Review Article

Virulence of Newcastle Disease Virus and Diagnostic Challenges

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Abstract | Newcastle disease (ND) is economically most important poultry disease and distributed worldwide causing devastating loses in poultry industry. Newcastle disease virus (NDV) has a wide host range and has been reported to infect more than 240 species of birds. In chickens virulence ranges from non-virulent, associated with asymptomatic enteric infections or unapparent or mild disease of respiratory tract (lentogenic strains), mild respiratory disease and moderate mortality rates (mesogenic strains) to severe disease with high mortality rates up to 100% (velogenic strains). Virulence of NDV strains depend greatly on the host response. Virulence of ND viruses is measured by scoring disease incidence after Intracerebral inoculation of day-old chickens. Highly virulent strains of NDV can be discriminated from low virulent strains by the presence of multibasic amino acid motif at the proteolytic cleavage site of the fusion (F) protein. Genetic classification has divided NDV into 2 classes (I and II), with class I composed of only 1 genotype (class I, genotype I) and with class II divided into 18 genotypes (class II, genotypes I–XVIII). Genotypes V, VI, and VII are virulent viruses and predominant genotypes circulating worldwide. Out of these, genotype VII is particularly important because it is associated with many or the most recent outbreaks in Asia, Africa and Middle East. For rapid and accurate diagnosis real time RT-PCR tests are equally or more sensitive than virus isolation and are always faster than virus isolation. Vaccination against ND is widely practiced. However, ND is still recognised to be endemic in many parts of the world, particularly in developing countries.

Keywords | NDV, Virulence, RRT-PCR

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INTRODUCTION

Newcastle disease is most economically important poultry disease and worldwide distributed causing devastating loses in poultry industry. Newcastle disease viruses (NDV) are by far the most important pathogen for birds of all types and in most countries infection with virulent forms represent a notifiable disease. Virulent Newcastle disease virus (NDV) is an office of international epizootics (oie) list A disease and subject to international regulations. NDV has a wide host range and has been reported to infect more than 240 species of birds (Alexander, 2003). Not only ND viruses show extremes of pathogenicity but also viruses of low virulence are enzootic in feral birds. In most countries, use of live vaccines is almost universal exacerbation of infections with viruses of low virulence may mimic disease produced by highly virulent virus (Alexander, 2003).

The clinical signs and symptoms vary widely in the birds infected with NDV and depends on factors such as the virus strain, host species, age of the birds, presence of other pathogen, environmental stress and the immunity status of the host. In chickens different strains of NDV cause sudden death with 100% mortality to sub clinical infection and the influence of species may be equally marked, such as the viruses causing severe disease in chickens and turkeys may cause few signs of disease in geese and ducks. Clinical signs that may be associated with ND are respiratory

distress, diarrhoea, cessation of egg production, depression, edema of head, face, wattle, nervous sign and death. Some, all or none of these signs may be present. It is very difficult to assess the exact prevalence of ND in the world at given time. In some countries or areas disease is not reported at all or only it occurs in commercial poultry, while its presence in village chickens or backyard flocks is ignored (Alexander et al., 2004). Countries that have long been recognised as free of ND, monitoring surveys often reveal symptomless infections with avirulent viruses which have presumably spread from waterfowl or other wild birds.

Avian paramyxoviruses are RNA viruses with helical capsid symentry, enveloped, negative sense and non-segmented single stranded genome. The length is approximately 15 kb and is divisible by six because the genome must be of polyhexameric length to replicate efficiently ('rule of six') (Peeters et al., 1999). It encodes for six proteins which in the 3' to 5' direction are nucleoprotein (NP), phosphoprotein (P) and the matrix protein (M) which lines the inner surface of the virus envelope, the surface glycoprotein hemagglutinin-neuraminidase (HN), which recognises and binds to sialic acid-containing molecules on the surface of the host cell, the fusion protein (F), which is responsible for the fusion of the viral envelope with the cell membrane, and the RNA dependent RNA polymerase (L, large gene), which together with the NP and P proteins are bound to the RNA genome to form the nucleocapsid (Steward et al., 1993). The RNA has molecular weight 5 x 10^8 which make up about 5% weight of the virus particle. The capsid of avian paramyxovirus is assmbled in the cytoplasm and enveloped by modified cell glycoprotein membrane due to budding from the cell surface. Two functional virus glycoproteins are inserted in envelop, one possess hemagglutination-neuraamindase (HN) activities, and other is fusion (F) protein. The surface projections on envelop approximately 8 nm long represent the HN molecules with the F molecule forming small projections. HN protein is responsible for attachment of the virus to the cell and F protein brings about fusion between cell and virus membrane to allow the genetic material to enter the cell for replication. The Newcastle disease were first reported during the mid-1920s at Newcastle–upon-Tyne, England (Snoeck et al., 2013). Few years later ND had spread throughout the world and became endemic in many countries. A virulent strain of NDV emerged between 1995 and 2000 affecting Double-Crested Cormorants in Canada. This strain causes significant mortality in juvenile cormorants and poses a risk to other avian species including poultry. India, being an endemic country for NDV, outbreaks still occurs in spite of regular vaccination programmes.

