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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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Abstract

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

Introduction

West Nile virus (WNV; family *Flaviviridae*; genus Flavivirus) is a member of the Japanese encephalitis virus group within the mosquito-borne flaviviruses. The Japanese encephalitis antigenic complex of viruses includes Cacipacore virus (CPCV), Koutango virus (KOUV), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), Alfuy virus (ALFV), St. Louis encephalitis virus (SLEV), Usutu virus (USUV), Yaounde virus (YAOV) and Kunjin virus (KUNV, which actually represents lineage 1b of WNV) (Thiel et al., 2005). WNV was first identified in Africa in 1937 (Smithburn et al., 1940) and subsequently, was detected in Europe, Asia, Australia, the American Continent and the Caribbean (Trevejo and Eidson, 2008). It has been proposed that one of the reasons for the rapid expansion is the high mobility of the virus in avian reservoirs (Malkinson and Banet, 2002; Rappole and Hubálek, 2003). A broad host and vector range was demonstrated for WNV (Balenghien et al., 2008), since it had been detected in at least 317 avian and 62 mosquito species (CDC). This virus is transmitted particularly by ornithophilic mosquitoes of the genus *Culex* within the bird populations, but some mosquito species are also capable of transmitting the virus to mammals such as horses, sheep or humans (Linke et al., 2007).

WNV has been present in Europe since decades (Hubálek and Halouzka, 1999). However, disease outbreaks were limited in time and geographic range, and presented as neurological disease in human beings or equines (Hubálek and Halouzka, 1999; Cantile et al., 2000; Krisztalovics et al., 2008). WNV-outbreaks affecting humans and horses are currently (since early August 2010) going on in Northern Greece (Website: Hellenic Centre for Disease Control and Prevention; Doudounakis, 2010). Starting in 2003, episodes of WNV-associated deaths in a flock of domestic geese and wild bird mortality have been reported from Hungary (Bakonyi et al., 2006; Erdélyi et al., 2007). The WNV strain involved in the goose outbreak in 2003 belonged to lineage 1, whereas wild bird mortality was due to a lineage 2 strain, which was for the first time identified in a goshawk in a national park in southeastern Hungary in

2004 (Bakonyi et al., 2006). In Austria, West Nile disease was never observed, only seroconversion to WNV had been detected in different animal species in the sixties and seventies of the last century (Aspöck et al., 1973; Grešikova et al., 1973, for review see Weissenböck et al., 2010). Attempts to identify WNV infections in equines and birds had been unsuccessful until summer 2008 (Nowotny, 2010).

The objective of this study was to describe a WNV outbreak in birds of prey in 2008 and 2009 in the eastern part of Austria.

Materials and Methods

Study site and sample collection

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

Pathomorphological and immunohistochemical analyses

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

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

Molecular analysis

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

Virus isolation

Virus isolation attempts were carried out using a pool of brain, liver, intestine and lung tissues in embryonated specific pathogen free chicken eggs (SPFE) (Lohmann, Cuxhaven, Germany) according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. The chorioallantoic fluid was harvested, analysed for WNV particles by electron microscopy, and used for further virus propagation in mouse neuroblastoma (NA) cell cultures. The cells were incubated in Eagle's MEM supplemented with 1% l-glutamine, 1% non-essential amino acids, 1% of 100mM sodium pyruvate, 158µg/ml neomycin, 80µg /ml bacitracin and fetal calf serum to reach a final concentration of 10%. Cell cultures were incubated at 37°C and 5% CO_2 and examined daily for evidence of viral cytopathic effect (CPE). Cell culture isolates were subjected to real-time RT-PCR (RT-qPCR) and sequence analysis.

Serological tests

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

grown as described for virus isolation (OIE Manual 2008, Chapter 2.01.13.B.1.ii). After incubation, the cells were added and further incubated at 37°C in an atmosphere of 5% CO_2. All samples were run in duplicate. Sera were considered positive after similar antibody titers in two test runs. The test serum results are expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. The start dilution of the serum samples was 1:10. If 50% of the wells with 1/10 diluted serum neutralised the virus, the titre is 10. For qualitative results, any neutralisation at a titre of 10 or above is considered to be positive.

Electron microscopic examinations

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

Results

Pathomorphological findings

The nutritional status of the 6 carcasses (two male, three female goshawks and one female gyrfalcon) was appropriate. An overview about the pathomorphological findings is given in Table 1. Gross pathology revealed no specific findings except for a mild hepato- and splenomegaly as well as moderate meningeal haemorrhages of the cerebellum in case 3. Histological, the most striking feature in all cases was a moderate multifocal to diffuse non-purulent meningoencephalitis with slight lymphocytic meningeal and perivascular infiltrates and formation of glial nodules (Fig. 2).

Varying amounts of viral antigen could be demonstrated by means of immunohistochemistry in the brain (neurons and their processes), spleen (histiocytes, dendritic cells, cells of the capsule), liver (hepatocytes, von Kupffer cells, intravascular mononuclear cells), eye (Str. nervosum retinae), heart (myocardial cells), kidney (tubular epithelial cells) and in some vegetative neurons of abdominal ganglia and the small intestine (Fig. 3 and Table 1). WNV antigen was also detected in the brain and the heart in one chicken embryo investigated immunohistochemically after death during the virus isolation trial.

