Review Article

Newcastle Disease Virus: Disease Appraisal with Global and Pakistan Perspectives

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INTRODUCTION

The Newcastle Disease (ND), locally called Ranikhet, is a highly contagious viral disease that affects domestic and wild bird species (Seal et al., 2000; Alexander, 2003). The disease was first described in 1926 from Newcastle–on–Tyne, England and Java, Indonesia (Alexander, 2001). It spread rapidly in Asia (Brandly, 1964), whereas, it took four decades to become panzootic (Alexander, 2012). Domestic poultry is considered highly susceptible to ND infection resulting in severe outbreaks worldwide. It represents major deplete in the economy of poultry rearing countries than any other viral disease (Alexander, 2003) especially in developing countries including Pakistan. To date, it occurs on at least six of the seven continents (Miller et al., 2012), enzootic in many countries of the world and is a constant threat to the poultry production worldwide, with variable outcomes in the form of outbreak and subsequent economic losses. Sporadic outbreaks had been seen in some of countries of European Union; however, since 2000 – 2009, virulent NDV (vNDV) for chickens has been detected in wild birds, pigeons and poultry (Alexander, 2012). The velogenic visceral tropic vNDV strains (a vNDV that affects visceral organs) termed as selective agent are not endemic in the US poultry, and the disease caused by them is often known as exotic ND (END) (USDA, 2006).

Clinically, the manifestation of ND depends largely upon the isolate(s) involved in disease outbreak (Alexander, 2003; Miller et al., 2010). Based on clinical signs, NDV strains are categorized into five different pathotypes; (a) viscerotropycelogenic (b) neurotropic velogenic (c) mesogenic (d) lentogenic or respiratory and (e) subclinical/asymptomatic or enteric (OIE, 2009). Among these, the vNDVs causes three well defined clinical forms: (a) neurotropic velogenic is characterized by high mortality along with respiratory and neurological symptoms with no gross lesions in the gastrointestinal tract (b) visceral tropic velogenic is characterized by an acute infection with high mortality along with multiple hemorrhagic lesions in the gastrointestinal tract (GIT) that includes proventriculus, intestine and cecal tonsils and (c) mesogenic which causes both respiratory and neurological signs with low mortality. Lentogenic NDVs are referred to as less virulent viruses that cause mild or no clinical symptoms in birds.

Newcastle Disease Virus; Taxonomy and Geographical Distribution

Newcastle disease virus (NDV) is a prototype of avian paramyxovirus serotype-1 (APMV–1). APMV–1 along with other nine avian paramyxovirus subtypes are classified in the genus Avulavirus within subfamily Paramyxovirinae, family Paramyxoviridae and order Mononegavirales (ICTV, 2009). This enveloped RNA virus has a negative–sense, single stranded, non–segmented genome of always either 15186, 15192 or 15198 nucleotides in length and follow the so called “rule of six” which is essential for viral replication (Kolakofsky et al., 2005). From 5’ to 3’ terminus, the NDV genome encodes for six structural proteins that include nucelocapsid protein (NP),


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The NDV strains are classified into four categories based upon pathogenicity in embryonated chicken eggs: velogenic [MDT (mean death time) >60 h], mesogenic (MDT 60–90 h), lentogenic (MDT <90 h) and avirulent (does not kill the embryos) (Beard and Hanson, 1984). The molecular mechanism behind this varying level of pathogenicity is known to be the amino acid sequence motif present in the protease cleavage site of the precursor fusion protein (F2) and subsequent abilities of the cellular proteases to cleave this F2 protein (Miller et al., 2010; Munir et al., 2012). The amino acid sequence for mesogenic and velogenic strains is 112R/K–R–Q–R/K–RJ 117 which is cleavable by a variety of cellular proteases and resulting in wider systemic infection involving respiratory, GIT and nervous system. The less virulent viruses such as lentogenic NDV strains have the sequence 112G/E–K/R–Q–G–E–RJ 117, which is cleavable only by trypsin like proteases and thus, limiting the infection only to respiratory. GIT (Miller et al., 2010). The virulent strains have phenylalanine instead of leucine in low virulent NDV at position I17 of F gene and basic amino acids surrounding Q I14, it has been shown to be necessary in determining the strain virulence using reverse genetics (de Leeuw et al., 2003).

