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Molecular and phylogenetic characterization based on the complete genome of a virulent pathotype of Newcastle disease virus isolated in the 1970s in Brazil

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ABSTRACT

Newcastle disease (ND) is caused by the avian paramyxovirus type 1 (APMV-1) or Newcastle disease virus (NDV) that comprises a diverse group of viruses with a single-stranded, negative-sense RNA genome. ND is one of the most important diseases of chickens, because it severely affects poultry production worldwide. In the 1970s, outbreaks of virulent ND were recorded in Brazil, and the strain APMV-1/Chicken/Brazil/SJM/75 (SJM) of NDV was isolated. This strain was characterized as highly pathogenic for chickens but not pathogenic for other bird species. Here we present the complete genome of NDV strain SJM and investigate the phylogenetic relationships of this virus with other NDV strains in terms of genome and proteins composition, as well as characterizing its evolution process. The NDV strain SJM is categorized as a velogenic virus and the complete genome is 15,192 nucleotides in length, consisting of six genes in the order 3′-NP-P-M-F-HN-L-5′. The presence of the major pathogenic determinant of NDV strains (^{112}R-R-Q-K-R↓F^{117}) was identified in the Fusion protein of the NDV strain SJM. In addition, phylogenetic analysis classified the NDV strain SJM as a member of class II, genotype V, and indicates that this virus help us in the understanding of the evolutionary process of strains belonging to this genotype. This study contributes to the growing interest involving the characterization of NDV isolates to improve our current understanding about the epidemiology, surveillance and evolution of the pathogenic strains.

1. Introduction

Newcastle disease (ND) is a highly contagious and widespread disease, which causes severe economic losses in domestic poultry, especially in chickens (Alexander, 2000; Sinkovies and Horvath, 2000). The World Organization for Animal Health (OIE) lists it as a notifiable disease and imposes restrictions and trade embargoes on countries and areas where outbreaks occur (OIE, 2009).

The causative agent of the disease is Newcastle disease virus (NDV) or avian paramyxovirus serotype 1 (APMV-1), which belongs to the genus *Avulavirus* within the subfamily *Paramyxovirinae* and family *Paramyxoviridae* (Lamb et al., 2005).

The NDV genome comprises a single-stranded, negative-sense RNA genome of ~15,200 nucleotides (nt) that contains six genes, which encode seven proteins. The nucleoprotein (NP gene), the phosphoprotein (P gene), a V protein resulting from mRNA editing of the P gene (Steward et al., 1993), the matrix (M gene), the fusion (F gene), the haemagglutinin–neuraminidase (HN gene) and the RNA-dependent RNA polymerase (L gene) proteins (Samson, 1988; Alexander and Senne, 2008).

Based on the analysis of the nucleotide sequence of the F gene, 19 different genotypes of NDV have been identified and classified into two classes. Class I, genotype I and class II, I–XVIII genotypes. Class I viruses are distributed worldwide and have been isolated mainly from waterfowl and shorebirds. Class II virus are typically found circulating within wild-bird and poultry species and have been divided into 18 genotypes (I–XVIII), with genotypes V–VIII being the predominant genotypes circulating in the world (Miller

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et al., 2009b, 2010; Diel et al., 2012a, 2012b; Courtney et al., 2013; Snoeck et al., 2013). Since ND was first described in 1926, three worldwide panzootics have occurred. The first panzootic (1926–1960) was caused by viruses belonging to genotypes II–IV, and the second (1960–1973) and the third (1970–1980) by genotypes V and VI (Alexander, 2003).

Virulent NDV (vNDV) strains are enzootic in several countries and have been responsible for outbreaks in at least six of the seven continents of the world (Miller et al., 2010). In Brazil, the first known outbreak of ND was described in 1953 (Santos, 1954), and the disease was endemic for nearly 20 years, with the occurrence of sporadic and isolated outbreaks. However, in the 1970s the disease re-emerged in the form of a highly pathogenic NDV (Flores et al., 2006). Over the next 20 years, vNDV was responsible for several outbreaks in different regions of Brazil until implementation of stricter control measures, including extensive vaccination with attenuated NDV strains (LaSota and B1 strains), reduced the number and severity of ND outbreaks (Orsi et al., 2010). Indeed, in 2003 the OIE recognized Brazil as free of pathogenic NDV in industrial poultry (Orsi et al., 2010). However, serological studies detected the activity of NDV infection among wild birds in 2003 and 2006 (Oliveira Júnior et al., 2003; Silva et al., 2006) and backyard birds in 2005 (Oliveira Júnior et al., 2005) and 2006 (Flores et al., 2006).