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

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

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

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

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

Virus isolation

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

ELISA and Virus Neutralisation Test

Serological results are summarised in Table 2.

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

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

Electron microscopic examinations

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

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

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

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

Discussion

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

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

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

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

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

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Legends for Figures and Tables:

Figure 1: Geographical locations of birds with lethal WNV infections

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

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

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

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

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

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

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

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

Table 1:

	Case 1 ***Accipiter gentilis*#**		**Case 2** ***A. gentilis*†**		**Case 3** ***A. gentilis*†**		**Case 4** ***Falco rusticolus*†**		**Case 5** ***A. gentilis*†**		**Case 6** ***A. gentilis*†**	
Organ	**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, chrorioid = chorioiditis.

450 Table 2:

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

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

Figure

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Figure2

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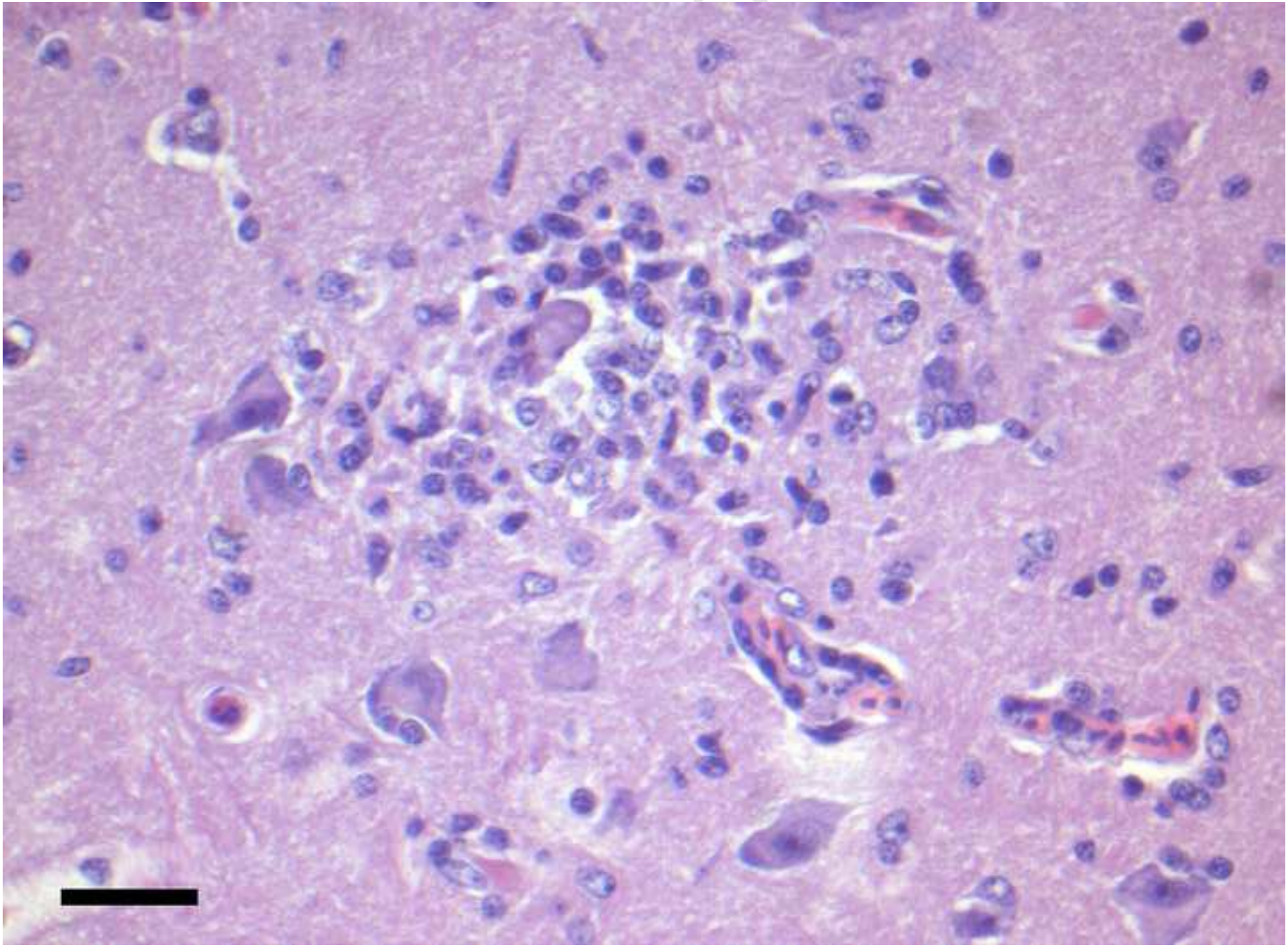

