CHARACTERISATION OF *NEWCASTLE DISEASE VIRUS* ISOLATES PREVALENT IN KERALA

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THESIS

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DEPARTMENT OF VETERINARY MICROBIOLOGY

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DECLARATION

I hereby declare that this thesis entitled **"Characterisation of *Newcastle disease virus* isolates prevalent in Kerala"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Characterisation of *Newcastle disease virus* isolates prevalent in Kerala"** is a record of research work done independently by Rashi U. (18-MVM-044) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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EXTERNAL EXAMINER

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Dedicated to Dr. S. B. Umesh
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INTRODUCTION

1. INTRODUCTION

Poultry industry is the most organised and fast growing segment of agricultural sector in India. The production of eggs and poultry meat in India has been rising at a rate of eight to ten per cent per annum. Now, India is the world's fifth and eighteenth largest producer of egg and broilers, respectively. One of the major constraints in poultry farming is production loss due to various infectious diseases, among which viral diseases are the most common ones. The loss is in the form of mortality, morbidity, decreased feed conversion, reduced production and loss of market value. Newcastle disease (ND) is one among OIE listed notifiable diseases which is a substantial threat to poultry industry (Alexander, 2000).

Newcastle disease is a highly contagious and catastrophic disease of poultry. High mortality and production loss in affected flocks is responsible for the severe economic loss to existing and developing poultry industries. Doyle (1927) published that a filterable virus, different from fowl plague had caused ND which was reported in 1926 in a farm near Newcastle-upon-Tyne, England and he named it as Newcastle disease virus (NDV). From then, it has been spread throughout the globe, recorded in six (Asia, Africa, Australia, Europe, North and South America) out of the seven continents of the world and is enzootic in many countries (Miller *et al*., 2010). The disease affects nearly 250 species of birds and all age groups of birds are susceptible to ND due to natural or experimental infections (Alexander, 1997). Depending upon pathotype of the virus and susceptibility of birds, mortality varies from zero to one hundred per cent.

The aetiology of ND is *Avian paramyxovirus*-1 (APMV-1), now officially named as *Avian orthoavulavirus*-1 (AOVA-1), belongs to the family *Paramyxoviridae*. The virus has approximately 15.2 kb long genome and codes for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN) and RNA-dependent RNA

polymerase (L). The pathogenicity of the virus is mainly due to the F glycoprotein, a product of F gene. The polybasic amino acid motif present in the fusion protein cleavage site (FPCS) in F gene attributes to virulent pathotypes and results in spread of virus systemically, whereas monobasic amino acid motif present in the FPCS is associated with avirulent pathotypes of the virus (Rott and Klenk, 1988).

The diagnosis of ND is mainly by isolation in embryonated chicken eggs (ECE) or in tissue/cell culture and identification of the virus by haemagglutination (HA) and haemagglutintion inhibition (HI) tests. Pathogenicity indices like mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) are used to determine the pathotype of the NDV isolates. Commonly employed serological tests for diagnosis of ND are HI and enzyme-linked immunosorbent assay (ELISA). Molecular methods such as reverse transcriptase polymerase chain reaction (RT-PCR) and real time PCR are being used for the rapid diagnosis of the disease (OIE, 2018). These methods coupled with sequencing and phylogenetic analysis is valuable in assessing global epidemiology (Cattoli *et al.,* 2011).

At present in Kerala, many cases of ND have been reported even in vaccinated birds. Therefore, isolation and characterisation of NDV from the affected birds is necessary for understanding the evolutionary kinetics and epidemiology. In this context, the present study is designed to characterise NDV isolated from various avian species in Kerala both by conventional and molecular methods with the following objectives:

1. Detection of NDV from clinical samples of various avian species using RT-PCR
2. Isolation of the virus in ECE and pathotyping by ICPI and MDT
3. Genotyping of the isolates by PCR and sequencing of the amplicons

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Newcastle disease is one of the most devastating diseases of poultry. It ranks 8th out of 71 diseases with regard to the number of wild faunas lost through disease, destruction or slaughter (Dimitrov *et al.,* 2016).

2.1 HISTORY

The first outbreak of ND occurred in 1926 in Java, Indonesia (Kraneveld, 1926) and in Newcastle-upon-Tyne, England (Doyle, 1927).

Doyle (1935) stated that within a very short period of time, in Java (Kraneveld, 1926), England (Doyle, 1927), Philippines (Rodier, 1928; Farinas, 1930), India (Edwards 1928; Cooper, 1931), Ceylon (Crawford, 1931) and Korea and Japan (Ochi and Hashimoto, 1929) outbreaks of virulent disease were recorded and all these were of similar pattern as ND. The author also affirmed that causative agents of these diseases were immunologically indistinguishable. This had lead to the recognition of the "Newcastle disease" as a separate entity.

Beach (1944) reported that a virus similar to NDV in serological tests had caused mild respiratory disease, with some exhibiting nervous symptoms in birds of the United States. Since then, numerous NDV isolates with lower pathogenicity, producing very mild or no evidence of the disease had been reported.

Four panzootics of ND had occurred since 1926. The first one appeared with emergence of ND and it had taken twenty years to become panzootic. The second was at the end of 1960s. The third one had occurred in 1980s, but it was first seen in the race and show pigeons which later spread to chicken and the fourth pandemic was ongoing and believed to have started from late 1980s, from a different genotype (Alexander *et al.,* 2004).

Due to the continuous evolution of the genome of NDV and expansion in geographic distribution, fifth panzootic is strongly expected (Miller *et al.,* 2015).

2.2 OCCURRENCE

2.2.1 Global

Epizootics of ND occurred on a regular basis in Asia, Africa, Central and South America, while sporadic cases were reported in Europe (Alexander *et al.,* 2004).

Kim *et al*. (2007) examined the genomic diversity of NDV isolates recovered from wild populations between 1986 and 2005 and also from live bird markets in the United States. All the isolates were found to be lentogenic pathotypes based on MDT, ICPI tests and analysis of FPCS region. The viruses exhibited broad antigenic and genetic diversity relating to both class I and class II, confirmed by complete F gene analysis. The authors also performed real time RT-PCR M-gene assay and monoclonal antibody (MAb) assay. Class I viruses were poorly or not detected.

Diel *et al*. (2012b) collected oral and nasal swabs from neurologically affected cormorants and gulls in the states of Minnesota, Massachusetts, New Hampshire and Maryland. Based on the F gene analysis, the isolates were found to be virulent and clustered with 2008 cormorants isolates, obtained during an outbreak in Minnesota and grouped in class II genotype V.

Wild migratory waterfowls were sampled during the year 2006 to 2010 in Finland and screened for NDV by Lindh *et al*. (2012). Out of the 715 samples, 39 strains of NDV were isolated and confirmed by RT-PCR targeting the F gene. All the isolates were lentogenic, grouped in class I or class II genotype I based on the sequence analysis.

Snoeck *et al*. (2013) sampled domestic birds (mainly chicken, also ducks, geese, guinea fowls and turkeys) from West and Central Africa. Out of 3610 samples, 157 were found to be positive for NDV by RT-PCR. Phylogenetic analysis based on F and HN gene sequences revealed that all the isolates belonged to genotype XIV and newly defined genotypes XVII and XVIII.

Choi *et al*. (2014) characterised 12 isolates of NDV obtained between 2007 and 2012 in Vietnam. The authors pathotyped the isolates using RT-PCR and obtained F0 cleavage site motif ^{112}RRRKRF^{117} in 10 isolates and motif ^{112}RRQKRF^{117} in other two isolates. The MDT of the isolates was also studied. Both the pathotyping tests proved all the isolates to be velogens. In phylogenetic analysis, all of them were similar to genotype VII strains.

Fuller *et al*. (2017) isolated NDV from outbreaks in Eastern Europe. The authors employed RT-PCR targeting F gene for the confirmation of the isolates. On phylogenetic analysis, it was deduced that the virus belonged to genotype VII and was related to NDV isolated from South-East Asia.

Velogenic pathotype of the NDV was found to be endemic in Asia, Middle East Africa, areas of Mexico, Central and South America, while lentogenic pathotypes were distributed worldwide (Getabalew *et al*., 2019).

Nagy *et al*. (2020) reported isolation of 13 virulent NDV isolates from vaccinated chicken flocks in Egypt. Analysis of complete F gene sequence showed that 11 isolates belonged to genotype VII 1.1 and two of them belonged to genotype II strains similar to LaSota and B1 vaccine strains, but contained F0 monobasic motif ^{112}GGRQGRL^{117}. This result confirmed the emergence of velogenic NDV isolates from lentogenic vaccine strain.

2.2.2 India

The first outbreak of ND in India occurred in July, 1927 in a place called Ranikhet, Uttarkhand (Edwards, 1928); hence the name Ranikhet disease is much more popular for ND. Since then, outbreaks were reported on a regular basis, leading to economic losses to poultry industry in spite of regular vaccination.

Kumanan *et al*. (1992) isolated velogenic NDV from chicken and Japanese quails, pathotyped by MDT, ICPI and IVPI tests. The authors also conducted MAb test for strain classification, all the isolates were found to be inhibited with MAb U85 in HI test and not by MAb 161 and MAb 7D4.

Nanthakumar *et al*. (2000) propagated NDV obtained from various parts of India and the one from Nepal in 11 day-old ECE. The RT-PCR was carried out targeting the F gene and the amplicons were subjected for nucleotide and amino acid analysis. Out of the five isolates, three were velogenic and had sequence of RRQK/RRF and two of them were lentogenic having GRQA/GRL sequence at the cleavage site.

An antigenically distinct NDV from an outbreak in racing pigeons was reported by Roy *et al*. (2000). The isolate differed from usual NDV in binding pattern with MAbs and was confirmed with World Reference Laboratory, UK. The pathogenicity index values for MDT, IVPI and ICPI were 60 h., >2.0 and 1.4, respectively indicating it to be a velogenic strain.

Ananth *et al*. (2008) isolated NDV from unvaccinated village chicken in South India. Based on ICPI test, two isolates were close to virulent strains while one was a lentogenic strain. All the isolates were confirmed by RT-PCR targeting FPCS of F gene.

The amino acid sequence analysis of the F gene, of NDV isolate from peacock revealed multiple basic amino acid sequence at FPCS and phenylalanine residue at

117, proving it to be velogenic. Based on phylogenetic analysis, the virus was grouped in genotype II of class II NDV (Vijayarani *et al*., 2010).

Tirumurugaan *et al*. (2011) isolated NDV from pooled spleen and brain samples of chicken and pigeon. Based on the ICPI, MDT and FPCS analysis, the isolates were found be virulent. Phylogenetic analysis of partial F gene and full length M gene, grouped the isolates to genotype IV.

Five NDV isolates obtained from outbreaks in Central India during the period from 2006 to 2012 were placed in genotype XII 1b based on phylogenetic analysis of amino acid sequence of FPCS region. All of them showed ^{112}RRQKRF^{117} at the cleavage site (Morla *et al*., 2016).

In South India, Gowthaman *et al*. (2019) obtained seven NDV isolates from different commercial farms where regular vaccination against ND was practiced. They were confirmed as NDV by HI test and RT-PCR. Mean death time test and FPCS analysis placed them in velogenic group. On the basis of phylogenetic analysis of full length F gene, three among seven isolates were clustered in subgenotype XIIIb viruses and the remaining clustered with novel subgenotype XIIIe of class II viruses posing threat to poultry industry.

2.2.3 Kerala

Sudharma (1981) studied the susceptibility of ducks to NDV and also their role in transmitting the disease to chicken. On experimental infection, one week-old ducklings died after showing symptoms while eight week-old duckling showed clinical infection at beginning and eventually recovered developing antibodies. Clinically normal and diseased ducks excreted the virus without showing symptoms.

Sulochana *et al*. (1981) investigated the role of Indian house crows in the epizootiology of ND. In HI test, about 38 per cent of the crows tested showed antibodies against NDV.

In Kerala, ND had been reported in different avian species like mynah (Sulochana *et al*., 1982), pigeons (Sulochana and Mathew, 1991) and Japanese quails (Mini *et al*., 2001).

Arun (2004) isolated NDV from seven pooled cloacal samples of chicken. All the isolates were pathotyped based on MDT and ICPI. The MDT values varied from 110-120 h. while ICPI values ranged from 0.00 to 0.31 and were categorised as lentogenic.

Raj *et al*. (2009) compared antibody titre of NDV in randomly collected blood sera and egg yolk of layers. Mean log_2 HI titre were 4.5 and 5.68 in sera and egg yolk, respectively. The two mean values were statistically significant and hence the authors concluded that egg yolk might be used as a sample for detecting NDV antibodies.

Reji *et al*. (2017) reported the concurrent infection of ND and salmonellosis in a pigeon loft in Thrissur, Kerala. The genome of the virus was detected directly from the tissue samples by RT-PCR.

Dhivahar *et al*. (2018) recorded a concurrent infection of ND with infectious bronchitis in pigeons showing diarrhoea and torticollis by RT-PCR.

2.3 EPIDEMIOLOGY

2.3.1 Host range

Though NDV was known to infect a wide range of birds, the disease had been reported in species ranging from reptiles to humans (Lancaster, 1966).

Virulent NDV infections were documented in budgerigars, canaries (Erickson *et al.,* 1977) and house crows (Sulochana *et al.,* 1981).

Kaleta and Baldauf (1988) reported the establishment of the infection either naturally or experimentally, in about 236 species of birds representing 27 out of the 50 orders of the class.

Chicken are the most seriously affected species by NDV. The disease in chicken varied from virulent to avirulent form (Alexander, 1988).

Wobeser *et al*. (1993) reported outbreaks of virulent NDV in cormorants, white pelicans and gulls in Western Canada, where as Panigrahy *et al*. (1993) reported ND in parrots.

Studies on ND in domestic turkeys in the USA suggested the close relationship of the virus isolated from the nearby cormorants, owing to the transmission of virulent virus to turkeys (Heckert *et al*., 1996).

Game birds such as partridges and pheasants were also susceptible to ND (Crespo *et al*., 1999; Kinde *et al*., 2005).

In addition to poultry, NDV had also been isolated from non-avian species. Infection of humans with NDV and development of conjunctivitis had been reported (Alexander, 2000).

Pigeons could be infected with NDV with minimal or no clinical disease (Wakamatsu *et al*., 2006). Newcastle disease in birds of order, *Columbiformes* was mainly due to the Pigeon paramyxovirus-1 (PPMV-1) which is another strain of APMV-1, originated in pigeons. The first outbreak was reported in Middle East during 1970s. Later in 1980s, it spread to Europe and later considered to be endemic worldwide (Cattoli *et al*., 2011).

Yuan *et al*. (2012) isolated the virus Xing10, which was proved as NDV. This virus was similar to vaccine strain LaSota and belonged to genotype II, possessing

MDT of 10 h., ICPI of 0.2 and IVPI of 0. Sharma *et al*. (2012) isolated NDV from two apparently healthy sheep after the sixth passage in vero cell line.

Infection with virulent form of NDV in waterfowls, such as geese (Xiang *et al*., 2020) and ducks (Zahid *et al*., 2020) was reported and these species acted as a source of the virus to domestic and backyard chicken.

