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Short Communication

Detection and Molecular Characterization of Virulent Newcastle Disease Virus in Ducks (*Anas platyrhynchos domesticus*)

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Authors' Contributions
AA and BZ provided material for experiments. BZ and JI collected the samples and performed experiments. AZ and SI did virus isolation and histopathology while BZ and II verified these. RA and HS executed the phylogenetic analysis. QA and RS analyzed the data.

Key words
Newcastle Disease Virus, Ducks, Haemagglutination Inhibition, Reverse Transcriptase Polymerase Chain Reaction

ABSTRACT

The present study was conducted to detect, isolate and characterize the Newcastle disease virus (NDV) in ducks. For this purpose, a total of 100 samples of cloacal and 100 oropharyngeal swabs of ducks (*Anas Platyrhynchos domesticus*) were collected from different villages of four tahsils of Sheikhupura during period (May to July 2017). NDV was isolated from field samples by inoculating the suspected samples into embryonated eggs of 9-11 days. Isolated virus was confirmed through haemagglutination and haemagglutination inhibition test. Virus isolation rate of NDV from cloacal swab was 12% (6/50) and from oropharygeal swab was 8% (4/50). The 238-bp region of F gene of NDV was amplified by using specific primers of reverse transcriptase-polymerase chain reaction (RT-PCR). Out of 50 field samples 11 cloacal swabs (34%), were positive and out of 50, nine oropharyngeal swabs (18%) were positive for NDV through RT-PCR. DNA sequencing was performed by amplifying the RT-PCR amplicon targeted the F gene. Phylogenetic tree was constructed. It was concluded that virulent strains were existed in ducks and more than one genotype of NDV was prevalent in ducks.

Newcastle disease is one of the extremely deadly diseases of the bird. ND virus belongs to genus Avula virus. ND is mainly caused by Avian paramyxovirus type 1(APMV-1) (Alexander and Gough, 2003). ND disease remains problem for developing world. ND is an important pathogen and can infect approximately 236 avian species (Aldous and Alexander, 2001). ND disease is a big challenge for poultry industry throughout the world. In the development of ND Vaccine and its diagnosis a lot of progresses have been made (Alexander, 2001; Goldhaft, 1980). Newcastle disease is an endemic disease and due to this disease National poultry sector has to face great economic losses (Sadiq, 2004). Most common and important symptoms and severe infection in ducks are reduction in egg laying capacity, respiratory failure, high level of mortality, and weight loss (Dawid *et al*., 2013). Although ND is a public problem worldwide yet it is concentrated at its peak in countries such as in Asia, Africa, Europe, America (OIC, 2012). ND is a negative sense RNA virus, non-segmented as well as single-stranded virus (Alexander and Senne, 2008; Kattenbelt, 2006). Newcastle disease is classified into three major groups based on the pathogencity, lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (high virulence). Velogenic strain are divided in a group of different neurotropic velogenic NDVs cause neurologic as well as respiratory signs, resulting mortality rate very high. Viscerotropic velogenic NDVs cause necro-hemorrhagic lessions with acute lethal infection in gastrointestinal tract (Seal *et al*., 2000).

Infected ducks with velogenic NDV could transmit virusinto the contact free range chickens (Otim *et al*., 2006). Newcastle disease virus is 15 kb long that includes phosphoprotein(P), nucleocapsid protein (NP), matrix protein (M), Ffusion protein (F) and hemagglutinin-

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neuraminidase (HN). Large RNA based polymerase protein (L) in order 3'-NP-PM-F-HN-L-5' (Alexender, 2003). A Specific motif at the cleavage site of the F protein is considered important factor of NDV Pathogenicity. Reverse-transcriptase PCR (RT-PCR) assays are now extensively applied for detection of Newcastle disease virus because this assay is less laborious as well as faster in performance (Chen *et al*., 2008; Panda *et al*., 2004; Alexender and Senne, 2008). The present project was designed to isolate the Newcastle disease virus from ducks by targeting the Fusion protein through RT-PCR. Isolated strains of NDV were further characterized and phylogenetic tree was constructed.

Materials and methods

A total of 100 samples of cloacal swabs and oropharyngeal swabs were collected from ducks (*Anas Platyrhynchos domesticus*), from different villages in four different Tahsil (Sheikhupura, Muridke, Sharqpor, Ferozwala) of district Sheikhupura. The four villages were named as Kotnoor shah, ila star shah, Khanpur, BurjAttari and Chak-23. Samples were collected from May to July 2017. The collected samples were preserved under refrigeration and were processed for molecular diagnosis and virus isolation.

