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Short communication

Usutu virus, Belgium, 2016

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ABSTRACT

During late summer 2016, in a northwest European region extending over Belgium, the Netherlands and the eastern border of the German state of North Rhine Westphalia, an outbreak of wild bird deaths occurred similar to those reported on the continent since 1996. Dead birds were necropsied and examined by complementary methods. Pathologic and immunohistological investigations strongly suggested an infection by Usutu virus. Subsequently, genomic segments of the said virus were detected, the virus was isolated and its complete genome was sequenced. The strain, designated Usutu-LIEGE, is a close phylogenetic relative of those isolated in Germany which form a distinct group within the USUV phylogeny, the so-called *Europe_3* lineage. Should this outbreak recapitulate the characteristics of those in southwest Germany in 2011 and in/around Vienna (Austria) in 2001, it is expected that specific avian populations in the affected area will face a significant reduction in size for a few years.

1. Introduction

Usutu virus (USUV) is a mosquito-borne, single-stranded RNA virus belonging to the Japanese encephalitis virus antigenic complex in the family *Flaviviridae* (Poidinger et al., 1996). The virus is primarily transmitted by ornithophilic mosquitoes among avian reservoir hosts (Nikolay et al., 2011). Prior to 1996, USUV had been isolated from some mosquito and bird species in Africa, but had never been associated with clinical signs or episodes of mass mortality (Nikolay et al., 2011). In early autumn 1996, an outbreak of wild bird deaths occurred in the provinces of Florence and Pistoia, in the Tuscany region of Italy. Mostly Eurasian common blackbirds (*Turdus merula*) were affected and USUV was identified as the causative agent (Weissenböck et al., 2013). In 2001, USUV emerged in and around Vienna, eastern Austria, where it caused an epidemic among birds of the orders Passeriformes and Strigiformes (Chvala et al., 2004; Weissenböck et al., 2002). In subsequent years, USUV was associated with episodes of massive die-off in Hungary (Budapest, 2003–2006 [Bakonyi et al., 2007]), Switzerland (Zurich, 2006 [Steinmetz et al., 2011]), northern Italy (Milan, 2006 [Manarolla et al., 2010]) and southwest Germany (2011–2014 [Becker et al., 2012; Ziegler et al., 2015]). A similar scenario started in August–September 2016 in the northwestern region of Europe including the German state of North Rhine Westphalia, the Dutch provinces of North Brabant, Limburg and Gelderland (Anonymous, 2016a) and the Belgian provinces of Limburg, Antwerp, Flemish Brabant (Anonymous, 2016b), Walloon Brabant, Liège and Hainaut (Desmecht et al., 2016). Here, we report the symptoms, the lesions and the detection of USUV antigens in the tissues of 3 common blackbirds, 1 robin (*Erythacus rubecula*) and 1 house sparrow (*Passer domesticus*) found moribund and referred to the Veterinary Faculty of University of Liège. Further, we report the genome sequence and phylogeny of the said USUV strain, designated Usutu-LIEGE.

2. The study

Clinical and postmortem examinations were performed on 5 wild adult birds referred to the authors by birdwatchers affiliated to the Natagora association (http://www.natagora.be/), a Belgian partner of BirdLife International (http://www.birdlife.org/), and by the Walloon Region's Surveillance Network for Wildlife Diseases (http://www.faunesauvage.be/). The birds had been found moribund in private gardens, 3 were found about 20–60 km of each other in the province of Liège, and the 4th and 5th about 90 and 150 km westward, in the Walloon Brabant and Hainaut province, respectively. Four birds were kept in a cardboard box for 1–2 h and died before they were admitted at the University Veterinary Hospital. The fifth remained in the garden for 3 days before being found dead. All 5 displayed non-specific clinical signs (immobility, ruffled plumage, half-closed eyes and anorexia), along with severe neurological signs (depression, incoordination, inability to fly, jerky movements, torticollis and nystagmus). The most

