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Detection and molecular analysis of West Nile virus infections in birds of prey in the eastern part of Austria in 2008 and 2009

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2 **Abstract**

3 The emergence of West Nile virus (WNV) was expected in Austria since the initial discovery
4 of the infection in neighbouring Hungary in 2003/2004. In 2008 six cases of West Nile
5 disease were diagnosed at the Institute for Veterinary Disease Control Mödling, Austrian
6 Agency for Health and Food Safety (AGES), involving five goshawks (*Accipiter gentilis*) and
7 one gyrfalcon (*Falco rusticolus*), which were found dead in the eastern Austrian federal states
8 of Lower Austria, Vienna and Styria, respectively. Pathomorphological and
9 immunohistochemical findings suggested a WNV infection. Virus was isolated in
10 embryonated specific pathogen free chicken eggs and propagated in mouse neuroblastoma
11 cells (NA), in which a cytopathic effect occurred. The virus was identified and characterised
12 by electron microscopic examination and molecular detection using RT-PCR, sequencing, and
13 phylogenetic analysis. The Austrian WNV sequences exhibited nucleotide identities of 99.9 %
14 to the lineage 2 WNV sequences described in Hungary since 2004. In addition, 71 sera of 14
15 different bird species were screened for the presence of WNV antibodies using a commercial
16 ELISA: 43,7 % of the tested samples showed antibody titres. Selected positive sera were also
17 subjected to WNV neutralisation tests, in which the ELISA results were verified in 66%. The
18 results of this study confirm unambiguously the presence of a lineage 2 WNV infection in
19 birds of prey in the eastern part of Austria.

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20 **Introduction**

21 West Nile virus (WNV; family *Flaviviridae*; genus *Flavivirus*) is a member of the Japanese
22 encephalitis virus group within the mosquito-borne flaviviruses. The Japanese encephalitis
23 antigenic complex of viruses includes Cacipacore virus (CPCV), Koutango virus (KOUV),
24 Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), Alfuy virus
25 (ALFV), St. Louis encephalitis virus (SLEV), Usutu virus (USUV), Yaounde virus (YAOV)
26 and Kunjin virus (KUNV, which actually represents lineage 1b of WNV) (Thiel et al., 2005).
27 WNV was first identified in Africa in 1937 (Smithburn et al., 1940) and subsequently, was
28 detected in Europe, Asia, Australia, the American Continent and the Caribbean (Trevejo and
29 Eidson, 2008). It has been proposed that one of the reasons for the rapid expansion is the high
30 mobility of the virus in avian reservoirs (Malkinson and Banet, 2002; Rappole and Hubálek,
31 2003). A broad host and vector range was demonstrated for WNV (Balenghien et al., 2008),
32 since it had been detected in at least 317 avian and 62 mosquito species (CDC). This virus is
33 transmitted particularly by ornithophilic mosquitoes of the genus *Culex* within the bird
34 populations, but some mosquito species are also capable of transmitting the virus to mammals
35 such as horses, sheep or humans (Linke et al., 2007).
36 WNV has been present in Europe since decades (Hubálek and Halouzka, 1999). However,
37 disease outbreaks were limited in time and geographic range, and presented as neurological
38 disease in human beings or equines (Hubálek and Halouzka, 1999; Cantile et al., 2000;
39 Krisztalovics et al., 2008). WNV-outbreaks affecting humans and horses are currently (since
40 early August 2010) going on in Northern Greece (Website: Hellenic Centre for Disease
41 Control and Prevention; Doudounakis, 2010). Starting in 2003, episodes of WNV-associated
42 deaths in a flock of domestic geese and wild bird mortality have been reported from Hungary
43 (Bakonyi et al., 2006; Erdélyi et al., 2007). The WNV strain involved in the goose outbreak in
44 2003 belonged to lineage 1, whereas wild bird mortality was due to a lineage 2 strain, which
45 was for the first time identified in a goshawk in a national park in southeastern Hungary in

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46 2004 (Bakonyi et al., 2006). In Austria, West Nile disease was never observed, only
47 seroconversion to WNV had been detected in different animal species in the sixties and
48 seventies of the last century (Aspöck et al., 1973; Grešikova et al., 1973, for review see
49 Weissenböck et al., 2010). Attempts to identify WNV infections in equines and birds had
50 been unsuccessful until summer 2008 (Nowotny, 2010).
51 The objective of this study was to describe a WNV outbreak in birds of prey in 2008 and 2009
52 in the eastern part of Austria.

53

54 **Materials and Methods**

55 **Study site and sample collection**

56 In mid-August 2008, a wild dead goshawk (*Accipiter gentilis*) was submitted to the Institute
57 for Veterinary Disease Control Mödling for post-mortem examination with the anamnesis of
58 sudden death after showing deviant behaviour caused by neurological symptoms. A WNV
59 neuroinvasive infection was presumed. The same tentative diagnosis was proposed one week
60 later for two additionally submitted goshawks, showing similar symptoms. In the same year,
61 also one out of ten dead gyrfalcons (*Falco rusticolus*) kept by a falconer in Vienna was
62 submitted for examination. In summer 2009, two goshawks from different locations were
63 submitted: one out of five dead goshawks from the east of St Pölten (Ollersbach), federal state
64 of Lower Austria, and one goshawk from the east of Styria. Besides specific analyses for
65 WNV routine testing for Avian Influenza and Paramyxovirus infections by real-time RT-
66 PCRs were carried out.