Figure3

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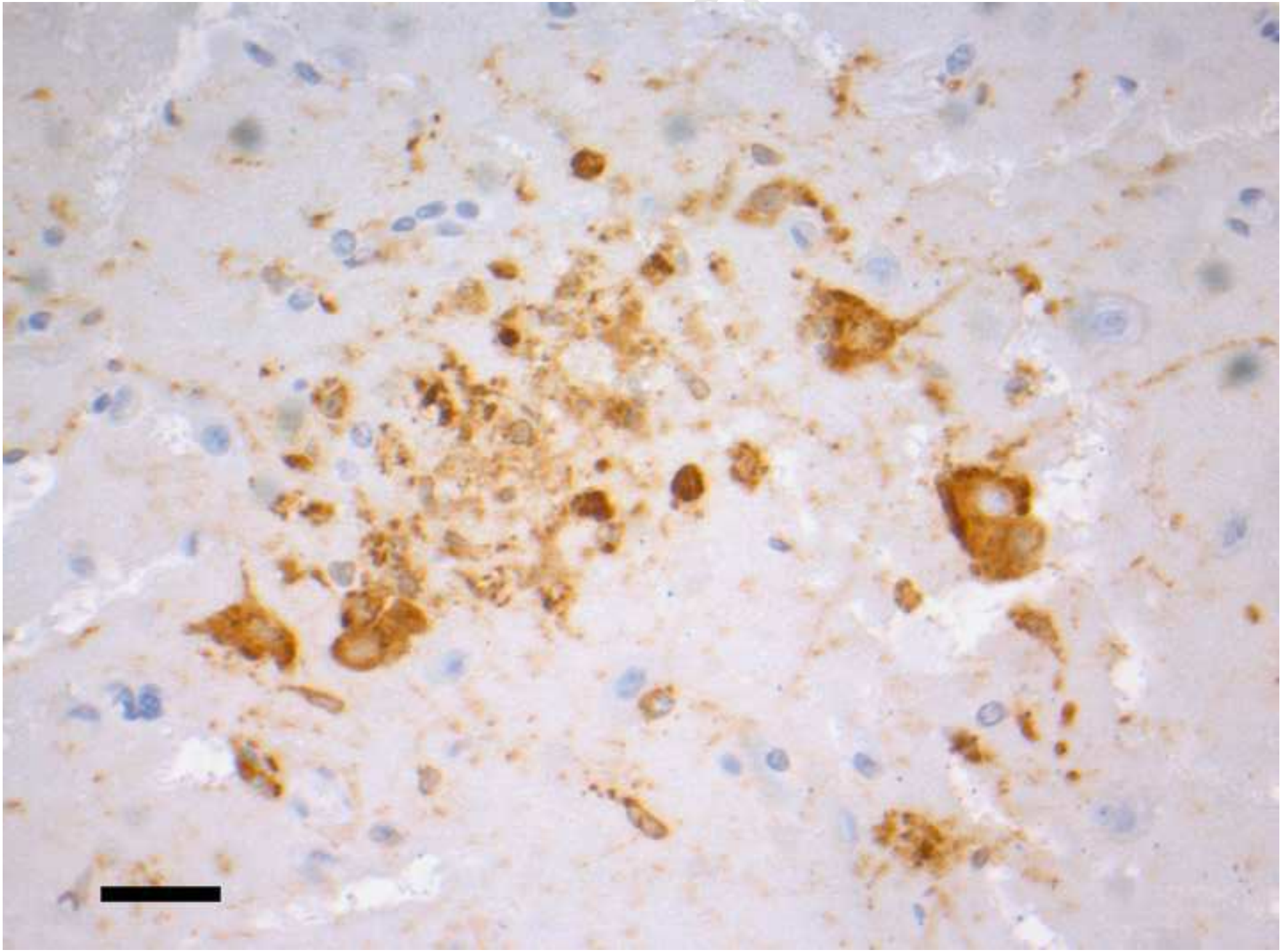