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stunning sign noticed by those who watched the diseased bird for 3 days was that it had stopped showing any sign of mistrust towards man. At necropsy, all 5 birds were in very good condition, with profuse subcutaneous and peritoneal fat. They had no significant gross lesions, except for a conspicuous, congestive hepatomegaly in the 3 blackbirds. The 2 halves of the brain/cerebellum and 2 pieces of the myocardium, liver, spleen and kidney were prepared separately, either being fixed in 10% neutral, buffered formalin and processed routinely for histopathology or immersed in DNA/RNA Shield™ and processed for RT-qPCR. Microscopic changes were shared by all the birds. In several areas of the cortex and brainstem, there was neuronal degeneration, neuronal necrosis and perineuronal clustering of glial cells reminiscent of satellitosis and neuronophagia (Fig. 1B). There were numerous foci of necrosis in both the liver (Fig. 1D) and the spleen (Fig. 1E). In the liver, a mononuclear leukocytic infiltration was duly noticed, but it remained discrete, suggesting a fulminant evolution of the necrotic process. Additionally, scattered cellular necrosis, again associated with minimal inflammatory reaction, was observed in the myocardium and kidney tubuli (Fig. 1F).

Then, tissue sections were stained by an immunoperoxidase method. A cocktail of monoclonal antibodies specifically raised against USUV was used as a source of primary antibodies (mAbs 4B9, 11B3 and 1E8, from J. Schmidt-Chanasit, by courtesy) and a horseradish peroxidase-labelled polymer conjugated to goat anti–mouse IgGs was used as the detection system (EnVision^{+} system-HRP, from Dako). Peroxidase was revealed by the bright red precipitate produced in the presence of 3-amino-9-ethyl-carbazole, and sections were counterstained with Gill's hematoxylin II. By doing so, USUV-specific antigens were detected in most tissues, most spectacularly in the liver (Fig. 2).

Further, a USUV-specific genomic segment was successfully detected using a reverse transcriptase (RT)-qPCR protocol (StepOnePlus real time PCR system; Applied Biosystems) targeting a 91 base pair region of the non-structural protein-1 (NSP-1) gene (Jöst et al., 2011). The presence of the said RNA segment was ascertained by the following Ct