67 **Pathomorphological and immunohistochemical analyses**

68 All six carcasses - five goshawks and one gyrfalcon - were subjected to post-mortem
69 examination including necropsy and histological analyses. For histological examination,
70 representative tissue samples were embedded in paraffin wax, and sections were cut at 3-4µm
71 and stained with haematoxylin and eosin. Immunohistochemical staining for the detection of

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72 WNV-antigen was performed using an avidin-biotin complex (ABC) detection system
73 according to the manufacturers instructions. The primary antibody was a polyclonal anti-
74 WNV mouse serum (dilution: 1:2500; kindly provided by Dr. B. Murgue, Institut Pasteur,
75 Paris), which has been previously shown to efficiently detect WNV antigen in positive control
76 tissues (Weissenböck et al., 2003).

77 **Molecular analysis**

78 Viral RNA was isolated with the RNeasy® Kit (Qiagen, Vienna, Austria) from a pool of
79 selected tissues including brain, spleen, lung, liver, intestine and trachea. Initial WNV-
80 specific analysis was performed using a modification of the method published by Linke et al.
81 (2007) with 5µl RNA and the SuperScript III™ Platinum® One-Step Quantitative RT-PCR
82 System (Invitrogen). For verification, a RT-PCR, based in the conserved NS5 region, which is
83 able to detect all members of the Japanese encephalitis virus antigenic group of flaviviruses
84 (Weissenböck et al., 2002) was applied. This RT-PCR was performed with the SuperScript
85 III™ Platinum® RT-PCR System (Invitrogen) by using 5µl RNA and 500nM of each primer
86 in a 25µl total reaction volume. The PCR products were analysed by electrophoresis on a
87 1.5% agarose gel and DNA was purified with the QIAquick® Gel Extraction kit (Qiagen).
88 Sequencing reaction was prepared with the Big Dye® Terminator v3.1 kit (Applied
89 Biosystems), and analysed in a 3130xl Genetic Analyzer (Applied Biosystems). WNV NS5
90 partial sequences of 897bp were aligned using the Bionumerics software v6.0 (Applied
91 Maths, Sint-Martens-Latem, Belgium). In order to investigate the genetic relationship of the
92 Austrian WNV samples to representative other WNV strains, a phylogenetic tree was
93 constructed by UPGMA analysis. WNV strains published in the NCBI GenBank representing
94 lineages 1, 2, putative lineages 3, 4 and 5, and Usutu Virus strains as outgroup were included.
95 Recent WNV strains characterised in humans and animals, and those isolated from birds in
96 Hungary (Bakonyi et al., 2006), Italy (Barzon et al., 2009), France and Spain (Sotelo et al.,
97 2009) were primarily considered.

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98 **Virus isolation**

99 Virus isolation attempts were carried out using a pool of brain, liver, intestine and lung tissues
100 in embryonated specific pathogen free chicken eggs (SPFE) (Lohmann, Cuxhaven, Germany)
101 according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. The
102 chorioallantoic fluid was harvested, analysed for WNV particles by electron microscopy, and
103 used for further virus propagation in mouse neuroblastoma (NA) cell cultures. The cells were
104 incubated in Eagle's MEM supplemented with 1% l-glutamine, 1% non-essential amino acids,
105 1% of 100mM sodium pyruvate, 158µg/ml neomycin, 80µg /ml bacitracin and fetal calf
106 serum to reach a final concentration of 10%. Cell cultures were incubated at 37°C and 5%
107 CO₂ and examined daily for evidence of viral cytopathic effect (CPE). Cell culture isolates
108 were subjected to real-time RT-PCR (RT-qPCR) and sequence analysis.

109 **Serological tests**

110 Seventy-one sera of fourteen different avian species were screened for the presence of WNV
111 antibodies (Table 2). Samples were collected from birds close to the location of the first
112 goshawk infections as well as from aviaries at the Research Institute of Wildlife Ecology,
113 University of Veterinary Medicine, Vienna. Antibodies to WNV were tested by Enzyme-
114 Linked ImmunoSorbent Assay (ELISA) using the ID Screen[®] West Nile Competition
115 Screening test (ID VET, Montpellier, France) according to the manufacturer's instructions.
116 When sufficient material was available, sera with positive or borderline ELISA results were
117 also tested by WNV neutralisation test, which was established as an in-house method. The
118 neutralisation test was performed in a 96-well plate format with Eagle's MEM, supplemented
119 with 1% l-glutamine, 5% Fungizone and 10 % fetal calf serum. Sera were inactivated for
120 30min at 56°C. Serum samples were diluted in a 50µl system in two-fold steps, starting with a
121 dilution of 1:10 to 1:1280. The 100TCID₅₀ titer of the stock solution (WNV isolate of the
122 goshawk, case 1) was 10^{-3.3} in 50µl. 50µl of the stock solution was added to each well except
123 the wells of the cell control. All samples were incubated for one hour at 37°C. NA cells were

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124 grown as described for virus isolation (OIE Manual 2008, Chapter 2.01.13.B.1.ii). After
125 incubation, the cells were added and further incubated at 37°C in an atmosphere of 5% CO₂.
126 All samples were run in duplicate. Sera were considered positive after similar antibody titers
127 in two test runs. The test serum results are expressed as the reciprocal of the dilution of serum
128 that neutralised the virus in 50% of the wells. The start dilution of the serum samples was
129 1:10. If 50% of the wells with 1/10 diluted serum neutralised the virus, the titre is 10. For
130 qualitative results, any neutralisation at a titre of 10 or above is considered to be positive.

