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Research paper

Epidemiological and genetic analysis of *Avian avulavirus-1* in Israel reveals parallel circulating strains and a new sub-genotype within genotype VI

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1. Introduction

Newcastle disease (ND) is a viral disease caused by virulent strains of *Avian avulavirus-1* (previously named avian paramyxovirus-1 or Newcastle Disease Virus). *Avian avulavirus-1* (AAvV-1) belongs to the Avulavirus genus of the family Paramyxoviridae (Afonso et al., 2016; Amarasinghe et al., 2017). *Avian avulavirus-1* is a non-segmented, single-stranded negative sense RNA virus. All AAvV-1 viruses belong to one serotype. Its genome size is 15,186 nucleotides (nt) ("historic" genotypes, 1930–1960), 15,192 nt ("later" genotypes, after 1960) or 15,198 nt (class I AAvV-1) (Czeglédi et al., 2006; Dimitrov et al., 2016). The genome codes for six proteins: the nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F) hemagglutinin-neuraminidase (HN), and RNA polymerase (L). The genetic classification of AAvV-1 isolates is based on the nucleotide sequence of the complete coding region of the fusion gene (Diel et al., 2012). Currently, there are two genetically divergent classes of AAvV-1. Class I isolates belong to a single genotype whereas class II isolates are divided into 18 genotypes (Dimitrov et al., 2016). *Avian avulavirus-1* infects wild and domestic avian hosts, which are infected through inhalation or ingestion. Clinical signs vary according to the virulence of the isolate and the susceptibility of the host. AAvV-1 viruses are classified to pathotypes according to their virulence: Asymptomatic, enteric and lentogenic (low virulence) strains typically cause subclinical infection or mild respiratory disease. Mesogenic strains (intermediate virulence) cause acute respiratory disease and neurologic signs with relatively low mortality rate. Velogenic strains (high virulence) cause severe disease with intestinal, respiratory and neurologic signs with high mortality rate (Swayne, 2013). The virulence classification is based on the amino acid composition at the cleavage site of the fusion protein or the intracerebral pathogenicity index (ICPI) (OIE, 2012). Low virulence strains are widely used as live vaccines and thus only velogenic and mesogenic strains are defined as virulent AAvV-1 and considered by the OIE (Office of International Epizootics) to be the cause of ND (OIE, 2012).

The first characterization of Newcastle Disease occurred in 1926 (or by some evidence even earlier) in Indonesia and England and today the disease is endemic in many countries across the world (Alexander, 2001; Alexander and Jones, 2008). Since domestic poultry are highly susceptible, ND affects the poultry industry worldwide. In developing countries, ND is reported as the single greatest constrain to traditional rural poultry production (Antipas et al., 2012; Awan et al., 1994; Spradbrow, 2001). In developed countries, ND has a great economic impact due to mortality, morbidity, cost of prevention and control and trade limitations (Cornax et al., 2012).

The first outbreak of Newcastle in Israel was recorded in 1937 (before the state of Israel was established), during the first panzootic of the disease, and since then Israel has been intermittently infected by the virus (Neriah, 2001). The control measures in Israel includes improved biosecurity, mandatory vaccination, declaration of infected areas, and until recently (February 2017), culling of infected flocks. These control measures had limited success.

A better understanding of the mechanisms driving the epidemiology are essential for the development of better means of prevention. The transmission patterns cannot be elucidated from the complex spatio-temporal patterns of the epidemics. Thus, genetic information has been gathered on a large number of samples over the last 10 years. The use of full viral genomes for high resolution phylogenetic tree construction, allows us to evaluate virus transmission between geographical regions and poultry sectors.

Since 2007, field strains isolated in Israel belong mainly to class II

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genotype VII, sub-genotypes VIIb (2011–2016), VIId (2007–2010) and VIIi (2011 − 2013) (Fuller et al., 2015; Haddas et al., 2014; Miller et al., 2015a,b).

Class II genotype VII is a diverse group of viruses, currently divided into sub-genotypes VIIa–VIIi. Viruses of genotype VII likely emerged in the Far East in the 1990s and have been associated with recurrent poultry outbreaks worldwide (Dimitrov et al., 2016). All genotype VII viruses are predicted to be virulent. Viruses of sub-genotype VIIb have been repeatedly recovered from gallinaceous poultry, domestic waterfowl, peri-domestic species and wild birds in China during 1998–2014 with additional reports in chickens from Vietnam in 2007 and Israel in 2011–2014. As evidenced from partial fusion gene sequences, viruses from sub-genotype VIIb were also associated with outbreaks in poultry in Europe, Turkey, South Africa, Mozambique, Kazakhstan, the Far East, the Middle East, and India in the 1990s and in the beginning of the 21st century (Dimitrov et al., 2016).

Sub-genotype VIId viruses have been recovered from poultry in China (1998–2013), South Korea (2000–2005), and Colombia (2006–2010). Additionally, sporadic reports of sub-genotype VIId viruses originate from samples collected from poultry in Israel, South Africa, Ukraine, and Venezuela during 2004–2009. Sub-genotype VIId viruses have also been recovered from wild bird in China and Serbia during 2006–2007 (Dimitrov et al., 2016).