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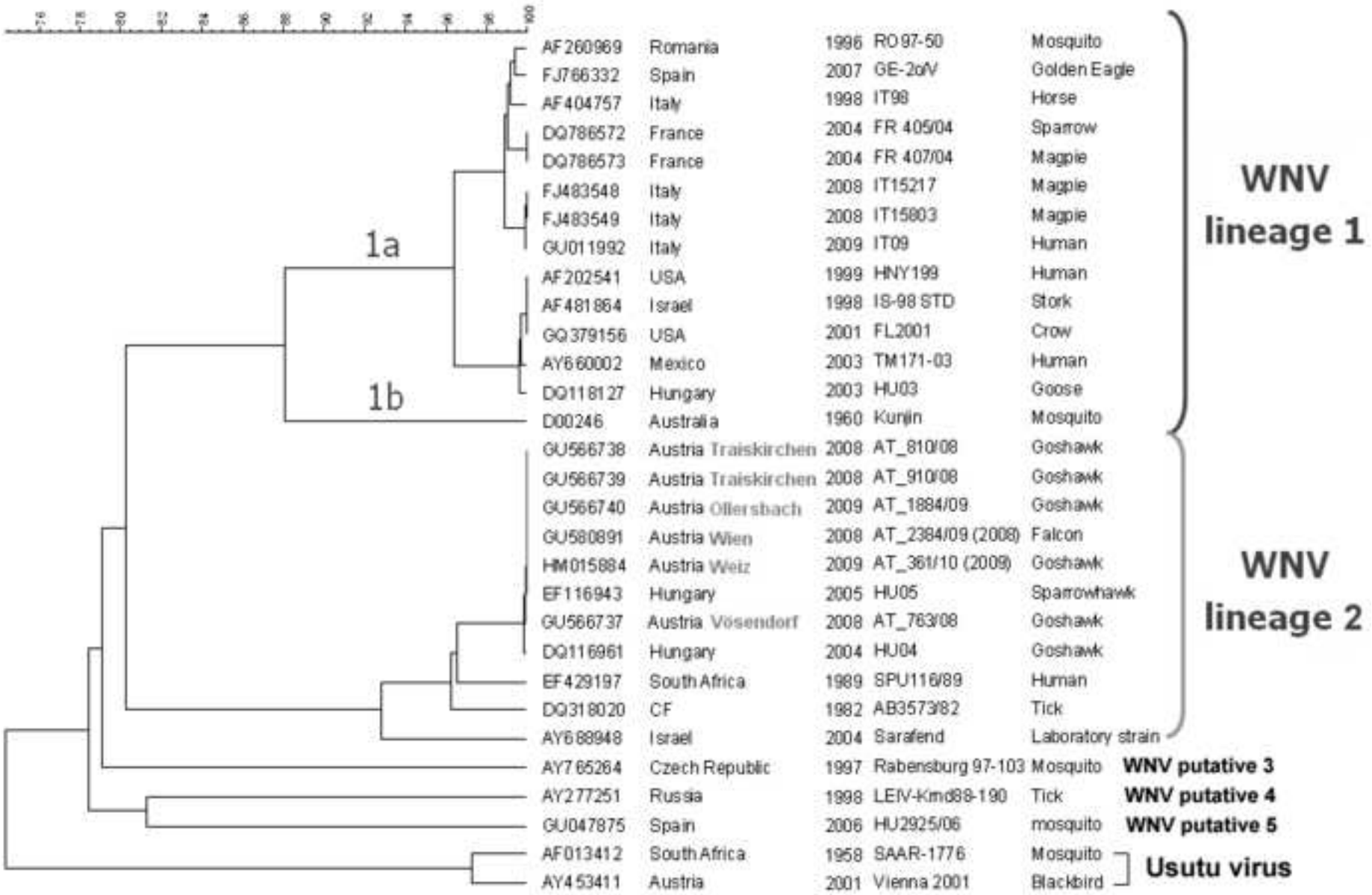

