDV-R	5'-TCATTGGTTGCRGCAATGCTCT -3'		

Agarose Gel Electrophoresis:

PCR products were subjected to agarose gel electrophoresis. For this 10 µl of PCR product along with 2 µl of 6X loading dye were mixed and loaded on 1.5% agarose gel along with 100bp ladder. The gel was run in 1X TAE buffer till the dye reach near other end. At the end, the gel was stained with ethidium bromide and observed under UV light.

Nucleotide sequence analysis:

The PCR product was purified and its nucleotide sequence was determined using both forward and reverse primers. The nucleotide sequence was analyzed using BLAST software and its homology was searched against available nucleotide sequences from GenBank. Phylogenetic analysis was performed using a partial nucleotide sequence of the fusion protein gene and phylogenetic tree was drawn on the basis of observed divergence using software DNAMAN by Lynnon Biosoft, Canada.

RESULTS AND DISCUSSION

Clinical signs and symptoms:

The infected broilers showed clinical symptoms of depression, dizziness, gasping, paralysis of neck, legs or wings and loss of appetite. Swelling of the eyes and discharge from eyes were also observed. Greenish yellow colored diarrhea was very prominent. Similar signs and symptoms were also reported by [1, 11].

Postmortem lesions:

Typical Postmortem lesions are shown in Figure 1. During postmortem examination of dead birds, it was observed that the necrotic lesions were present in the mucosa of intestine, proventriculus and gizzard. Hemorrhagic lesions were very prominent in the mucosa of the proventriculus. "The air sacs were filled with whitish translucent material, but lungs were normal in size. Enlarged spleen and liver were also observed". The similar postmortem lesions in birds [16 and 23].