Although all the different NDV strains belong to the APVM–I, concerns about antigenic variation and genetic diversity of the strains are emerging (Aldous et al., 2003; Alexander et al., 1997). Two different systems of classification of NDV exist, which is based on genome size, the NDV strains can be classified into six lineages and 13 sub-lineages, in which three additional sub-lineages were, added (Snoeck et al., 2009). Alternatively, based on genome size, the NDV strains can be classified into two classes; class I and II. Class I carries strains of NDV with large genomes (15986 nt), which are avirulent in chickens, and class II strains with shorter genomes and includes lentogenic, mesogenic and velogenic strains of NDV. Based upon the partial sequence of the F gene, class II viruses are further divided into 6 genotypes (I–VII), a genotypes VI and VII, which are genetically diverse are further classified into eight (a–h) and five (a–e) sub-genotypes, respectively (Herczeg et al., 1999; Aldous et al., 2003; Cezegledi et al., 2006; Munir et al., 2012). The NDV strains belonging to class II have been isolated from the vast majority of clinical outbreaks in chicken; however, class I have been isolated from waterfowl worldwide. Most of the class I NDVs are known to be non-pathogenic to chicken, yet there has been a report of class I originated outbreak in chicken in Ireland (Alexander et al., 1992), suggesting the ability of these avirulent strains to become virulent in chicken with yet unknown mechanism (Collins et al., 1998).

NDV is prevalent worldwide; however, the distribution of pathotypes is different in different countries. Velogenic NDV strains are prevalent in Central and South America, Mexico, Asia, and Middle East, whereas, lentogenic strains are reported worldwide and pigeon adopted mesogenic pathotype (pigeon paramyxovirus) does not known to infect poultry readily (OIE, 2009). More specifically, among the prevalent NDV strains throughout the globe, genotypes V, VI, VII, and VIII are the predominant with only virulent viruses. The genotype V was originally emerged from South and Central America in 70s and caused outbreaks in Europe and part of American continent (Ballagi–Pordany et al., 1996; Wise et al., 2004a; Perozo et al., 2008). Genotype VI emerged in 60s in Asia, remained continued in circulation and subsequent disease reporting until 80s, was replaced with more common genotype VII (Mase et al., 2002) and is still prevalent. Among the sub-genotypes VIa through VIIg of genotype VI, VIIb has been more commonly isolated from pigeons. Genotype VII was initially divided into two sub-genotypes: VIIa and VIIb. Both emerged from Far East around 90s, however, VIIa spread to Europe (Herczeg et al., 1999) and Asia whereas, VIIb spread to South Africa (Aldous et al., 2003). The two sub-genotypes of VII are further divided into VIIc, d, and e, which have been reported from China, Kazakhstan and South Africa (Bogoyavlenskiy et al., 2009; Wang et al., 2006), and VIII, g, and h, which represent African isolates (Sneock et al., 2009). The isolates from Far East and Israel have been categorized as VIIId and from Iran and sub-continent as sub-genotype VIIb. Apart from this, Aldous et al. (2003) have reported VIIa from the field outbreaks in the Middle East, Europe, Taiwan and India. Genotype VIII viruses have been circulating in South Africa since 60s (Abolnik et al., 2004a) and continue to circulate in Southeast Asia. Genotype IX represents a unique NDV first reported from Vietnam in 2001, which has spread to Europe, Asia and recently to the Middle East region (Herczeg et al., 1999) and, yet now, the members of this genotype are continued to be occasionally isolated from China (Wang et al., 2006). Genotype X viruses have been isolated and reported from Taiwan in 1969 and 1981 (Tsai et al., 2004). New genotypes (XI–XV) are specifically reported from Africa and Asia. With the exception of genotype IV reporting since 1989, viruses from most genotypes are still continue to be circulated and subsequently reported at the present time around the globe.

