VETERINARSKI ARHIV 93 (1), 65-70, 2023 DOI: 10.24099/vet.arhiv.1862

Biological characterization of isolated pigeon Paramyxovirus-1 and its pathogenicity analysis in SPF chickens

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ZHANG, Y., S. REN, X. YUAN, J. YANG, W. A. MENG: Biological characterization of isolated pigeon Paramyxovirus-1 and its pathogenicity analysis in SPF chickens. Vet. Arhiv 93, 65-70, 2023.

ABSTRACT

Pigeon paramyxovirus type 1 (PPMV-1) is considered as an antigenic variant of Newcastle Disease leading to high mortality and significant economic losses to the poultry industry. However, the pathogenicity of PPMV-1 to chickens is still unclear. Herein, we reported the biological characterization of the isolated PPMV-1 from the diseased pigeons suspected to Newcastle Disease and studied its pathogenicity in SPF chickens. The phylogenetic tree and evolution distances revealed that the new isolate belonged to a VI.2.1.1.2.2 sub-genotype of NDV. Despite the cleavage motif of the F protein containing the ^{112}RRQKR↓F^{117} sequence associated with virulent NDV strains, and the value of MDT and ICPI of the isolate showing mesogenic characteristics, the challenge trial showed that the isolate had weak pathogenicity to chickens while causing lesions in multiple tissues and organs in pigeons.

Key words: PPMV-1; VI.2.1.1.2.2 sub-genotype; genetic characterization; pathogenicity

Introduction

Newcastle disease (ND), caused by the Newcastle Disease virus (NDV), is highly contagious and devastating in many species, including chickens and pigeons, and is a heavy threat to the poultry industry around the world (ALEXANDER et al., 2012). On the basis of their nucleotide sequences, NDV isolates are currently categorized phylogenetically into two classes (class I and class II). Class I lineage viruses comprise a single genotype and are frequently isolated from wild birds and domestic waterfowl, while Class II viruses have many species which can be divided into 20 genotypes (DIMITROV et al., 2019). Viruses of Class II genotype VI have a high host preference for Columbidae (including doves and pigeons) infected with an ND-like infectious disease, and are commonly referred as pigeon paramyxovirus-1 (PPMV-1) (CHONG et al., 2014; QIU et al., 2017). The PPMV-1 is a member of Avulavirus genus, of the Paramyxoviridae family. The genome of PPMV-1 is approximately 15kb, and encodes six structural proteins including fusion (F) protein,

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ISSN 0372-5480
Printed in Croatia

hemagglutinin-neuraminidase (HN) protein, matrix (M) protein, nucleocapsid (NP) protein, and large (L) protein. Affected pigeons have clinical signs such as dysentery, tremor, unilateral or bilateral paralysis, and head twisting and neck skewing in the chronic and late epidemic stages. Pigeons are considered to be not only the pathogen carrier of NDV, but also represent an important hidden risk of disease transmission. There have been many different studies on the pathogenesis of PPMV-1 in chickens (OLSZEWSKA-TOMCZY et al., 2018; KOMMERS et al., 2002). Some studies have found that pigeons infected with PPMV-1 have caused ND outbreaks in unvaccinated chickens, while others believe that this pathogenicity has not been found in chickens (ALEXANDER et al., 1984; KOMMERS et al., 2002, TORO et al., 2005).

In this study, a PPMV-1 strain, designated Pigeon/China/SD0315/2018, was identified for the first time from racing pigeons with obvious respiratory problems and a high mortality rate in the Shandong Province, a high-density chicken breeding area in China. Rapid assessment of the virulence and pathogenicity of the pigeon-originated isolate is essential to control the potential risks of transmission in chickens. We determined the biological characteristics of the isolate and its ability to cause infection in SPF chickens and pigeons separately in this study. We further thoroughly researched the genetic diversity and Phylogenetic information on the basis of the complete F gene sequence of this strain, in order to understand the gene distribution of the isolate.