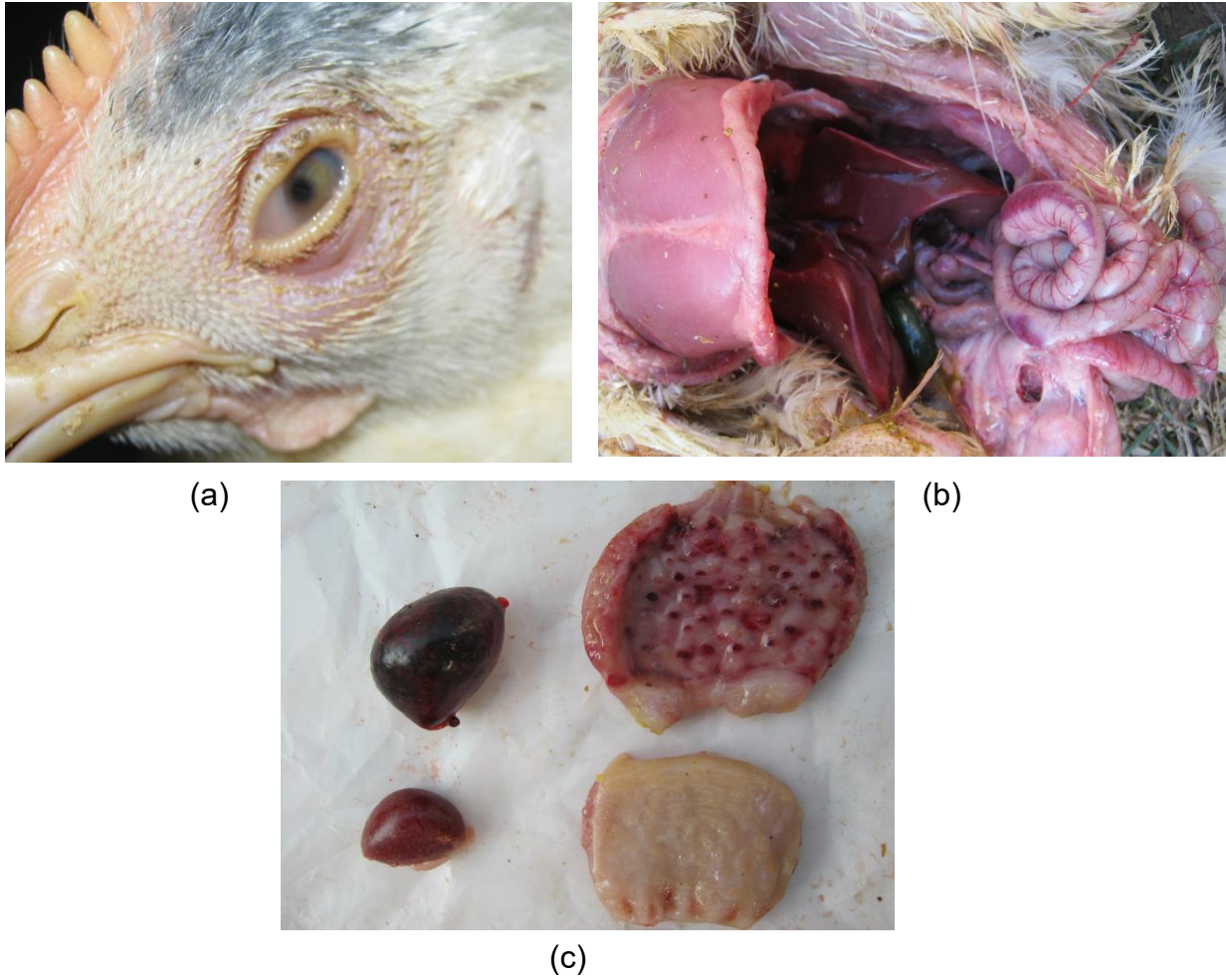


Fig 1: (a) Typical conjunctivitis (b) Postmortem examination showing hemorrhages of intestine (c) Enlarged spleen and hemorrhages in mucosa of proventriculus, along with normal organs for comparison.

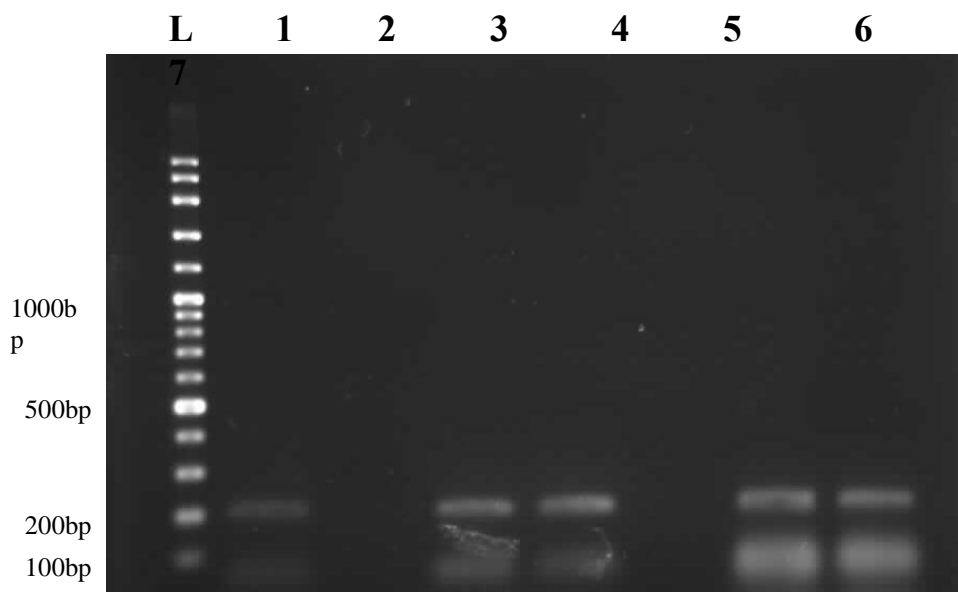


Fig 2: Agarose gel electrophoresis showing RT-PCR results.

Lane L: 100bp ladder

Lane 1,3,4,6,7: Positive results, showing 202 bp PCR product

Lane 2,5: Negative results, showing no amplification

Detection of NDV by Haemagglutination Assay:

Haemagglutination assay (HA) was performed to detect the presence of NDV in suspension of infected homogenized tissues. Results are shown in Table 2. HA is not the best method to detect the virus when it is in trace amounts. When the

homogenized viral suspension was allowed to multiply in allantoic fluid of embryonated eggs, then HA can detect successfully the virus in allantoic fluid. [5] also found that HA is one of the rapid and successful techniques to detect the NDV.