Figure5a

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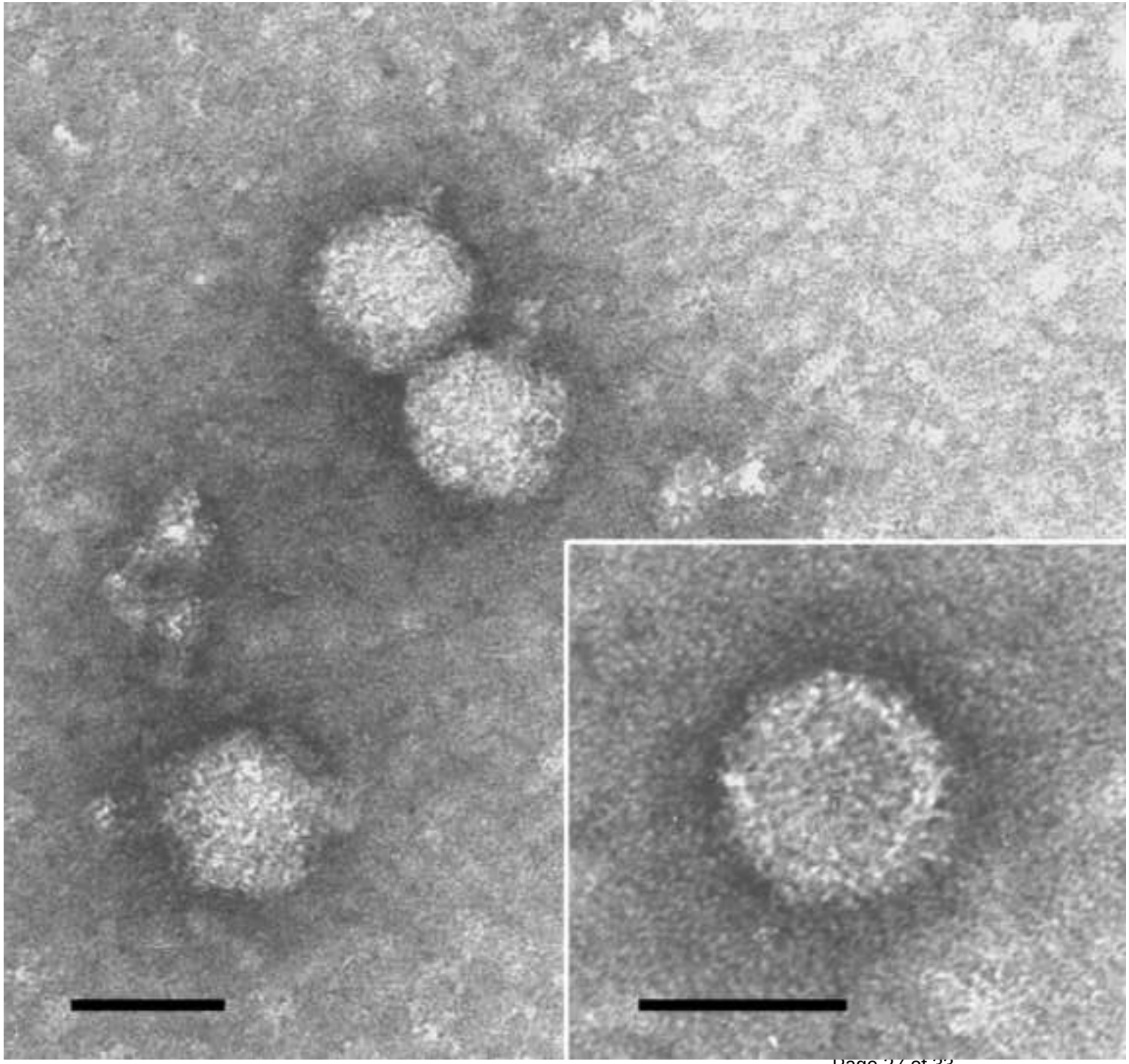