131 **Electron microscopic examinations**

132 For negative staining, spleen, pancreas, cerebrum, cerebellum, and mesencephalon of
133 goshawks and the embryos of the infected egg cultures were grounded in sodium phosphate
134 buffer (1 part tissue : 10 parts buffer), and further processed by UV irradiation, immersion in
135 Alcian Blue and ultracentrifugation. The supernatants of the centrifuged (4°C, 1300g for
136 15min) suspensions were ultracentrifuged with a Beckman Airfuge for 15min (91124 x g at
137 20psi) on carbon coated Pioloform copper grids. Grids were stained with 0.5% aqueous uranyl
138 acetate and 0.5% aqueous phosphotungstic acid. The same procedure was performed with
139 allantoic fluid and cell culture supernatant. For analysis of ultrathin sections the above
140 mentioned organs of the carcasses, organs (spleen, brain, heart) of the embryos and the cells
141 from the inoculated NA cell cultures were fixed by immersion in cold Karnovsky solution
142 (4°C, pH 7.3), post-fixed with cold phosphate buffered osmium tetroxide, embedded in Epon
143 and polymerised at 60°C during 2 days. Infected cell cultures were investigated for flavivirus
144 assembly after 18, 24 and 48h post inoculation. Semithin sections of 0.5µm thickness were
145 stained with Richardson's solution, the 70nm thick ultrathin sections with methanolic uranyl
146 acetate and lead citrate. All samples were analysed in a TEM (Zeiss 906) at 80kv.

147

148 **Results**

149 **Pathomorphological findings**

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150 The nutritional status of the 6 carcasses (two male, three female goshawks and one female
151 gyrfalcon) was appropriate. An overview about the pathomorphological findings is given in
152 Table 1. Gross pathology revealed no specific findings except for a mild hepato- and
153 splenomegaly as well as moderate meningeal haemorrhages of the cerebellum in case 3.
154 Histological, the most striking feature in all cases was a moderate multifocal to diffuse non-
155 purulent meningoencephalitis with slight lymphocytic meningeal and perivascular infiltrates
156 and formation of glial nodules (Fig. 2).
157 Varying amounts of viral antigen could be demonstrated by means of immunohistochemistry
158 in the brain (neurons and their processes), spleen (histiocytes, dendritic cells, cells of the
159 capsule), liver (hepatocytes, von Kupffer cells, intravascular mononuclear cells), eye (Str.
160 nervosum retinae), heart (myocardial cells), kidney (tubular epithelial cells) and in some
161 vegetative neurons of abdominal ganglia and the small intestine (Fig. 3 and Table 1). WNV
162 antigen was also detected in the brain and the heart in one chicken embryo investigated
163 immunohistochemically after death during the virus isolation trial.

164 **RT-qPCR, RT-PCR, sequencing and phylogenetical analysis of NS5 sequences**

165 The five goshawks and the gyrfalcon were tested positive by RT-PCR for WNV. The WNV
166 real-time RT-PCR revealed higher virus load in the organ pool of the first bird (quantification
167 cycle (Cq) value of 13.1) compared to the single organ samples spleen and brain (Cq values
168 around 15), whereas the Cq values obtained for the organ pool samples of the other five birds
169 varied between 20.2 and 33.8 in the different samples.

170 The nucleotide sequences of the 1084bp long specific PCR product of the partial NS5 of the
171 six Austrian WNV isolates analysed in this study were very similar. Pairwise alignment
172 revealed one mismatch position (99.88% identity) between the first goshawk (AT_763/08)
173 and the other five bird cases, which were completely identical at the nucleotide level. As
174 expected, the highest sequence homology (99.77%) was found to WNV strains of lineage 2
175 detected in birds of prey in Hungary, i.e. strains Hu/04 and Hu/05 (DQ116961-goshawk and

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176 EF116943-sparrow-hawk), respectively. According to our analysis, Austrian and Hungarian
177 WNV strains as well as isolates from Central and South Africa belong to the same sub-cluster
178 (Fig. 4). Neither WNV strain of the lineage 1, found in Italy in 2008 and 2009, nor the
179 putative lineage 3 isolated in 1997 in Czech Republic (Bakonyi et al., 2005; Bakonyi et al.,
180 2006) could be detected.

181 The NS5 partial sequences of the WNV strains detected in Austria were submitted to the
182 GenBank of the National Centre for Biotechnology Information (NCBI) under the following
183 accession numbers: GU566737-GU566740 and HM015884 for the 5 goshawk cases and
184 GU580891 for the gyrfalcon. Further alignment with other representative WNV sequences of
185 different lineages and two Usutu virus strains published by the NCBI GenBank (Fig. 4) was
186 carried out.

187 **Virus isolation**

188 During the first passage of samples in SPFE, one chicken embryo died on third, fourth and
189 fifth day of incubation. Two chicken embryos per day died on the fourth, fifth and sixth day
190 of incubation of second passage. Three days after inoculation with CAF from the inoculated
191 SPFE a general CPE in the NA cell culture was seen. The CPE was characterized by the
192 appearance of rounded, refractile cells followed by complete destruction of the cell
193 monolayer.