GENOTYPING AND PATHOTYPING

Based on severity of disease in chickens, NDV strains and isolates have been grouped into four pathotypes (velogenic, mesogenic, lentogenic and asymptomatic), that relate to the disease signs produced in infected fully susceptible chicken. Velogenic NDV strains are highly pathogenic/virulent causing mortality rates up to 100%. Velogenic strains are further classified in to viserotropic velogenic NDV, which produces acute lethal infections in which hemorrhagic lesions are prominent in the gut and neurotropic velogenic NDV, which produces high mortality preceded by respiratory and neurological signs, gut lesions are conspicuously absent. The mesogenic NDV, produeces low mortality, acute, respiratory disease and nervous sign in the some birds. The lentogenic NDV, which produces mild and unapparent respiratory infection. The asymptomatic enteric NDV, which are avirulent viruses that appears to replicate primarily in the gut. These groups are not completely clear cut and some overlapping between the signs associated with the different groups has been reported.

A widely accepted system used to implement virulence classification is the intra-cerebral pathogenicity index (ICPI) in day-old chicks, which yields a numeric score on a 0–2 scale, with scores close to 2 being typical of very virulent strains (Alexander et al., 2008; OIE). According to the World Organization for Animal Health (former Office International des Epizooties [OIE]), virulent NDV strains (notifiable to the international community) are those viruses that have an ICPI equal to or greater than 0.7, or an amino acid sequence of the fusion protein cleavage site with at least 3 arginine or lysine residues between positions 113 and 116 and a phenylalanine residue at position 117 (OIE). Although all NDV strains belong to a single serotype (serotype-1), there is large genetic variability among NDV isolates (Miller et al., 2009a, 2010; Afonso et al., 2013). A recent implementation of this genetic classification has divided NDV into 2 classes (I and II), with class I composed of only 1 genotype (class I, genotype I) and with class II divided into 18 genotypes (class II, genotypes I–XVIII) (Courtney et al., 2012; Snoeck et al., 2013). While class I encompasses only avirulent NDV strains (except for 1, APMV-1/chicken/Ireland48/904) found mainly in waterfowl, class II includes both virulent and avirulent strains (Miller et al., 2009a, 2010; Afonso et al., 2013).

Virulent NDV strains have a polybasic amino acid configuration that allows cleavage of the fusion protein by furin-like proteases found ubiquitously in the organism, allowing for systemic viral spread. The sequences of the F protein cleavage site (FPCS) is a well characterized, major determinant of NDV pathogenicity in chickens. On the basis of the phylogenitic analysis with partial hypervariable nucleotide sequences of the F gene, NDV strains have been classified in to ten genotypes (I- X) (Alexander, 2003). Genotype VI and VII are further divided in to seven and eight sub genotype respectively. Despite the

extensive use of vaccines, several outbreaks of NDV has been reported. Vaccines cannot reasonably be expected to protect hundred percentage of the flock under commercial poultry conditions. The actual protection obtained will be determined by the combination of all the factors which can affect vaccine efficacy. However, vaccine efficacy will never be greater than the maximum obtainable under experimental conditions for a given vaccine. Genotypes V, VI, and VII are virulent viruses and predominant genotypes circulating worldwide. Of these, genotype VII is particularly important because it is associated with many or the most recent outbreaks in Asia, Africa and Middle East (Liu et al., 2003). Subsequently, subgenotypes, VIIa–VIIe represent isolates from China, Malaysia, Kazakhstan and Kyrgyzstan (Bogoyavlenskiy et al., 2009 and Wang et al., 2006) and VIIf–VIIh represent African isolates (Snoeck et al., 2009). More recently, Miller et al. (2014) identified virulent Newcastle disease virus (NDV) isolates from new sub-genotypes within genotype VII are rapidly spreading through Asia and the Middle East causing outbreaks of ND characterized by significant illness and mortality in poultry, suggesting the existence of a fifth panzootic. The complete genome sequence (15,192 nucleotides in length) of a NDVstrain (NDV/Chicken/Nagpur/01/12) was isolated from vaccinated chicken farms in India during outbreaks in 2012 and is classified as genotype VII in class II (Gogoi et al., 2015).

