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Short communication

Repeated isolation of virulent Newcastle disease viruses in poultry and captive non-poultry avian species in Pakistan from 2011 to 2016

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ABSTRACT

Virulent viruses of the panzootic Avian avulavirus 1 (AAvV-1) of sub-genotype VIIi were repeatedly isolated (2011–2016) from commercial chickens and from multiple non-poultry avian species in Pakistan. These findings provide evidence for the existence of epidemiological links between Newcastle disease outbreaks in commercial poultry and infections with virulent AAvV-1 strains in other avian species kept in proximity to poultry. Our results suggest that the endemicity of Newcastle disease in Pakistan involves multiple hosts and environments.

1. Introduction

Newcastle disease (ND) is a highly contagious and fatal disease affecting poultry and a wide range of wild birds worldwide that is caused by infections with virulent strains of Newcastle disease virus (NDV) (Miller et al., 2010; Dimitrov et al., 2016c). Recently, the International Comittee On Taxonomy of Viruses amended the taxonomy of genus *Avulavirus* of the *Paramyxoviridae* family. Newcastle disease virus and all avian paramyxoviruses 2-13 were renamed to avian avulavirus (AAvV) 1 to 13, respectively (https://talk.ictvonline.org/taxonomy). For the purpose of this paper and for consistency with previous publication, further below we have used the taxa name Newcastle disease virus. Despite intensive vaccination, endemicity of ND is a significant problem across Asia, Africa, and Central America. Recent reports have documented that some of the newly identified viruses of sub-genotype VIIi are rapidly spreading from Southeast Asia, into the Middle East, to Eastern Europe and North Africa and can cause mortality in poorly vaccinated poultry (Miller et al., 2015b; Rehmani et al., 2015; Dimitrov et al., 2016c). The presence of virulent viruses in vaccinated birds in commercial farms (Rehmani et al., 2015) and their constant evolution over time (Miller et al., 2009) suggest the existence of a high environmental viral load with continuous replication of these virulent NDV strains in endemic countries. However, the nature of the endemicity and the mechanisms of panzootic viral spread for NDV are largely unknown.

For the successful control of ND it is important to identify the factors that contribute to its endemicity. The spillover (defined here as the bi-directional transmission of closely related NDV) of NDV between poultry and wild bird species has been reported previously (Vidanovic et al., 2011; Cardenas Garcia et al., 2013; Ayala et al., 2016). Exotic birds kept in captivity and pet birds have also been infected with virulent NDV strains (Nolen, 2002; Pedersen et al.,

Abbreviations: AIV, avian influenza virus; AAvV, Avian avulavirus; ICPI, intracerebral pathogenicity index; ND, Newcastle disease; NDV, Newcastle disease virus; UVAS, University of Veterinary and Animal Sciences.

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2004) and are considered a biosecurity threat to domestic and commercial chickens. Avian influenza studies have identified wild bird species that could be considered "bridge hosts" for the transmission of viruses between poultry and wild birds (Caron et al., 2014; Caron et al., 2015). We have recently shown that backyard chickens are an important component in the circulation of genotype VII virulent NDVs in Bulgaria and Ukraine (Dimitrov et al., 2016b).

Pakistan presents a unique opportunity to study mechanisms of viral maintenance and spread in endemic countries. In Pakistan, ND was first detected in 1963 (Khan and Huq, 1963) and since then outbreaks have been observed repeatedly in both commercial and backyard poultry flocks. There is a wide variety of non-poultry bird species (both free-living and kept in captivity) in Pakistan; however, limited information is available concerning the potential role of these avian species in the dissemination of NDV. To understand the relationship among the circulating viruses and to identify the avian species and the husbandry systems that might contribute to ND endemicity, we have isolated and characterized NDV from different avian species and production systems in Pakistan over a five-year period. Here, we describe the repeated isolation of highly related virulent NDV strains from poultry, non-poultry species kept in captivity, and wild birds (chickens, pheasants, peafowls, pigeons, exotic parakeets [Australian parakeets locally known as Bajri], and Black Swan) at multiple locations and in different types and sizes of flocks in Pakistan between 2011 and 2016.

