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Genetic characterization and evolutionary analysis of 4 Newcastle disease virus isolate full genomes from waterbirds in South China during 2003–2007

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ABSTRACT

Complete genomes of four Newcastle disease virus (NDV) strains, isolated from ducks and wild birds in Guangdong province of China from 2003 to 2007, were sequenced and analyzed in this study. Pathogenicity tests in chicken embryos and chickens illustrate that D3 and R8 are lentogenic, and W4 and P4 are mesogenic strains. Phylogenetic analysis using all six genes provides a high resolution profile for genotype designation as genotype I for D3 and R8 strains and genotype VI for W4 and P4 strains. In addition, molecular dating based on different genes suggests that D3 and R8 diverged from their common ancestor at around 1998; W4 and P4 diverged from their common ancestor at around 1999. Subsequent selective pressure analysis displayed specific traits of genes evolution in all 4 strains since their divergence from the recent common ancestor. Furthermore, the geographic origins of 4 strains were deduced to be from Europe via two independent introduction events by phylogeographical analysis. This provides insights to the potential influence of waterfowl migration on NDV epidemiology.

1. Introduction

Newcastle disease (ND) is a highly contagious viral infection commonly found in most avian species (Miller et al., 2009a). ND virus (NDV) is classified as a member of genus *Avulavirus* within family *Paramyxoviridae* (Mayo, 2002). As a negative-sense, single stranded, non-segmented enveloped RNA virus, NDV genome is approximately 15.2 kb long, and codes for nucleocaspid proteins (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and RNA polymerase (L) (Miller et al., 2009a). The proteolytic cleavage site of the F_0, which is the precursor of F glycoprotein, can be cleft by trypsin-like enzymes, and cleft products mediate the fusion of viral and cellular membranes helping the virus particle entry into host cells (Rott, 1979). Low virulent NDV has monobasic fusion cleavage site motifs along amino acid (aa) positions 112–117 of the F protein; while virulent viruses have multiple motif (Glickman et al., 1988). A wide range of pathogenicity levels has been documented for NDV (Alexander, 2000): pathogenicity tests classified strains of NDV into three categories: lentogenic (apathogenic), mesogenic (intermediately pathogenic) and velogenic (highly pathogenic) (Hanson, 1980).

Newcastle disease is one of the most devastating poultry infections due to its global distribution and economical threat (Miller et al., 2009a). Among NDV

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strains, the virulent forms which exhibit intracerebral pathogenicity index (ICPI) of ≥0.7 impose a major economic concern for poultry producers worldwide (OIE, 2009). In the past decade, the disease became endemic in 57% of the poultry-rearing countries, other 23% suffered one or several introductions of the virus (Czegledi et al., 2006). Today, NDV is listed by the World Organization for Animal Health, and any episodes of velogenic ND infection must be notified to the Organization abiding the Terrestrial Animal Health Code (OIE, 2009). Another reason why ND is considered a serious infectious disease is its pervasiveness in a wide range of domestic and wild birds. Viable hosts include domestic fowl, turkeys, pigeons and parrots; and milder forms of the disease are found in ducks, geese, pheasants quail, guinea fowl, and canaries (Cross, 1991; Liu et al., 2008). Wild birds, in particular migratory waterfowls, are often regarded as a potential reservoir of NDV. Although the role of wild birds in the epidemiology of NDV remains unclear, the susceptibility of wild birds to infection with NDV has brought forth the hypothesis about the possible role of these free-flying birds in the origin and spread of disease over long distances (Müller et al., 1998; Qureshi, 1988; Vickers and Hanson, 1982a). This hypothesis is partly supported by the isolation of NDV strains from waterfowl, which are mostly lentogenic and some potentially virulent (Astorga et al., 1994; Bahl et al., 1977; Pfitzer et al., 2000; Rosenberger et al., 1975; Spalatin and Hanson, 1975; Takakuwa et al., 1998; Vickers and Hanson, 1982a,b).