194 **ELISA and Virus Neutralisation Test**

195 Serological results are summarised in Table 2.

196 From the 71 serum samples tested; 31 samples (43,7% of the living birds) showed a positive
197 reaction in the ELISA, which seemed to be not only specific for WNV as mentioned by the
198 manufacturers. Positive reactions were found in two chickens (*Gallus gallus domesticus*), two
199 graylag goose (*Anser anser*), ten bearded vultures (*Gypaëtus barbatus*), twelve Ural owls
200 (*Strix uralensis*), one common buzzard (*Buteo buteo*), two golden eagles (*Aquila chrysaëtos*),

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201 and two goshawks (*Accipiter gentilis*). 66% of the positive ELISA reactions could be
202 confirmed in the NT (see Table 2).

203 **Electron microscopic examinations**

204 Virions were found in negative staining in the suspension of organ tissue of goshawks, in the
205 allantoic fluid and brain tissue of infected chicken embryos (45-55nm in diameter). Virions
206 were enveloped, icosahedral in shape and resembled structurally viruses of the family
207 Flaviviridae (Fig. 5a).

208 In ultrathin sections prepared from tissue of infected goshawks, flavivirus particles were
209 frequently seen in the cerebellum, liver, exocrine pancreas and spleen, which exhibited
210 multiple small foci of necrotic apoptotic lymphoid cells and deposition of fibrin.

211 Virus particles detected in the bird organs measured about 50nm in diameter and showed
212 typical flaviviral morphology, that is, a dense, round core surrounded by a thin, diffuse outer
213 layer. Viruses were often found solely in tissue cells, especially in pancreatic acinar cells (Fig.
214 5c). Packages of viruses, especially in cytoplasmic vacuoles, were rarely seen, however, most
215 frequently detected in hepatocytes (Fig. 5d) and perikarya or neuronal processes of brain
216 tissue (Fig. 5b). Viral accumulations were also rare in heart, brain and splenic tissue of
217 infected embryos.

218 Neuroblastoma cells (Fig. 6a-c), investigated after 18 and 24 hours, revealed a complex of
219 vesicular membrane structures of 80-100 nm diameters. Virus particles were observed within
220 the lumen of these membrane cisternae which were part of the endoplasmatic reticulum (ER)
221 and Golgi network (Fig. 6b-insertion). In autolytic neuroblastoma cells (48h post infection)
222 virions were accumulated in large vesicles (Fig. 6c).

223

224 **Discussion**

225 Virus isolation and serological tests proved the virus nature of the pathogen. Electron
226 microscopic analysis revealed flavivirus infection in several organs of goshawks. Analysis of

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227 virus infected neuroblastoma cell culture showed membrane structures identical to vesicular
228 packets found in connection with *Dengue virus*- and *Kunjin virus*-replication (Mackenzie and
229 Westaway, 2001; Welsch et al. 2009). The pathomorphological and immunohistochemical
230 findings agree with the report of lineage 2 WNV cases in goshawks from Hungary (Erdélyi et
231 al., 2007). Molecular biological diagnostics confirmed the infection of WNV lineage 2, and
232 sequence analysis showed 99.77% homology to the Hungarian isolates. High identity to the
233 Hungarian lineage 2 West Nile virus strain was also obtained from the West Nile viruses
234 detected in a pool of *Culex* mosquitoes collected in Greece (Papa, 2010). In Austria, WNV
235 lineage 2 was the causative agent of the observed clinical signs and the death of the birds.
236 Fortunately, in Austria, compared to other European countries like Italy, Hungary and Greece
237 (Kecskeméti et al., 2007; Rizzo et al., 2009; Doudounakis, 2010), no transmission to
238 mammals like horses, sheep and humans were reported by now and the infection seems to be
239 limited to birds of prey.

240 The pathological lesions and the virus distribution of the present WNV lineage 2 infections
241 largely resemble the observation from cases of lineage 1 WNV infections from North
242 America (Steele et al., 2000; Wünschmann et al., 2005). Here the circulating strain caused a
243 hitherto unprecedented epornithic with selective vulnerability of certain bird species, e.g. the
244 American crow (*Corvus brachyrhynchus*). In this bird species a significantly enhanced
245 virulence has been demonstrated experimentally (Komar et al., 2003), which has been
246 recently associated with a point mutation resulting in an amino acid change in the NS3 protein
247 of the North American WNV strain (Brault et al., 2007). The selective vulnerability of birds
248 of prey for the lineage 2 virus circulating in Central Europe is obvious and suggests an
249 underlying similar molecular mechanism, which is still unknown. It also showed that birds of
250 prey, and particularly goshawks, seem to be a biological indicator for the activity of the WNV
251 lineage 2 in Austria.