Viruses of sub-genotype VIIi have primarily been recovered from chickens in Indonesia, Israel, and Pakistan during 2010–2013 (Dimitrov et al., 2016).

In addition to genotype VII viruses, there were sporadic isolations of genotype VI viruses in Israel from domestic pigeons and wild birds. Viruses of class II genotype VI, previously called pigeon paramyxovirus serotype 1 or PPMV-1, are considered to be panzootic and are highly diverse genetically. The different sub-genotypes of genotype VI are detected primarily in birds of the family Columbidae (Dimitrov et al., 2016).

2. Materials and methods

2.1. Newcastle disease case definition

Data on diagnosed ND cases were collected from the database of the Poultry Health Laboratories of Israel Egg and Poultry Board (Berman and Samberg, 1991). This database records veterinary data on all commercial flocks and some backyard poultry and wild birds. According to the Israeli regulation, a flock is defined as all the poultry in all the houses on one farm. All poultry in one farm must be of a single sector type and a single age and be raised by a single operator (Israel Ministry of Agriculture and Rural Development, 1981). In this study we refer to flocks diagnosed with ND during 2007–2016. A ND outbreak was defined according to the OIE guidelines (OIE, 2012). The diagnoses of cases were based on virus isolation and a real time reverse transcriptase polymerase chain reaction (rRT-PCR) or rRT-PCR test alone, regardless of clinical signs. The rRT-PCR method differentiates virulent viruses from low-virulence (vaccine) viruses (Fuller et al., 2009; Wise et al., 2004).

2.2. Virus detection

Virus detection was done by real time reverse transcriptase PCR (rRT-PCR). Total RNA was extracted from suspension of tracheal and cloacal swabs using the QIAmp Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The rRT-PCR assay that targeted the matrix (M) gene was performed according to Wise et al. (Wise et al., 2004), and sub-typing by rRT-PCR that targeted the fusion (F) gene was performed according to Fuller et al. (Fuller et al., 2009).

2.3. Virus isolation

Virus isolation was done in the Poultry Health Laboratories. Virus isolation was performed by injection of a suspension of tracheal and cloacal swabs into the allantoic cavity of 9–11-day-old Specific pathogen free (SPF) embryonated chicken eggs (OIE, 2012). The swabs were tested in pools of 5 swabs (1–2 pools for every source) and each pool was injected into 5 eggs. The virus isolation was followed by a hemagglutination assay (HA) and rRT-PCR on allantoic fluid in order to confirm the diagnosis and differentiate virulent (velogenic) viruses from low-virulent (lentogenic) viruses.

2.4. AAvV-1 isolates

AAvV-1 isolates in allantoic fluid were obtained from the Poultry Health Laboratories of Israel Egg and Poultry Board and from the Division of Avian Diseases of Kimron Veterinary institute, Israel. One hundred and seventy-two isolates were selected from the available stock without prior knowledge on their genotype. Except few isolates with known epidemiological connections, the isolates were chosen randomly to represent all poultry sectors and all geographical regions. From these isolates we obtained 162 whole genome sequences. One of the isolates (no. 104) was a mix infection of two field strains from different genotypes and both sequences were included in the study. 11 isolates produced poor-quality sequences or were mixed infection with vaccine strains and therefore were not included in the study.

2.5. Viral RNA purification

Avian Avulavirus-1 RNA purification was based on previously published method (Jakhesara et al., 2014). Briefly, after an initial centrifugation at 10,000*g* for 15 min to remove tissues and cells, 6 ml of allantoic fluid (in PBS buffer) was ultracentrifuge at 70,000 g for 3 h at 4 °C. Following centrifugation, the supernatant was decanted and the pellet containing the virus was resuspended. The viral suspension was transferred to an Eppendorf tube and incubated in DNase-RNase buffer to decrease host DNA and RNA. RNA purification was performed using the Trizol LS kit (Thermo Fisher, Cat 10296028) according to the manufacturer's instructions. Viral RNA was stored at − 80 °C.

2.6. Pre-sequencing DNA preparation

After the Viral RNA purification, tree different methods were used to prepare DNA before sequencing: specific primers (SP), sequence independent single primer amplification (SISPA) and direct RNA library preparation.

2.6.1. Sequence specific primer amplification of AAvV-1 RNA

A reverse transcription primers and PCR primers were designed to amplify 3.5 kb overlapping amplicons of the AAvV-1 genome. The reverse transcription was performed using Agilent's Accuscript high fidelity Reverse Transcriptase (Cat. 600089) with modifications. The Sequence specific primer amplification is described in detail in the Supplementary data.

2.6.2. Sequence independent single primer amplification (SISPA)

The SISPA protocol was performed according to the literature (Djikeng et al., 2008) with modifications described in the Supplementary data.

2.6.3. RNA library preparation

RNA libraries were prepared from purified RNA by HayLabs using the Ion ress™ RNA library prep kit (Life Technologies).