Three factors that may increase the risk of an outbreak and predict the need to conduct studies on the evolutionary mechanisms affecting NDV genome are: (1) only a few nucleotide changes in the fusion gene are sufficient to change NDV from low virulence to high virulence, (2) there are large and highly mobile reservoirs of low virulence viruses in nature that may come into contact with poultry, and (3) billions of doses of low virulence live vaccine virus are inoculated into poultry annually, thereby likely releasing the vaccine virus into the environment.

NDV vaccine strains of genotype I and II are being used to control clinical disease during the outbreak. Additionally, control of risk factors including immunosuppressive agents, biosecurity breaks, inadequate management practices and harsh environment together is required to diminish the economic impact of ND outbreaks.

Virulent isolates from outbreaks in Australia were shown to be genetically similar to the viruses of low virulence that were known to be previously circulating in the country (Kattenbelt et al., 2006). These endemic low virulence viruses require only two point mutations to become virulent (Westbury, 2001). In addition to field conditions, some low virulent NDV shown to have the capacity to become virulent under experimental condition (de Leeuw et al., 2003; Shengqing et al., 2002; Zanetti et al., 2008). For the purpose of disease prevention it would be ideal to predict the potential of each genotype of low virulence NDV to mutate into a virulent form (Miller et al., 2010). The highly pathogenic form of ND is a serious problem, either as an enzootic disease or as a cause of regular, frequent epizootics throughout Africa, Asia, Central America and parts of South America (Copland, 1987; Spradbrow, 1988; Rweyemamu et al., 1991; Alders and Spradbrow, 2001). In Europe sporadic epizootics occurring despite vaccination programmes (Kaleta and Heffels-Redmann, 1992). In Western Europe there was a marked increase in reported outbreaks during the early 1990s were reported. Between 1995 and 1999, outbreaks of NDV were reported in the all areas of Western Europe that had been declared free of ND. Two outbreaks of virulent ND occurred in Australia in 1998 and further outbreaks were reported in 1999 and 2000 (Kirkland, 2000; Westbury, 2001). The genome sequence of a new strain of NDV (chicken/Pak/Quality Operations Lab/SFR-611/13) is reported from a vaccinated chicken flock in Pakistan in 2013 and has panzootic features is classified in subgenotype VIIi of genotype VII, class II (Wajid et al., 2015).

MOLECULAR BASIS OF PATHOGENICITY OF ND

The molecular approach for NDV identification and pathotyping using reverse transcriptase PCR followed by direct sequencing and analysis of fusion protein gene cleavage site is currently used for NDV research and surveillance (Aldous et al., 2003). NDV particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious while replication (Rott and Klenk, 1988). This post translation cleavage is mediated by host cell proteases (Nagai et al., 1976a). Trypsin is capable of cleaving F0 for all NDV strains and *in vitro* treatment of noninfectious virus will induce infectivity (Nagai et al., 1976b). Rott (1985) reported the cleavability of the F0 molecule was directly related to the virulence of viruses *in vivo*. It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues. This allows these viruses to spread throughout the host, damaging vital organs. In contrast F0 molecules in viruses of low virulence appear to be restricted in their sensitivity to host proteases resulting in restriction of these viruses to grow only in certain host cell types. Collins et al. (1993) carried out initial studies comparing the deduced amino acid sequences at the cleavage site of the F0 precursor of a number of virulent and avirulent ND strains. After that large number of studies have confirmed the presence of multiple basic amino acids at that site in virulent viruses. Usually the sequence has been 113/117 RQK/RR↓ F0 in virulent viruses have a basic amino acid at position 112 as well. In contrast, viruses of low virulence usually have

the sequence 113/117K/RQG/ER↓. The major influence on the pathogenicity of NDV is depend on the amino acid motif at the F0 cleavage site, the presence of basic amino acids at positions 113, 115 and 116 and phenylalanine at 117 in virulent strains means that cleavage can be effected by protease or proteases present in a wide range of host tissues and organs. For viruses of low virulence, cleavage can occur only with proteases recognizing a single arginine, i.e. trypsin-like enzymes. Such viruses are therefore restricted in the range of sites where they are able to replicate in areas with trypsin-like enzymes, such as the respiratory and intestinal tracts, whereas virulent viruses can replicate in a range of tissues and organs resulting in a fatal systemic infection (Rott, 1979).