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252 The recent clinical findings of WNV infections in goshawk and gyrfalcon not far from the
253 eastern border of Austria to Hungary (Bakonyi et al., 2006) demonstrated that an introduction
254 of this virus into Austria was only a question of time. The same WNV strain detected in 2008
255 in the south of Vienna was identified one year later in a goshawk showing clinical symptoms
256 in Ollersbach / St. Pölten (federal state of Lower Austria) and in another goshawk at the
257 eastern part of Styria (district of Weiz), indicating an expansion of the area of WNV activity
258 to the west and south. It is not possible to trace exactly the introduction routes of WNV to
259 the Austrian bird of prey population. To our knowledge there have been no reports on WNV
260 detection in birds or other animals in Austria before, only seroconversion was demonstrated in
261 a few horses imported from Eastern Europe (Weissenböck et al., 2003; Weissenböck et al.,
262 2010). Birds are the natural reservoir of the virus, and migratory birds are playing probably a
263 major role in introduction or re-introduction of the virus in a given area. The majority of
264 WNV transmission between birds occurs through mosquito bites. However, transmission
265 through faeces (Kipp et al., 2006) as well as oral transmission should not be discounted and
266 suggestions for this route of transmission have been found in birds of prey in Spain, Hungary,
267 Israel and North America (ECDC). As a consequence of the introduction of WNV to Austria,
268 surveillance programs, which have been considered an effective tool in early detecting of new
269 emerging bird diseases like Avian Influenza, have been promptly established based on virus
270 detection in wild birds with special attention to birds of prey (Falconiformes) and
271 Passeriformes (crows and raven) in Lower Austria and Burgenland, respectively. Serological
272 screenings of birds in the areas at risk will also give an overview about the epidemic situation.
273 It is likely that WNV is becoming a permanently established pathogen with the tendency to
274 disperse, at least in the eastern (and possibly south-eastern) part of Austria, since the same
275 virus strain has been detected in two consecutive years. Transmission by mosquito vectors
276 plays a major role in WNV epidemiology. Hence, when mosquito-borne pathogens have been
277 introduced to a certain area, transmission can be sustained provided suitable vectors are

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278 present. Potential vectors of WNV, especially *Culex sp.* and *Aedes sp.*, belong to the most
279 prevalent mosquito species in Austria. A key parameter for a permanent residence or even
280 further spread of the disease, in the context of climate change, is the extended chance of the
281 pathogen to circulate between vectors and hosts (De la Rocque et al., 2008, Morand and
282 Guéguan, 2008, Reiter, 2008). As future climatic conditions (Stone, 2008) and vector
283 populations seem to be ideal for the establishment of newly introduced mosquito-borne
284 diseases in Austria, West Nile fever will presumably become a permanent disease in our
285 region, as the introduction of the related flavivirus Usutu virus to eastern Austria resulted in
286 permanent residence of the virus in this area (Weissenböck et al., 2002, Weissenböck et al.,
287 2003, Weissenböck et al., 2010). Furthermore, in the eastern part of Austria the climatic
288 conditions are comparable to western Hungary, where WNV also established itself as resident
289 pathogen with continuous dispersal into new areas (Bakonyi et al., 2006).

290

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295

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435

436 Legends for Figures and Tables:

437 Figure 1: Geographical locations of birds with lethal WNV infections

438

439 Figure 2: Goshawk, WNV-encephalitis, brain. Neuronal necroses surrounded by glial nodules.
440 Microphoto, H&E, bar = 25µm.

441

442 Figure 3: Goshawk, WNV-encephalitis, brain. High amounts of WNV-antigen in neurons and
443 their processes. Microphoto, ABC-technique, bar = 25µm

444

445 Table 1: Pathomorphological findings and WNV antigen distribution in selected tissue
446 samples.

447

448 Figure 4: Phylogenetic tree of partial NS5 sequences of recently found WNV isolates from
449 lineage 1 (clades 1a and 1b), lineage 2 and the proposed putative lineages 3 and 4, including

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450 the WNV strains isolated in five goshawks and one falcon in Austria (GU566737-GU566740,
451 HM015884 and GU580891), respectively. Branch lengths are proportional to the number of
452 nucleotide changes (genetic distances). Scale bar shows the percentage of base substitutions in
453 the sequence. The bootstrap analysis revealed that all WNV isolates from Austria are grouped
454 within the lineage 2 with the Hungarian strains (not shown). Note: CF, Central African
455 Republic.

456

457 Table 2: Prevalence of West Nile virus – neutralising antibodies among living birds, sampled
458 in Lower Austria nearby finding place of the first WNV diseased goshawk and from aviaries
459 at the Research Institute of Wildlife Ecology in Vienna. 38,7% of the positive ELISA-
460 samples (ID Screen[®] West Nile Competition Screening test) were subsequently checked by
461 WNV neutralisation test.

462

463 Fig. 5: *Accipiter gentilis* (Goshawk): Negative staining: a) Flavivirus particles from spleen
464 suspension. bar = 50nm; b-d) Epon sections of affected organs, arrows point to virus particles
465 b) Cerebellum: virus particles in vacuoles of perikarya and neuronal processes (insert), bar =
466 100nm c) exocrine part of the pancreas: virus particles in cytoplasm (left insert) and between
467 ER of acinar pancreatic cells (right insert), bar = 85nm d) liver: virus particles in vacuolar
468 system of hepatocytes, bar = 100nm

469

470 Fig.6: Neuroblastoma cell: a) WNV 2-infected cell – after 18h postinfection, bar = 1µm;
471 insert: dilated ER – 18h, bar = 100nm; b) vesicular membrane structure complex (vm),
472 autolytic body (a); insert: virus particle inside membrane cisterna – 24h, bar = 100nm; c)
473 autolytic cell: accumulation of virions (arrow) in large vesicles – 48h, bar = 100nm