Materials and Methods

Virus Isolation and Identification. In March 2018, we collected 140 tracheal swabs from a racing pigeon flock in Shandong Province, China. There were 500 pigeons in the racing pigeon flock, of which 300 pigeons showed obvious clinical signs, including neurological signs, and obvious respiratory problems. All the swab samples were stored in phosphate-buffered saline (PBS) and inoculated into the allantoic cavity of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. A total of 140 eggs were used in this test. The allantoic fluids were harvested for testing by hemagglutinin (HA) assay. The allantoic fluids were diluted in PBS to different gradients (2, 4, or 6 $\log_{10}$). 10-day-old SPF chicken embryos were inoculated into the allantoic cavity per 0.2 mL by diluting the virus solution mentioned above, to evaluate the virus titration (EID_{50}) by the Reed-Muench method (LIANG et al., 2021).

Viral RNA Extraction and RT-PCR. The viral RNA was extracted from the haemagglutination-positive allantoic fluid using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and transcribed into cDNA with a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher, Shanghai, CA, China). The complete genetic sequence of the fusion (F) gene was determined using the protocols described previously (WANG et al., 2015). A set of F gene-specific primers (Fw: 5'-ttgcttatagttagttcgcctgtc-3'; Rv: 5'-acccgtgtattgctctttgg-3') were used in this study. The positive PCR-amplified products were cloned into pMD19-T (Takara Bio Inc.) and sequenced at Shanghai Biotech Co., Ltd.

Phylogenetic Analysis and Molecular Characterization. Nucleotide sequence editing, analysis and the deduction of amino-acid sequences were conducted using the DNASTAR software. For phylogenetic analysis, all NDV genetic sequences used in this research were obtained from the NCBI database. Phylogenetic and homology analysis of the amino-acid of the F gene were conducted using MEGA 6.0 software, employing the neighbour-joining method and a 1000-bootstrap analysis.

Pathogenicity and serological study. The virulence of the isolate was determined by mean death time (MDT) tests in 10-day-old embryonated SPF chicken embryos, and intracerebral pathogenicity index (ICPI) tests in 1-day-old SPF chickens, according to the OIE manual (OIE, 2012). Specifically, a MDT up to 60 h was characterized as velogenic, from 61 to 90 h as mesogenic, and more than 90 h as lentogenic. ICPI below 0.7 were considered to be of low virulence, and those with an ICPI equal or greater than 0.7 were considered to be virulent (SUAREZ et al., 2019).

A challenge experiment was carried out to detect the pathogenicity of the isolated virus taken from pigeons and chickens. Twenty healthy

28-day-old pigeons and the same number and age SPF chickens were randomly divided into 2 groups each. In groups A and C, ten pigeons and ten SPF chickens were inoculated with 10^6 EID_{50} of the strain via the ocular-nasal route with a volume of 0.2 mL. In groups B and D, 10 pigeons and 10 SPF chickens were inoculated with 0.2 mL of PBS as a mock-infected control. All the birds were raised separately, and symptoms (such as lethargy, anorexia and feather fluffing) were monitored three times a day for 14 days. The dead birds were autopsied immediately to determine the lesions. On the 14th day post-infection (dpi), serum samples were collected from all the surviving birds and tested for NDV antibodies by HI assay using four HA units of the isolates.

Results

Virus Isolation and Identification. After three passages in SPF chicken embryos, one haemagglutination-positive sample, named SD0315, was identified as PPMV-1 by RT-PCR assays. The HA titre of the allantoic fluids ranged from 4log2 to 6log2 . The complete sequence of the F gene was transferred to the GenBank database (accession no. MK896851).

The infectivity of the isolate determined by the Reed-Muench method was 4.0 log10 EID_{50} mL^{-1}.