The fifty collected samples of cloacal swab and oropharyngeal swabs were processed for virus isolation. Swabs were dipped in tube contained PBS solution to the 1ml as well as antibiotics (10,000 1U/mL penicillin, 1 mg/ml streptomycin sulphate, 1 mg/ml gentamicin sulphate). For isolation of NDV virus, 9 day old embryonated chicken eggs were inoculated through allantoic cavity at dose rate of 0.2 ml of NDV inoculum having titer of (ELD50 $10^{7.32}$ /100µl) (0.1ml virus suspension + 0.1ml antibiotic mixture) (Lanre *et al*., 2011; Mabiki *et al*., 2013). Allantoic fluid was examined by using egg Candler (bright light in dark room), and mortality of embryonated inoculated eggs observed after 24hrs to 48hrs of incubation (Rena and Peter, 2015). Allantoic fluid was also confirmed through RT-PCR.

Haemagglutination inhabitation test was performed to each row of microtiter plate. 25µl of phosphate buffered saline and 25µl of the chicken serum was also dispensed into the first well only. Serial dilutions of the sera were made. A volume of 25µl of NDV containing 4 haemagglutination units was dispensed into all the wells. A positive control reference serum was included on the last row of the micro plate. At room temperature plates were incubated for 30 minutes and 25µl of 1 % chicken RBC was dispensed into each well. The plates were shaken gently and incubated at room temperature. The test was read after 45 minutes. End point was indicated by a red button of RBC at the bottom of the well while a negative test was indicated by a matt at the bottom of the well (Bouzari *et al*., 2014).

The fifty clocal and oropharyngealswabs were directly processed for molecular detection. The viral RNA of the ND viruswas extracted from FavorPrep™Viral Nucleic Acid Extraction Kit (Favorgen, FisherBiotech, Australia). The RNA was extracted in 50 µl of elution buffer and used as a template directly for RT-PCR assay and stored at -80ºC until further use.

A commercial cDNA synthesis kit (Fermantas,USA) was used to make cDNA. The procedure was adopted from instruction manual of manufacturer. To amplify a 238 bp fragment of F gene hypervariable region, Forward primer5'GCCCAGAGTCTACACCAT -3' and Reverse primer 5'- CCCGGATTATGTCTTTGA -3' were used. The amplification products were detectedby gel electrophoresis in 1.5% agarose gel in TAE buffer. Gels were run for 1.5 h at 80 Volts, stained with ethidium bromide (0.5µg/ml), exposed to ultraviolet light and photographed (Visi-Doc-It system, UVP, UK). A commercial 100-bp DNA ladder (Fermentas) was used as molecular-weight marker in each gel (Zhang *et al*., 2011).

DNA sequencing was performed by amplifying the RT-PCR amplicon targeted the F gene. Five positive amplicon (ND1, ND2, ND3, ND4 and ND5) were sequenced. Phylogenetic tree was constructed. The data was analyzed by using NCBI BLAST and Bioedit software 7.2 V. Electropherograms of NDV samples obtained were compared with reference sequence present at NCBI. Any mutations present in the sequences were noted. All the 5 NDV sequences were aligned with each other to find the homology by use of online Clustal W software and the phylogenetic trees were constructed by use of Phylogenetic software MEGA 7. The reported sequences of different countries including Pakistan were retrieved from the NCBI and were compared with the sequences of NDV obtained in this research work. Phylogeny was formed to conclude the origin of NDV obtained from Pakistani ducks included in this study.

Results and discussion

A total of 100 cloacal and oropharyngeal field samples from ducks were collected. Virus isolation rate of NDV from cloacal swab was 12% (6/50) and oropharnygeal swab was 8% (4/50). For NDV isolation samples were inoculated in embryonated eggs through Allantoic cavity route. Embryonated eggs that showed mortality after 48 hours of incubation were selected for collection. In second and third passages 100% mortality observed in inoculated embryonated eggs after this time period due to virulent NDV (Zhang *et al*., 2011). NDV was confirmed after each passage from triturated embryo and allantoic fluid