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Table 1:

| Organ | Case 1 <i>Accipiter gentilis</i> [#] | | Case 2 <i>A. gentilis</i> [†] | | Case 3 <i>A. gentilis</i> [†] | | Case 4 <i>Falco rusticolus</i> [†] | | Case 5 <i>A. gentilis</i> [†] | | Case 6 <i>A. gentilis</i> [†] | |
|-----------------|--|------|---|-----|---|-----|--|-----|---|-----|---|-----|
| | PMF | IHC | PMF | IHC | PMF | IHC | PMF | IHC | PMF | IHC | PMF | IHC |
| Brain | np. enc. | *+++ | np. enc. | + | np. enc., men. haem. | +++ | np. enc. | ++ | np. enc. | +++ | np. enc. | ++ |
| Spleen | sm., necr. | +++ | NT | NT | sm. | NT | NAD | + | sm., necr. | +++ | NAD | ++ |
| Liver | hm., chst. int. hep. | +++ | hm., chst. | - | hm., chst. | + | hm. | + | int. hep. | + | hm. chst. siderosis | ++ |
| Heart | NAD | - | hp. | + | NAD | ++ | NAD | ++ | np. myoc. | + | np. myoc. | ++ |
| Kidney | NAD | - | NAD | + | tn., tdil. | - | NAD | NT | int. nep. & fibr., tdil. | + | NAD | NT |
| Lung | NAD | - | anthracosis | - | gn. pneum., anthracosis | - | edema | NT | edema | - | NAD | NT |
| Small i. | NAD | - | NAD | + | NAD | + | NAD | NT | NAD | + | np. gangl. | + |
| Testes | NAD | NT | | | NAD | - | | | | | | |
| Ovary | | | NAD | NT | | | NAD | NT | NAD | NT | NAD | NT |
| Salpinx | | | NAD | - | | | NAD | NT | NAD | NT | NAD | NT |
| Eye | NT | NT | np. iridoc. | - | NT | NT | NT | NT | np. iridoc. & chorioid. | ++ | NT | |

= wild bird, † = aviary, * amounts of antigen: +++ = high, ++ = moderate, + = low, - = no antigen detected, NT = not tested, grey field = sex specific organ not existing

Abbreviations: PMF = pathomorphological findings, IHC = immunohistochemistry, np = non purulent, enc = encephalitis, men. haem. = meningeal haemorrhages, sm = splenomegaly, necr = necrosis, NT = not tested, NAD = no abnormalities detected, hm = hepatomegaly, chst = intrahepatic cholestasis, int = interstitial, hep = hepatitis, hp = hydropericardium, myoc = myocarditis, tn = tubulonephrosis, fibr = fibrosis, tdil = dilatation of renal tubuli, nep = nephritis, gn = granulomatous-necrotizing, pneum = pneumonia, small i = small intestine, gangl = intramural ganglionitis, irodoc = iridocyclitis, chorioid = chorioiditis.

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450 Table 2:

| Species | Locality | No. bird samples | Status | ELISA No. pos./No. neg. | WNV-NT No. pos./No. neg. |
|---|---------------|------------------|--------------|----------------------------|-----------------------------|
| <i>Gallus gallus domesticus</i> (Chicken) | Lower Austria | 10 | free range | 2/8 | n* = 1 1/0 |
| <i>Anas platyrhynchos domesticus</i> (Domesticated Muscovy duck) | Lower Austria | 4 | free range | 0/4 | - |
| <i>Anser anser</i> (Greylag Goose) | Lower Austria | 2 | free range | 2/0 | n = 1 0/1 |
| <i>Columba livia domestica</i> (Domestic pigeon) | Lower Austria | 13 | domesticated | 0/13 | - |
| <i>Corvus frugilegus</i> (Rook) | Lower Austria | 1 | wild | 0/1 | - |
| <i>Accipiter gentilis</i> (Goshawk) | Lower Austria | 1 | wild | 0/1 | - |
| <i>Accipiter gentilis</i> (Goshawk) | Lower Austria | 2 | aviary | 1/1 | n = 1 1/0 |
| <i>Accipiter gentilis</i> (Goshawk) | Vienna | 2 | aviary | 1/1 | n = 1 1/0 |
| <i>Gypaëtus barbatus</i> (Bearded Vulture) | Vienna | 12 | aviary | 10/2 | n = 3 2/1 |
| <i>Buteo buteo</i> (Common Buzzard) | Vienna | 1 | aviary | 1/0 | NT [#] |
| <i>Aquila chrysaëtos</i> (Golden Eagle) | Vienna | 2 | aviary | 2/0 | n = 1 1/0 |
| <i>Strix uralensis</i> (Ural Owl) | Vienna | 18 | aviary | 12/6 | n = 4 2/2 |
| <i>Strix varia</i> (Barred Owl) | Vienna | 2 | aviary | 0/2 | - |
| <i>Aegolius funereus</i> (Tengmalm's Owl) | Vienna | 1 | aviary | 0/1 | - |