During the recent years, from 2009 to date, a number of vNDVs have been isolated from commercial, rural and wild birds; some of them are completely characterized while some have been studied for F protein gene based genotypic characterization. Nearly all the studies described the isolates as vNDVs and raised concerns about substantial genetic variability from previously reported isolates, subsequent sensitivity of antigen diagnostics assays that have been validated previously. Khan et al. (2010) have clustered together the NDV’s isolates as velogenic and phylogenetically close to Japanese isolates (1989/1999). Munir et al. (2011) have performed complete genome sequence analysis of NDV and, based on phylogenetic analysis of HN protein from NDV belonging to Sweden, China, India and Russia, described the isolate as genotype VIIb with substantial genetic difference to be regarded as VIIb and 89% genome similarity to isolates from one of the neighboring countries, the China. In another study, Munir et al. (2012) have evaluated the NDV isolates from commercial and backyard poultry and found a new sub–lineage (SI) within lineage 3; the genetic difference from previously reported isolates was much more than those reported by Cattoli et al. (2010) from West and Central Africa. From these studies, it can be concluded that multiple velogenic isolates of genotype VII similar to those previously reported from Asian countries are prevailing in the Pakistan and require future studies to elucidate the mechanism of this emergence. Furthermore, these NDV strains undergone substantial antigenic drift to avoid detection from previously validated molecular diagnostic assays (Khan et al., 2010; Munir et al., 2012).

**Epidemiology and Transmission**

The clinical outcome of NDV varies from sub-clinical infection to 100% mortality. This variability depends largely upon viral strain involved, the species of bird and its immune status, age and the conditions under which the birds are being reared (Jindal et al., 2009). Co-infection with other microbes may also aggravate even the mildest form of NDV infection similar to...
vNDV infection. Consequent to this, no disease signs may be considered as pathognomonic for definite diagnosis of the disease (Alexander, 2011). The vNDV strains may produce peracute as well as acute infections where the clinical outcome may include sudden death. Typically, the vNDV strain result in depression, prostration, ruffled feathers, most of the time greenish diarrhoea, and oedema of the head involving eyes and nostrils, and nervous signs such as torticollis, aberrant circling movement, and wing paralysis may occur in later stages of the disease. The flock mortality may reach up to 100%; however, the percent mortality is relatively higher in younger or immunologically naïve birds than older birds (Alexander, 2011). Mesogenic viruses result in severe respiratory symptoms followed by nervous system involvement with mortality ≥ 50%. The viruses of low virulence or lentogenic strains may cause mild respiratory disease in chicken and turkeys, however, co-infection along with poor management practices may lead to clinical outcome comparable to vNDVs (Alexander, 2011).

A wide range of birds and other species including mammals are susceptible to NDV infection. The most commonly infected includes chickens, turkeys, pigeons and ducks; chickens are more susceptible with severe clinical outcome while turkeys are not known to show severe form of NDV (Jindal et al., 2009; OIE, 2009). The wild birds especially the ducks are generally considered natural reservoir of lentogenic NDVs and exhibit no or very few clinical signs even after infection with strains that are otherwise lethal to chicken (Jindal et al., 2009; Zhang et al., 2011). These lentogenic viruses are known to become velogenic after mutation, upon establishing the infection in domestic poultry. Human may also become infected manifesting swelling, redness and excessive lacrimation from eyelid and conjunctivitis (OIE, 2009).

The principal routes of NDV transmission are ingestion (feco–oral) and/or inhalation with direct contact to secretion of infected animal. Respiratory secretions and feces are generally considered the main source of NDVs from infected birds. The virus remained in continuous shedding during the incubation period (2–15 days), clinical disease and even for sometime in convalescence period. The hatching chicks may also get infected via egg, however, vertical transmission of highly virulent NDVs is still unknown. Mechanical transmission of NDVs through flies is also not established (OIE, 2009).

DIAGNOSIS
The NDV is tentatively diagnose in Pakistan based on the clinical outcome, postmortem lesions particularly pin-point hemorrhages in proventriculus and hemmorhages in cecal tonsils, mortality pattern, vaccine history. However, geometric mean titer (Haemagglutination inhibition test), and virus isolation are being considered the gold standard method in identifying ND outbreaks and subsequent biological and molecular characterization of the genotype involved.

