ELECTRON MICROSCOPIC DEMONSTRATION OF A REOVIRUS-LIKE AGENT IN CARRION CROWS (*CORVUS CORONE*) ASSOCIATED WITH CLINICAL SYMPTOMS SIMILAR TO WEST NILE VIRUS INFECTION

Elektronenmicroscopische detectie van een reovirusachtig agens bij zwarte kraaien (Corvus corone) geassocieerd met klinische symptomen lijkend op West Nile virusinfectie

J. Mast[1], T. van den Berg[1], C. Letellier[1], P. Kerkhofs[1] , H. Zeller[2], G. Meulemans[1]

[1] Veterinary and Agrochemical Research Centre, Groeselenberg 99, B-1180 Brussels, Belgium
[2] Pasteur Institute, Lyon, France

ABSTRACT

During the first two weeks of March 2004, 80 carrion crows (*Corvus corone*) were found dead in a park in Brussels (Belgium). At necropsy, splenomegaly, hemorrhagic enteritis with duodenum lesions and cerebral or meningeal hemorrhages were observed reminiscent of West Nile virus infection. By real-time (RT)-PCR (reverse transcription polymerase chain reaction), no amplification of the West Nile disease virus-specific 3'UTR RNA sequence was observed, while viral isolation from brain homogenate was also negative. Toxicological examination revealed no toxins. Transmission electron microscopy demonstrated intracytoplasmic, reovirus-like particles in ultra-thin sections of the spleen and duodenum. Proliferation of these virions in cultured chicken embryonic hepatocytes was evidenced by their cytopathogenic effect and their presence in infected cells and their supernatant.

SAMENVATTING

Tijdens de eerste twee weken van maart 2004 werden er 80 zwarte kraaien (*Corvus corone*) dood aangetroffen in een park in Brussel (België). Bij de lijkschouwing werden splenomegalie, hemorragische enteritis met duodenumletstels en cerebrale of meningeale bloedingen waargenomen die deden denken aan West Nile virusinfectie. Met realtimePCR (real-time omgekeerde transcriptie polymerasekettingreactie) werd geen amplificatie van de West Nile virusspecifieke 3'UTR RNA-sequentie waargenomen terwijl ook de virusisolatie van gehomogeniseerde hersenen negatief was. Toxicologisch onderzoek toonde geen toxinen aan. Transmissie-elektronenmicroscopie toonde intracytoplasmatische, reovirusachtige partikels aan in ultradunne secties van de milt en het duodenum. De vermeerdering van dit virus in culturen van embryonaire hepatocyten van de kip werd bewezen op basis van hun cytopathogeen effect en hun aanwezigheid in geïnfecteerde cellen en hun supernatans.

INTRODUCTION

The outbreaks of West Nile Virus (WNV) in Romania, Italy and France (Zeller & Schuffenecker, 2004) and of Usutu Virus (USUV) in Austria (Weissenböck *et al.* 2002) compel us to remain vigilant for arthropod-borne viral diseases in Western Europe, outside their typical geographic and climatological habitat. Infections of most wild bird species are probably asymptomatic: in France, cases in humans and horses were not associated with any abnormal mortalities of wild birds. A recent serological survey in the United Kingdom evidenced the presence of WNV, USUV and Sindbis virus in the wild bird population without any signs of disease or mortality (Buckley *et al.* 2003). In certain species, however, outbreaks of disease have been recorded: in Austria, the emergence of USUV was associated with high mortalities in the blackbird population *(Turdus merula)*, whereas in the USA, the American crow (*Corvus brachyrhynchos)* is considered an indicator species for WNV because it is highly sensitive to this virus. Its death rate has been shown to be instrumental in following the epidemic and the epizootic spread of the virus across the North American continent (Yaremych *et al.* 2004). For surveillance of WNV in the northeastern US, the dead crows have served as "neon needles

in a haystack"– indicators of viral activity that call attention to themselves. The surveillance factor most closely associated with the number of human cases was the dead crow density (Eidson, 2001). For this reason, a sudden episode of multiple mortalities of carrion crows in a frequently visited public park in the centre of a city like Brussels (Belgium) immediately raised public health concerns, and all the more so because the observed pathological findings were consistent with the disease caused by WNV (WND) in avian species. Contra-indications were that WNV is not endemic in Belgium, and the fact that the climatological conditions of frost and some snow during the period of the crow mortalities reduce the numbers of arthropod carriers of arboviruses, including WNV. However, after the initial North American outbreak in 1999, WNV overwintered in New York, with mid-winter infections discovered in hibernating mosquitoes (Nasci *et al.* 2001) and in a fresh carcass of a red-tailed hawk (*Buteo jamaicensis*; Garmendia *et al.*, 2000).