ROLE OF ND VACCINES IN THE EVOLUTION OF VIRULENT NDV

Mostly reservoir of virulent Newcastle disease virus (vNDV) is the vaccinated poultry population there is evidence that wild birds may represent natural reservoirs of mesogenic viruses (Aldous et al., 2007; Czegledi et al., 2006). Wild water fowl and shore birds are infected with a large and diverse group of avirulent viruses that normally do not produce any clinical signs in poultry. Phylogenetically related vNDV of genotype V have been isolated from double-crested cormorants (*Phalacrocorax auritus*) from 1975 through 2008 and they have been implicated in earlier ND outbreaks (Allison et al., 2005; Blaxland, 1951; Heckert et al., 1996). Virulent pigeon paramyxovirus-1 (PPMV-1) isolates, which are clinically neurotropic in chickens, were first isolated in 1981 in pigeons (*Columba livia*), and continue to circulate in feral birds of the Columbidae family worldwide (Kaleta et al., 1985; Kim et al., 2008; Mase et al., 2009).

The vNDV isolated from cormorants and pigeons are considered mesogenic because their ICPI values in chickens vary from > 0.7 to< 1.5 and they do not usually cause significant disease in poultry. However, recently the U.S. designated all NDV with ICPI values > 0.7 or containing an amino acid sequence consistent with virulent strains of NDV as virulent and classified them as selected agents, to follow the World Organization for Animal Health (OIE) and the European Union standards (OIE). Weingartl et al. (2003) reported that the cormorant and pigeon viruses continue to evolve and display year-to-year genomic changes and no significant changes in virulence have been observed in wild-bird samples. The almost exclusive predominance of low virulence class I and mesogenic viruses of class II, genotypes V or VI in cormorants and pigeons, in contrast to the prevalence of viscerotropic vNDV (class II, genotypes V–X) in vaccinated poultry (Czegledi et al., 2006), it suggests that the vaccination induces immune pressure for selecting variant forms of vNDV.

Antigenic and phylogenetic distances between vaccine strains and current circulating virulent strains may play important role in the evolution of virulent NDV (Miller et al., 2007). Many studies have been reported that current vaccines prevent disease but cannot stop viral shedding (Kapczynski and King, 2005; Miller et al., 2009a; Utterback and Schwartz, 1973). In addition, there are reports of using genotype-matched vaccines which can significantly reduce viral shedding. Two antigenically matched vaccines have demonstrated increased capacity to prevent viral shedding of viruses of genotype VII and V, respectively (Hu et al., 2009; Miller et al., 2009b). Existence of field-isolated variants that escape vaccination has also been reported (Cho et al., 2008).

It has been observed that current ND vaccines fail to protect against morbidity and mortality caused by new variants from genotype VII, is a contraversial. Liu et al. (2003) reported that the chickens vaccinated with either a live or a killed oil-emulsion LaSota (genotype II) vaccine, were fully protected against heterologous challenge strains of genotypes VIg, VIb, VIId and IX. Similar findings were observed by using two commercial vaccine strains in specific pathogen free (SPF) chickens against two virulent challenge viruses of genotype VII (Jeon et al., 2008). Despite the controversy, enough evidence exists to suggest that NDV variants may be evolved in poultry as a result of suboptimal vaccination. Regardless of genotype differences between worldwide circulating NDV strains, all NDV isolates belong to the same serotype. If the vaccination given correctly, ND vaccines prepared with any NDV should protect poultry from clinical disease and mortality in the event of a virulent challenge (OIE).

DIAGNOSTIC CHALLENGES

Diagnosis of Newcastle disease is generally carried out by isolation of NDV in SPF embryonating chicken eggs (ECE), confirmation by reverse transcriptase polymerase chain reaction (RT-PCR) or by real-time RT-PCR (RRT-PCR) and by serology using the hemagglutination-inhibition (HI). All NDV isolates are known to replicate in ECE and the MDT to kill the embryo varies depending on the virulence of the virus. The HI test is used to identify a virus as NDV. A panel of Monoclonal antibody (mAb) testing can be used to characterize NDV. Most of the mAb were developed and optimized to recognize class II viruses and fail to recognize viruses of class I (Collins et al., 1998; Kim et al., 2007b). Enzyme linked immunosorbent assay (ELISA) are also used to assess antibody response following vaccination, but have limited value in surveillance and diagnosis because of the use of vaccines in domestic poultry.