2.3.2 Transmission of the disease

Jones *et al*. (1973) detected NDV in 64 metre downwind from infected premises.

Capua *et al.* (1993) isolated a virulent NDV from embryonated chicken eggs, suggesting vertical transmission of the virus.

Alexander (2000) reviewed that secondary spread of ND might occur due to the movement of persons and equipment from infected to non-infected area.

Inhalation of droplets containing the virus and ingestion of faecal contaminated materials were the primary routes of transmission of ND (Seal *et al*., 2000).

Roy and Venugopalan (2005) reported isolation of a virulent NDV from dead-in-shell embryos and day-old hatchlings.

Alexander (2012) suggested that migratory birds might be responsible for the primary introduction of the virus in Great Britain and also stated that wild birds and waterfowls might be the reservoir hosts for lentogenic NDV, which on mutation could be converted into virulent virus causing disease in domestic birds.

Attenuated and killed vaccines used for vaccination against ND might be also responsible for the transmission of disease, if the vaccine virus was not properly attenuated or inactivated (Hines and miller, 2012).

Conteh *et al.* (2020) reported that housing of different species of birds susceptible to NDV in the same shed might lead to the widespread of disease in the study area.

Xiang *et al.* (2020) reported horizontal transmission of virus within the species and between species, among chicken and geese housed together by direct contact. The virus was pathogenic to chicken but not to geese. The virus was also transmitted from vaccinated birds to unvaccinated ones.

2.4 AETIOLOGY

Newcastle disease, an affliction which causes severe losses in both commercial and backyard poultry production was caused by NDV (Alexander, 1997). The virus was able to infect almost all orders of avian species, but the severity of the disease varies with the strain of virus and the host affected. Even low virulent strains of NDV may produce severe respiratory disease when co-infected with other organisms or due to the adverse environmental conditions (OIE, 2018).

2.4.1 Taxonomy

The name Newcastle disease virus was given by Doyle in 1927 based on the place of origin. Tumova *et al*. (1979) suggested a synonym APMV-1 for NDV to distinguish it from other avian paramyxovirus serotypes.

Strains of AOAV-1 were genetically classified into two genotype classes: Class I and Class II. Class I consisted of low virulent viruses mostly found in wild birds, while Class II consisted of viruses ranging from low virulent to high virulent, affecting wide range of species (Diel *et al*., 2012a).

International Committee on Taxonomy of Viruses (ICTV) renamed NDV formally called APMV-1, as *Avian avulavirus* 1 (AAvV-1) in 2016 and further in 2018 as *Avian orthoavulavirus* 1 (AOAV-1). The virus belongs to the newly formed

genus *Orthoavulavirus*, classified under the subfamily *Avulavirinae* of the family *Paramyxoviridae*. *Avulavirinae* consists of three genera, namely *Metaavulavirus, Orthoavulavirus* and *Paraavulavirus* within which twenty different serotypes of avian avulaviruses (Avian paramyxoviruses) are grouped. Serotype 1 consists of viruses causing ND.

Dimitrov *et al*. (2019) proposed a new classification of the NDV and also guidelines for naming them. According to this, Class I has one genotype and Class II consists of 20 genotypes some of which are further classified into sub genotypes.

2.4.2 Properties of the virus

2.4.2.1 Morphology

Newcastle disease virus is a pleomorphic, enveloped virus about 100-300 nm in diameter (Kingsbury and Granoff, 1970).

Virions are covered with glycoprotein spikes and contain herringbone shaped nucleocapsid which are 600-800 nm in length, 18 nm in diameter and are helically symmetrical (Murphy *et al.,* 1999).

Envelope consists of fusion protein (F) and haemagglutinin-neuraminidase protein (HN). The ribonucleoprotein complex is made up of nucleocapsid protein (NP) bound to genomic RNA to which RNA-dependent RNA polymerase (L) and phosphoprotein (P) are attached forming core of the virion. Matrix protein (M) is present just below the envelope (Fenner, 2011).

The HN protein form homotetrameric spikes and F protein form homotrimeric spikes, which project from the surface of the virus and bind with host cell receptors (Fenner, 2017).

2.4.2.2 Genome

Kolakofsky *et al*. (1974) stated that molecular weight of NDV genome would be 5.2 -5.7×10^6 Daltons.

The genome of the virus is antisense, non-segmented, single stranded RNA and contains one of the three genome sizes *viz*., 15,186, 15,192, and 15,198 nucleotides-nt (Dubois-Dalcq *et al.,* 1984). It encoded for six proteins *viz.,* NP, P, M, F, HN and L (Chambers *et al.,* 1986).

The NDV genome adhered to the “rule of six” *i.e.,* genome nt sequences are present in multiple of six, since NP protein binds effectively with six nucleotides of genome during replication (Calain and Roux, 1993).

All the viral genes were monocistronic, encoding single structural protein except P gene, which encodes for one structural (P) and two nonstructural (V and W) proteins (Steward *et al*., 1993).

Typically, genome at 3’end consists of 55 nt leader and its 5’ end consists of 114 nt trailer regions (Mohamed *et al*., 2009). It was not polyadenylated at the 3’ end and does not contain 5’ cap, but contains functional 3’ and 5’ noncoding elements (Fenner, 2017).

2.4.2.3 Proteins

The M protein present between lipid membrane (envelope) and nucleocapsid controls the viral RNA synthesis and helps in assembling the virion by interacting with actin, on host cellular membrane (Peeples and Bratt, 1984).

The F protein, synthesised as an inactive F0 protein has to be cleaved by cellular host proteases at FPCS to form F1 and F2 proteins and further these have to be linked by disulfide bond to form functional F1-F2 active form. The amino acid

sequence at FPCS region varies among the NDV strains and was responsible for the virulence of the virus (Toyoda *et al*., 1987; Glickman *et al*., 1988).

In electron micrograph, NP protein holds a herringbone like structure. It encases genomic RNA and protects it from degradation by the action of nucleases (Errington and Emmerson, 1997).

Mebatsion *et al*. (1999) suggested that M protein might be responsible for maintaining the spherical structure of the nucleocapsid and considered as the highly conserved protein in pramyxoviruses (Seal *et al*., 2000).

The P protein, the co-factor for the L protein when one and two guanine residues were inserted to the conserved RNA editing site of the P protein yields V and W proteins, respectively. Carboxy-terminus of V protein was known to have anti-interferon activity, thus interfering with the host innate immune response (Park *et al*., 2003).

The P-L complex was responsible for the replication of the whole genome. In addition, shifting of transcription to replication was regulated by the complex formed by P and NP proteins (Jahanshiri *et al*., 2005).

The RNA dependent RNA polymerase (L protein), the largest protein forms complex with P protein, catalyses viral mRNA synthesis and assists in RNA replication (Lamb and Parks, 2007).

The HN protein, a multifunctional protein aids in receptor recognition, interacts with F protein and helps in fusion, has sialic acid binding site and hence capable of agglutinating erythrocytes. It possesses neuraminidase activity and can cleave the sialic acid receptor which helps in viral release after replication. It plays an important role in tissue tropism. This protein has an antibody binding site and takes part in humoral immunity (Iorio and Mahon, 2008).

Nucleocapsid, P and L proteins associate to form ribonucleoprotein complex (RNP) which act as a template for the genome replication as well as mRNA synthesis (Dortmans *et al*., 2010).

Paldurai *et al.* (2014) reported that NDV strains possessing identical FPCS substantially vary in virulence. The authors concluded that other parts of the F protein also might be responsible for the virulence.

Fusion of F protein spike with host cell membrane brings conformational change to F protein causing binding of HN protein to host cell receptor aiding in viral entry in to the host cell (Fenner, 2017).

2.4.2.4 Physico-chemical properties of the virus

Elford *et al*. (1948) observed that NDV was stable at pH 5.5 to 7.5.

In 1967, Nakajima and Obara, proposed mean value of RNA content in NDV to be 0.72 per cent (determined by optical density method) and protein and lipid content to be 67.17 per cent and 40.96 per cent, respectively. The authors also reported the sedimentation co-efficient of RNA to be 55S.

Thermo-stability at 56°C of different NDV strains varied from 6-120 min. generally five minutes for lentogenic and 30-120 min. for virulent strains (Hanson, 1972).

Newcastle disease virus was found to be sensitive to detergents, lipid solvents, formaldehyde and oxidising agents (Ganar *et al.,* 2014).

Mohamed (2019) studied the effect of pH and moisture of poultry manure on survivability of virulent NDV. Shifting of pH to alkaline, dryness and lowering the moisture in the poultry manure took long time (33 days) to inactivate the virus.

2.4.2.5 Biological properties of the virus

The HN protein was found to be responsible for agglutination due to its binding with sialic acid receptor of red blood cells (Bang and Libert, 1952).

Newcastle disease virus could agglutinate erythrocytes of mammalian origin apart from chicken erythrocytes (Ratanparkhe *et al.,* 1982)

Kumanan *et al.* (1992) reported that strain differentiation was possible based on the ability of the virus to agglutinate equine erythrocytes. Fewer lentogenic strains were able to agglutinate erythrocytes from equine while velogenic strains did not.

The neuraminidase activity of HN protein helped in clumping progeny virus by degrading sialic acid receptor (Lamb and Kolakofsky, 1996).

Lamb *et al*. (2006) demonstrated the fusion of F protein to host cell followed by haemolysis and was found to occur at neutral pH.

Jin *et al.* (2016) studied the contribution of length diversity of HN protein in NDV virulence, replication and biological activities. The ICPI and MDT were not altered by the mutation in length of HN protein. *In vitro* studies revealed increased haemagglutination titre and receptor binding ability with virus containing extended HN protein.

Level of HI antibodies indirectly correlated with the immunity. The presence of low HI titre indicated strong immunity and neutralising antibodies against the F and HN proteins provided the functional measure of protection (Fenner, 2017).

Lentogenic virus with monobasic amino acid at FPCS exhibited reduced capacity in multicycle replication while velogenic and mesogenic NDV with polybasic amino acid at FPCS had greater capacity in syncytia formation and multicycle replication (Burman *et al.,* 2020).

2.5 PATHOGENESIS

The pathogenesis of the disease was studied experimentally by inoculating the virus into adult chicken and pigeons. The infected birds developed clinical signs by three to four days and died by four to five days after infection (Roy *et al.,* 2000).

De Leeuw *et al*. (2003) reported that low virulent NDV had monobasic amino acid sequence at C-terminus of F2 protein and also leucine at N-terminal of F1 protein, because of which the F0 protein could be cleaved only by extracellular proteases like trypsin, commonly present in the respiratory and intestinal tracts. Virulent NDV had multiple basic amino acid sequence at C-terminus of F2 protein and a phenylalanine at N-terminus of F1 protein and this could be cleaved by ubiquitous furin like proteases, present in most of the tissues intracellularly.

Multiple factor such as host species, age, stress, immune status, secondary infection, environmental condition, route of transmission, amount of the virus transmitted and strain of the infecting virus played an important role in the pathogenicity (Alexander *et al*., 2004).

Panda *et al*. (2004) employed reverse genetics to convert the sequence motif of low virulent NDV into that of virulent one. This increased ICPI values from 0/0.01 to 1.12-1.28. The opposite was also true, when Hu *et al*. (2009) modified virulent NDV cleavage site to a low virulent NDV strain and ICPI value reduced to 0.1 from 1.89.

Many PPMV-1 possessing multiple basic amino acid sequence showed low virulence phenotype upon ICPI testing, proving that other factors were also involved in virulence and pathogenicity of NDV (Dortmans *et al*., 2010).

Kim *et al*. (2014) reported the importance of HN protein in the virulence of NDV. By extending C-terminal of HN gene of virulent Indonesian NDV isolate from

571 amino acids to 577 and 616 amino acids, there was marked reduction in viral pathogenicity.

The V protein of NDV was known to interfere with type I and II interferon response in host. Qiu *et al*. (2016) conducted a study on the V protein deficient NDV. These viruses were unable to target STAT-1 protein and therefore were incapable to block interferon signalling pathway in host, making them less virulent.

2.6 DIAGNOSIS

2.6.1 Clinical signs

Beard and Hanson (1984) classified NDV into five pathotypes based on the predominant signs in affected chicken. They are Velogenic Viscerotropic NDV/ Doyle’s form (VVNDV), Velogenic Neurotropic NDV/ Beach’s form (VNNDV), Mesogenic NDV/ Beaudette’s form, Lentogenic NDV/ Hitchner’s form and Asymptomatic enteric NDV.

McFerran and McCracken (1988) considered greenish-yellow diarrhoea, signs of depression, oedema of head and wattle, nervous signs like torticollis and paralysis and respiratory signs as generalised symptoms of ND. The authors stated that these signs were not universal and no gross lesions could be considered as pathagnomonic for any form of ND.

Lentogenic NDV generally did not cause disease in adult chicken and even with experimental infection no clinical signs were observed (Hamid *et al*., 1990), but might cause respiratory disease in very young birds.

Nakamura *et al*. (1994) observed mild respiratory clinical signs, drop in egg production along with misshapen eggs in birds affected with Beaudette’s form.

Ostriches were usually presented with nervous signs and mortality occurred in young birds (Alexander, 2000).

Secondary bacterial and concurrent viral infections complicated the mesogenic viral infection resulting in severe disease (Alexander and Senne, 2003).

Kommers *et al*. (2003) stated that main signs in Doyle's form included anorexia, conjunctival swelling, ruffled plumage, prostration, tremors, diarrhoea and weakness.

Clinical signs in different avian species differed and also depended on breed of the bird. Descending order of birds showing most clinical signs to least signs are chicken, turkeys, pigeons and ducks. The disease in pigeons was usually characterised by tremors (Wakamatsu *et al*., 2006).

Non-vaccinated pheasants showed similar clinical signs as chicken with high susceptibility to infection (Aldous and Alexander, 2008).

Terregino and Capua (2009) reported that neurological signs like head twitch, opisthotonus, tremors and paralysis were generally observed in birds affected with VVNDV. Respiratory signs like open mouth breathing and gasping were also considered as prominent features.

Susta *et al*. (2010) instilled VVNDV through eye-drop in chicken and the clinical signs were limited to open mouth breathing and respiratory signs were rarely observed.

On experimental infection with mesogenic NDV, very minimal clinical signs were observed. Milder neurological signs were seen in rare cases (Susta *et al*., 2011).

Desingu *et al.* (2016) observed nervous signs like inco-ordination, wing paralysis, tremors, torticollis and circling along with varying respiratory distress in peafowls affected with virulent NDV.

Xiang *et al.* (2020) on experimental inoculation of virulent NDV into chicken observed neurological signs like head twitch, muscular tremors and paralysis after 6 to 11 days post-inoculation (PI). Some birds showed diarrhoea, depression and ruffled feathers.

2.6.2 Pathology

Multi-focal haemorrhages throughout the serosal layer of intestine, multi-focal necrosis of gut associated lymphoid tissue, disseminated foci of necrosis in spleen along with 'old faithful' lesions such as haemorrhages in caecal tonsils, ulceration and multi-focal haemorrhages in the junction between proventriculus and gizzard were highly suggestive of VVND (McDaniel and Orsborn Jr, 1973).