The present case illustrates how the given situation could be handled efficiently by combining a very specific nucleic acid amplification test – to obtain a negative diagnosis for WND - with the non-directed approach of diagnostic electron microscopy that indicated the probable etiological agent.

CASE REPORT

During the first two weeks of March 2004, a total of 80 carrion crows (*Corvus corone*) were found dead in a public park in the centre of Brussels (Belgium; 50°52'N, 4°22'E). Two times, five of these birds were submitted to the Veterinary and Agrochemical Research Centre (Brussels, Belgium) for further examination. At necropsy all examined crows demonstrated splenomegaly, 60% of the birds showed hemorrhagic enteritis, particularly at the level of the duodenum, and 50% of the birds demonstrated cerebral or meningeal hemorrhages.

Brain's homogenate extracts from 5 crows were investigated for WNV using a real-time RT-PCR specific for the 3'UTR non-coding RNA sequence of lineage 1 of WNV as described by Lanciotti *et al.* (2000). No specific amplification occurred while virus isolation was also negative. Toxicological examination for heavy metals and pesticides in the Centre d'Informations Vétérinaires en Pharmaco-Toxicologie (University of Liège, Belgium) revealed no specific toxins.

Specific pathogen-free (SPF) embryonated eggs were inoculated with spleen extracts by allantoic and yolk sac injection following current methods. The yolk sac inoculation killed the embryos and *Chlamydophila psittaci* was isolated, while no etiological agent was observed in the allantoic fluid by direct negative staining with uranyl acetate and transmission electron microscopy (TEM).

Representative parts from six spleen and three duodenum samples were preserved in a cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2% paraformaldehyde and processed for routine embedding in epoxide resin and transmission electron microscopy (TEM) as described by Kimpe *et al.* (2003). The available tissues already showed strong signs of decomposition so that initially, standard histopathological examination was not envisaged. By TEM, groups of a few intracytoplasmic particles were observed in ultra-thin epoxide sections of the spleen of two of six affected birds and several groups of large numbers of identical particles in sections of two out of three duodenum samples (Figure 1). The observed particles had the size and morphological characteristics of reoviruses: they were not enveloped, had an isometric, round appearance and comprised a core and an outer capsid. This capsid shell was composed of two layers, had a regular surface structure and was approximately 75 nm in diameter.

To isolate the virus, chicken embryonic hepatocytes were inoculated with spleen and brain homogenates of one bird, as described by Decaesstecker *et al.* (1986). Proliferation of virus from the spleen extract was evidenced by its cytopathogenic effect and by the presence of identical reovirus-like particles in the cytoplasm of infected cells (Figure 2) and in the supernatant (Figure 3). When 3-week-old specific pathogen-free chicks held in isolation units were inoculated with this cell culture-produced reovirus, no clinical signs of infection were observed, indicating host specificity.

DISCUSSION

The observation of a sudden mortality of carrion crows that showed very similar pathological findings as observed by Steele *et al.* (2000) in cases of avian WNV infection, compelled researchers to institute a WND-specific diagnostic. These tests were negative, but a Chlamydophila psittacci was isolated by inoculation of the yolk of embryonated chicken eggs. C. psittaci can have played a role in the pathogenesis of the disease in the crows. However, because a significant proportion of wild birds are subclinical carriers of *C. psittaci* (Olsen *et al.* 1998) and because *Chlamydophila spp*. have a wide host range (Kaleta and Taday, 2003) while no other bird species seemed to be affected, it can be assumed that *C. psittaci* was not the primary or only etiological agent.

The demonstration and isolation of a reovirus-like agent by electron microscopy does not necessarily imply

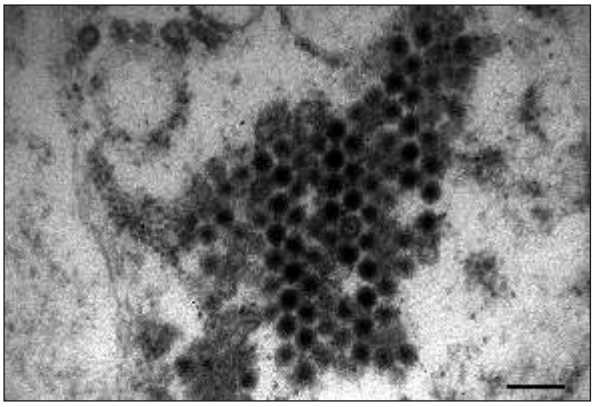

Figure 1. Micrograph demonstrating reovirus-like virions in ultra-thin sections of the duodenum of an affected crow. Bar 200 nm.