* n = number of samples tested positive in the ELISA, NT[#] = not tested



Figure 2
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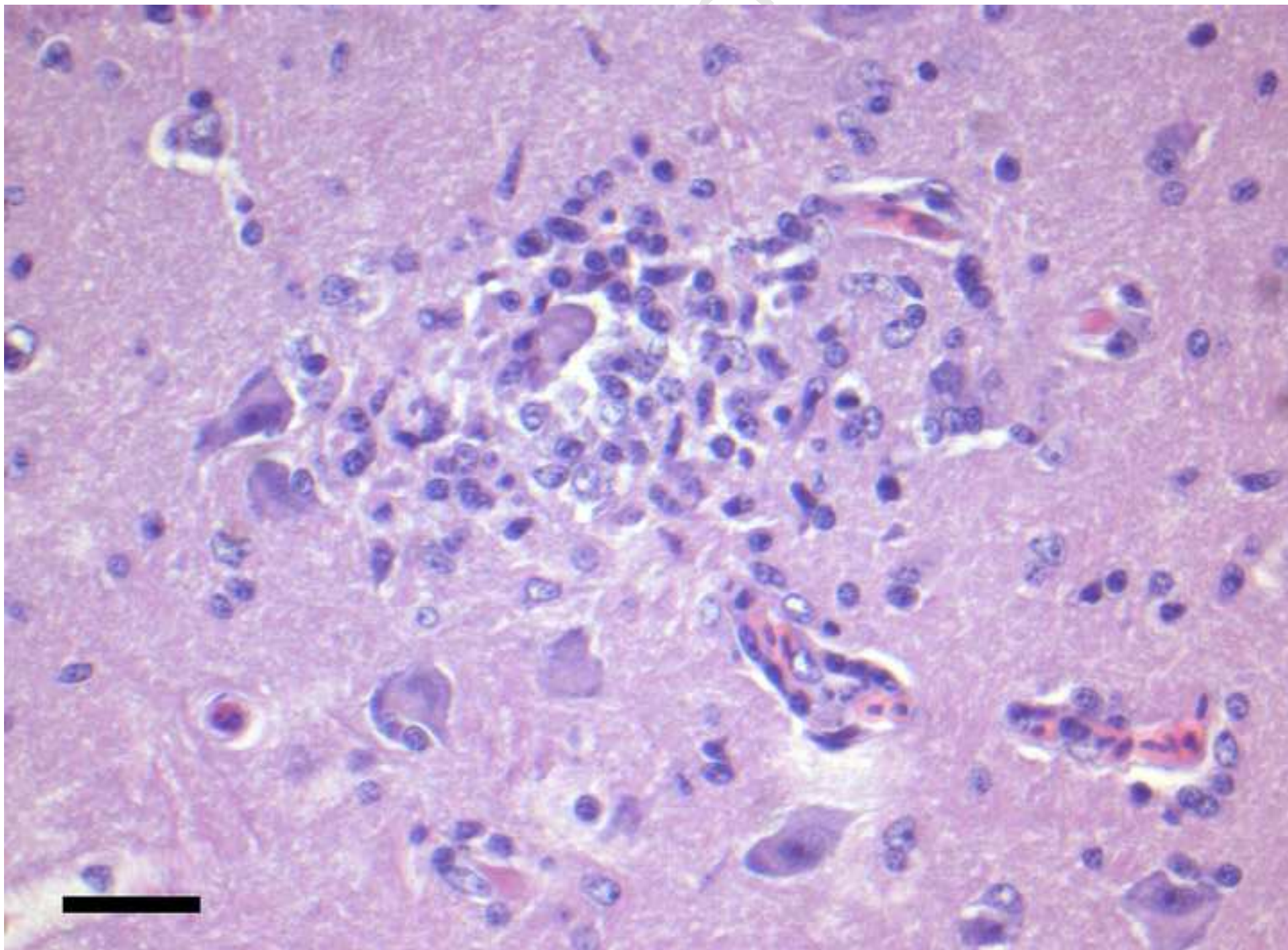


Figure3
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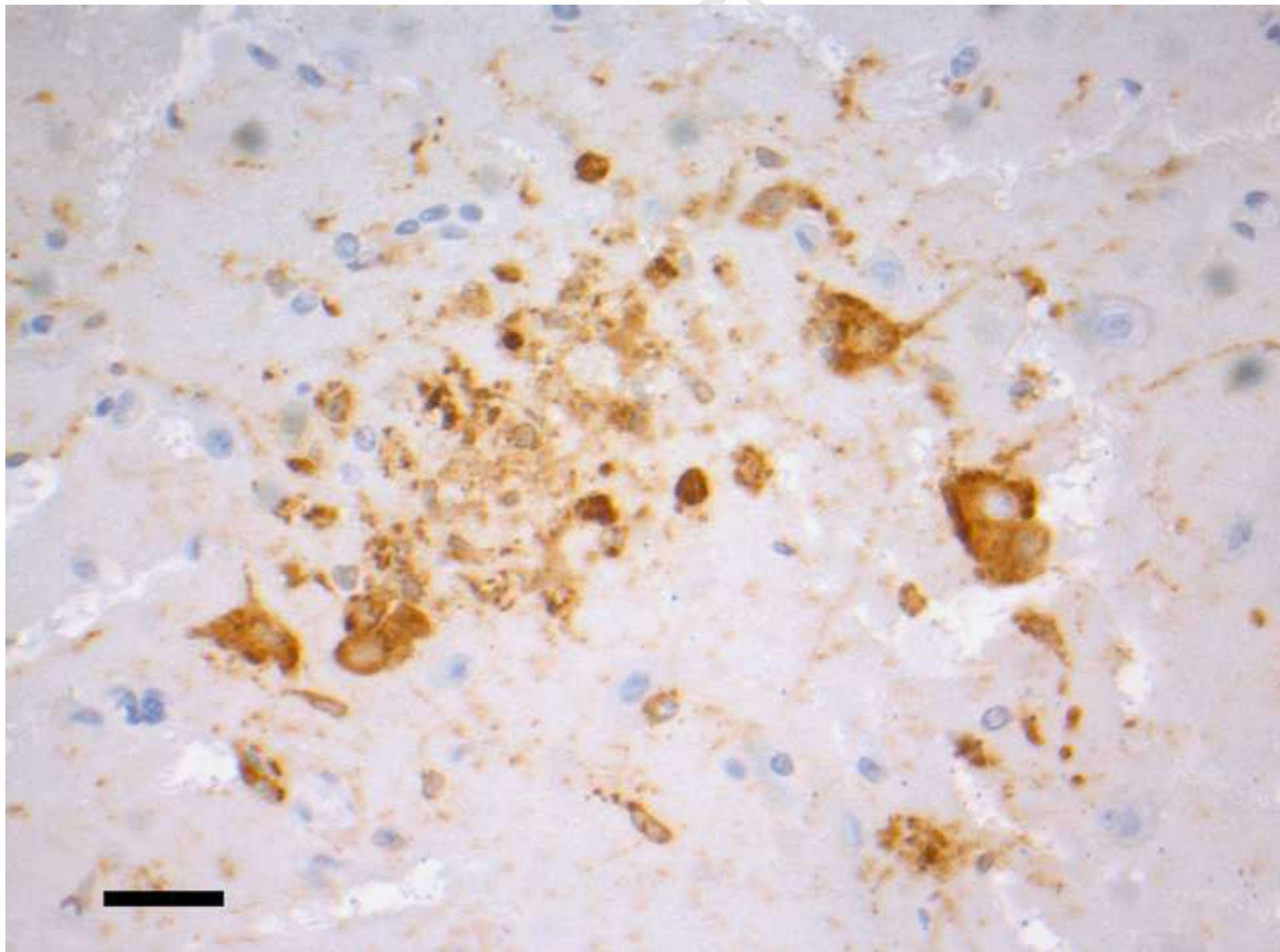


