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Canine distemper outbreak in raccoons suggests pathogen interspecies transmission amongst alien and native carnivores in urban areas from Germany

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ABSTRACT

From December 2012 to May 2013, an outbreak occurred among urban wild carnivores from Berlin. We collected 97 free-ranging raccoons from the city area. PCR assays, histopathology and immunohistochemistry confirmed canine distemper virus (CDV) infection in 74 raccoons. Phylogenetic analysis of haemagglutinin gene fragments (1767 nucleotides) of CDV isolated from four raccoons showed close relation to CDV isolates from foxes from Germany and a domestic dog from Hungary; all belonging to the "Europe" lineage of CDV. These study results suggest an inter-species transmission of CDV as the origin for the outbreak among the raccoon population. Implications for domestic pets and suggested interspecies transmission between urban wildlife and raccoons are discussed. This is the first major outbreak of CDV amongst free-ranging raccoons in Europe.

1. Introduction

Raccoons can easily adapt to sylvatic, rural or urban habitats. In their native North American habitat, raccoons are known to respond positively to urban environments with high population densities in cities (Prange et al., 2003). In Germany, North American raccoons were locally introduced in 1934, but to date are widely distributed throughout the country (Michler and Michler, 2012). In the region where the first introduction occurred, population density of this synanthropic carnivore now reaches up to 100 animals/km^2 in semi-urban areas (Michler et al., 2004). Other wildlife carnivores like red foxes (*Vulpes vulpes*), stone martens (*Martes foina*) or European badger (*Meles meles*) have also successfully adapted to the urban environment and share this habitat. High wildlife density in these areas can increase disease transmission (Ditchkoff et al., 2006) and all mentioned carnivore species could potentially transmit infectious pathogens to other wildlife species, domestic animals or humans—and vice versa.

Canine distemper virus (CDV) is a highly contagious pathogen, which may cause lethal systemic disease in dogs and other carnivores. The virus belongs to the genus Morbillivirus within the virus family *Paramyxoviridae*. CDV occurs worldwide and has a relatively broad host range infecting members of the families *Canidae*, *Procyonidae*, *Mustelidae*, *Hyaenidae*, *Ursidae*, *Viveridae* as well as some

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Felidae (Deem et al., 2000). Outbreaks in captive Rhesus macaque have been also reported (Sakai et al., 2013). The prevalence of CDV in the world dog population is high in spite of common vaccination in developed countries. Fatal outbreaks of CDV infection have been reported in lions (*Panthera leo*) (Roelke-Parker et al., 1996), endangered species like the African wild dog (*Lycaon pictus*) (Goller et al., 2010), and critically endangered species like the Iberian lynx (*Lynx pardinus*) (Meli et al., 2010). Canine distemper is an acute to chronic systemic disease. The transmission of the virus occurs primarily by close contact via aerosols or oral, respiratory and ocular fluids (Williams, 2001). Primary infection is established at mucosal membranes and lymphoid tissues, followed by viraemic spread leading to lymphopenia and immunosuppression (von Messling et al., 2003).

The CDV genome encodes two membrane glycoproteins, namely haemagglutinin (H) and fusion–protein (F), an envelope-associated matrix (M) protein, the polymerase-associated phosphoprotein (P), the viral RNA polymerase (L) and the nucleocapsid protein (N) which encapsulates and protects the viral genomic RNA (Hirama et al., 2004). The H protein mediates the attachment to the cellular receptors as the first step of infection. Being a major target for the host's immune response this protein displays a considerable genetic diversity (von Messling et al., 2001; Pomeroy et al., 2008). Molecular analyses of the H gene have been widely used to investigate the genetic relationship among virus strains (Hirama et al., 2004). Analyses have shown that the heterogeneity of the H gene generally reflects the geographic distribution of virus isolates (Martella et al., 2006). Based on H gene analyses, CDV strains cluster in nine lineages: America-1, America-2, Asia-1, Asia 2, Artic-like, South America, Southern Africa, Europe and Europe wildlife (Martella et al., 2006; Nikolin et al., 2012).