Table 2: Comparison of results of Haemagglutination assay (HA) and RT-PCR

Test		Haemagglutination Assay		RT-PCR	
		Direct tissue suspension	Allantoic Fluid	Direct tissue suspension	Allantoic Fluid
Clinical samples	Tracheal swabs	-	+	+	+
	Serum	-	-	-	-
	Fecal	-	+	+	+
	Proventriculus	+	+	+	+
	Spleen	-	+	+	+

Post-mortem samples	Lungs	-	-	+	+
	Intestine	+	+	+	+

Confirmation of NDV by RT-PCR:

RT-PCR using NDV specific primers analyzed different clinical and postmortem samples. Results of comparison of both techniques are shown in Table 2. It was found that the virus was present in most of the infected samples except the serum of infected birds. It might be because virus presence in the blood is for short periods during infection. RT-PCR is very sensitive technique to detect the presence of NDV in different tissue samples, even if the virus was present in minute quantity [2, 24]. Moreover, [25] “ reported one-step RT-PCR test coupled with restriction enzyme assay (REA) as fast and specific method for the detection and typing of APMV-1 from field samples”.

Nucleotide sequence analysis

During multiple sequence alignment (MSA) it was found that, our isolates have high homology (98%)

with other reported NDV isolates. Phylogenetic tree and multiple sequence alignment are shown in Figure 3 and Figure 4. Phylogenetic analysis revealed that our isolate was closely related with viscerotropic velogenic types of NDV, which are highly pathogenic Newcastle disease virus. It was found that our isolate (ND-NIAB-Pak) was grouped in a different cluster, which was differentiated from other reported NDV isolates. Moreover, it was revealed that our isolate is closely related with isolates of NARC-Pak, Israel, Kudus and Sragen, while it was distantly related with isolates of Iran, Guangdong, Japan, India and USA. [5, 26-27] did epidemiological investigations of NDV by phylogenetic analysis using a partial nucleotide sequence of a fusion protein gene. Our findings are in line with their results.

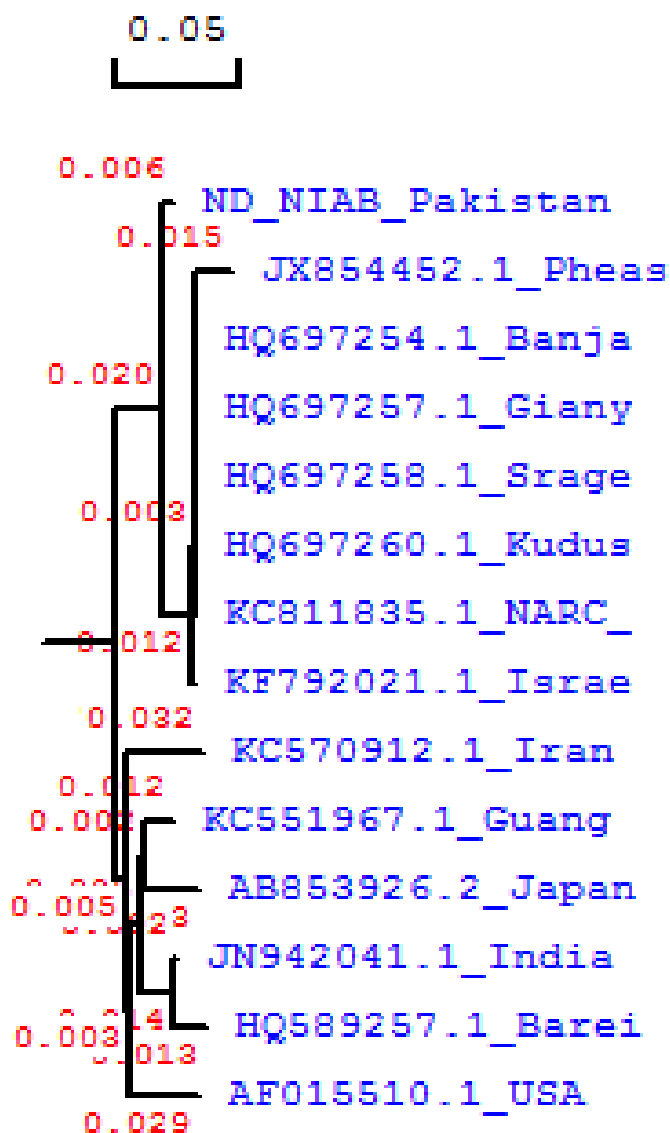


Fig 3: Phylogenetic tree showing relationships among reported isolates of NDV and new Pakistani isolate (ND - NIAB) based on partial nucleotide sequence of Fusion gene.