In China, Newcastle disease first appeared in 1946 and became endemic regionally (Liu et al., 2003). In the past three decades, implementation of intensive vaccination program in large-scale poultry operations and village poultry farming had curtailed the number of ND episodes (Liu et al., 2003). Yet since the late 1990s, epizootic occurrences of velogenic ND cases in vaccinated chicken flocks have been reported in southern China (Liu et al., 2003), with speculation of failed vaccination program, possibly due to a number of reasons like incompatibility between circulating and vaccine stains (Miller et al., 2009a), inappropriate vaccination procedures and generation of novel genotype under the condition of high immune pressure (Liang et al., 2002). Genotype VII NDV viruses were the predominant circulating genotype in China, which may possibly be responsible for disease outbreaks in chicken flocks in the past decade (Liu et al., 2003). In parallel, some ND outbreaks in waterfowl geese with severe clinical signs were also reported starting in 1997 in southern and eastern China (Jinding et al., 2005; Wan et al., 2004), a region which provides most domestic waterfowls in the country (Liu et al., 2008). Observing the avian ecology, Guangdong province is an important site for migratory waterfowl (Peng et al., 2003). The co-existing economic and ecological roles of Guangdong province in poultry industry and site of migratory waterfowl winter-residence highlight a risk of intra- and interspecies ND transmission. Legitimacy of this concern is conferred by previous studies (Okazaki et al., 2000; Sakai et al., 2007; Takakuwa et al., 1998).

In this report, sequences from 4 NDVs isolated in South China (Guangdong Province) during 2003–2007 were obtained for evolutionary and molecular analysis. This study also provides a suggestion on NDV data collection and analysis to enhance future virus evolutionary analysis.

2. Materials and methods

2.1. NDV isolates

The four isolates reported in this research were obtained from different live hosts in South China (Guang-dong Province): P4 was isolated from wild pigeon in 2003; W4 was isolated in 2005 from white-breasted waterhen (*Amaurornis phoenicurus*)—a waterbird whose breeding habitat is marshes across south Asia from India and Sri Lanka to south China and Indonesia; R8 was a genotype I apathogenic strain derived from *Rallus aquaticus* in 2005, a small wetland bird whose breeding habitat is marshes and reedbeds across Europe and Asia; D3 was isolated from feral migratory ducks at Guangdong (one of major migration sites for migratory wintering waterfowl) during the 2007 winter season.

Virus isolation was done by chicken embryo inoculation (CEI) in the allantoic sac. To identify the presence of NDV, haemagglutination inhibition activity test against NDV specific antiserum was conducted in anti-fetal (AF) sera. Positive results proved NDV identity rather than of avian influenza virus (AIVs), APMVs and egg drop syndrome (EDS) virus. AF samples infected with different isolates were harvested by passage in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs.

2.2. Pathogenicity test in chickens

The pathogenicity levels of the four viruses were determined by three different internationally accepted pathogenicity tests. These included the mean death time (MDT) test in sixty 9-day-old SPF embryonated chicken eggs, the intracerebral pathogenicity inoculation (ICPI) test in ten 1-day-old chicks, and the intravenous pathogenicity inoculation (IVPI) test in ten 6-week-old chickens (Alexander, 1989).

2.3. Complete genome amplification of these four virus strains by RT-PCR

Total RNAs were isolated from these four NDV-infected AF of 9-day-old specific pathogen free (SPF) chicken embryos, using an RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. Reverse transcription (RT) was performed using Super-Script II reverse transcriptase (Invitrogen, Carlsbad, CA).

Each complete genome was amplified in five overlapping fragments; the sequences of the 3′ and 5′ termini of these viral genomes were amplified by 3′ and 5′ RACE (Krishnamurthy and Samal, 1998; Kumar et al., 2008). PCR reactions were performed and products were purified using the QIAquick gel extraction kit according to manufacturer's instructions (QIAGEN).

Overlapping DNA fragments were cloned into the pCR-XL-TOPO vector (Invitrogen) the recombinant plasmids were purified using the QIAGEN Plasmid Maxi Kit (Qiagen),