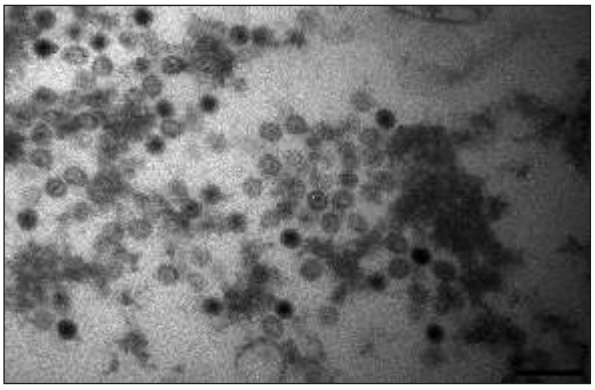

Figure 2 . Micrograph of the cytoplasm of cultured chicken hepatocytes inoculated with spleen extract. The infected cells show proliferation of identical virions as observed in the spleen and duodenum of dead crows. Bar 200 nm.

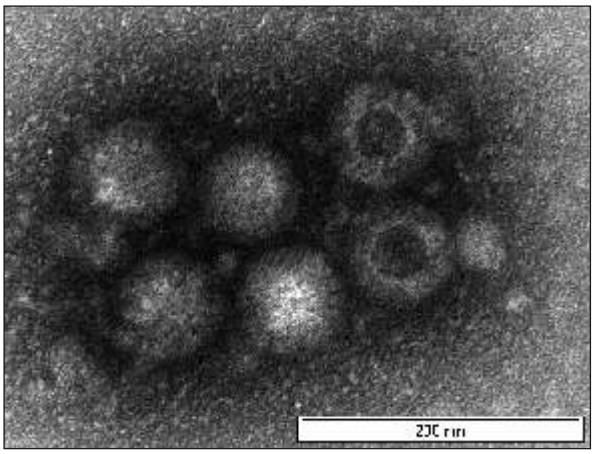

Figure 3. Micrograph of particles in the supernate of chicken hepatocytes inoculated with spleen extract after paraformaldehyde fixation and direct negative staining with 2% uranyl acetate. The particles have the size and morphological characteristics of virions of a reovirus. Bar 200 nm.

that this particular agent is the etiological agent of the disease. Among reovirus strains, a range of virulence exists, from highly virulent to virtually harmless (Jones, 2002). Because in the field no other avian species seemed to be affected and because experimentally infected SPF chickens did not show clinical signs – a fact indicating a limited host range –, infection trials of the host species remain to be done to formally prove its importance as a crow pathogen. This experiment would be difficult to realize since specific pathogen free crows, or crows that are (sero)-negative for the yet uncharacterized agent, are difficult to obtain.The following arguments lend support, however, to the assumption that in this case the reovirus-like agent is at least associated with the disease: (i) high numbers of virions were found in the enlarged spleen and at the sites of hemorrhagic duodenal lesions in multiple birds by the relatively insensitive technique of TEM; (ii) the finding of virions in the spleen suggests a systemic infection; (iii) identical pathological lesions were observed in virulent chicken reovirus strains (van Loon *et al.* 2001) causing high morbidity and mortality.

One can only speculate concerning the origin of the observed reovirus-like agent. Plausibly, the virus is endemic to the Belgian crow population and outbreaks go for the most part unnoticed. This hypothesis is supported by the finding that also in American crow populations a deadly reovirus is present: some dead American crows (*Corvus brachyrhynchos)* collected in WND monitoring efforts were diagnosed with a reovirus which was assumed to have contributed to population change in crows in 2003 in Seattle. Independently, surveillance unrelated to WNV revealed an unusually high mortality among American Crows in Snohomish, King, Pierce, and Thurston counties caused by a previously unidentified reovirus-like organism (Washington State Department of Health, 2004).

A second possibility is that the virus originates from another bird species and crosses the species barrier. In this perspective, the observation might be relevant that in the spring of 2004 a reovirus-like agent decimated the free-living ring-necked parakeet (Psittacula krameri) populations in the United Kingdom (Anonymus, 2004), while in the Brussels park where the crow mortality occurred, crows and free-living parakeets are in close contact because they share the same feeding places. It should be stressed, however, that no mortality was recorded in the Belgian parakeet population.

In conclusion, the recent introduction of WND in New York followed by rapid dissemination over the United States and the reappearance of WNV in the south of France in 2000 after 35 years of absence (Murgue *et al.* 2001) compel us to improve monitoring of the field situation.

Without 'accessory knowledge', a correct differential diagnosis becomes impossible, and so-called 'red herrings' - like the detection of *C. psittaci* in this example - will obscure a correct diagnosis of a possibly lethal infectious agent. The detection of reovirus in crows and its further characterization increases the knowledge of the specific pathogens of crows and may improve the use of corvids as indicator species for WNV infection.

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