Phylogenetic Analysis. A phylogenetic tree was constructed according to the complete coding sequence of the F gene of the 82 NDV isolates, using the MEGA 6.0 program (Fig. 1). The results demonstrated that the SD0315 strain (accession no. MK896851) belongs to a VI.2.1.1.2.2 sub-genotype, differing greatly from the LaSota strain. Moreover, the amino acid distances between the SD0315 strain and LaSota strain was 0.192, which also indicates that they belong to different genotypes. The amino acid sequence deduced from the cleavage site of the F gene protein of the isolate was determined to be ^{112}RRQKR↓F^{117}, a motif that is commonly found in strains that are highly virulent in chickens (ZHANG et al., 2010).

Pathogenicity in Infected Pigeons and Chickens. The SD0315 strain had a MDT value of 67 h and the ICPI value of the chickens was 1.3, confirming that the virus belongs to a mesogenic strain.

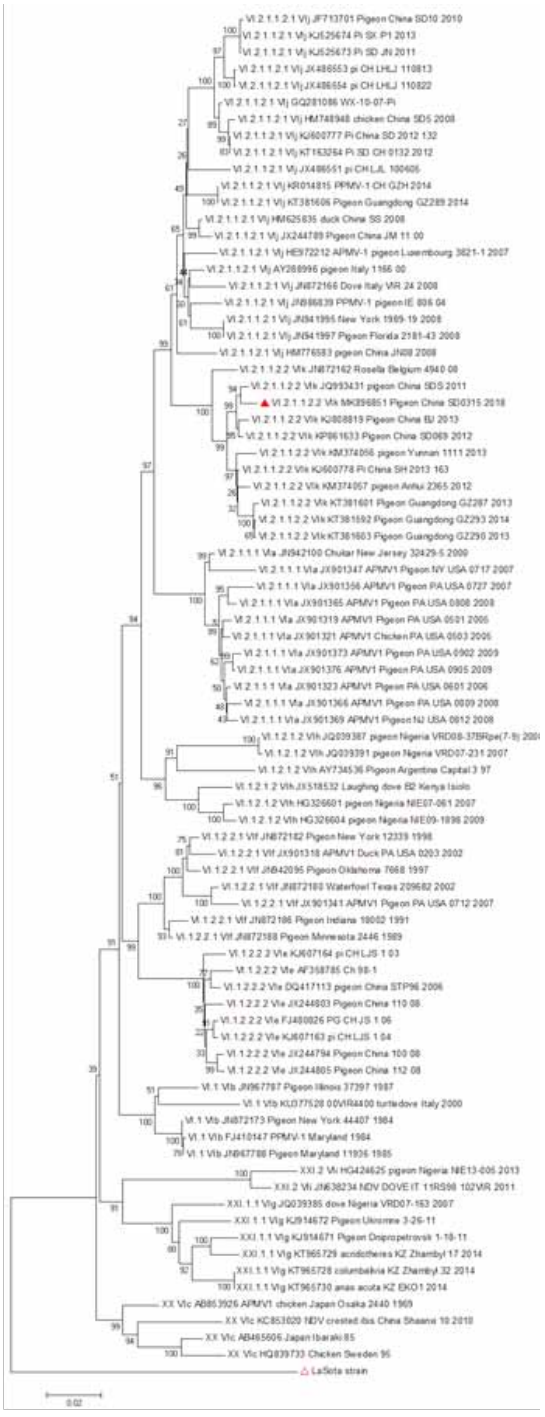

Fig. 1. Phylogenetic tree of the SD0315 strain based on the F gene sequences. The sequence of the SD0315 strain in this Figure is marked with a red triangle; other strains used in this study were found on the National Centre for Biotechnology website. The phylogenetic tree was generated using MEGA 6.0 software, employing the neighbour-joining method and a 1000 bootstrap analysis. The scale bar is 0.02.