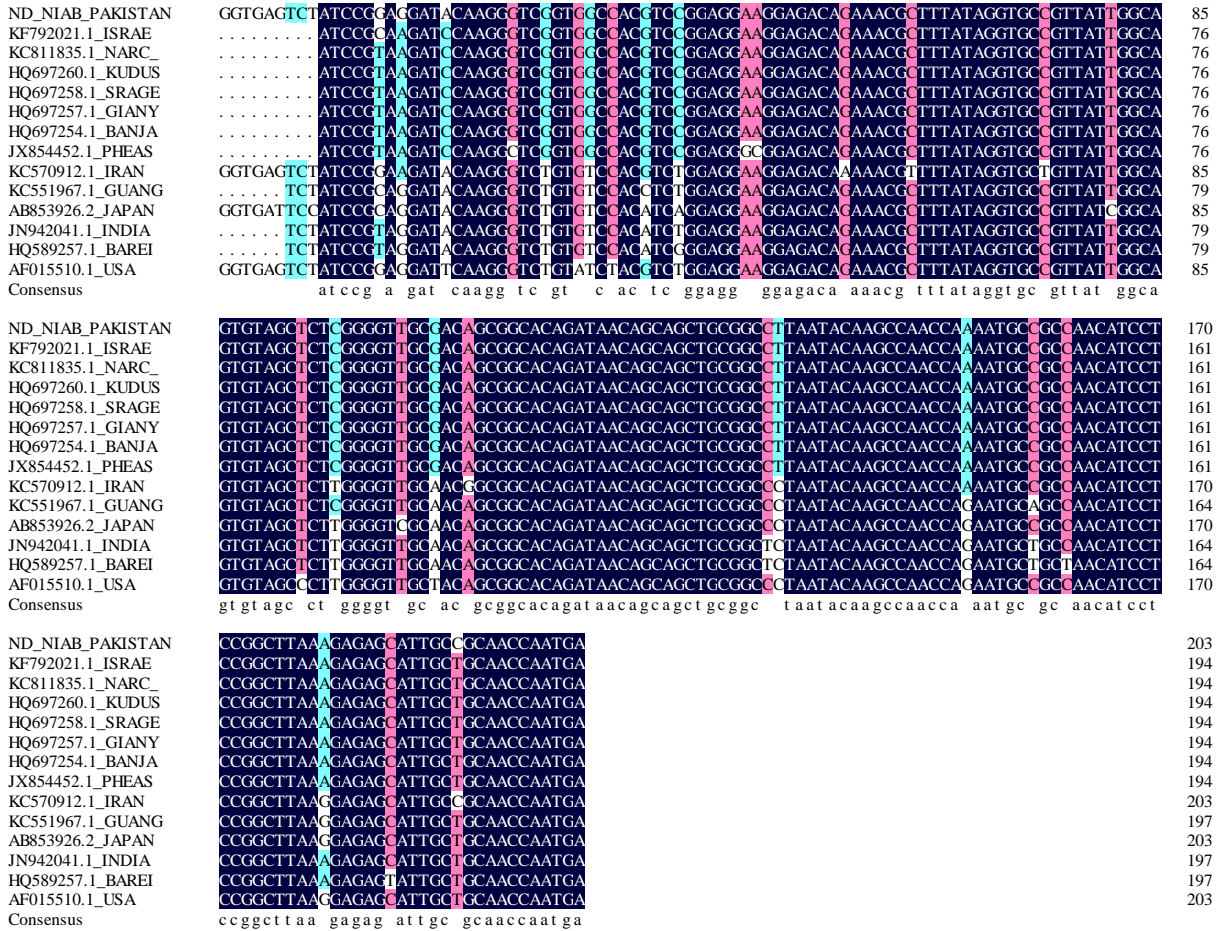


Fig 4: Multiple sequence alignment of partial fusion gene (203bp) of different reported NDV isolates along with NDV isolate of Pakistan (ND-NIAB-Pak).

CONCLUSION

It was concluded the virus was present in most of the infected samples except the serum of infected birds. The multiple sequence alignment (MSA) exhibited, that these isolates have, high homology (98%) with other reported NDV isolates. Phylogenetic analysis revealed that our isolate is closely related with viscerotropic velogenic types of NDV, which are highly pathogenic Newcastle disease virus.

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