Figure4
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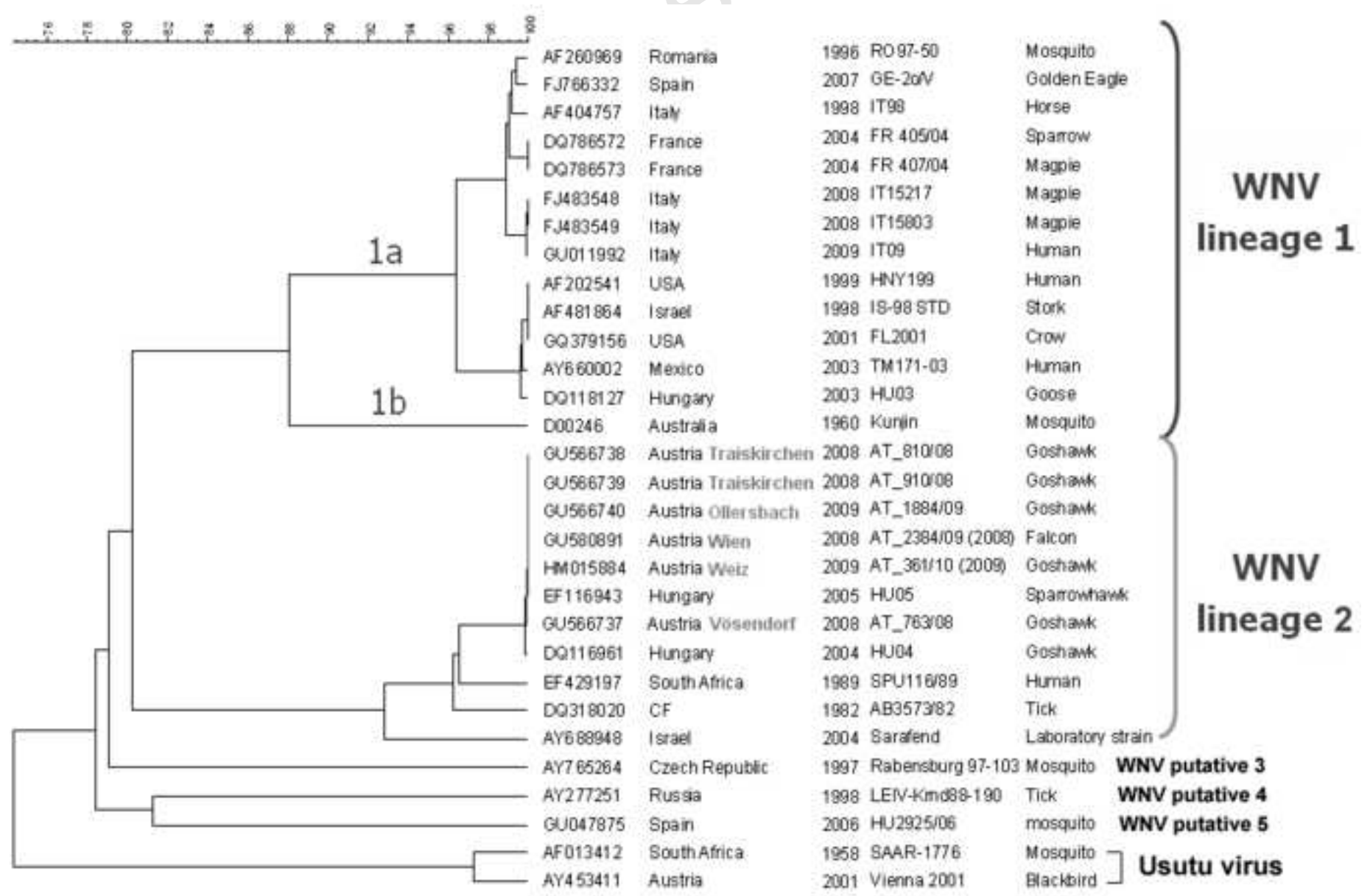


Figure5a
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