Pigeons infected with the SD0315 strain displayed neurological signs, open-mouth breathing, reduced feed intake, and head and neck distortion (Fig.2B). Necropsy revealed cerebral congestion and bleeding (Fig.2C), and severe haemorrhages in the trachea and lungs (Fig.2D; Fig.2E). Occasionally, yellow staining of the liver, accompanied by haemorrhagic foci, was also observed (Fig. 2F). The mortality rate in pigeons inoculated with the SD0315 strain was 40% and the mean death time of the inoculated pigeon group was 4.5 days. By contrast, the pigeons in the control group did not show obvious clinical symptoms.

On the 14th day after the challenge, serum was collected from the living pigeons, and the HI test was conducted to detect the NDV antibody. All serum samples from the surviving pigeons in the challenge group were positive for the NDV HI antibody, and the average HI titre was 7.2 log2.

Conversely, the chickens challenged with the SD0315 strain were alive and showed mild respiratory symptoms during the observation period. The average HI titre of all serum samples from the chickens in the challenge group was 4.2 log2.

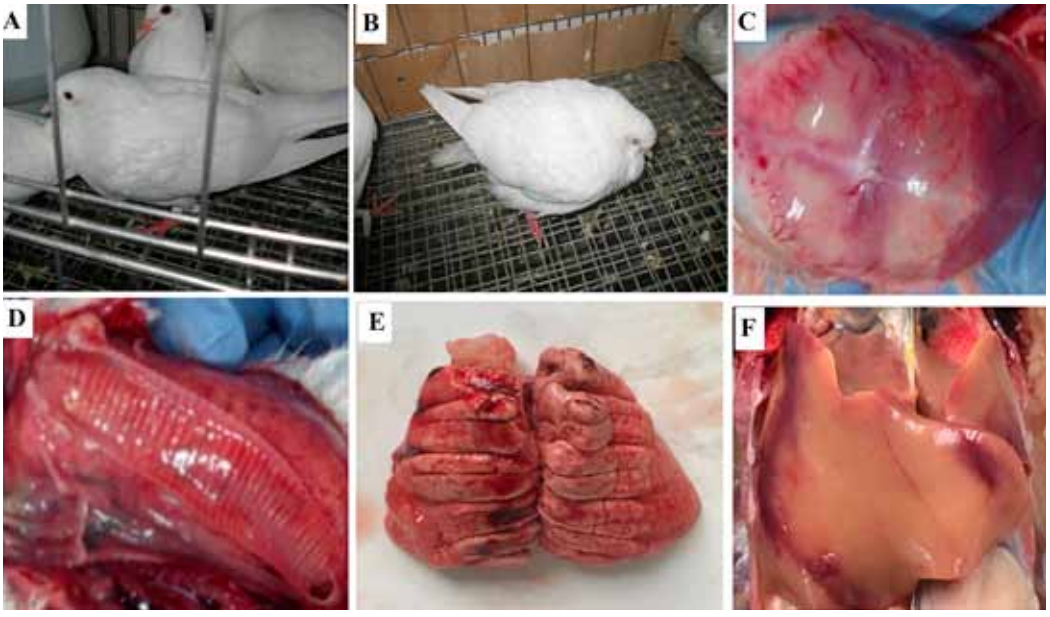

Figure 2. Clinical symptoms and gross lesions of SD0315-infected pigeons and uninfected controls.
(A) Healthy pigeons from the control group. (B) Lethargy, and ruffling of neck feathers seen in an infected pigeon. (C) Brain hyperaemia and haemorrhage. (D) Severe haemorrhage in the trachea. (E) Severe haemorrhage in the lung. (F) Yellow staining of the liver accompanied by haemorrhagic foci.