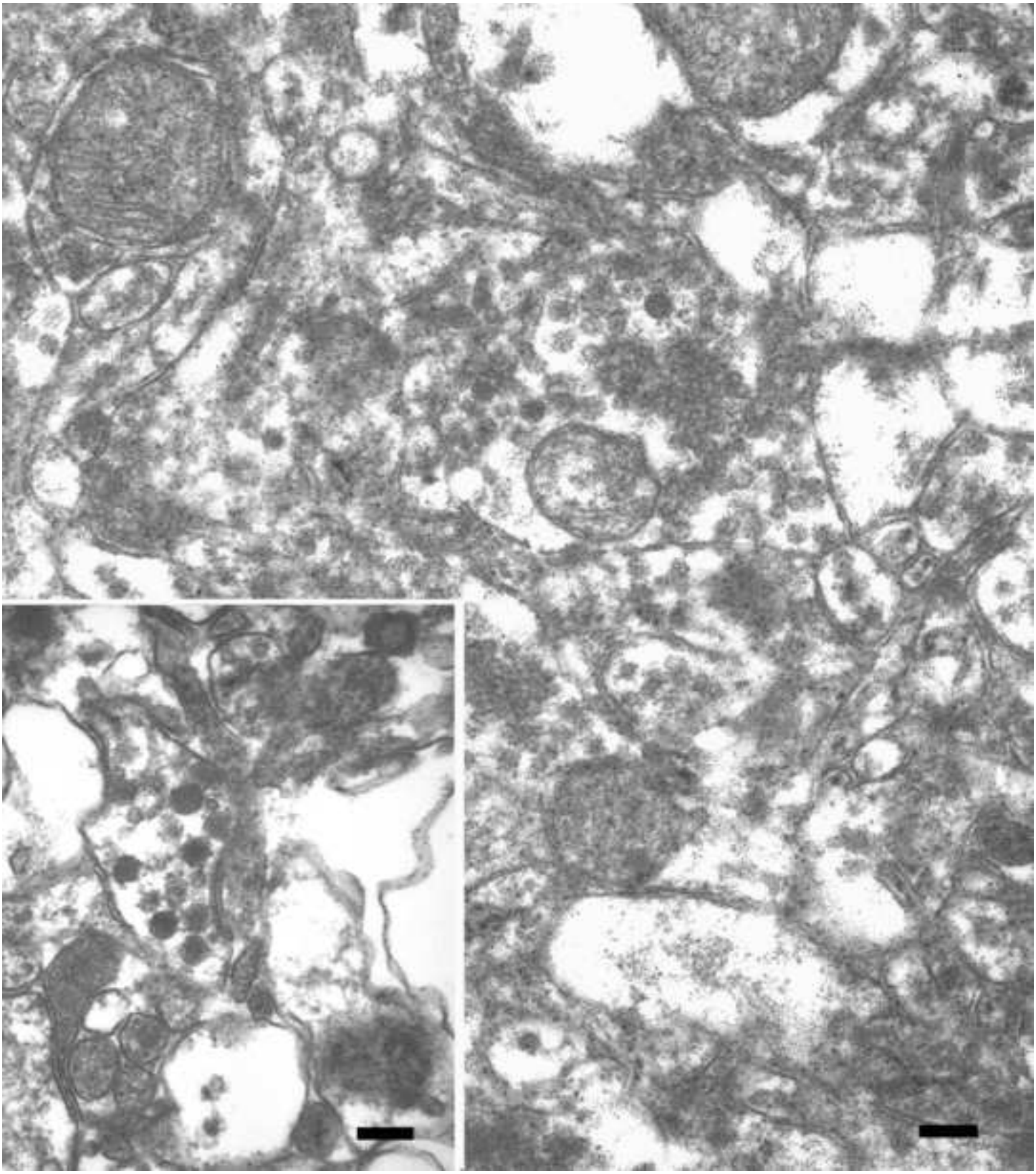

Figure5c

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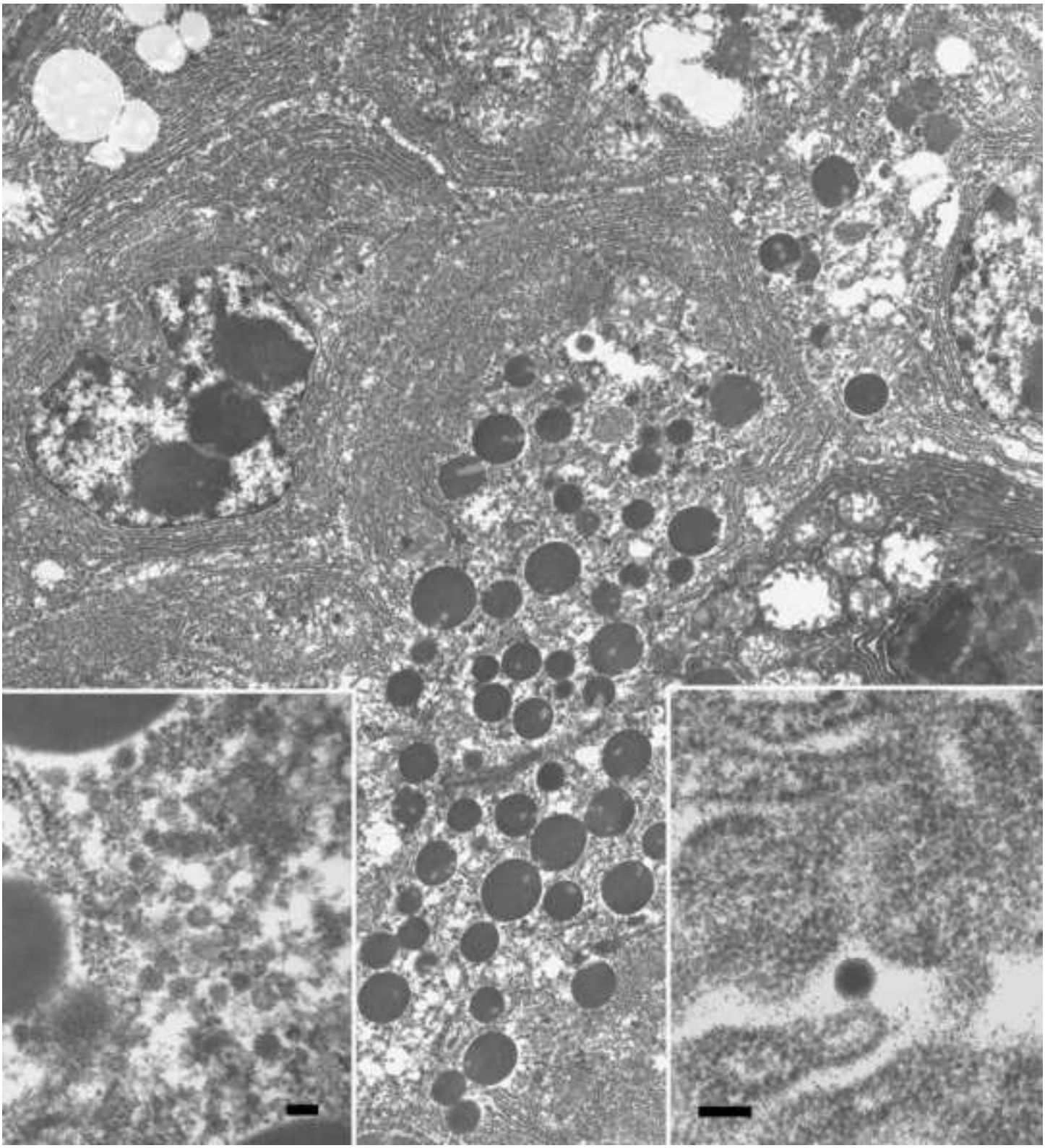