Gross lesions were minimal with Beaudette's form. Experimental eye-drop instillation of mesogenic NDV revealed mild splenomegaly and mild degree of conjunctivitis (Brown *et al*., 1999).

Hooper *et al*. (1999) reported gross lesions like reddening of trachea and chronic non-suppurative tracheitis in commercial broilers infected with lentogenic NDV.

Roy *et al.* (2000) carried out necropsy of birds which were dead due to virulent NDV. The authors observed petichiae in proventriculus and caecal tonsils and mild congestion of intestine in chicken and pigeons, respectively.

Gross lesions were usually minimal or absent in Beach's form (Kommers *et al.,* 2003).

In asymptomatic enteric ND referred as AEND, replication of the virus was limited to gastro-intestinal tract with no symptoms and lesions (Hines and Miller, 2012).

Gowthaman *et al.* (2013) conducted systematic necropsy examination in dead turkeys and Japanese quails affected with NDV. Congestion of meninges, catarrhal tracheitis, haemorrhagic lungs and cloudy air sacs were observed in turkeys, while Japanese quails revealed severe congestion in internal organs and diffuse ulcers in the intestine.

Morla *et al.* (2016) reported post-mortem findings of the birds which were dead due to virulent NDV. The main lesions were haemorrhages at tip of the proventriculus and caecal tonsils and congestion of spleen. Some birds showed congestion in brain.

Alazawy and Al Ajeeli (2020) conducted necropsy in chicken suffering from velogenic ND. Petechial haemorrhages in proventriculus, inflammation of trachea, severe haemorrhages in intestine and spleen were observed.

2.6.3 Virus isolation and identification

Isolation of the virus was considered as the “gold standard” test for definitive diagnosis of ND (Alexander, 2000).

Faecal/ cloacal swabs and tracheal/ oro-pharyngeal swabs from live birds, tissue from dead birds are the samples of choice for the isolation of NDV. Tissue samples should include liver, spleen, lung, kidney, intestine and caecal tonsils collected separately or pooled. In addition, brain must also be collected and was not mixed with other tissues (Pedersen, 2011).

The suspension of homogenised tissues and swabs added with antibiotics are inoculated in nine to eleven day-old ECE via allantoic route or inoculated in cell

culture of avian or non-avian origin, for isolation of NDV. It was the prescribed test for international trades (OIE, 2018).

2.6.3.1 Virus isolation in embryonated chicken eggs

Cunningham (1960) described method of inoculation of NDV into allantoic cavity of nine day-old ECE. After harvesting the allantoic fluid, it has to be tested for HI for confirmation.

Blaskovic and Styk (1967) isolated the virus by inoculation of the filtrate of suspected clinical tissue sample into specific pathogen free (SPF) ECE by allantoic route. The authors considered a sample as negative for NDV at least after three blind passages.

Mortality of the inoculated embryo was influenced by the strain of the virus, age of the embryo and concentration of the inoculum. Death often occurred in three to five days PI. The embryos died very quick when inoculated via amniotic and yolk sac routes (Cattoli *et al*., 2011).

Jahan *et al*. (2013) isolated NDV in SPF-ECE by inoculating intra-allantoically. The authors observed mortality of embryos after 48 h. PI and most of the embryos died within 120 h. PI. It was also found that virus infectivity titre was high after 96 h. PI.

Balachandran *et al*. (2014) inoculated NDV in to nine day-old ECE by intra-allantoic route and observed mortality, haemorrhages throughout the body of embryo along with haemorrhages at occipital region three to seven days PI.

Qosimah *et al*. (2018) studied the effect of NDV on embryonic length, embryo mortality, pathological changes and protein profile when inoculated intra-allantoically in to nine to eleven day-old ECE in different level of NDV titre.

Embryos were shorter, dead in 48 h. PI and had severe haemorrhages on all body surfaces, predominantly on occipital region when compared with non infected ECE.

2.6.3.2 Virus isolation in cell culture

Beard *et al*. (1970) developed a protocol for the isolation and identification of NDV simultaneously in one culture step. Chicken embryo fibroblast (CEF) cell culture was used for the study and the results were compared with HI, haemadsorption of selected erythrocytes and elution rate of haemadsorbed virus.

Cytopathic effects (CPE) such as formation of syncytia, disruption of monolayer and plaque of diameter 0.5-4.0 mm which are clear, dull or very dark could be formed when cell lines were infected with velogenic or mesogenic NDV strains. But, the same was true for the lentogenic virus, when culture medium was supplied with Mg2+ ions and trypsin or diethylaminoethyl dextran at the rate of 0.01 mg/mL (McGinnes *et al*., 2006).

Ravindra *et al*. (2009) observed CPE such as rounding, syncytia, detachment of the monolayer cells and cell death 48 h. PI of NDV in to vero cell lines. In addition to this, the electron microscopic examination of the virus infected cells revealed cytoplasm vacuolation, membrane blebbing, nuclear envelope breakdown and nuclear condensation.

Terregino and Capua (2009) reported that a wide variety of cell cultures both of avian and non-avian origin could be used for propagation of APMV-1 strains. The most widely used ones were CEF, chicken embryo kidney cells, chicken embryo liver cells, African green monkey kidney cells and avian myogenic and chicken embryo related cells.

Some strains of PPMV-1 and APMV-1, non pathogenic Ulster strain could only be isolated through cell culture and not through ECE (Dortmans *et al*., 2011).

Nath *et al.* (2016) plaque purified allantoic fluid containing NDV using CEF cell culture at 0.01 multiplicity of infection.

Al-Shammari *et al.* (2020) studied CPE caused by NDV in vero and HeLa cell lines. Vero cell lines exhibited rounding while HeLa presented syncytia formation along with cytoplasm vacuolation and membrane blebbing 24 h. PI.

2.6.4 Virus identification by haemagglutination and haemagglutination inhibition tests

Burnet (1942) reported agglutination activity of NDV. Infected amniotic/ allantoic fluid were able to agglutinate erythrocytes from several species and this activity was inhibited, when specific antiserum was used.

The HA property was due to the HN protein of NDV (Russell, 1988) and HI activity could be used for confirmation of NDV using specific antiserum (Alexander, 2000).

Isolates negative for HA test should be passaged again at least once. Bacteria and some viruses like influenza virus might cause agglutination; hence contamination of amnio-allantoic fluid (AAF) should be checked by culture method and evaluated by HI test using specific antiserum (Hines and Miller, 2012).

Several researchers had used HA and HI tests for identification and confirmation of NDV after isolation (Roy *et al*., 2000; Vijayarani *et al*., 2010; Desingu *et al*., 2016; Fuller *et al.,* 2017).

2.6.5 Pathotyping of the virus

Historically, three *in vivo* tests had been used extensively to determine the pathogenicity of NDV. These tests included determination of i) MDT in ECE, ii) ICPI and iii) IVPI (Alexander, 1988).

Many workers have differentiated NDV isolates based on the pathogenicity indicies (Kumanan *et al*., 1992; King and Seal, 1998; Mathivanan, 2002).

Several PPMV-1 strains possessing polybasic amino acid sequence at FPCS region had been reported with lentogenic MDT values and mesogenic ICPI values and these values were increased with multiple passages in chicken (Kommers *et al*., 2001).

Yu *et al*. (2001) conducted intra-cloacal inoculation test for differentiating velogenic NDV isolates into viscerotropic velogenic and neurotropic velogenic. The scores were given based on the observations noticed during necropsy.

Virulent NDV outbreaks need immediate attention and are required to be notified to the OIE; hence, pathotyping of NDV isolates is necessary (Petrini and Vallat, 2009).

Many molecular based assays had been developed for determination of amino acid sequence at FPCS region of F gene in the virus, which in turn could be used for pathotyping NDV isolates (Samal *et al*., 2011).

The IVPI and MDT in ECE had been used overtime but by international agreement, virulence of the virus should be assessed based on ICPI test (OIE, 2018).

2.6.5.1 Mean death time

Newcastle disease virus isolates with MDT less than 60 h., 60-90 h. and more than 90 h. were classified under velogenic, mesogenic and lentogenic pathotypes, respectively (Alexander, 1998).

Vijayarani *et al*. (2010) conducted MDT in ECE for pathotyping of NDV isolated from a peacock. The authors reported a value of 47 h. in order to prove an isolate to be velogenic.

For ascertaining MDT, virus was inoculated in to nine to eleven day-old ECE and the MDT was calculated as the average time in hours, taken by mean lethal inoculum of the virus to kill all the inoculated ECE (Munir *et al*., 2012).

Gowthaman *et al*. (2013) recorded an outbreak of ND in commercial chicken farmed along with other species, especially turkeys and Japanese quails. The MDT of the isolates from turkeys and Japanese quails were between 38-60 h. The virus isolate from chicken showed MDT of >90 h. They concluded that difference might be due to the presence of potential antibodies against NDV in chicken and disease in turkeys and Japanese quails might be due to vaccination failure.

Choi *et al.* (2014) employed MDT for pathotyping of NDV and the reported value was <60 h. which grouped the isolate into virulent NDV.

Chowdhary *et al.* (2020) conducted MDT for pathotyping NDV isolated from chicken and pigeon. The values for chicken and pigeon isolates were found to be 51.43 h. and 92 h., proving them to be velogenic and mesogenic, respectively.

2.6.5.2 Intracerebral pathogenicity index

Time taken for day-old chicks to die or show symptoms after intracerebral inoculation of the virus was used in calculating ICPI (Hanson and Brandly, 1955).

Lentogenic virus had ICPI less than 0.7, while for mesogenic strains, value ranged from 0.7-1.5 and virulent strains had values more than 1.5 (Alexander, 1998).

Mathivanan (2002) differentiated the NDV isolates from guinea fowl based on ICPI and MDT. The reported values were 1.8 and 54 h., respectively and classified the isolate as velogenic.

Alexander *et al*. (2004) reported ICPI as one of the virulence criteria required for reporting NDV to OIE.

Diel *et al.* (2012b) reported the ICPI values of NDV isolated from cormorants to be 1.38-1.55, proving them to be virulent.

The definitive assessment of NDV virulence is to be made on the ICPI value. The isolate showing ICPI value >0.7 should be considered as virulent (OIE, 2018).

Nagy *et al.* (2020) pathotyped NDV by MDT and ICPI tests and the corresponding values were <36-48 h. and 1.88-2.00, classifying the isolate in the velogenic group.

2.6.5.3 Intravenous pathogenicity index

The IVPI was determined based on the time taken for six week-old chicks to show signs of paralysis or die after intravenous injection of NDV isolate. The values for lentogenic strain is 0, for mesogenic strain, it ranges from 0.05 to 0.31 and value range from 0.5 to 1.5 for velogenic strain (NRC, 1963).

King and Seal (1998) conducted IVPI test for NDV isolated from turkey and captive birds. The authors obtained value of 0.00 and 0.39 and grouped them as lentogenic and mesogenic strains, respectively.

Qin *et al.* (2008) employed IVPI test for pathotyping 25 NDV isolates. Based on IVPI value, 24 isolates were velogenic and one isolate was found to be lentogenic. The results correlated with the ICPI values.

Desingu *et al.* (2016) employed IVPI as one of the criteria for pathotyping NDV. The virus was inoculated into six week-old SPF chicks. The authors obtained a value close to three which was typical for velogenic strains.

Al-Shammari *et al.* (2020) carried out IVPI test in six week-old chicken for pathotyping of NDV isolate. The IVPI index of 2.56, proved the isolate as velogenic.

2.6.6 Serological tests

Serology was mostly employed in diagnostic laboratories for quantification of humoral immune response following vaccination (Thayer and Beard, 1998).

The most commonly used serological tests for detection and quantification of antibodies to NDV were HI and ELISA (Alexander, 2012).

Haemagglutination inhibition test, plaque neutralisation test, virus neutralisation test (VNT), agar gel immunodiffusion (AGID), single radial immunodiffusion and ELISA were the serological techniques available. Among these, HI test and ELISA were capable of measuring antibody titres (Hines and Miller, 2012).

2.6.6.1 Enzyme-linked immunosorbent assay

Swain *et al*. (1998) used two MAbs raised against HN protein of NDV in mouse and conducted Dot-ELISA and AGPT. Both the MAbs reacted specifically with all the NDV isolates in Dot-ELISA.

Makkay *et al*. (1999) reported that ELISA based on recombinant NP protein expressed in insect cells was able to detect viral antibodies and this test differentiated vaccinated birds from infected ones.

De Sousa *et al*. (2000) developed a liquid phase blocking ELISA (LPB-ELISA) using polyclonal immunoreagents for detection and quantification of antibodies to NDV from serum of ostriches and rheas and compared it with the HI test. Both the tests yielded almost similar results. The former was easy to perform and cheaper when compared with ELISA using MAbs.

Mohan *et al*. (2006) developed singe serum dilution ELISA using recombinant HN protein as antigen for serological profiling of NDV. The test was simple and 35-40 samples could be tested and quantified with accuracy while only 8-

10 samples were examined at a stretch in a microtitre plate with serial dilution. Das and Kumar (2015) used a similar method using P protein as antigen.

Zhoa *et al*. (2018) employed recombinant full length NP protein expressed in bacterial cells as antigen and developed antibody detection ELISA for differentiating infected from vaccinated animals (DIVA) property of the test.

2.6.6.2 Haemagglutination inhibition test

Allan and Gough (1974) described the standard HI test for NDV. Log_2 of the reciprocal of the serum dilution giving 50 per cent HA was taken as the titre.

Brugh and Beard (1980) developed a practical method for collecting and processing whole blood samples using filter paper in order to facilitate large scale testing programmes for NDV. Haemagglutination inhibition test was used for detection of antibodies eluted from the filter paper and simultaneously collected serum samples. The authors obtained similar results with both the collection methods.

Sulochana *et al*. (1981) used HI test for detection of antibodies to NDV in Indian house crows. The antibody titre upon experimental infection with NDV isolates was found to rise from day 7, reached maximum in 21st day and declined thereafter.

Haemagglutination inhibition test was employed by many workers for detection and quantification of antibodies to NDV (Kumanan *et al*., 1990; Tewari *et al*., 1992; Roy *et al*., 2000; Rahman *et al*., 2017).

The presence of NDV might be regarded as positive, if the HI titre is 16 or more against 4HA unit of the antigen (OIE, 2018).

2.6.7 Molecular detection of the virus by reverse transcriptase - polymerase chain reaction

Jestin and Jestin (1991) developed a RT-PCR for identification of NDV for the first time. Viruses were grown in SPF eggs and infected allantoic fluids were used for extraction of the virus. A 238 base pair F gene sequence was amplified using universal primers. The amplified PCR products were subjected for two per cent agarose gel electrophoresis and stained with ethidium bromide. All the isolates were amplified and detected by the assay.

Kant *et al*. (1997) employed RT-PCR to detect NDV in homogenised tissue samples. Two oligonucleotide primers targeting FPCS region of F gene of either virulent or non-virulent NDV strains, respectively were used for differentiating NDV. All virulent and some non-virulent isolates were detected by the test. The authors opined that sensitivity of the assay required 10^5 EID_{50}/ mL of virus concentration.