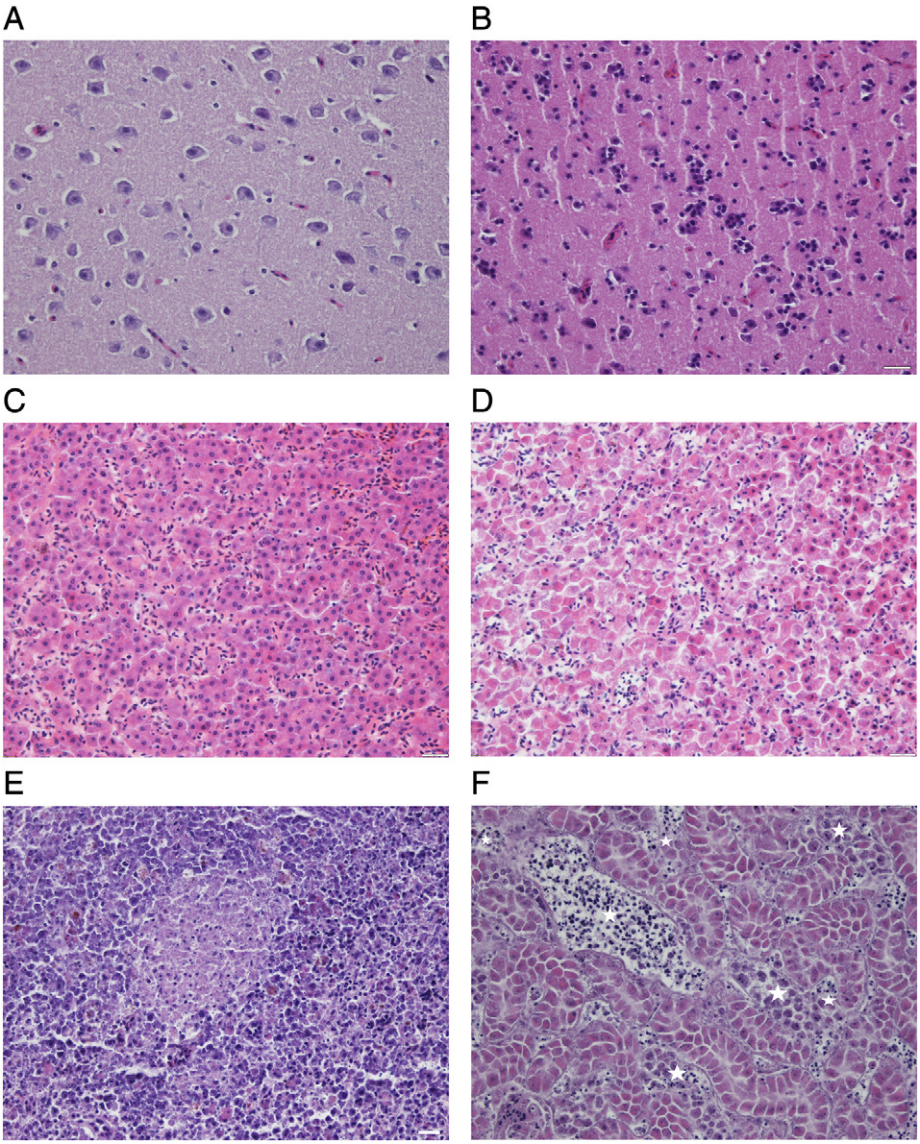

Fig. 1. Natural Usutu virus infection, Common blackbird (*Turdus merula*). Cerebrum (A, B), liver (C & D), spleen (E) and kidney (F) from control, USUV-negative (A, C) and principal, USUV-positive (B, D, E, F) birds. Diffuse, moderate gliosis, satellitosis (the multiple foci of proliferated neuroglia around degenerating neurons) and neuronophagia in principal bird (B). Massive hepatic necrosis in principal bird, with individualized hepatocytes showing hypereosinophilic cytoplasm and nuclear pycnosis and surrounded by cellular debris (D). A well-demarcated focus in the spleen is shown (E), where the predominant cytomorphology is consistent with a coagulation necrosis. In the kidney of another principal bird (F), most tubules display epithelial modifications characteristic of postmortem decay (caryolysis); besides, it is easily seen in a few scattered tubuli (stars) that the epithelium has undergone a complete process of necrosis and exfoliation in the lumina, only basement membranes are preserved. Hematoxylin & eosin, magnification 400×. Scale bar: 20 μm.

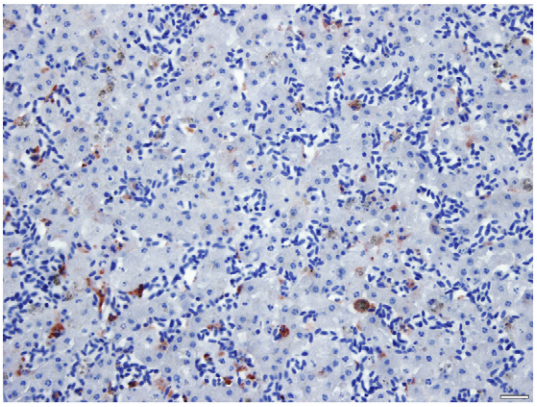

Fig. 2. Natural Usutu virus infection, Common blackbird (*Turdus merula*). Immunohistochemistry of USUV-infected liver using a cocktail of USUV-specific murine monoclonal antibodies (from J. Schmidt-Chanasit, by courtesy). Numerous USUV-positive hepatocytes (red-brown precipitates) are readily detectable. Mayer's hematoxylin, magnification 400×. Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

values: 17,.8 ± 0.01 (blackbird#1), 15.99 ± 0.01 (#2), 19.84 ± 0.10 (#3), 32.77 ± 0.21 (robin) and 33.50 ± 0.11 (house sparrow). In order to confirm the presence of the USUV genome within the tissues, extracted samples were also analyzed for the presence of flavivirus RNA by using a modified pan-flavivirus reverse transcription PCR targeting a segment of the NSP-5 gene (Becker et al., 2012). Direct sequencing of the retrieved amplicons confirmed the presence of USUV-specific nucleic acid sequences. A phylogenetic tree was inferred using the Bayesian Markov chain Monte Carlo approach available in BEAST v1.8.3. Analysis was performed under the best fit nucleotide substitution model identified as the TN93 + Γ using jModelTest 2 (Darriba et al., 2012). These analyses of the partial NS5 gene sequences revealed the close relationship of the Usutu-LIEGE strain with those from Germany which form a distinct group within the USUV phylogeny ("Europe 3" lineage, Fig. 3).