After identification, pathotyping of isolates is required to determine virulence characteristics. The methods used to

pathotype newly isolated strains of the virus include the intracerebral pathogenicity index (ICPI) test, MDT and determining the amino acid motif at the cleavage site of the fusion protein. As pathotyping tests are time consuming and expensive and serological tests are complicated by the universal use of live-virus vaccines in poultry, rapid nucleic acid based assays have been developed. RT-PCR, matrix gene assay and multiple single-tube, sensitive, rapid real-time reverse transcription polymerase chain reaction(RRT-PCR) assays have been developed in the last decade around the world to detect the viruses circulating in those locations (Antal et al., 2007; Fuller et al., 2009; Pham et al., 2005; Tan et al., 2004; Wise et al., 2004). Depending on assay conditions, these RRT-PCR tests can be equally or more sensitive than virus isolation and are always faster than virus isolation and have been adopted as the standard method for surveillance in the U.S.

REAL TIME PCR FOR THE DETECTION OF NDV

The primers and probe for the M-gene assay were designed to detect the highly conserved matrix (M) gene of NDV and, as such, detects most NDV genotypes of class II, regardless of pathotype. However, due to the heterogeneous genetic nature of this virus, class I viruses, tested often fail to be detected (Kim et al., 2007a). Evaluation of the nucleotide sequence alignment of the M-gene assay probe site of class I and II viruses revealed a high number of mismatches between the two classes, and this is likely the reason that the class I viruses escape detection by this assay.

Kim et al. (2008) developed a new matrix-polymerase multiplex RRT-PCR for the detection of a broad range of class I and II NDV isolates. A conserved region from the polymerase (L) gene of class I NDV genome was identified and used in the design and evaluation of a multiplex RRT-PCR assay (L-TET) that identifies a broad range of NDV. Although the fact that viral nucleic acids were not detected in all the samples that were positive in virus isolation, it suggests that RRT-PCR cannot replace virus isolation completely for individual sample basis.

The ability to detect virulent viruses quickly is key to containing an outbreak. The F gene probe created specifically to detect virulent NDV from field swabs during the outbreak of 2002 in the U.S., is widely used because it was field validated. It is imperative that diagnostic laboratories using the USDA validated or other fusion protein based PCR assays continue to monitor genomic changes and re-design alternate primers and probes to prevent the failure of detection of PPMV-1. Alternatively, the use of virus isolation in eggs in conjunction with the PCR assays will identify these isolates with embryo mortality.

Random Priming Methods

The high capacity for mutation in RNA viruses (genetic drift) and the large diversity of NDV genotypes often makes it difficult to predict the genetic composition of new isolates. Random genome sequencing represents an unbiased and thorough alternative that has been widely used to characterize the genomes of large DNA viruses (Afonso et al., 2006), but has only recently been used with NDV (Djikeng et al., 2008). A protocol that follows two basic principles: (i) random amplification of total RNA, and (ii) random selection of colonies followed by sequencing and assembly has been developed. This protocol, a modification of the sequence-independent, single-primer amplification, SISPA method, first developed by Reyes and Kim (1991), and recently adapted for NDV entails the directional ligation of an asymmetric primer at either end of a blunt-ended DNA molecule. The method described is of great utility in generating whole genome assemblies for viruses with little or no available sequence information, viruses from greatly divergent families, previously uncharacterized viruses, or to more fully describe mixed viral infections (Djikeng et al., 2008).

CONCLUSION

New genotypes of NDV are reported to be circulating worldwide. To date, 18 class II genotypes of NDV have been described. Due to increase in genomic diversity of NDV poses several problems for prevention and control of ND. The genotypes involved in the most recent outbreaks worldwide are genotypes V (Central and South America), VI (Europe) and VII (China, South Africa and India). Regardless of genotype differences between worldwide circulating NDV strains, all strains of the ND virus belong to one serogroup, so proper vaccination protects poultry from clinical disease of ND. If the vaccination given correctly, it should protect poultry from clinical disease and mortality in the event of a virulent challenge. Generally, vaccine failure occurs due to factors of the immune status of the host, the improper storage of vaccine (live vaccine are thermolabile, poor cold chain reduces titter of vaccine), improper vaccination and pathogenic virus strain. Inspite of taking all the measures, sometime vaccine failure has occurred. For such outbreaks the reason are still unknown. Biosecurity of commercial poultry facilities is an important step in preventing transmission of NDV and avoid heavy economic losses. Genotype or subgenotype specific of NDV strains prevalent in that region are included in the vaccination schedule. A rigorous epidemiological surveillance is required to decide the actual impact of the disease in poultry population.

CONFLICT OF INTEREST

There is no conflict of interest.

AUTHORS CONTRIBUTION

All authors contributed equally for the preparation of this paper. Chawak M.M. gave technical and conceptual advice for writing this paper.

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