Kho *et al*. (2000) coupled RT-nested PCR with ELISA as detecting system for identification of NDV. The authors used two nested primers which were highly specific for F gene of NDV of all the pathotypes. Nested PCR was found to be 100 times more sensitive than the non nested PCR. When ELISA was applied as detection system, the sensitivity increased 10 times than that of gel electrophoresis detecting system.

The common forward primer and distinct reverse degenerate primers targeting F gene encoding FPCS region were used in RT-PCR to differentiate avirulent and virulent NDV strains (Tiwari *et al*., 2004).

The RT-PCR was performed for detection of NDV in both allantoic fluid obtained from NDV inoculated SPF-ECE and tissue samples obtained from NDV infected chicken experimentally. The assay detected the virus in both allantoic fluid and tissue samples (Smietanka *et al*., 2006).

Haque *et al*. (2010) isolated NDV and identified using HI test and RT-PCR. Reverse transcriptase polymerase chain reaction was conducted for both the RNA targeting FPCS region of F gene from clinical samples and isolated laboratory samples (allantoic fluid and infected cultural fluid). The assay revealed equal sensitivity and specificity.

Liu *et al*. (2011) developed a multiplex RT-PCR for detection and differentiation of NDV into class I and II strains. Two separate sets of primers specific to F gene of either class I or class II were used in the assay.

The RT-PCR and restriction enzyme analysis were employed for detecting and differentiating APMV-1 and PPMV-1. Primers used were targeting the F gene and eight restriction enzymes were used for enzymatic digestion of the amplicons. The assay differentiated APMV-1 and PPMV-1 accurately at genomic level (Naveen *et al*., 2013).

Gowthaman *et al*. (2016) isolated and characterised NDV from an emu. They performed RT-PCR targeting F partial nucleotide sequence of F gene including FPCS region.

Virulence of NDV could be assessed by amplifying F gene containing FPCS using RT-PCR. Demonstration of the multiple basic amino acids in the amplified product confirmed virulent NDV infection (OIE, 2018).

2.6.8 Molecular characterisation of the virus and phylogenetic analysis

Collins *et al*. (1993) based on the molecular level phylogenetic studies, determined amino acid sequence ^{112}R/K-R-Q-K/R-R^{116} for virulent and mesogenic strains, while ^{112}G/E-K/R-Q-G/E-R-L^{117} for lentogenic NDV strains.

Jestin *et al*. (1993) first reported that molecular technique could identify and characterise NDV. The authors described that nested RT-PCR could amplify F0 gene

region including FPCS region directly from infected tissue samples. Nucleotide sequence of the product was also determined and used it to assess virulence of the virus.

Both traditional and molecular techniques were used by Marin *et al*. (1996) for identification of NDV. The techniques revealed similar results, but molecular techniques were able to differentiate the minute genetic heterogeneity in lentogenic field strains while traditional techniques failed to do so.

King and Seal (1997) conducted biological and molecular characterisation of NDV. They amplified F gene and sequenced the amplified product. Phylogenetic analysis revealed lentogenic strains were of different lineage from the virulent strains.

The RT-PCR targeting F gene was carried out for detection and characterisation of NDV isolated from chicken. The PCR products were subjected for nucleotide sequencing and further phylogenetically analysed. Based on this, isolates were grouped as II, III, VI and VII of class II NDV (Qin *et al*., 2008).

Tan *et al.* (2008) isolated three NDV having amino acid motif ^{112}GRQGRL^{117} and by pathotyping they were identified as virulent. But, phylogenetic analysis revealed all the three isolates to be related to LaSota. The authors concluded that these viruses might have originated from the vaccine virus under host immune pressure.

Diel *et al*. (2012a) proposed a unified classification system for NDV using complete F gene coding sequence. This led to the classification of NDV into two classes consisting genotypes and subgenotypes. Dimitrov *et al*. (2019) updated this classification.

Perozo *et al*. (2012) isolated NDV from a field outbreak. The researchers employed RT-PCR and complete coding region of F gene was amplified and

subjected for sequence analysis. Based on the phylogenetic analysis, the isolate was grouped in genotype VII of class II NDV.

The emergence of new variant of genotype XIII NDV was reported from North-East India. The complete genome of the isolate was sequenced and characterised. The strain showed ^{112}RRQKRF^{117} in its FPCS region of F gene. On phylogenetic and evolutionary analysis, the isolate clustered with the strains of XIII and showed distance of 9.2 per cent and 11.2 per cent with subgenotype XIIIa and XIIIb virus, respectively. The result confirmed that the isolate was independent among genotype XIII viruses and was tentatively grouped in a separate subgenotype XIIIc group (Nath and kumar, 2017).

Ansori and Kharisma (2020) conducted nucleotide sequence and phylogenetic analysis targeting the F gene. The most virulent NDV strains were having ^{112}KRQKRF^{117} while avirulent strains possessed motif ^{112}GKQGRL^{117} and ^{112}GRQGRL^{117}.

2.7 CONTROL

2.7.1 Vaccines

For the PMV-1 serotype (NDV) the working hypothesis, control policies practiced for the past 50 years had been that, no significant antigenic variation occurred between strains and isolates. Vaccines derived from a single strain would protect against all virulent field viruses (Allan *et al*., 1978).

Live virus vaccines are the most regularly used ones worldwide, formulated from the strains isolated in 1940s and 1960s. LaSota, B1 and VG/GA were prepared from the virus circulating in poultry (Meulemans, 1988).

Boursnell *et al*. (1990) developed a fowlpox virus vector-based vaccine, expressing NDV F or HN protein and was proved to protect chicken against virulent NDV.

Bensink and Spradbrow (1999) reported that LaSota was the most broadly used vaccine strain for its superior immunogenicity, while B1 based vaccine are highly attenuated with no post-vaccine respiratory reaction. V4 and I2 had greater thermo-stability and could tolerate slight temperature variation in the absence of cold chain.

Recombinant Herpes Turkey virus-ND vaccine was able to protect birds from clinical disease and mortality from ND (Sonoda *et al*., 2000). Huang *et al*. (2004) developed LaSota vector-based vaccine expressing VP2 protein of infectious bursal disease virus (IBDV) which provided protection against both NDV and IBDV.

Komorov and Mukteswar mesogenic strain vaccines developed in India were used as live vaccines. They are usually administered as booster vaccine priming with lentogenic strains (Senne *et al*., 2004). The VG/GA vaccine stimulated gut mucosal immunity as they were more enterotropic (Perozo *et al*., 2008).

All live attenuated vaccines irrespective of site of administration were known to induce both systemic and mucosal immune response similar to infection (Rauw *et al*., 2009). They were suitable for mass application via drinking water making them relatively inexpensive (Geus *et al*., 2012). Hundred per cent protection was achieved with mean EID_{50} of 10^2-10^8 virus load (Cornax *et al*., 2012).

Inactivated vaccines were the earliest strategy in vaccination. Inactivation was done by physical or chemical methods. Most popular and commonly used chemicals for inactivation are binary ethylenimine and formaldehyde (Razmaraii *et al*., 2012).

Kim *et al*. (2014) developed virulent NDV vector-based vaccine expressing H5 protein of avian influenza virus.

Many experimental vaccines such as virus-like particle vaccine (McGinnes *et al*., 2010), antigen-antibody complex vaccine (Kapczynski *et al*., 2012) and nanoparticle vaccine (Dai *et al*., 2015) have been developed overtime giving maximum protection against ND, but cost involved in the production and availability of vaccine globally is uncertain (Dimitrov *et al*., 2017).

Yang *et al.* (2020) developed a nanoparticle inactivated NDV vaccine using chitosan, hydroxypropyltrimethyl ammonium chloride chitosan and sulphated chitosan as adjuvants. Humoral and cellular immune responses were measured and compared with commercial inactivated oil emulsion vaccine. The former provoked better cellular immunity, but low level of humoral immunity when compared to the latter vaccine.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

All the reagents used in the study were of molecular biology grade, obtained from Sigma Aldrich (USA), Hi-Media (India), Sisco Research Laboratories (SRL) private limited and Merck GeNei (Bangalore). Glassware were from Borosil and plasticware from Tarson. The facilities in the Department of Veterinary Microbiology and Central Instruments Laboratory, College of Veterinary and Animal Sciences (CVAS), Mannuthy were utilised for the study. Permission to carry out experimental work on animals was obtained from Institutional Animal Ethics Committee (IAEC).

3.1 COLLECTION OF CLINICAL SAMPLES

3.1.1 Materials

1. Tissues
2. Sterile swabs
3. Phosphate buffered saline (PBS 1X) (0.15 *M*, pH 7.2)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Potassium dihydrogen phosphate anhydrous	0.2 g
Disodium hydrogen phosphate anhydrous	0.92 g

Dissolved in one litre distilled water and mixed. The pH was adjusted to 7.2 using 1*N* sodium hydroxide (NaOH) and sterilised by autoclaving at 121°C for 15 min. and stored at 4°C.

4. Antibiotic- antimycotic solution containing,

Penicillin G	1,00,000 Units/µL
Streptomycin	100 mg/µL
Amphotericin B	200 µg/µL

Ten microlitre of the solution was added to one microlitre of the suspension to obtain a final concentration of 10,00,000 units of penicillin, one gram of streptomycin and two milligram of amphotericin B.

3.1.2 Method

Pooled tissue samples consisting of lung, kidneys, caecal tonsils, spleen, liver and heart were collected in sterile vials containing PBS from recently dead and ailing birds with gross lesions/clinical signs suggestive of ND brought to the Departments of Veterinary Microbiology and Veterinary Pathology, CVAS, Mannuthy. Brain and intestine were collected separately and stored at -20°C. Tracheal and cloacal swabs were also collected in PBS from the birds showing clinical signs suggestive of ND presented to the Teaching Veterinary Clinical Complex, CVAS, Mannuthy for treatment. The swabs were stored at -20°C until processed.

3.2 PROCESSING OF CLINICAL SAMPLES

The tissue samples were homogenised with a sterile mortar and pestle, part of the homogenised tissue was used for RNA extraction. Twenty per cent suspension (w/v) from the remaining part of homogenised tissues was prepared in sterile PBS, centrifuged at 3,000 rpm for 10 min. and the supernatant was collected in sterile RNAase free vials. It was then filtered through 0.22 µm sterile disposable syringe filter. The filtrate after adding antibiotic - antimycotic solution was incubated at 37°C for 30 min. and later kept at -20°C until used for virus isolation.

The tracheal and cloacal swabs collected were squeezed against the wall of the collection tube by a sterile pipette. A part of the fluid material was taken for RNA extraction. Remaining part was centrifuged at 1,000 x g for 20 min. To the supernatant, antibiotic - antimycotic solution was added, incubated at 37°C for 30 min. and later kept at -20°C until used for virus isolation. Five-fold higher concentration of the antibiotic-antimycotic solution was added for cloacal samples.

3.3 DIRECT DETECTION OF NEWCASTLE DISEASE VIRUS FROM CLINICAL SAMPLES BY RT-PCR

3.3.1 Ribonucleic acid extraction

The total RNA was extracted by TRIzol method (Nanthakumar *et al.,* 2000) from the triturated tissue samples, tracheal and cloacal swabs and LaSota vaccine.

3.3.1.1 Materials

1. Biosafety cabinet II
2. RNase Zap RNase decontamination solution
3. RNase decontamination reagent
4. Centrifuge tubes (2 mL)
5. Diethyl pyro carbonate (DEPC) treated water
6. TRIzol reagent
7. Chloroform (AR)
8. Isopropanol (AR) (100 per cent)
9. Ethanol (75 per cent)

Ethanol	75 mL
DEPC treated water	25 mL

Stored at 4°C

3.3.1.2 Preparation for ribonucleic acid isolation

The isolation was done in biosafety cabinet II. The surface and work table were wiped clean with 70 per cent ethanol, followed by RNase ZAP (Sigma) and rinsed with RNase free water. Ultra violet lamp was switched on for 20 min. prior to isolation of RNA. All plasticware used *i.e.*, pipette tips and microcentrifuge tubes were certified by manufacturers as RNase free (Invitrogen, USA). All the solutions

and buffers used for RNA isolation were prepared using 0.1 per cent DEPC treated water.

3.3.1.3 Method

Processed tissue samples (0.25 mL) were pipetted into a two millilitre RNase free microcentrifuge tube, 0.75 mL of TRIzol LS reagent was added and mixed by pipetting the suspension up and down several times and incubated at room temperature for five minutes. Chloroform (0.2 mL) was added to the tube followed by vigorous shaking for 15 sec. and incubated for 10 min. at room temperature. The contents were centrifuged at 12,000 x g for 15 min. at 4ºC. The mixture was separated in to a lower red phenol chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase was carefully transferred to a fresh tube.

To the aqueous phase, 0.5 mL of isopropanol was added, vortexed and incubated at room temperature for 10 to 15 min. followed by centrifugation at 12,000 x g for 10 min. at 4ºC. The supernatant was discarded from the tube leaving only the RNA pellet. The pellet was washed with one millilitre of 75 per cent ethanol and centrifugation was performed at 7,500 x g for five minutes at 4ºC and the supernatant was discarded. The pellet was air dried for five to ten minutes and then suspended in DEPC treated water (20 to 50 µL). The extracted RNA from samples and LaSota vaccine was stored at -70ºC.

Concentration and purity of the obtained RNA were measured using spectrophotometer (NanoDrop 2000C). The purity of RNA stock was estimated by finding the ratio between the OD readings at 260/230 nm and 260/280 nm. The samples showing OD value between 1.8 and 2 were chosen for further studies.

3.3.2 Reverse transcription

After isolating RNA from samples and from LaSota vaccine, reverse transcription reaction was carried out. The resultant cDNA was immediately used as template for PCR or stored at -70ºC for subsequent use.

3.3.2.1 Complementary deoxyribonucleic acid synthesis

First strand of complementary DNA (cDNA) was synthesised using iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instruction. The reactions were set up in 0.2 mL PCR tubes as shown in table 1. Master mix was prepared excluding template RNA and nuclease free water. These were added individually to each reaction tube based on the concentration of RNA obtained.

The contents of the tube were mixed gently and spun briefly. The reaction mix was set up initially at 25ºC for five minutes for priming, followed by 46°C for 20 min. for reverse transcription, after which inactivation was done at 95°C for one minute. After synthesis, cDNA was stored at -70ºC until further use.

Table 1. Optimised concentration of reagents for first strand cDNA synthesis

Sl. No.	Components	Volume
1	Template RNA	100 fg- 1 µg of total RNA
2	5X reaction buffer	4 µL
3	Reverse transcriptase	1 µL
4	Nuclease free water	To make up to 20 µL
	Total	20 µL

3.3.3 Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction was used for the detection of NDV from the processed clinical samples.

3.3.3.1 Materials

1. Emerald Amp GT PCR master mix (Takara Bio company)
2. Primers (Table 2)
3. Nuclease free water
4. Template (cDNA)

3.3.3.2 Designing and synthesis of primers

The primers specific for the F gene were used for the detection of NDV. The sequences and other parameters of the primers are depicted in table 2. Primers were custom synthesised commercially (Sigma Aldrich) and obtained in lyophilised form.