Brain and liver samples were then processed for next-generation sequencing in order to establish the genome sequence of the Usutu-LIEGE strain. Briefly, 500 mg of tissue were homogenized in 1 ml of 1× DNase buffer (Life Technologies, Ghent, Belgium) for 5 min at 30 Hz using a TissueLyser II device (Qiagen, Hilden, Germany). After centrifugation for 10 min at 11,000*g*, supernatant was collected

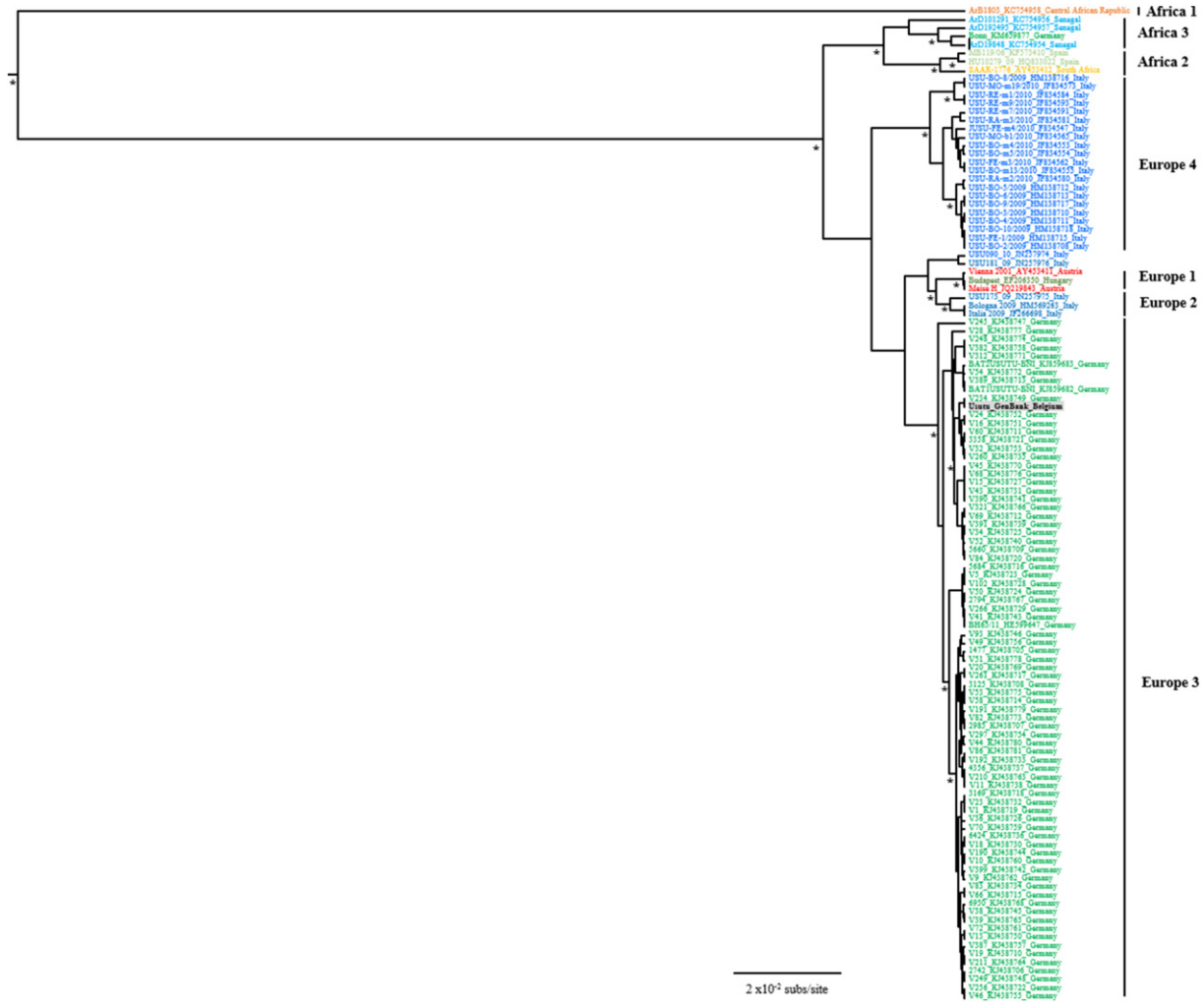

Fig. 3. Bayesian phylogenetic tree of USUV showing the phylogenetic placement of the Usutu-LIEGE virus strain compared with known USUV strains. The phylogenetic tree was inferred on the basis of the partial non-structural gene NS5 sequences with the use of the Bayesian Markov chain Monte Carlo method implemented in the BEAST v1.8.3. Statistical support of grouping from Bayesian posterior probabilities (clade credibilities ≥ 90%) is indicated with an asterisk. The taxon information includes the virus abbreviation, GenBank accession number, and country of origin. Branches are colored according to country of origin and the USUV sequence generated in this study is bolded. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and filtered using a 0.2 μm filter (Pall Corporation, Newquay, United Kingdom). Turbo™Dnase (Life Technologies, Ghent, Belgium), Benzonase® nuclease (Sigma-Aldrich, Diegem, Belgium) and RNase A/T1 (Thermo Scientific, Waltham, USA) were added to the elution at a 1:50 dilution. The mixture was stored for 1 h at 37 °C. Total RNA from viral particles was extracted using a combination of TRIzol reagent (Thermo Scientific, Waltham, USA) and NucleoSpin RNA Virus kit (Macherey-Nagel, Düren, Germany), according to manufacturer's instructions. RNA was retrotranscribed using SuperScript® III Reverse Transcriptase (Thermo Scientific, Waltham, USA) and random hexamers and the second strand synthesis was performed using NEBNext® mRNA Second Strand Synthesis Module (New England Biolabs, Ipswich, USA). Libraries were prepared using Ion Plus Fragment Library Kit and sequencing was performed with the Ion Torrent PGM technology (Life Technologies, Ghent, Belgium). Full genome assembly and sequence analysis were performed with Geneious 8.1.8 (Biomatters, Auckland, New Zealand). Near full-length genome sequence of strain Usutu-LIEGE was successfully obtained (Genbank accession no. KY263626). The single open reading frame encodes a polyprotein of 3415 aa and the nucleotide and amino acid