2. Materials and methods

2.1. Sample collection, clinical observation and pathogenicity tests

Samples from sick or dead birds from broiler and layer commercial poultry farms, along with additional information, were collected from flocks experiencing either above average mortality or appearance of ND-like clinical signs. The samples from non-poultry species were collected from birds kept in captivity in zoo or farm exhibitions, as family pets, or as racing birds, except for one free-living pigeon. The samples from the Zoo Park were collected during routine visits by the Veterinary Officer from zoo birds with clinical signs of ND under quarantine at the zoo. Backyard samples were collected across different neighborhoods, no specific criteria for selection of study sites existed, and sampling was performed upon signals of increased poultry mortality by owners and field veterinarians. The samples background data (e.g. dates and locations of sample collection, host species, husbandry types, flock size and age of birds, number of dead birds) were collected using standard form (template is available upon request).

In total, between 2011 and 2016, 52 NDV (21 from non-poultry species and 31 from poultry) were isolated at the University of Veterinary and Animal Sciences, Lahore, Pakistan (UVAS). The viruses from poultry were isolated from either vaccinated commercial flocks or non-vaccinated backyard chickens. Detailed information on the isolates is presented in Supplemental Table S1. Twenty one of the isolates (marked with superscript "a" in Supplemental Table S1), that have not been already analyzed at UVAS, were submitted to the Southeast Poultry Research Laboratory (SEPRL) of the USDA in Athens, GA and 20 of them underwent evaluation to establish intracerebral pathogenicity index (ICPI) values following routine procedures (OIE, 2012). Following the same procedure, the pathogenicity of eight additional viruses was assessed by intracerebral inoculation of NDV- and avian influenza virus (AIV)-free 1-day-old chickens at UVAS (marked with superscript "b" in Supplemental Table S1).

2.2. RNA extraction and sequencing

For the samples analyzed at SEPRL viral RNA was isolated from the allantoic fluid using TRIzol LS reagent (Invitrogen, USA) and the QIAamp RNA viral mini kit (Qiagen, USA) and further processed by next-generation sequencing as reported by Dimitrov et al. (2017). Thirty one samples were analyzed at UVAS and viral RNA was extracted from infected allantoic fluid using the TRIzol LS reagent (Invitrogen, USA) following the manufacturer's instructions. Sequencing of the complete coding region of the fusion (F) protein gene was performed as described previously (Munir et al., 2010; Miller et al., 2015a; Miller et al., 2015b).

2.3. Phylogenetic analyses

All available complete F-gene coding sequences of class II NDV (n = 1542) were downloaded from GenBank (Benson et al., 2015) as of September 2016 and analyzed together with the sequences obtained in the current study. A smaller dataset of closely related previously characterized NDV (n = 19) and the isolates sequenced here (n = 52), were further analyzed phylogenetically using MEGA6 (Tamura et al., 2013). For comparison purposes, five additional sequences of more distant genotype VII viruses were also added. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-3 model, selected by corrected Akaike Information Criterion, with 1000 bootstrap replicates as implemented in MEGA6 (Tamura, 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.4724]). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 nucleotide sequences (Supplemental Table S2). Codon positions included were 1st, 2nd, 3rd, and non-coding. All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

3. Results

3.1. Clinical signs and in vivo characterization

Number of dead birds and clinical signs varied widely in different flocks and species with some large vaccinated chicken flocks and non-vaccinated pet birds showing high numbers of survivors (Supplemental Table S1). Infected birds of different species were of different ages and isolated at different locations (Fig. 1). Infected birds presented with respiratory, neurological and/or enteric clinical signs typical for ND. In pheasants respiratory signs and greenish diarrhea were observed. Diseased peafowls showed nervous signs with torticollis, tremors, disorientation and weakness and a few birds also had wing and leg paralysis. Upon post mortem examination hyperemic and hemorrhagic spleens were found. In parakeets no clinical signs except sudden death were observed. Infected pigeons had tremor and torticollis that are typical for the disease in this species (Vindevogel and Duchatel, 1988). The infected Black Swan had depression and greenish watery diarrhea along with nervous signs including circular movements of the neck and head 4–5 h prior to death. Birds in vaccinated poultry flocks showed clinical signs typical for ND (Miller and Koch, 2013).

In pathogenicity studies performed with SPF chickens (Supplemental Table S1) 20 selected viruses presented ICPI assay values between 1.75 and 1.96, typical for velogenic NDV (Alexander and Swayne, 1998) and demonstrating that there was no increase in virulence of the NDV strains obtained from the non-poultry species. Host-related patterns relating to the ICPI values were not observed.