Table 2. Sequences of primers used for RT-PCR targeting F gene

Sl. No.	Primer name	Primer sequence (5'-3')	Product size	Reference
1	NDV UP	GGA GGA TGT TGG CAG CAT T	320 bp	Pang *et al.* (2002)
2	NDV DOWN	GTC AAC ATA TAC ACC TCA TC		

3.3.3.3 Dilution and storage of primers

The stock primers were centrifuged in a microcentrifuge for about 30 sec. to prevent the loss of contents. Nuclease free water as specified by the manufacturer was added to obtain a stock concentration of 100 p*M*/µL. Stock solutions were incubated

at room temperature for one hour and working solution was prepared in sterile 1.5 mL microcentrifuge tubes at a concentration of 10 p*M*/µL and stored at - 20 ºC.

3.3.3.4 Optimisation of polymerase chain reaction conditions

The optimisation of PCR conditions was achieved through gradient PCR (MJ Mini Bio- Rad thermal cycler). For these, modifications in different time-temperature combinations of annealing and extension steps were used. The temperature gradient which provided the best results for amplification was selected for all downstream use. The reaction was carried out in 0.2 mL PCR tubes and details of PCR reaction mix are provided in table 3. Complementary DNA from LaSota vaccine was used as positive control. Negative control was made up with nuclease free water. The master mix prepared was spun briefly. Polymerase chain reaction was performed in a MJ Mini thermal cycler (Bio-Rad, USA) using the programme described in table 4.

Table 3. Optimised concentration of PCR reagents for the amplification of F gene (320 bp)

Sl. No.	Constituents	Volume (µL)
1	Template cDNA	3
2	Forward primer (10 p*M*/µL)	1
3	Reverse primer (10 p*M*/µL)	1
4	Emerald Amp GT PCR master mix	6.25
5	Nuclease free water	1.25
	Total	12.5

Table 4. The PCR conditions optimised for the amplification of F gene (320 bp)

Sl. No.	Step	Temperature (°C)	Time	
1	Initial denaturation	94	10 min.	
2	Denaturation	94	45 sec.	35 cycles
3	Annealing	55	1 min.	
4	Extension	72	1 min.	
5	Final extension	72	10 min.	

3.3.4 Submarine agarose gel electrophoresis

3.3.4.1 Materials

1. Ethidium bromide stock solution

Ethidium bromide (SRL)	10 mg
Triple distilled water	1 mL

The solution was mixed and stored in amber coloured bottle at 4°C.

2. Tris borate EDTA (TBE) buffer (10X) pH 8.2

Tris base	108.0 g
Boric acid	55.0 g
0.5 *M* EDTA, pH (8.0)	40.0 mL

The volume was made up to one litre by adding triple distilled water. It was autoclaved at 121°C and 15 lbs pressure for 15 min. and stored at room temperature. The stock solution was diluted to 1X before use.

3. Agarose gel (1 per cent)

Agarose low EEO (SRL)	1.0 g
Tris base ethylenediamine tetraacetic acid (EDTA) buffer	100 mL

4. Molecular weight marker

   DNA ladder 100 bp (SRL)

3.3.4.2 Method

The PCR product was detected by electrophoresis in one per cent agarose gel in TBE buffer (1X). Agarose was dissolved in TBE buffer by heating. When the mixture was cooled to around 50ºC, ethidium bromide was added to a final concentration of 0.5 µg/mL. The clean, dry gel platform edges were sealed with adhesive tape and the comb was kept in proper position before pouring agarose. Once the gel was set, the comb and adhesive tapes were removed gently and the tray containing the gel was placed in buffer tank. Buffer (1X TBE) was poured until the gel was completely covered.

Five microlitre of the PCR product of samples, positive control, negative control and DNA ladder were loaded in the respective wells. Electrophoresis was carried out at 50 V and 16 mA until the dye migrated two-third of length of the gel. The gel was visualised under UV transilluminator and the results were documented in a gel documentation system (Bio-Rad, USA).

3.4 ISOLATION OF NEWCASTLE DISEASE VIRUS IN EMBRYONATED CHICKEN EGGS

Nine to eleven day-old ECE were supplied by the Hatchery Unit, University Poultry and Duck Farm, College of Veterinary and Animal Sciences, Mannuthy.

3.4.1 Materials

1. Suspension of processed tissue samples and tracheal/cloacal swabs (3.2)
2. Biosafety cabinet II
3. Nine to eleven day-old ECE
4. Egg candler
5. Egg drilling machine
6. One millilitre syringe with a 3 cm long 26 G needle (sterile)
7. Wax to seal the eggs

3.4.2 Method

Embryonated eggs (nine to eleven day-old) were candled and those with viable embryo were selected. Air space was pencil marked and head of the embryo was located. A position was marked just opposite of the embryo head about 0.5 cm above the air space. Three eggs were taken per sample and one egg as control. The egg shell over the air space was sterilised with 70 per cent alcohol and a small hole was drilled at marked position by egg driller. The area was re-sterilised with 70 per cent alcohol, 0.1 mL of inoculum was drawn in a one millilitre syringe and the needle was plugged through the hole in the shell directly into the allantoic cavity. Control eggs were inoculated with 0.1 mL of PBS. The hole was sealed with wax and incubated in upright position at 37°C. Eggs were candled twice daily to observe the viability of the embryo. The eggs were turned three times a day.

3.5 HARVESTING OF AMNIO-ALLANTOIC FLUID

3.5.1 Materials

1. Biosafety cabinet II
2. Inoculated ECE
3. Sterile Petri-dishes
4. Sterile scissors, small curved
5. Sterile, fine pointed forceps
6. Sterile storage vials

3.5.2 Method

Embryos that died within 24 h. PI were considered as nonspecific and those which died after 24 h. were chilled overnight at 4°C. The embryos found viable even after five days PI were killed by chilling at 4°C overnight. Eggs were harvested individually. Egg shell was cut below the pencilled line marking the air space. Amnio-allantoic fluid (AAF) was pipetted out into the appropriately labelled storage vial using sterile Pasteur pipette. The embryo was taken out and kept in a sterile Petri-dish and observed for any lesions by comparing each embryo with negative control. The AAF was tested for HA activity by spot agglutination. To 20 µL of AAF, equal volume of 10 per cent chicken RBC was added. For negative control, 20 µL of PBS was added instead of AAF. Subsequently, three more passages were done before discarding the samples as negative.

3.6 CONFIRMATION OF NEWCASTLE DISEASE VIRUS BY HAEMAGGLUTINATION INHIBITION TEST

The presence of NDV in harvested AAF was demonstrated by HA test and further confirmation was made by HI test using NDV positive serum (FAO, 2002).

3.6.1 Haemagglutination test

3.6.1.1 Materials

1. Alsever's solution

Citric acid	0.055 g
Sodium citrate	0.8 g
D-Glucose	2.05 g
Sodium chloride	0.42 g
Distilled water to make up to 100 mL	

The pH was adjusted to 7.2 and sterilised by autoclaving at 116°C for 10 min. and stored at 4°C.

2. Harvested AAF (sample)
3. Negative and positive control samples
4. One per cent chicken erythrocytes

Blood was collected from the wing veins of three chicken in equal amount of Alsever's solution. It was centrifuged at 500 x g for 10 min. The packed cell volume of the pellet of RBC after washing thrice with PBS was measured and 10 per cent RBC stock solution was prepared and stored at 4°C. One per cent RBC working solution was made from the stock at the time of test.

5. 96 well V-bottom microwell plates with lid
6. PBS [3.1.1.3]

3.6.1.2 Method

Twenty-five microlitre of PBS was added into each well of the microwell plate. Twenty-five microlitre of AAF was placed in the first well of each row of column 1. Using a multichannel pipette, two-fold serial dilutions was carried out up

to column 11 of each row. Later, 25 µL of one per cent RBC was added to each well. To the column 12, 25 µL PBS was added and the wells in this column were RBC control wells. Positive and negative controls were also maintained in the last two rows (row G and H) of the plate, respectively. The solution was mixed by tapping the plate gently. Lid was placed on the plate. The plates were incubated for 45 min. at room temperature. The results were read by tilting the plate and observed for the presence or absence of mat formation. End point was noted *i.e.,* last well to show complete haemagglutination.

Haemagglutination negative: A sharp button of RBC at the bottom of the well

Haemagglutination positive: A hazy film of RBC, no button or a very a small button at the bottom of the well

Results were expressed as HA titre *i.e.,* reciprocal of the dilution that contains end point.

3.6.2 Haemagglutination inhibition test

3.6.2.1 Materials

1. 96 well V-bottom microwell plates with lid
2. PBS [3.1.1.3]
3. One per cent washed RBC [3.6.1.1.4]
4. Sample antigen diluted to four HA units per 25 µL
5. Standard positive serum against NDV maintained in the Department of Veterinary Microbiology, CVAS, Mannuthy

3.6.2.2 Method

Beta method of HI test was followed. Twenty-five microlitre of PBS was dispensed into each well of the microwell plate. Twenty-five microlitre of standard positive serum against NDV was added to the first well and two-fold serial dilutions

of the serum were made across the plate using a multichannel pipette along the row until column 11. Later, 25 µL of the sample antigen (4 HA unit) was added to each well. The reagents were mixed by gently tapping the sides of the plate. Plate was covered with a lid and allowed to stand for 30 min. at room temperature. After incubation, 25 µL of one per cent washed RBC was added to each well including the control wells in the last column. Virus control, RBC control, serum control were also kept.

Virus control (12th column) - 25 µL PBS+ 25 µL of viral antigen + 25 µL of washed RBC

Serum control (Row G) - 25 µL PBS + 25 µL serum + 25 µL washed RBC from column 9 to 12

RBC control (Row H) - 50 µL PBS + 25 µL washed RBC from column 9 to 12

The reagents were mixed by gently tapping the sides of the plate. It was covered with a lid and was incubated for 45 min. at room temperature. Endpoint was determined *i.e.,* the last well where there is complete inhibition of haemagglutination. Results were expressed as HI titre *i.e.,* reciprocal of the dilution that contain end point.

The AAF of the samples confirmed for NDV by HI test were filtered through 0.22 µm sterile syringe filter and stored at -70°C without the addition of antibiotic-antimycotic solution for ICPI test.

3.7 DETECTION OF NEWCASTLE DISEASE VIRUS FROM HARVESTED AMNIO-ALLANTOIC FLUID BY RT-PCR

The harvested AAF was subjected to RNA extraction by TRIzol method as described in 3.3.1. The cDNA was synthesised using Bio-Rad cDNA synthesis kit (3.3.2) and RT-PCR was performed (3.3.3). The PCR products were detected by electrophoresis in one per cent agarose gel in TBE buffer (1X) as mentioned in 3.3.4.

3.8 PATHOTYPING OF NEWCASTLE DISEASE VIRUS

The ICPI and MDT were calculated for the isolates as per OIE (2018) and FAO (2002), respectively.

3.8.1 Intracerebral pathogenicity index test

3.8.1.1 Materials

1. Normal saline

Sodium chloride	8.5 g
Distilled water	1000 mL

The solution was sterilised by autoclaving at 121°C for 15 min. and stored at 4°C.

2. AAF
3. Day-old chicks

Day-old Gramasree chicks were procured from the Hatchery unit, University Poultry and Duck Farm, College of Veterinary and Animal Sciences, Mannuthy.

4. Tuberculin syringe and needle
5. 70 per cent ethyl alcohol

3.8.1.2 Method

Fresh AAF with a HA titre $\geq 4 \log_2$ was diluted 1/10 in sterile saline without antibiotics. Diluted virus (0.05 mL) was injected intracerebrally into day-old chicks. Ten birds per isolate were inoculated. The control group birds (n=10) were inoculated with 0.05 mL of normal saline. The birds were observed every 24 h. for eight days. At each observation, the birds were scored: zero, if normal, one, if sick, and two, if dead. (Birds that are alive but unable to eat or drink were killed humanely and scored

as dead at the next observation. Dead individuals were scored as two at each of the remaining daily observations after death). The ICPI was calculated which is the mean score per bird per observation over the eight-day period.

3.8.2 Mean death time

3.8.2.1 Materials

1. Fresh AAF of isolates
2. Normal saline [3.8.1.1.1]
3. Materials [3.4.1.2 to 3.4.1.7]

3.8.2.2 Method

Fresh AAF added with antibiotic - antimycotic solution was diluted in sterile saline to give a ten-fold dilution series between 10^{-6} and 10^{-9}. For each dilution, 0.1 mL was inoculated into the allantoic cavity of each of five, nine to eleven day-old ECE and incubated at 37°C. The remaining virus dilutions were retained at 4°C and another five eggs with 0.1 mL of each dilution were inoculated eight hours later and placed at 37°C. Each egg was examined twice daily for seven days and the time of any embryo deaths were recorded. The minimum lethal dose was calculated as the highest virus dilution that causes all the embryos inoculated with that dilution to die. The MDT was determined which is the mean time in hours for the minimum lethal dose to kill the embryos.

3.9. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION TARGETING FUSION PROTEIN CLEAVAGE SITE REGION OF F GENE

3.9.1 Materials

1. Emerald Amp GT PCR master mix (Takara Bio company)

2. Primers (Table 5)
3. Nuclease free water
4. Template cDNA

3.9.2 Designing and synthesis of primers

The primers targeting the FPCS region of the F gene were used for the sequencing. The sequences and other parameters of the primers are depicted in table 5. Primers were custom synthesised commercially (Sigma Aldrich) and obtained in lyophilised form.

Table 5. Sequences of primers used for RT-PCR targeting FPCS region of F gene

Sl. No.	Primer name	Primer sequence (5'-3')	Product size	Reference
1	FPCS sense	CCT TGG TGA ITC TAT CCG IAG	254 bp	Ananth *et al*. (2008)
2	FPCS antisense	CTG CCA CTG CTA GTT GIG ATA ATC C		

3.9.3 Dilution and storage of primers

The dilution and storage of the primers specific for FPCS region of F gene was done as mentioned in 3.3.3.3.

3.9.4 Optimisation of polymerase chain reaction conditions

The optimisation of PCR conditions was achieved through gradient PCR (MJ Mini thermal cycler, Bio- Rad, USA). For these, modifications in different time-temperature combinations of annealing and extension steps were used. The

temperature gradient which provided the best results for amplification was selected for all downstream use. The reaction was carried out in 0.2 mL PCR tubes and details of PCR reaction mix are provided table 6.

Complementary DNA from LaSota vaccine was used as positive control. Negative control was made up with nuclease free water. The master mix prepared was spun briefly. Polymerase chain reaction was performed in a MJ Mini thermal cycler (Bio-Rad, USA) using the programme (Table 7).