Isolation of Virus
Isolation and subsequent identification of genotype of NDV are mandatory for international trade as well as optimization of conventional and advanced molecular diagnostic tools (OIE, 2008). The NDV may be isolated during incubation period, clinical stage of the disease and even early convalescence period from respiratory and fecal secretion and tissues such as brain, trachea, lungs, spleen, proventriculus, cecal tonsils and intestine. Specified pathogens free (SPF) embryonating chicken eggs of age 9–11 days are the best and economical culturing system for NDV isolation, particularly for developing countries like Pakistan. Unfortunately, no SPF chicken is available in Pakistan; however, this problem is solved in getting embryonating eggs from flocks with (a) low or no antibody titer to NDV, (b) route of virus inoculation, chorio–allantoic route is preferred than yolk sac where maternal antibodies can interfere with inoculants and (c) harvesting the allantoic fluid well before 15 days of embryo age when absorption of NDV antibodies from egg yolk get starts, if embryo is survived with inoculating virus. Likewise, eggs and immunologically naïve chickens are being used to biologically characterize the isolated NDVs through different assays that include (Embryo Infective Dose) EID50, Intracerebral Pathogenicity Index (ICPI), Intravenous Pathogenicity Index (IVPI) and Mean Death Time (MDT) as per international standards and protocols available. Different cell lines are being used worldwide to isolate the NDV and further biological analysis, however, no such known facility is in practice in Pakistan.

Antigen Detection Methods, Molecular Tools
With the advancement of conventional and molecular diagnostic tools worldwide and due to the availability of appropriate tools (software and equipment) in many of the diagnostic laboratories and research institutes in Pakistan, clinical to laboratory base diagnosis has been improved. The major advantage is the diagnosis of single organism or multiple organisms causing the similar symptoms without the initial culturing of the organism; e.g., multiplex PCR that can differentiate organisms in one go. In this regards, the role of Higher Education Commission of Pakistan and foreign funding agencies particularly the US, either directly or indirectly, can’t be ignored. However, educational and focused training on the use and application of these developed and validated protocols as per the needs of the country are still required. This is of particular importance due to constant changes and mutations in the circulating viruses of the country and established assays fail to detect the pathogens as has been noticed in NDV (Munir et al., 2012).

In principal, the molecular diagnosis of NDV can be divided into two (a) detection of NDV using primers against relatively conserved regions of genome such as, NP, L and M gene and (b) detection of virulent NDVs using hypervariable region of F gene including the region of cleavage site. Targeting the conserved, NP, M and L gene, detection of all the groups, genotypes and lineages is not always possible. Moreover, detection of virulent NDVs is relatively difficult using primers for the F gene when considering the geographical variations (Hoffmann et al., 2009).

Reverse Transcriptase Polymerase Chain Reaction
Among the various molecular diagnostic techniques developed for NDV, polymerase chain reaction is the most sensitive and effective assay. Using NDV specific primers from already published literature worldwide, most of different veterinary laboratories and research institutes in Pakistan are identifying the NDV strains. Nevertheless, most of work related to antigen characterization, its sequencing and genome analysis is being carried out in collaboration with foreign organizations/OIE reference lab (Khan et al., 2010; Munir et al., 2011; Munir et al., 2012). Routine serological techniques and virus isolation are being employed to samples submitted for isolation and identification of virus but these are not equally suitable in decomposed tissues and organs. Using the blood as clinical specimen, NDVs have been identified and characterized even from NDV suspected and/or diseased flock through PCR (Munir et al., 2012). This technique is equally suitable for isolates, clinical secretions and/or discharges, as well as decomposed organs/tissues. The only limitation is the
posibility to fail detection due to targeted–gene mutation of NDV. This requires continuous validation of established assays and frequent updation of the personnel. From the available information, universal primers may be used widely for targeted regions of NDV (Barbezange and Jestin, 2002). However, if epidemiological as well as prevailing genotypic and pathotype information is required, a 373bp sequence of F protein followed by sequence analysis is being used worldwide (Aldous et al., 2003; Munir et al., 2012). From the nucleotide information available at NCBI, the primers can be designed using freely available software and can be used successfully in the lab and this is the area where scientist/researcher at universities and government institutes should collaborate.