The strain APMV-1/Chicken/Brazil/SJM/75 (SJM) of NDV is the only vNDV strain available from the 1970 outbreaks in Brazil. This strain produces severe lesions and high mortality in experimentally infected chickens, whereas in other non-galliform birds species it replicates and is excreted without pathological effects (Campioni et al., 2012; Martins et al., 2012; Denadai et al., 2011; Carrasco et al., 2008; Nishizawa et al., 2007; Paulillo et al., 2005; Lima et al., 2004). These findings suggest that wild birds may still be reservoirs of this virus and contribute to virus dissemination. Indeed, vNDV strains that emerged in South and Central America in the 1970s have been linked to the outbreaks in Europe and the United States in the same period, through imported exotic birds (Ballagi-Pordany et al., 1996; Miller et al., 2010).

Therefore, although it is plausible that an epidemic outbreak of NDV is unlikely to occur in Brazil in the short term, the threat in the medium and long term must not be underestimated. This requires not only constant epidemiological vigilance but also efforts to increase the current understanding about the relationships of NDV strains relevant to Brazil and other worldwide circulating strains. To this end, accurate molecular characterization and phylogenetic analysis of NDV isolates, including the SJM strain, are warranted. Indeed, extensive molecular and phylogenetic study of this strain as well as its relationship with other worldwide circulating strains and its possible role in the evolution of NDV genotypes remain undefined.

Here we present the complete genome of NDV strain SJM and investigate the phylogenetic relationships of this virus with other NDV strains in terms of genome organization and proteins signatures, as well as characterizing its evolution process.

2. Materials and methods

2.1. Isolation and propagation of virus

The SJM, a highly pathogenic NDV strain for chickens, was isolated in 1975 from a poultry farm located in the state of Rio de Janeiro, Brazil. This virus has an Intracerebral Pathogenicity Index (ICPI) of 1.78 and a Mean Death Time (MDT) of 48 h (Lima et al., 2004; Carrasco et al., 2008). The NDV strain SJM isolate was propagated by inoculation in nine-day-old embryonated SPF chicken eggs. The embryos were incubated at 37 °C for 40 h; subsequently, a sample of allantoic fluid was extracted, clarified by centrifugation and stored at −70 °C (Sousa et al., 2000).

2.2. RNA isolation and sequencing

Total RNA was extracted from allantoic fluids using TRIzol LS (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. *De novo* sequencing of SJM was carried out using the Illumina HiscanSQ. The library was constructed using TruSeq® RNA Sample Prep kit v2 (Illumina®), and cluster formation of the library cDNA templates was performed with the TruSeq PE Cluster kit v3 (Illumina®) and the Illumina cBot workstation, using conditions recommended by the manufacturer. Paired end 100 base pair (2×100 bp) sequencing by synthesis was performed with TruSeq SBS kit v3 (Illumina®) on an Illumina HiscanSQ using protocols defined by the manufacturer.

Base call conversion to sequence reads was performed using CASAVA 1.8.3 (Illumina®). Virus assembly was performed using CLC Genomics Workbench 6.5.1. The genome annotation was performed using Prokka 1.5.2 (Prokka: Prokaryotic Genome Annotation System, http://vicbioinformatics.com/).

2.3. Phylogenetic analysis and estimating evolutionary distances

Alignment and comparison of the nucleotide and amino acid sequences between SJM and selected strains representing established NDV genotypes were performed using the software ClustalW 2.2 with iteration in each alignment step (Thompson et al., 1994).

Phylogenetic analysis was performed using the MEGA5 software (MEGA, version 5.2.2) (Tamura et al., 2011). To select the best-fit models of DNA evolution the jModelTest 2 software were used (Darriba et al., 2012). The SJM genomic sequence was compared against 129 complete and near-complete reference genome sequences (only sequences >15,180 nt) of viral strains from class I and II (genotypes I–XIII and XVI) available at GenBank. The F gene sequence of SJM was analyzed with 100 sequences of the full F gene from class I and II (genotypes I–XVIII) published in GenBank in order to construct the Fusion gene tree. The evolutionary history was inferred by the Maximum Likelihood method based on the General Time Reversible (GDR) model (Tavaré, 1986), with standard errors being calculated based on 1000 bootstrap replicates and expressed based on the number of nucleotide substitutions per site. The codon positions included in the analysis were the 1st, 2nd, 3rd, and noncoding. All positions containing gaps and missing data were eliminated from the data set (the "complete deletion" option). The name and numbers used in the phylogenetic trees represent the name and accession numbers in GenBank. Estimation of the evolutionary distances between strains of NDV was performed by the maximum composite likelihood method in MEGA5.

2.4. Nucleotide sequence accession number

The annotated sequence of strain APMV-1/Chicken/Brazil/SJM/75 (SJM) has been deposited at DDBJ/EMBL/GenBank under the accession number KJ123642.

3. Results

3.1. Genome analysis and deduced proteins

A summary of the genomic features of NDV strain SJM is presented in Table 1. These characteristics are similar to those presented by other virulent APMV-1. Comparisons of nucleotide and amino acid sequences between NDV strain SJM and selected class

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