Table 6. Optimised concentration of PCR reagents for the amplification of FPCS region of F gene (254 bp)

Sl. No.	Constituents	Volume (µL)
1	Template cDNA	3
2	Forward primer (10 p*M*/µL)	1
3	Reverse primer (10 p*M*/µL)	1
4	Emerald Amp GT PCR master mix	6.25
5	Nuclease free water	1.25
	Total	12.5

Table 7. The PCR conditions optimised for the amplification of FPCS region of F gene (254 bp)

<table>
<tr><th>Sl. No.</th><th>Step</th><th>Temperature (°C)</th><th colspan="2">Time</th></tr>
<tr><td>1</td><td>Initial denaturation</td><td>94</td><td colspan="2">5 min</td></tr>
<tr><td>2</td><td>Denaturation</td><td>94</td><td>30 sec.</td><td rowspan="3">25 cycles</td></tr>
<tr><td>3</td><td>Annealing</td><td>57.5</td><td>30 sec.</td></tr>
<tr><td>4</td><td>Extension</td><td>72</td><td>1 min.</td></tr>
<tr><td>5</td><td>Final extension</td><td>72</td><td colspan="2">7 min.</td></tr>
</table>

3.9.5 Submarine agarose gel electrophoresis

The PCR products were detected by electrophoresis in one per cent agarose gel in TBE buffer (1X) as mentioned in 3.3.4.

3.10 SEQUENCING AND PHYLOGENETIC ANALYSIS OF FUSION PROTEIN CLEAVAGE SITE REGION OF F GENE

3.10.1 Sequencing of fusion protein cleavage site region of F gene

The FPCS region of F Gene amplified using primers with amplicon size of 254 bp was sequenced. Sequencing was performed by automated sequencer using Sanger’s dideoxy chain termination method at Agrigenome, Cochin. The nucleotide sequences and predicted amino acid sequences were analysed by various bioinformatics tools. The different tools used are presented in table 8.

3.10.2 Phylogenetic analysis

For phylogenetic analysis, sequences of other NDV isolates were selected from GenBank, NCBI. The obtained sequences were aligned with downloaded sequences using Cluster W programme of MEGA X software. Maximum likelihood method was used to interpret evolutionary history with 1000 bootstrap replications.

Table 8. Tools used for sequence analysis

Sl. No.	Bioinformatics Tool	Purpose and Web address
1	Sequence Manipulation	To evaluate potential PCR primers based on melting temperature, % GC etc
2	Sequence Manipulation Suite: Reverse Complement	To convert a DNA sequence into its reverse-complement counterpart (http://www.bioinformatics.org/sms2/rev_comp.html)
3	EMBOSS: merger	To merge two overlapping nucleic acid sequences into one (http://emboss.bioinformatics.nl/cgi-bin/emboss/merger)
4	Basic Local Alignment Search Tool (BLAST)	To obtain similar sequences to any given sequence of DNA, RNA or protein from the database (http://www.ncbi.nlm.nih.gov/blast)
5	Transeq (EMBOSS)	For translating nucleotide sequence to corresponding peptide sequence (https://www.ebi.ac.uk/Tools/st/)

RESULTS

4. RESULTS

4.1 COLLECTION OF CLINICAL SAMPLES

Recently dead/ ailing birds with gross lesions/clinical signs suggestive of ND, presented to the Departments of Veterinary Microbiology and Veterinary Pathology, CVAS, Mannuthy formed the subject for the study. Tissue samples were collected from 55 birds. Out of 55 samples, 10 were from dead and 45 from ailing birds. Tissues like lung, kidneys, caecal tonsils, spleen, liver and heart from each bird were pooled and collected in PBS (pH 7.2). Brain and intestine were collected separately. The dead birds showed lesions like pinpoint haemorrhages in the summit of papillae of proventriculus and caecal tonsils (Fig. 1). Splenomegaly and congestion of the lungs were also observed. The symptoms exhibited by the sick birds were droopiness, respiratory distress and diarrhoea. Some of them exhibited torticollis (Fig. 2).

Tracheal and cloacal swab samples were also collected from the sick birds (n=8) showing clinical signs suggestive of ND which were bought to the Teaching Veterinary Clinical Complex, CVAS, Mannuthy. The clinical signs reported by the owners were off-feed and droopiness. The birds were weak and inactive. The details of the samples collected are given in table 9.

4.2 DIRECT DETECTION OF NEWCASTLE DISEASE VIRUS FROM CLINICAL SAMPLES BY RT-PCR

4.2.1 Ribonucleic acid extraction

The tissue samples, tracheal and cloacal swabs were processed. A part of the processed sample was taken for extracting RNA by TRIzol method.

The concentration of all the samples were more than 100 ng/µL and the mean ratio of OD of extracted RNA sample at 260/230 nm and 260/280 nm were 1.9±0.05 and 1.9±0.1, respectively.

Table 9. Details of the samples collected

Sl. No.	Condition of bird and source of sample	Species						Type of sample collected
		Chicken	Quail	Pigeon	Duck	African love bird	Total	
1	Dead birds brought to the Department of Veterinary Pathology	10	-	-	-	-	10	Tissue samples
2	Ailing birds brought to the Department of Veterinary Microbiology	33	8	-	3	1	45	Tissue samples
3	Sick birds brought to the Teaching Veterinary Clinical Complex	-	-	7	-	1	8	Tracheal and cloacal swabs
Total		43	8	7	3	2	63	

4.2.2 Reverse transcription

The total RNA obtained was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, USA). One microgram of total RNA was used for cDNA synthesis. The synthesised cDNA was used as template for RT-PCR.

4.2.3 Reverse transcriptase polymerase chain reaction

The standardisation of PCR condition was done using cDNA synthesised from LaSota vaccine. Gradient PCR using NDV UP and DOWN primers targeting F gene revealed best amplification at an annealing temperature of 55°C. This temperature was taken for PCR assay employing clinical samples. An amplicon of 320 bp was generated when NDV UP and DOWN primers were used for PCR and detected in agarose gel electrophoresis (Fig. 3).

Newcastle disease virus could be detected in five pooled tissue samples collected from ailing birds. Out of ten samples collected from dead birds, three were found to be positive. All the eight positive tissue samples were from chicken and tissue samples collected from quail, duck and African love bird were negative for NDV. None of the swabs collected were positive for the virus.

4.3 ISOLATION OF NEWCASTLE DISEASE VIRUS IN EMBRYONATED CHICKEN EGGS

A part of the clarified suspension obtained on processing the tissue samples and swabs (3.2) were inoculated into nine to eleven day-old ECE via allantoic route. All the inoculated embryos were alive after 24 h. PI. The death of the embryos was observed after three days PI. The AAF was harvested and tested for spot HA. Eight samples that were positive in RT-PCR were also positive for HA (Fig. 4). The dead embryos revealed lesions like generalised congestion of the body, petechiae on the

occipital region and congestion of chorio-allantoic membrane from the first passage onwards. The lesions were more prominent in subsequent passages (Fig. 5).

Those AAF without haemagglutination activity were discarded only after three blind passages and considered as negative.

Tissue samples positive for NDV were named as NDV-S5, NDV-S6, NDV-P2, NDV-S9, NDV-P4, NDV-S17, NDV-S18 and NDV-P6 for future references.

4.4 CONFIRMATION OF NEWCASTLE DISEASE VIRUS BY HAEMAGGLUTINATION INHIBITION TEST

4.4.1 Haemagglutination test

The AAF samples collected were tested for HA activity using chicken erythrocytes. All the eight AAF samples showed HA (Fig. 7) and their titre varied from 4 $\log_2$ to 10 $\log_2$.

4.4.2 Haemagglutination inhibition test

All the eight AAF samples showing HA activity were subjected to HI test using NDV specific antiserum for confirmation. Four HA unit of all the samples were calculated, prepared and the test was conducted. Haemagglutination activity of all the eight samples was inhibited by NDV specific antiserum (Fig. 8) and their HI titre values are shown in table 11.

4.5 DETECTION OF NEWCASTLE DISEASE VIRUS FROM HARVESTED AMNIO-ALLANTOIC FLUID BY RT-PCR

Total RNA was extracted from NDV confirmed AAF samples by TRIzol method. The concentration of all the samples were more than 150 ng/µL and the

mean ratio of OD at 260/230 nm and 260/280 nm were 2.0±0.1 and 1.9±0.12, respectively.

The total RNA obtained was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, USA). One microgram of total RNA was used for cDNA synthesis. The synthesised cDNA was used as template for RT-PCR using ND UP and DOWN primers targeting the F gene. All the eight tissue samples which were positive in RT-PCR were also positive when the harvested AAF from the embryo of corresponding tissue samples were subjected to RT-PCR using the same set of primers. Amplicons of 320 bp obtained after RT-PCR were detected in agarose gel electrophoresis (Fig. 6).

Table 10. Details of samples positive in direct RT-PCR and virus isolation

Sl. No.	Type of samples	No. of samples collected	Samples positive in direct RT-PCR	Samples positive in virus isolation	Samples positive in RT-PCR from AAF
1	Tissue samples (Dead birds)	10	3	3	3
2	Tissue samples (Ailing birds)	45	5	5	5
3	Tracheal and cloacal swab (Sick birds)	8	-	-	-

4.6 PATHOTYPING OF NEWCASTLE DISEASE VIRUS BY INTRACEREBRAL PATHOGENICITY INDEX AND MEAN DEATH TIME

4.6.1 Intracerebral pathogenicity index test

The ICPI of the eight isolates were assessed by intracranial inoculation of 1:10 diluted AAF in 10, day-old chicks per isolate. The birds were observed every 24 h. for symptoms and death. The chicks showed symptoms like huddling, depression, drowsiness and nervous symptoms like backward movement and tilting of head. These birds were taken as sick for observation; the birds which showed paralysis and were unable to drink and eat were killed humanely and taken as dead from next observations (Fig. 9). The value of ICPI varied from 0.75- 1.53.

All the chicks inoculated with NDV-S5, NDV-S17, NDV-S18 and NDV-P6 were dead within eight days. Total of eight birds each died which were inoculated with NDV-S6 and NDV-P2 during the course of time. Nine out of ten birds inoculated with NDV-S9 and NDV-P4 were dead within eight days. The ICPI of the samples are depicted in table 12.

Based on ICPI test, NDV-S5, NDV-S17 and NDV-S18 were found to be velogenic and NDV-S6, NDV-P2, NDV-S9, NDV-P4 and NDV-P6 were mesogenic pathotype.

4.6.2 Mean death time

The MDT test was conducted for eight isolates in nine to ten day-old ECE by inoculating intra-allantoically. Serial ten-fold dilutions of the isolates were made and dilutions from 10^{-6} to 10^{-9} were used for inoculation. The value of MDT of the isolates ranged from 54 h. to 79.2 h. The MDT data are shown in table 12.

Table 11. HA and HI titre of NDV isolates

Sl. No.	Isolates	HA titre (Log_2)	HI titre (Log_2)
1	NDV-S5	9	4
2	NDV-S6	10	4
3	NDV-P2	8	4
4	NDV-S9	6	4
5	NDV-P4	5	4
6	NDV-S17	4	4
7	NDV-S18	4	4
8	NDV-P6	4	4

Table 12. Pathogenicity indices of NDV isolates

Sl. No.	Isolates	ICPI	MDT, h.	Pathotype
1	NDV-S5	1.5	60	Velogenic
2	NDV-S6	0.98	62.4	Mesogenic
3	NDV-P2	0.75	75.6	Mesogenic
4	NDV-S9	1.35	62.4	Mesogenic
5	NDV-P4	0.9	73.2	Mesogenic
6	NDV-S17	1.51	58.8	Velogenic
7	NDV-S18	1.53	54	Velogenic
8	NDV-P6	1.03	79.2	Mesogenic

Based on MDT, samples NDV-S5, NDV-S17 and NDV-S18 were classified under velogenic pathotype. NDV-S6, NDV-P2, NDV-S9, NDV-P4 and NDV-P6 were grouped in mesogenic pathotype.

4.7. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION TARGETING FUSION PROTEIN CLEAVAGE SITE REGION OF F GENE

The standardisation of PCR condition was done using cDNA synthesised from LaSota strain vaccine. Gradient PCR using FPCS sense and antisense primers targeting FPCS region of F gene revealed the best amplification and yielded amplicons of 254 bp at an annealing temperature of 57.5°C. All the eight samples were amplified using the standardised assay and the obtained PCR products were detected in agarose gel electrophoresis (Fig. 10).

4.8 SEQUENCING AND PHYLOGENETIC ANALYSIS OF FUSION PROTEIN CLEAVAGE SITE REGION OF F GENE

4.8.1 Sequencing of fusion protein cleavage site region of F gene

The PCR products of 254 bp obtained were sequenced by Sanger's dideoxy chain termination method and merged using EMBOSS merger. On blasting, the BLASTn hits confirmed that obtained sequence were of FPCS region of F gene (Table 13). All the isolates showed similar nucleotide sequence at FPCS region. Upon conversion of nucleotide sequence to amino acid sequence, all the isolates revealed ^{112}GRQGRL^{117} amino acid motif at FPCS region.

4.8.2 Phylogenetic analysis of fusion protein cleavage site region of F gene

The isolates showed 100 per cent sequence homology with United States (U139691 and U22285) isolates, Brazilian (MN599095) isolate and 99 per cent sequence homology with vaccines strains such as LaSota (MH392212), B1 and

VG/GA strain of NDV. The isolates showed 98.82 per cent similarity with Indian isolates obtained from backyard poultry in North India (MK796810 and MK796808), Jammu (MH577764), Hissar (KX011038), Gujarat (KM056358, KM056357 and KM056354) and Maharashtra (KJ621048, KJ621043 and KJ621042).

Phylogenetic analysis was carried out using ‘MEGA X’ programme of Lasergene software using the Maximum Likelihood method. Phylogenetic tree is depicted in fig. 11. On phylogenetic analysis of FPCS region of F gene, all the isolates clustered with each other along with the United States isolates and related to NDV class II genotype II strains with bootstrap value of 1000. They were grouped differently from vaccine strains (VG/GA and B1) but were found to originate from same ancestor. All the other Indian isolates and LaSota vaccine strains which showed sequence homology were grouped separately and were distinct from the isolates obtained.