(AF) through plate haemagglutination inhabitation test (Rasool *et al*., 2015; Khan *et al*., 2012). Egg infectivity dose (ELD50) was estimated through the described procedure described by Reed and Muench (1938). Infectivity titer was 10 /100µl ($10^{7.32}$) which also reported that NDV could be propagated in embryonated chicken eggs (Lanre *et al*., 2011; Mabiki *et al*., 2013). Newcastle disease virus was confirmed from field oropharyngeal and cloacal swab samples and AF after each passage through the reverse transcriptase polymerase chain reaction (RT-PCR), primers of 238 bp were used. Out of 50 field samples 11 cloacal swabs (34%), were positive and out of 50, nine oropharyngeal swabs (18%) were positive for NDV through RT-PCR (Supplementary Fig. 1). BK specific primers confirmed the NDV in F gene tissue samples (Shabbir *et al*., 2013). Molecular identification of NDV was done by using RT-PCR and it is more sensitive method. To identify the strain of NDV existed in field, DNA sequencing was done using five amplicons (ND1, ND2, ND3, ND4 and ND5) targeted F genes. The phylogenetic tree represent that sequence No5 has closeness with already reported sequences of virulent strain of NDV Shandong, India and Egypt. This sequence has some closeness with the virulent NDV (Supplementary Fig. 2). Sequences of F region of NDV of other countries like China, Russia, Spain. Israel, Chimalhualan, Finland, Muktesar, Mexico, Egypt, Belize, USA, Mexico, Shanxi, Liaoning. Beijing, Shandong, Guangxi, Luxemboung, India, Egypt, Hellongjian, Malko Tarnrovo retrieved from NCBI. Virulent NDV has very good similarity with the NDV isolates obtained from NARC Pakistan. Similarly, NDV 3 is also surrounded by China, India and Egypt isolates and virulent NDV and Pakistani NARC sequences are very close neighbors with each other but are far neighbors of sequences 3 and 5. The virulent NDV strain was isolated from healthy ducks (Wajid *et al*., 2016; Dai *et al*., 2014). On the other hand, NDV isolates No 2&4 are close neighbors without any gap and these isolates are surrounded by China in phylogenetic tree while the sequence 1 is the most recent one and surrounded by already reported Pakistani sequences and new vaccines should be prepared for these isolates. Evolutionary analysis was conducted in MEGA7 (Fig. 1). The analysis involved 131 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 157 positions in the final dataset. The similarity matrix of all the five NDV isolated from ducks clearly shows that each sample is identical (ID) with its own sequence only, while it has different similarity matrix with each other. The sample No 1 has 0.828 value of similarity index with NDV No2 which shows 82.8%. The each similarity matrix value should be multiplied with 100 to get the %age similarity index and all five samples have variations of similarity ranging from 75%-97% (Fig. 2). The Clustal W alignment of all 5 NDV isolates also showing that all six NDV have a wide difference in similarity among each other, representing the fact the NDV F gene is undergoing in the process of mutations very rapidly (Supplementary Fig. 3) (Zhang *et al*., 2011).

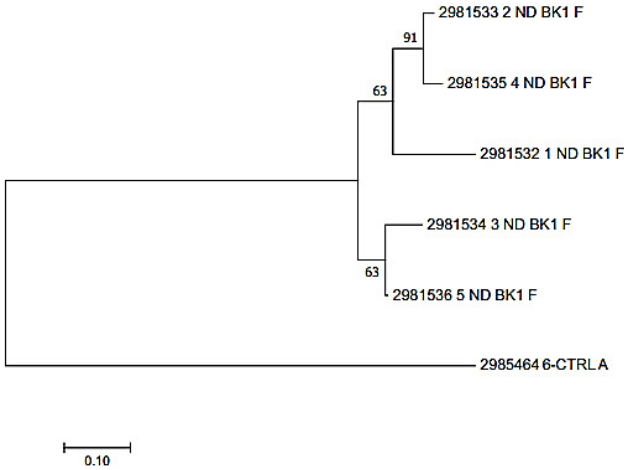

Fig. 1. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.61196123 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 212 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Conclusion

It is concluded virulent strain of Newcastle disease virus existed in domestic ducks of district Shiekhupura. More than one genotype of NDV prevalent in ducks and carrier ducks are the one of the source of transmission of virus in backyard chicken. The current research indicated more work should be done in NDV endemic areas. Due to rapid mutations in NDV, even vaccinated birds get infections with NDV and shows mortality. So there is need to make new vaccines.

Sequence Identity Matrix
Input Alignment File: Untitled5

Seq->	2981532_1_ND_BK1_F_	2981533_2_ND_BK1_F_	2981534_3_ND_BK1_F_	2981535_4_ND_BK1_F_	2981536_5_ND_BK1_F_	2985464_6-CTRL_A
2981532_1_ND_BK1_F_	ID	0.820	0.763	0.806	0.750	0.092
2981533_2_ND_BK1_F_	0.820	ID	0.813	0.933	0.826	0.092
2981534_3_ND_BK1_F_	0.763	0.813	ID	0.802	0.910	0.095
2981535_4_ND_BK1_F_	0.806	0.933	0.802	ID	0.821	0.092
2981536_5_ND_BK1_F_	0.750	0.826	0.910	0.821	ID	0.097
2985464_6-CTRL_A	0.092	0.092	0.095	0.092	0.097	ID

Fig. 2. The similarity matrix of all the five NDV isolated from ducks.

Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2020.52.1.sc1

Statement of conflict of interest

The authors declare no conflict of interest.

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