In North America CDV is considered enzootic in raccoons and occurs regularly in sylvatic and urban raccoon populations (Roscoe, 1993). In 2007 a distemper outbreak was recorded in the raccoon population in a National Park in North-eastern Germany (Michler et al., 2009; Nikolin et al., 2012). Phylogenetic analyses placed the involved virus within the "Europe wildlife" lineage. CDV isolated from foxes from the same geographical area one year later belonged to the "Europe" lineage suggesting a different origin for this outbreak.

From December 2012 to May 2013, an outbreak of CDV occurred in wildlife carnivores in greater Berlin metropolitan area. We investigated raccoon carcasses from this outbreak aiming to isolate and characterize the causative CDV by a phylogenetic analysis of the H-gene.

2. Materials and methods

2.1. Study area and carcass collection

In connection to a larger study on raccoon diseases in Germany, carcasses of 97 raccoons were retrieved for post mortem examination during a suspected CDV outbreak in wildlife carnivores (red foxes and raccoons) from December 2012 to May 2013 from Berlin metropolitan area, North-eastern Germany. During necropsy organ samples were taken for virological and histological investigations.

2.2. RNA extraction and reverse transcription nested-PCR (RT-PCR)

Tissue samples of brain, lung and spleen were initially frozen at −20 °C at the Veterinary Investigation Centre (Landeslabor Berlin-Brandenburg) and after transfer to the Leibniz Institute for Zoo and Wildlife Research, stored at −80 °C. Still being frozen organ samples from each raccoon were pooled to extract RNA using the RNeasy Kit (Qiagen GmbH, Germany) according to manufacturer's instructions. For detection of CDV reverse transcription nested-PCR was performed following an in-house protocol essentially as described earlier (Becher et al., 1997) using two sets of primers targeting a conserved region of the N-gene (see Supplemental material for primers sequences). Reverse transcription of 2.5 μl RNA was performed after heat-denaturation (3 min/94 °C, 2 min/4 °C) in the presence of 0.5 μl reverse and forward primer (50 mM) by adding RT mix (final concentration 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM dNTPs), 10 U RNaseOUT™ recombinant Ribonuclease Inhibitor (Invitrogen)) and 50 U reverse transcriptase (SuperScriptII, Invitrogen) for 30 min at 45 °C. Reverse transcription was terminated by heating to 80 °C for 2 min. Subsequently 1 U Taq Polymerase (Biotherm) was added together with PCR mix (final concentration 25 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 1.8 mM dNTPs, 0.1% Triton X 100, 0.02% BSA). Amplification conditions were: 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. Nested PCR was done using 2.5 μl of the first round product, 0.5 μl of both forward and reverse primer (50 μM), 2 mM dNTPs and 1 U Taq Polymerase (Biotherm) in PCR buffer (Biotherm). Amplification conditions were: 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s.

Fragments of the expected size were detected by gel electrophoresis and selected amplicons verified by sequencing. Negative controls for carryover contamination were included in the extraction protocol and RT-PCR. In order to assure integrity of the sample RNAs a house keeping gene (beta-actin) was amplified (see Supplemental material for primers sequences).

2.3. Histology and immunohistochemistry

In order to confirm molecular investigations for CDV infection respective samples of brain, lung, liver, spleen and lymph nodes were taken for patho-histological investigations. Tissues were fixed in 4% formalin, embedded in paraffin, processed routinely and stained with haematoxylin and eosin stain (HE).

To demonstrate the presence of CDV in lesions via immunohistochemistry, a mouse anti-canine-distemper-virus antibody (AbD Serotec MCA1893, Serotec, Düsseldorf, Germany) was employed. Tissue sections were deparaffinised and slides were processed in an automated immunohistochemistry stainer (DAKO, Hamburg, Germany) through the following steps: (a) thirty-minute incubation with 1:2000 dilution of mouse anti-canine-distemper-virus antibody; (b) Tris–HCl buffer washing; (c) twenty-minute incubation with biotinylated secondary antibody (Zyto-Chem-Plus-AP-Kit,