Table 13. Representative sequence of FPCS region of F gene (254 bp)

Representative sequence
> NDV-S5, Newcastle disease virus, F gene, FPCS region, Kerala isolate, Partial CDS, 254 bp Ccttggtgagtctatccggaggatacaagagtctgtgactacatctggagggggagacaggggcgccttataggcgcc attattggcggtgtggctcttggggttgcaactgccgcacaaataacagcggccgcagctctgatacaagccaaacaaaat gctgccaacatcctccgacttaaagagagcattgccgcaaccaatgaggctgtgcatgaggtcactgacggattatcccaa ctagcagtggcag

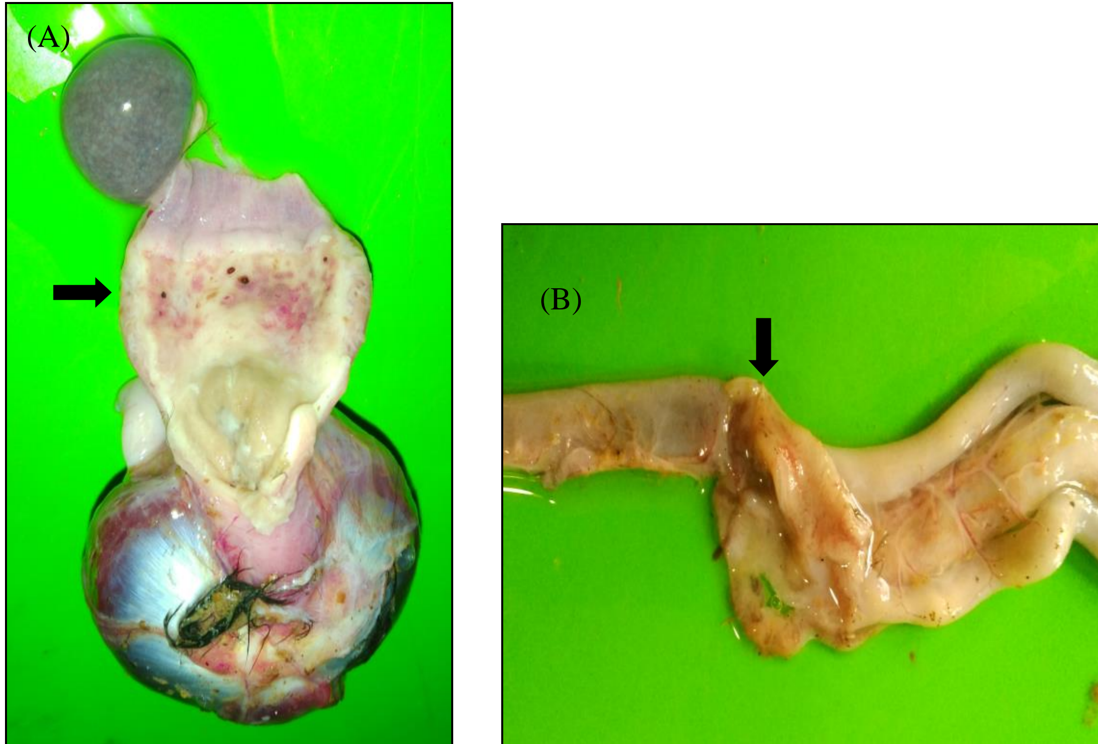

Fig. 1. Post-mortem findings suggestive of ND (A) Pinpoint haemorrhages in the summit of papillae of proventriculus (B) Haemorrhages in caecal tonsils

Fig. 2. Torticollis exhibited by ND suspected bird

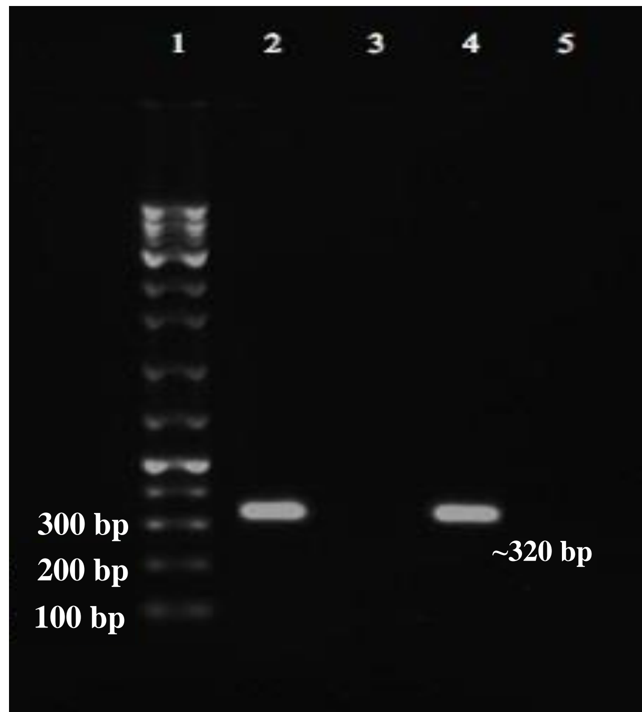

Fig. 3. Agarose gel electrophoresis of RT-PCR amplified products of F gene from tissue cDNA, 320 bp (Representation)

Lane 1: 100 bp DNA Ladder
Lane 2: Positive control
Lane 3: Negative control
Lane 4: NDV positive tissue sample

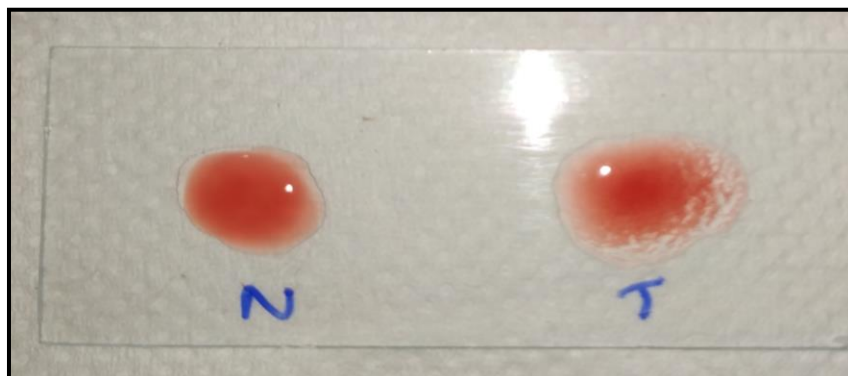

Fig. 4. Spot haemagglutination test

N: Negative control
T: Harvested AAF

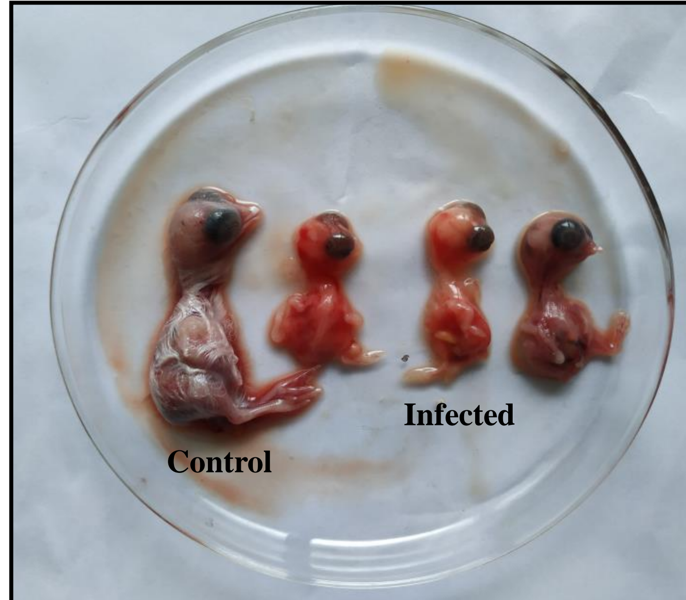

Fig. 5. Characteristic post-inoculation changes of the embryo (Nine to eleven day-old)

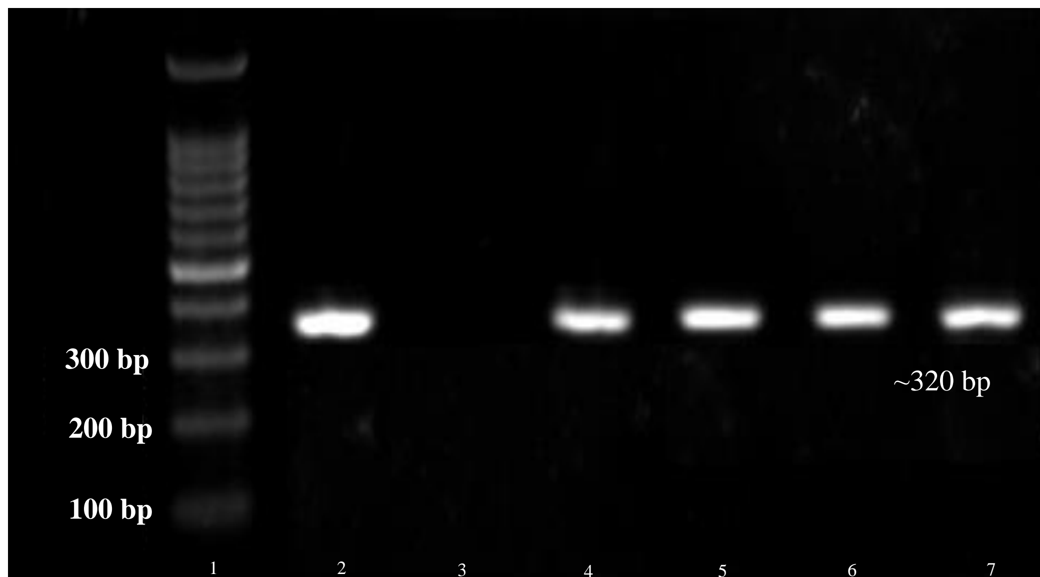

Fig. 6. Agarose gel electrophoresis of PCR amplified products of F gene from AAF cDNA, 320 bp (Representation)

Lane 1: 100 bp DNA Ladder
Lane 2: Positive control
Lane 3: Negative control
Lanes 4 to 7: NDV positive AAF

1	2	3	4	5	6	7	8	9	10	11	12
(1 $\log_2$)	(2 $\log_2$)	(3 $\log_2$)	(4 $\log_2$)	(5 $\log_2$)	(6 $\log_2$)	(7 $\log_2$)	(8 $\log_2$)	(9 $\log_2$)	(10 $\log_2$)	(11 $\log_2$)	(RC)

Fig. 7. Haemagglutination test (Representation)

Rows A to D upto column 11: NDV isolates **Column 12: RBC control (RC)**

Row G: Positive control **Row H: Negative control**

1	2	3	4	5	6	7	8	9	10	11	12
(1 $\log_2$)	(2 $\log_2$)	(3 $\log_2$)	(4 $\log_2$)	(5 $\log_2$)	(6 $\log_2$)	(7 $\log_2$)	(8 $\log_2$)	(9 $\log_2$)	(10 $\log_2$)	(11 $\log_2$)	(VC)

Fig. 8. Haemagglutination Inhibition test (Representation)

Rows A to D upto column 11: NDV isolates **Column 12: Virus control (VC)**

Row G: Serum control **Row H: RBC control**

Fig. 9. Bird showing torticollis after intracranial inoculation of NDV

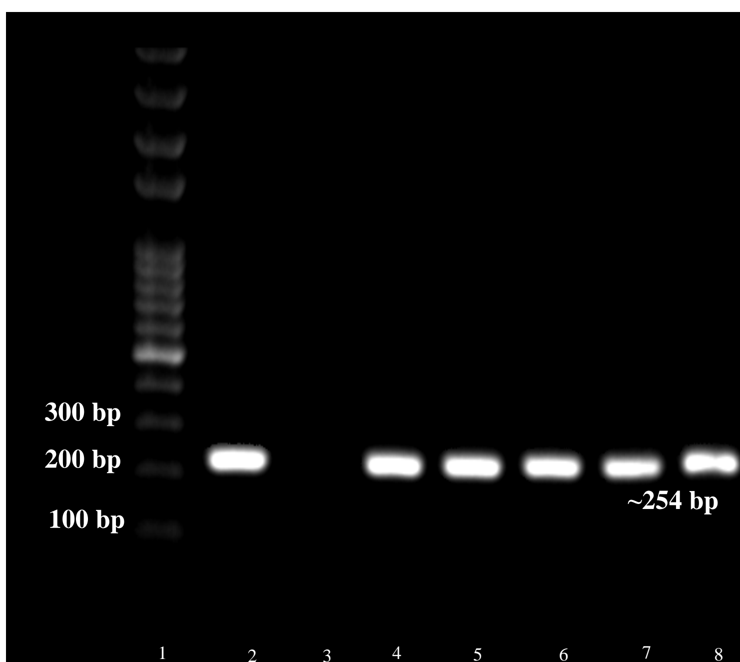

Fig. 10. Agarose gel electrophoresis of PCR amplified products of FPCS region of F gene from AAF cDNA, 254 bp (Representation)

Lane 1: 100 bp DNA Ladder
Lane 2: Positive control
Lane 3: Negative control
Lanes 4 to 8: NDV positive AAF

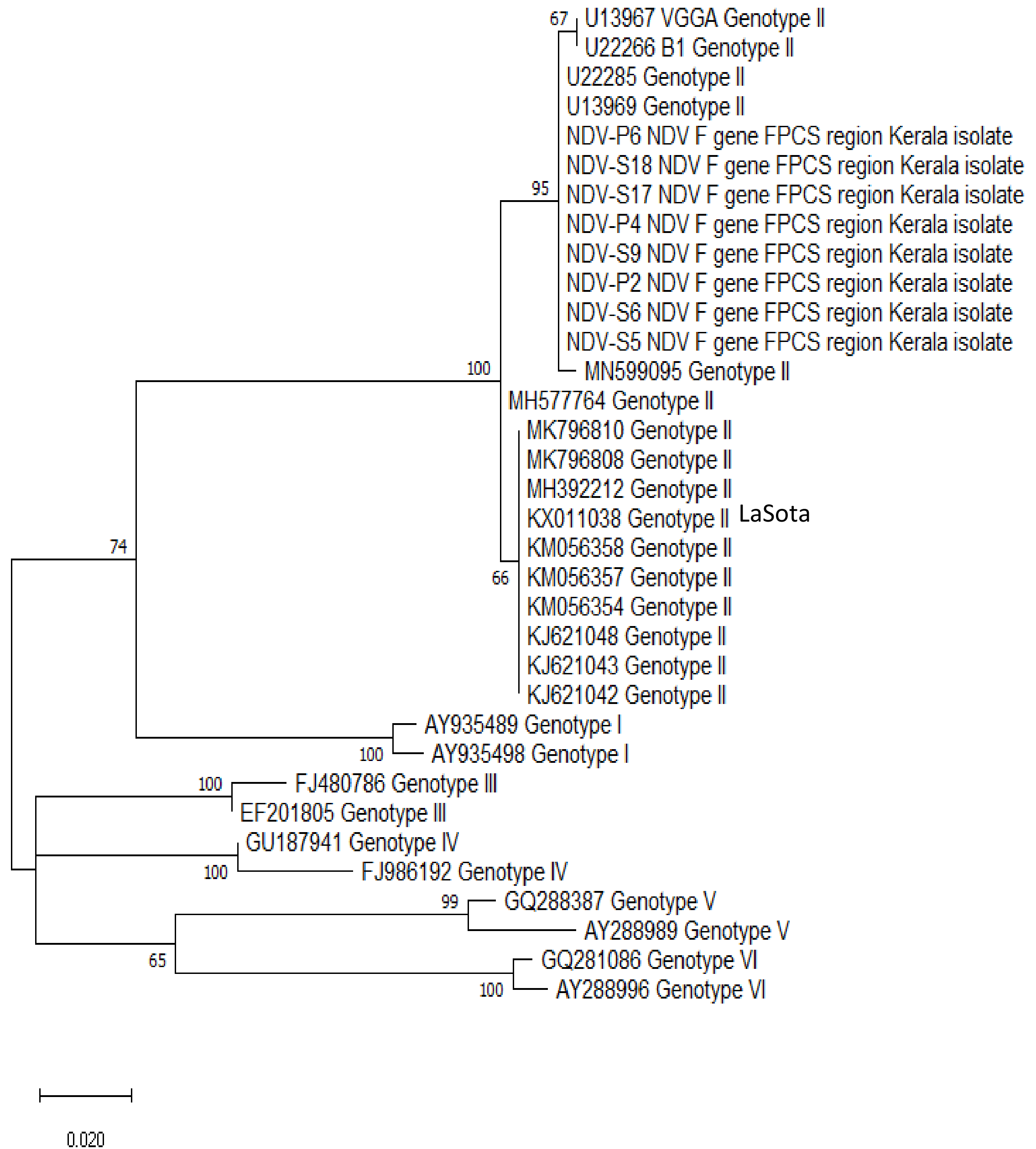

Fig. 11. Phylogenetic tree based on FPCS region of F gene

Phylogenetic analysis was done in MEGA X software, using the Maximum Likelihood method based on the General Time Reversible model, and Gamma distributed with invariable sites (G + I). The codon positions 1st, 2nd, 3rd and non-coding were included. All positions containing gaps and missing data were eliminated (Desingu *et al.,* 2016).