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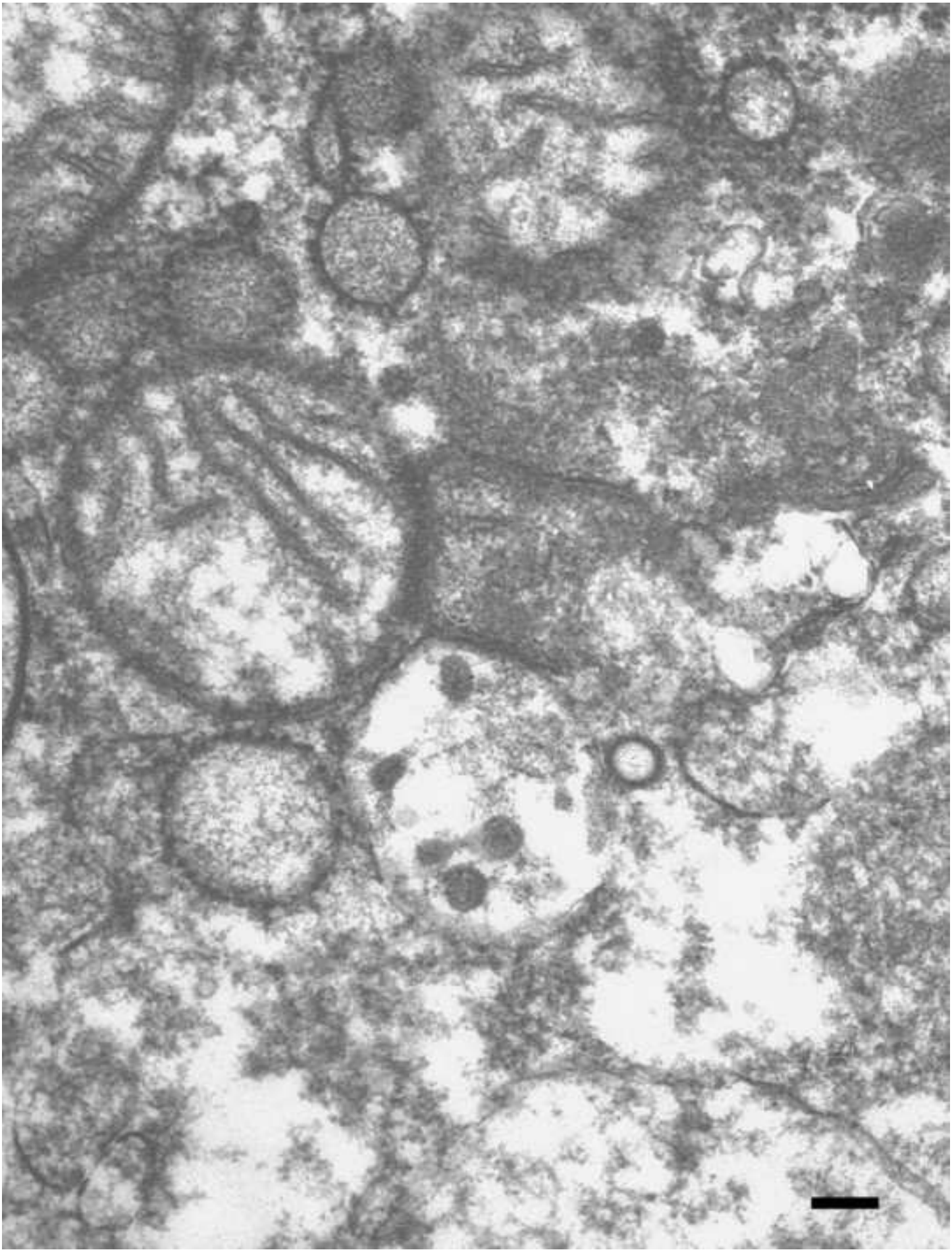

Figure6a

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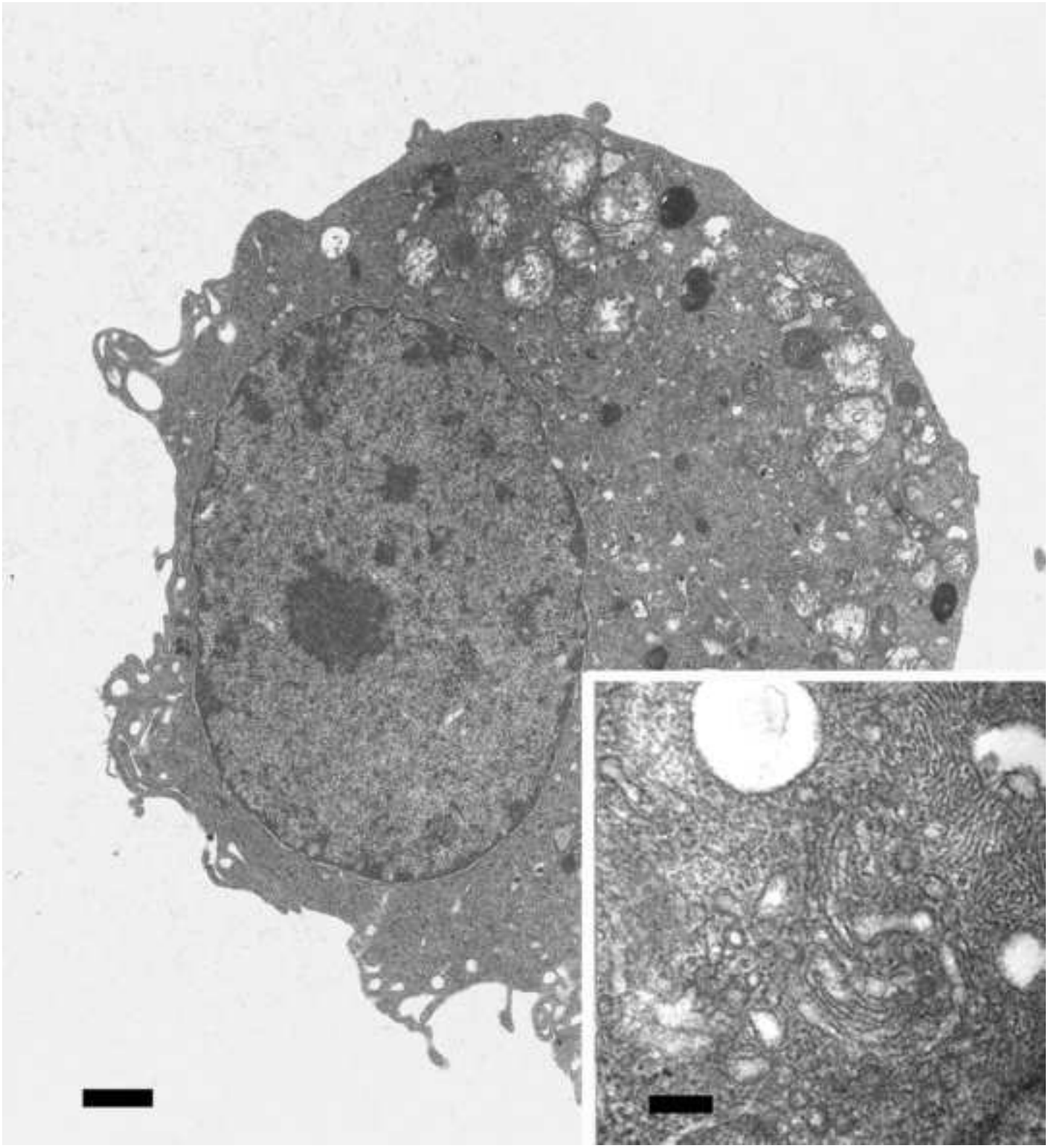