Discussion

Genotype VI comprises a large number of highly diverse viruses, and has been further divided into many sub-genotypes (VI.2.1.1.1, VI.1, XX, VI.1.2.2.2, VI.1.2.2.1, XXI.1.1, VI.1.2.1.2, XXI.2, VI.2.1.1.2.1, VI.2.1.1.2.2, XXI, XXI.1.2) (DIMITROV et al., 2019). These genotypes of viruses are especially important because they are usually associated with doves and pigeons, and are therefore likely to be introduced into poultry flocks (ALEXANDER et al., 2011). In this study, a VI.2.1.1.2.2 sub-genotype PPMV-1, which was first isolated from a rosella in Belgium in 2008 (Rosella/Belgium/494/08), was isolated from a racing pigeon flock in the Shandong Province, China. Since 2011, VI.2.1.1.2.2 strains isolated from pigeons emerged in many parts of China, such as Liaoning, Shandong, Shanghai, Inner Mongolia, Anhui, and Yunnan (XUE et al., 2017),

and caused major economic losses to the poultry industry. Therefore, it is necessary to continue the epidemiological surveillance of the pigeon NDV.

The pathogenicities of the mesogenic SD0315 strain towards pigeons and chickens were significantly different. Haemorrhaging from multiple organs was observed in pigeons, especially in the brain, small intestine and lungs, while mild clinical signs were seen in chickens. This is consistent with the findings of other researchers, who found that NDV VI genotype isolates from pigeon samples do not usually cause any clinical signs in chickens, and are not transmitted to naïve chickens even after a challenge using a high infective dose (FERREIRA et al., 2019). This may be related to the species, because chickens are not the natural host of pigeon Newcastle disease virus. Although the SD0315 strain did not show any pathogenicity towards chickens, we are not sure whether the virus would infect chickens after continuous passages in chickens. This issue deserves our continuous attention. In recent years, typical cases of ND in chicken farms have rarely been isolated in China, while PPMV-1 has been reported frequently (WANG et al., 2015; WEI et al, 2018; HE et al., 2020). Whether the occurrence of PPMV-1 will cause an outbreak of chicken Newcastle disease virus is still worthy of vigilance. Thus, further ecological monitoring of poultry and wild birds is recommended.

Author contributions

Data curation, Yuxia Zhang; Funding acquisition, Kai Meng, Xiaoyuan Yuan and Wu Ai; Investigation, Sufang Ren and Yuxia Zhang; Writing-original draft, Kai Meng; Writing-review & editing, Wu Ai and Kai Meng.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript. This work was supported by the Natural Science Foundation of Shandong Province (ZR2021MC060), the Agricultural Scientific and Technological Innovation Project of Shandong Academy of Agricultural Sciences (CXGC2022A25), Young Talents Training Program of Shandong Academy of Agricultural Sciences.

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Received: 25 January 2022
Accepted: 6 June 2022

ZHANG, Y., S. REN, X. YUAN, J. YANG, W. A. MENG: Biološka karakterizacija izolata golubljeg paramiksovirusa 1 i analiza njegove patogenosti u SPF pilića. Vet. Arhiv 93, 65-70, 2023.

SAŽETAK

Golublji paramiksovirus tipa 1 (PPMV-1) smatra se antigenskom varijantom njukaslske bolesti koja uzrokuje visoku smrtnost i znatne ekonomske gubitke u peradarskoj industriji. Patogenost virusa PPMV-1 u pilića međutim još uvijek nije jasna. U ovom je radu provedena biološka karakterizacija PPMV-1 izoliranog iz golubova za koje se sumnja da su oboljeli od njukaslske bolesti te je istražena njihova patogenost u SPF pilića. Filogenetsko stablo i evolucijske udaljenosti otkrili su da novi izolat pripada podgenotipu VI.2.1.1.2.2 virusa njukaslske bolesti (NDV). Unatoč motivu u mjestu cijepanja F-proteina koji sadržava sekvenciju 112RRQKR↓F117 povezanu s virulentnim sojevima NDV-a, vrijednosti MDT-a i ICPI-ja izolata pokazale su mezogene značajke. Ovo probno istraživanje je pokazalo da su izolati PPMV-1 slabo patogeni u pilića, dok u golubova uzrokuju lezije na više organa i tkiva.

Ključne riječi: PPMV-1; podgenotip VI.2.1.1.2.2; genska karakterizacija; patogenost