**Real Time Polymerase Chain Reaction**

Real time PCR is much sensitive and specific than the conventional polymerase chain reaction; however have most of its uses only in diagnostic lab. The said technique identifies and quantifies the virus in a clinical specimen and isolate simultaneously. The USDA validated real time PCR protocols are available and are being used by a number of laboratories worldwide. It is difficult to develop a single rRT–PCR to detect all prevailing avian paramyxoviruses. Thus, RT–PCRs based on the F–gene can be performed in parallel or in the same tube to achieve amplification of the cleavage site of at least all NDV's. However, hypervariable nature of this part of NDV genome may result in failure of the amplification of new virus isolates as evident in the current studies on Pakistan isolates (Khan et al., 2010; Munir et al., 2012). The ability of a probe to hybridise to a specific product is very sensitive to mutations (Kim et al., 2006), however, to overcome this problem, several probes in one reaction has been used (Aldous et al., 2004) which can better tolerate mutations.

SYBR–green, an intercalating dye has been used and validated for NDV–specific rRT–PCR (Tan et al., 2004) and this approach has further been used for pathotyping (Pham et al., 2003b). As the SYBR–green emit signals by intercalating double stranded DNA and thus, the non–specific amplification may contribute to false positive results. However, the specificity of the amplicon can be checked afterwards by melt curve analysis and/or gel electrophoresis and comparison with controls.

**Haemagglutination Inhibition Test: Virus Identification and Serological Monitoring**

Pakistan is a developing country where molecular diagnostic tools, though practice in some of the government as well as private labs in capital and provinces, and relevant consumables are not equally available to all veterinary institutes. Therefore, virus identification is not always being carried out using RT–PCR and real time PCR. The most widely used method available nearly in all the veterinary laboratories are the Haemagglutination Inhibition test (HI); both in research and NDV diagnostics. This test is being used equally to identify the suspected ND isolates as well as, pre– and post–vaccination titres for monitoring the immune response of birds.

For virus identification, the isolate is being processed for Haemagglutination (HA) test first and on the basis of potential to cause agglutination of chicken RBCs, the corresponding 4HA unit of the isolate is being calculated. This 4HA unit suspected NDV is then allowed to interact with commercially available monovalent antisera against all the prevailing and well known haemagglutinating viruses such as influenza strains and NDVs. Based on HI titer with respect to the controls, the virus is being identified as NDVs or else. Normally, the test is being run in duplicate and repeated twice to see the concordance in results.

For serological monitoring, the application of HI are in two ways (a) measuring the serological status of day old chicks so that vaccination schedule pertaining to ND vaccine can be determined and (b) post–vaccine, measuring the antibody titer vaccinated birds and deciding whether and when to vaccinate the birds again. For both purposes, the sera of the birds are collected and submitted to nearby and relevant veterinary diagnostic private/government lab. The Poultry Research Institute, Punjab, and Directorate of Animal Disease Surveillance and Reporting System, Punjab has at least one diagnostic lab in each district of Punjab province. Likewise, HI facilities are available in most of the labs in other provinces of Pakistan. Using two fold serum dilution and 4HA unit NDV, the individual sample’s HI titer is being observed and subsequent GMT is calculated as per Brugh's table. Generally, the GMT ≥ 128 is considered protective to NDV; however, from the recent high mutations that are well enough to be considered the circulating vNDVs as new sub-genotype (VIIb) and new sub-lineage (Si), it is very likely that a relatively high level of antibody titer is being required for the birds to remain protective from vNDV field strains.

**Enzyme Linked Immunosorbent Assay**

Commercially available simple ELISA as well as competitive ELISA (cELISA) kits are in use in some of the labs; most are for research purpose only. As whole of the virus is being used as antigen in simple ELISA kits, it can detect antibodies to all proteins of the virus particle. In cELISA kits, some part of the virus is being used as antigen and thus can be used specifically to identify the immune response against that particular part. Nevertheless, none of the ELISA kits can differentiate between vaccinated and naturally infected birds, until live vaccine is being in practice. Though the test is much sensitive and specific one than HI, it is not widely used in Pakistan due to high cost, time consuming, technically labour, and equipment requirements (e.g. ELISA reader).