Figure6b

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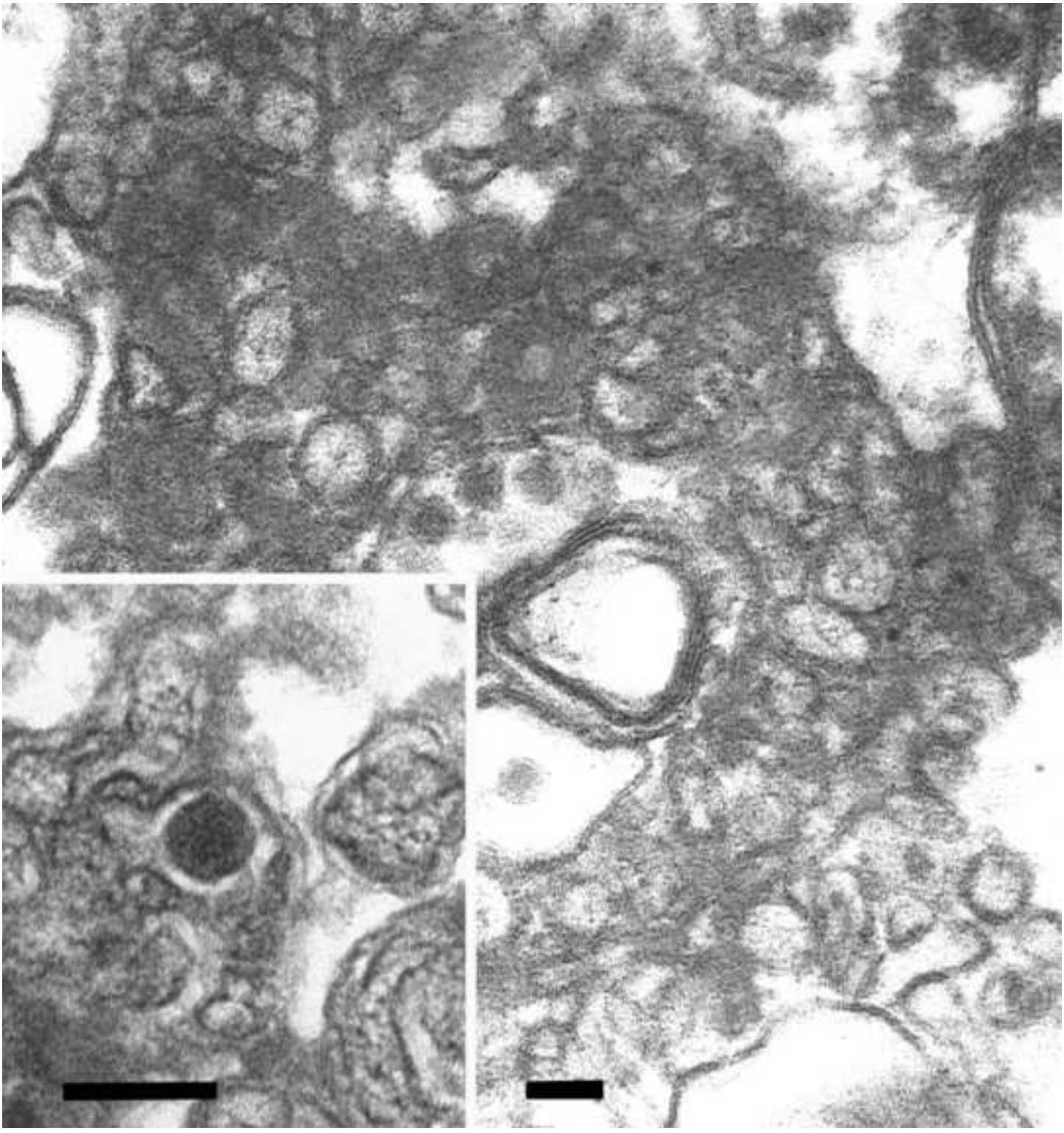

Figure6c

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