DISCUSSION

5. DISCUSSION

Newcastle disease virus, a member of family *Paramyxoviridae,* is a highly infectious pathogen capable of causing disease in a wide range of avian species. It is a constant threat to poultry industry as it causes high morbidity and mortality in affected flocks (Hines and Miller, 2012). Rapid detection and identification of the viral agent is necessary for controlling the spread of the disease (Ganar *et al.,* 2014). In Kerala, despite vaccination measures taken for controlling the disease, there are many reports of ND outbreaks. Hence, the present study is aimed at isolation of NDV and pathotyping of the isolates. It also aims at identifying the genotype of the virus prevalent in the region.

5.1 COLLECTION OF CLINICAL SAMPLES

The sample of choice for virus isolation includes tissue samples (lung, kidney, intestine, caecal tonsils, spleen, brain, liver and heart tissues) from recently dead or moribund birds, both tracheal/oropharyngeal and cloacal swabs from live birds associated with clinical disease in PBS of pH 7.0-7.4 (OIE, 2018). Hence, the same samples were collected from recently dead and ailing bird with gross lesions/ clinical signs suggestive of ND. Tracheal and cloacal samples were also collected from live birds showing signs suggestive of ND. Similar procedure was also followed by Chowdhary *et al.* (2020).

5.2 DIRECT DETECTION OF NEWCASTLE DISEASE VIRUS FROM CLINICAL SAMPLES

5.2.1 Ribonucleic acid extraction

Ribonucleic acid was extracted from the processed tissue samples, tracheal and cloacal swabs by TRIzol method (Nanthakumar *et al.,* 2000). The concentration and purity of the RNA were measured using spectrophotometer (NanoDrop 2000C).

The concentration of all the samples were more than 100 ng/µL and the mean ratio of OD at 260/230 nm and 260/280 nm were 1.9±0.05 and 1.9±0.1, respectively.

5.2.2 Reverse transcription

The total RNA obtained was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, USA). One microgram of total RNA was used for cDNA synthesis. The synthesised cDNA was used as template for RT-PCR.

5.2.3 Reverse transcriptase polymerase chain reaction

Rapid identification of the virus is a pre-requisite for confirming the disease. Conventional methods like isolation and determination of ICPI require minimum of five days. Hence, Kant *et al.* (1997) detected NDV by RT-PCR with the RNA obtained directly from tissue sample. Smietanka *et al*. (2006) reported a high degree of correlation between RT-PCR conducted directly in tissue samples and virus isolation for detection of NDV.

In the present study, RT-PCR was employed using the RNA obtained directly from the tissue samples. The primers (NDV UP and DOWN) targeting F gene were used. A 320 bp PCR product was obtained at an annealing temperature of 55°C. Eight out of sixty-three samples were found to be positive for NDV. Haque *et al.* (2010) also employed primers targeting the F gene for detection of NDV from clinical samples.

5.3 ISOLATION IN EMBRYONATED CHICKEN EGGS

Virus isolation and identification is considered as the gold standard test for the diagnosis of ND (Alexander, 2000). Jahan *et al.* (2013) reported high viral infectivity titre in allantoic fluid when the virus was inoculated by allantoic route. In this study, the tissue and swab samples were inoculated in nine to eleven day-old ECE intra-

allantoically. The embryo deaths were observed three days PI from the first passage. Similar observations were made by Vijayarani *et al.* (2010). The AAF was harvested after five days PI and tested for HA. The samples which were positive by RT-PCR were positive in virus isolation test.

The embryos showed lesions like generalised congestion of the body, petechiae on the occipital region and congestion of chorio-allantoic membrane from the first passage. These finding are in accordance with those of Balachandran *et al*. (2014) and Qosimah *et al*. (2018).

5.4 CONFIRMATION OF NEWCASTLE DISEASE VIRUS BY HAEMAGGLUTINATION INHIBITION TEST

The AAF was subjected to HA and further HI test for confirmation of the presence of NDV. Eight of them agglutinated chicken erythrocytes, subsequently were inhibited by the known specific NDV antiserum which is confirmatory for NDV (Alexander, 2000). Similar method was employed to detect the presence of NDV in allantoic fluid by Vijayarani *et al*. (2010), Desingu *et al.* (2016) and Fuller *et al*. (2017).

5.5 DETECTION OF NEWCASTLE DISEASE VIRUS FROM HARVESTED AMNIO-ALLANTOIC FLUID

Jestin and Jestin (1991) developed an RT-PCR assay for detection of NDV in allantoic fluid. The authors reported that this method might be useful for identification of new isolates. The RNA was extracted from the eight AAF fluid samples possessing HA activity by TRIzol method (Diel *et al.,* 2012b). Concentration and purity of the obtained RNA were measured using spectrophotometer (NanoDrop 2000C). The concentration of all the samples were more than 150 ng/µL and the mean ratio of OD at 260/230 nm and 260/280 nm were 2.0±0.1 and 1.9±0.12, respectively. The RNA was converted into cDNA and RT-PCR was performed

targeting F gene using primers (NDV UP and DOWN) which amplified 320 bp product.

The assay detected the presence of NDV in all the eight samples. The results were similar to reports of Haque *et al.* (2010) in which RT-PCR showed equal sensitivity and specificity in detecting NDV from all the RNA isolated from tissue, allantoic fluid and cell culture. The RT-PCR targeting F gene for detection of virus from allantoic fluid was employed by Choi *et al.* (2014) and Gowthaman *et al.* (2019).

5.6 PATHOTYPING OF NEWCASTLE DISEASE VIRUS BY INTRACEREBRAL PATHOGENICITY INDEX AND MEAN DEATH TIME

Pathotyping is necessary for definitive assessment of the virulence of viral isolates (OIE, 2018). Velogenic and mesogenic NDV isolates show similar sequence at FPCS region and molecular method cannot be used for differentiating pathotypes (Balachandran *et al.,* 2014). Thus, ICPI test and MDT in ECE have been employed in this study for assessing the pathotype of the viral isolates obtained.

The ICPI value of the isolates in this study varied from 0.75 to 1.53. All of them belonged to virulent type (OIE, 2018). In the present study, three isolates (NDV-S5, NDV-S17 and NDV-S18) were classified as velogenic and five isolates (NDV-S6, NDV-P2, NDV-S9, NDV-P4 and NDV-P6) were classified as mesogenic similar to Alexander (1998) where isolates with ICPI value ranging from 0.7 to 1.5 were classified as mesogenic viruses and those with ICPI value more than 1.5 were classified as velogenic.

Mean death time in ECE is the other commonly employed test for pathotyping of NDV virus. The MDT of eight isolates varied from 54 h. to 79.2 h. The isolates NDV-S5, NDV-S17 and NDV-S18 were classified as velogenic and NDV-S6, NDV-

P2, NDV-S9, NDV-P4 and NDV-P6 were classified as mesogenic. This is in agreement with Hanson and Brandly (1955).

Pathotyping of the viral isolates based on ICPI matched with the corresponding results obtained in MDT test. Similar results were reported by other authors when both MDT and ICPI values were employed for pathotyping of NDV (Tan *et al.,* 2008; Desingu *et al.,* 2016; Nagy *et al*., 2020).

5.7 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION TARGETING FUSION PROTEIN CLEAVAGE SITE OF F GENE

In the present study, primers (FPCS sense and FPCS antisense) targeting FPCS region of F gene were used in RT-PCR to amplify the desired region for molecular characterisation (Alexander, 2000). The eight NDV positive samples were amplified in RT-PCR yielding 254 bp amplicons at annealing temperature of 57.5°C. These samples were sent for sequencing and for further analysis of virulence of the virus. The presence of multiple basic amino acids at FPCS region is one of the criteria for defining ND caused by virulent APVM-1 (OIE, 2018). The same region was used for molecular characterisation of NDV isolates by Ananth *et al.* (2008). Along with analysis of the F gene, M gene (Kim *et al.,* 2007) and HN gene (Tan *et al.,* 2008) were also employed for molecular characterisation of NDV.

5.8 SEQUENCING AND PHYLOGENETIC ANALYSIS OF FUSION PROTEIN CLEAVAGE SITE REGION OF F GENE

On sequencing the PCR products obtained after amplifying FPCS region of F gene it was found that all of them contained similar sequences at FPCS region and corresponding amino acid sequence at this region revealed ^{112}GRQGRL^{117} motif in all the isolates. They contained monobasic amino acid at FPCS region of F gene. On phylogenetic analysis, all the isolates were related to NDV genotype II of class II strains and clustered with each other along with the United States isolates. The

isolates showed 98.82 per cent sequence homology with all the other Indian isolates and LaSota vaccine strains though they were distinct and were grouped separately.

Ananth *et al.* (2008) reported that lentogenic strain with monobasic amino acid sequence at FPCS region and having low values of pathogenicity indices belonged to genotype II of class II viruses. Velogenic strains containing polybasic amino acids motifs and pathogenicity indices corresponding to velogenic values were grouped in genotype II of class II viruses (Vijayarani *et al.,* 2010). But in this study, contrary to previous studies, virulent viruses possessing high ICPI (0.75-1.53) and MDT (54 h.-79.2 h.) with monobasic amino acid sequence were isolated. Similar results were also reported by Tan *et al.* (2008) and Nagy *et al.* (2020), where in the authors isolated velogenic viruses possessing monobasic amino acid sequences.

In this study, the mutation elsewhere in the genome might be responsible for the increase in the virulence of the isolates (De Leeuw *et al.,* 2003) and the isolates must have been generated from nature indicating emergence of virulent virus from lentogenic viruses under host immune pressure (Tan *et al.,* 2008). Though the sequence of FPCS region showed similarity with the lentogenic strains there is significant difference in virulence. This implies that additional factors other than F protein might have contributed to the virulence of NDV (Paldurai *et al.,* 2014).

From this study, it can be deduced that, there is continuous evolution of the strains of NDV. Periodic surveillance of the disease and complete characterisation of the virus is necessary for understanding the strains circulating in the region. This is necessary for planning vaccination programme with the proper strain of virus for controlling the disease.

SUMMARY

6. SUMMARY

Newcastle disease is a highly infectious, OIE notifiable disease having devastating effect on poultry industry. The disease is caused by Newcastle disease virus which affects almost all avian species worldwide and has been able to cause several panzootics. Isolation and identification along with molecular techniques for detection and characterisation of the virus have been employed for the past several years. The present study was conducted for isolation, identification and characterisation of the NDV isolates in Kerala.

Sixty-three samples (55 tissues, eight tracheal and cloacal swabs) were collected from recently dead and ailing birds showing lesions and signs suggestive of ND, presented to the Departments of Veterinary Microbiology and Veterinary Pathology and from Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Mannuthy. The samples collected were initially subjected for RT-PCR targeting F gene of the virus. Subsequently, isolation of the virus was also attempted in nine to eleven day-old ECE. Out of 55 tissue samples, eight samples were found to be positive for NDV in RT-PCR targeting F gene and none of the swabs were positive.

The AAFs of the same eight samples showed HA activity and were inhibited by NDV specific antiserum confirming the presence of the virus. They were also tested for NDV by RT-PCR using same set of primers employed for direct detection of the virus from tissue samples.

Pathotyping of the virus was done by ICPI and MDT tests. The ICPI test was carried out in day-old chicks. The diluted viruses were inoculated intracerebrally and observed for signs of infection and death for eight days. The ICPI values of the isolates ranged from 0.75 to 1.53. Mean death time was assessed for all the eight isolates in ECE. The value of the MDT varied from 54 h. to 79.2 h. Based on ICPI

and MDT values, three of the isolates were grouped as velogenic and the remaining five isolates were grouped as mesogenic.

The RT-PCR targeting FPCS region of F gene was carried out for molecular characterisation and phylogenetic analysis of the isolates. Based on the sequence results, all the isolates possessed ^{112}GRQGRL^{117} monobasic amino acid motifs at FPCS region and on phylogenetic analysis, all of them were grouped in genotype II of class II viruses.

In the present study, NDV was isolated using ECE from all the samples that gave positive amplicons by RT-PCR. It clearly reveals that RT-PCR could be employed for rapid detection of NDV directly from tissue samples at the time of an outbreak. Also, conventional method of isolation using ECE and confirmation by HI can be considered as an equally sensitive method. Owing to the rapidity in detection, RT-PCR could be used and in laboratory lacking the facilities, embryo inoculation technique can be employed for diagnosis of ND. From the study, it was also found that there might be an emergence of virulent virus from lentogenic strains due to host immune pressure in the vaccinated chicken. Hence, there is a need for frequent surveillance of the disease and characterisation of the virus isolates so that effective vaccination programme with suitable strain of virus can be employed for control and prevention of the disease.

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CHARACTERISATION OF *NEWCASTLE DISEASE VIRUS* ISOLATES PREVALENT IN KERALA

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ABSTRACT

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ABSTRACT

Newcastle disease caused by *Newcastle disease virus* (NDV) is one of the highly pathogenic viral diseases of poultry and is considered to cripple poultry industry globally. The diagnosis of the disease is based on virus isolation and characterisation and detection of the genome of the virus by molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR). Samples collected from dead and ailing birds showing lesions and signs suggestive of ND formed the materials for the study. Out of the 63 samples collected, eight revealed the virus in RT-PCR targeting the F gene, directly from the tissue samples. The NDV could be isolated in embryonated chicken eggs from the samples which yielded positive amplicons by RT-PCR. All the viral isolates showed haemagglutination activity (4 to 10 log_2) and was inhibited by the NDV specific antiserum. The HI titre for all the isolates was found to be 4 log_2. The ICPI and MDT indices were determined. The ICPI values were in between 0.75 to 1.53 and MDT values varied from 54 h. to 79.2 h. Based on the intracerebral pathogenicity index and mean death time, three of the isolates were classified under velogenic group and the remaining five under mesogenic group. The RT-PCR assay targeting the fusion protein cleavage site of the F gene was carried out and the specific amplified products were sequenced and phylogenetically analysed. All the isolates were of similar sequences and contained ^{112}GRQGRL^{117} monobasic amino acid motifs at FPCS region, which is peculiar to lentogenic viruses. On phylogenetic analysis, all the isolates were grouped in genotype II of class II viruses. In this study, NDV isolates with lentogenic FPCS region belonging to velogenic and mesogenic pathotypes were obtained.

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