**PROPHYLAXIS AND CONTROL**

Vaccination is the only measure to prevent the epizootics worldwide particularly in vNDV endemic countries. Vaccines type and its administration schedules varies worldwide and depends largely upon the potential threat involved, virulence of the field challenge virus, type and schedule of production. Even then, outbreaks have been reported and vNDVs have been isolated and reported to public databases, e.g., Netherlands in 1992 to 1993, the UK in 1997, the USA in 2002 (Alexander, 2003) and Pakistan (Khan et al., 2010; Munir et al., 2011; Munir et al., 2012). Low virulent NDVs have been used to prevent infection from vNDVs circulating in the environment, however, it is still a matter of question whether or not these vaccine strains do produce effective immune response or they provide the prevailing NDV strains to evolve as a result of escape mutants and subsequent evolution. Vaccination of large number of chicken simultaneously via spray or drinking water produce variable immune response, providing an opportunity to vNDVs to replicate and subsequent effects in the form of outbreak and genome evolution. From the recent reports, it may be concluded that, using currently available vaccines, better protection can be obtained against challenge viruses isolated from 1930s to 70s (Herts33/56 and California 71) and which are genetically closer or similar to vaccine strains than those isolated recently which are relatively distinct from vaccine strains (Netherlands 93, Pakistani strains) (Czegledi et al., 2006; Munir et al., 2012). Simultaneous administration of live and inactivated ND vaccine is shown to confer effective protection/immune response against virulent NDV and has been employed successfully in
areas of intense poultry production (Sene et al., 2004). The only limiting factor in the use of live vaccine is the ability to interfere with surveillance and laboratory diagnosis of vNDVs. However, real time PCR assays that can differentiate low virulent and vNDVs in the face of an outbreak can be optimized and practiced. Taken together, failure of previously used live vaccines in protecting the birds from current field isolates and conferring protective response by the use of live and inactivated vaccine, urged the need to know the antigenic relationship between past and current isolates, and the corresponding match between isolate and vaccine strains on the level of protection conferred against disease, shedding and viral transmission. Since the effectiveness of vaccine (killed or live or both) is ultimately determined by halting the epizootics, vaccine development should be more focused toward controlling the infection and shedding rather than disease. The presence of vNDV strains requires continuous monitoring and control measures even in countries where they are endemic because the existence of the virus severely impacts commercial productivity and the international trade in poultry and poultry products.

Both poor biosecurity and field strain-unmatched NDV vaccine are responsible for the current scenarios of ND in Pakistan. Without doubt, NDV strains exist as single serotype and vaccination with one strain protects the birds against all genotypes. However, extensive vaccination (thrice or even four times) in bird's life 0-5-6 weeks results in un-necessary pressure on the immune status and thus exposing to the clinical infections of ND. In this regards, immune response evaluation in terms of maternal antibodies and subsequent vaccine schedule in consultation with veterinarian is highly recommended followed by continuous monitoring weekly especially in countries like Pakistan. Moreover, immediately 2-5 days post ND vaccination, IBD vaccine is known to decrease the NDV antibodies markedly and thus it should be avoided (unpublished data). Simultaneous or with the interval of one day, birds should be vaccinated with live and killed vaccine; live vaccine should be given before killed. The farmers should employ all in and all out system, one way traffic or vehicle movement in the farm, shed specific labors, use of powdered disinfectants than liquid so that proper contact time is given to disinfectant to act on virus, proper and deep disposal of dead birds, avoid wild birds movement, separate the shed specific labors, use of powdered disinfectant to act on virus, proper and deep disposal of dead birds, birds should be vaccinated with live and killed vaccine; live vaccine should be given before killed. The farmers should employ all in and all out system, one way traffic or vehicle movement in the farm, shed specific labors, use of powdered disinfectants than liquid so that proper contact time is given to disinfectant to act on virus, proper and deep disposal of dead birds, avoid wild birds movement, separate the shed specific labors, use of powdered disinfectant to act on virus, proper and deep disposal of dead birds.

Strict biosecurity measures along with high antibody titer should be maintained in order to keep the farm unaffected and safe from disastrous effects of ND.

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