conservation is >99.5% compared with the 2 closest phylogenetic relatives (gi|347977232 and gi|658508263). Comparison of the Usutu-LIEGE polyprotein sequence with these latter showed 2 nonsynonymous, unique mutations, both surprisingly located close to each other within the NS5-encoding gene. The flavivirus NS5 protein encodes the RNA dependent RNA polymerase which, together with other NS proteins and the underlying UTR skeletal backbone, is a critical component of the virus replication complex. The two consecutive substitutions in a conserved domain of this polymerase in the case of Usutu-LIEGE (I3116T and H3175Y) could impact on viral replication efficiency via perturbation of the viral replication complex, a possibility that deserves further investigations.

3. Conclusion

In this study, clinical, pathological and molecular investigations led to the discovery of the new USUV strain that emerged in Belgium in the summer/fall 2016. Whether the Usutu-LIEGE strain is archetypal of those associated with the massive die-off recorded at the same time in the Netherlands and in North Rhine Westphalia (Germany) remains to be confirmed. Its near complete genome sequence was determined and the specific amino acid substitutions characteristic of its polyprotein-encoding sequence are listed. Phylogenetic analyses revealed a close relationship of the Usutu-LIEGE strain with those from Germany which form a distinct group within the USUV phylogeny, the so-called *Europe_3* lineage.

There are some evidences that former outbreaks of USUV in Europe caused regional declines and temporary local eradications of European Blackbird populations. The 2011 outbreak in southwestern Germany (Becker et al., 2012) caused a 74% decline of local Blackbird breeding pairs near the city of Heidelberg. A garden bird watch project also revealed noticeable declines in and around the outbreak region (Bosch and Schmidt-Chanasit, 2011). In countries where dead Blackbirds tested USUVpositive, the breeding population declined by 34% (Ziegler et al., 2015). Further, model calculations predict periodic USUV outbreaks and in worstcase scenarios declines of Blackbird populations of up to 24% (Brugger and Rubel, 2009). Should this outbreak recapitulate the same characteristics, it is expected that specific avian populations in the affected area will face a significant reduction in size for a few years.

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