Short Report: Isolation of Usutu Virus in Germany

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Abstract. Usutu virus (USUV) is a mosquito-borne flavivirus that emerged 2001 in Austria and caused deaths in wild birds. In Germany, 70,378 female mosquitoes were captured in 2009 and 2010 and assayed for USUV. Virus was isolated in cell culture from one pool of *Culex pipiens pipiens* mosquitoes trapped exclusively in August 2010 in Weinheim, Germany. Subsequent phylogenetic analysis demonstrated a close relationship between the isolated USUV strain from Germany and a USUV strain from Austria, which was detected in a dead blackbird in 2004.

Usutu virus (USUV) is an arthropod-borne, single-stranded RNA virus and belongs to the *Japanese encephalitis* virus group within the family *Flaviviridae*. This virus was first isolated in 1959 from *Culex neavei* mosquitoes collected in Ndumu, Natal, South Africa.¹ Vectors of USUV are ornithophilic mosquitoes of the genera *Culex*, *Coquillettidia*, *Mansonia*, and *Culiseta*.¹

Wild birds are the principal reservoir for USUV and migratory birds play a key role in the introduction of USUV into new areas. Outside Africa, USUV emerged in 2001 in Vienna, Austria and caused deaths in blackbirds (*Turdus merula*) and great gray owls (*Strix nebulosa*).² In 2002, USUV was still circulating in Austria, demonstrating that USUV has managed to overwinter in a local bird-mosquito cycle in central Europe.³ More recently, USUV-specific RNA or antigen was also detected in birds or mosquitoes in Hungary, Switzerland, Italy, and Spain.^{4–6} In the summer of 2009, USUV-related illness were reported in two immunocompromised patients in Italy.^{7,8} Despite these cases, the potential of USUV to cause human disease needs further investigations.

In 2002, an extensive analysis of serum samples from healthy birds captured in the United Kingdom was performed and demonstrated that a significant number of the serum samples contained neutralizing antibodies against USUV.⁹ This study was subsequently followed by a seroconversion study of chickens from the time of hatching until the age of eight or nine months and seroconversion to USUV was detected.¹⁰ Neutralizing antibodies against USUV were also demonstrated in serum samples of wild birds captured during 2002–2005 in Germany.¹¹ In the United Kingdom and in Germany, no obvious reduction in the bird population was observed during this period. However, it was not known if USUV circulate in German mosquito populations. Therefore, the natural vectors of USUV in Germany also remain unknown.

Mosquitoes were collected during July–September 2009 and April–September 2010 at 11 sites in Germany (Figure 1). Trappings were made with CO₂-baited encephalitis vector surveillance traps (BioQuip, Compton, CA) and at the Weinheim trapping site (Figure 1) with gravid traps designed according to the Centers for Disease Control and Prevention (Atlanta, GA)

gravid trap model 1712 (John W. Hock Company, Gainesville, FL). All collected mosquitoes were frozen at –70°C and transported to the laboratory.

Mosquito identification, RNA extraction, and virus isolation were performed as described.^{12,13} Extracted RNA was analyzed by using a newly designed USUV-specific real-time reverse transcription–polymerase chain reaction (RT-PCR) with primers USUTU F (5′-CGTTCTCGACTTTGACTA-3′, nucleotide positions 3294–3311; nucleotide positions are given according to numbering in USUV strain Vienna, GenBank accession no. AY453411), USUTU R (5′-GCTAGTAGTAGTTCTTATGGA-3′, nucleotide positions 3384–3364), probe USUTU P (5′-FAM-ACCGTCACAATCACTGAAGCAT-BHQ1-3′, nucleotide positions 3325–3346, FAM, 6-carboxy-fluorescein; BHQ-1, black hole quencher 1). The target was a 91-nucleotide region of the nonstructural protein 1 gene. Real-time RT-PCR was used as described.^{12,13}

A total of 70,378 female mosquitoes (16,057 in 2009 and 54,321 in 2010) were captured, identified to species, and pooled according to species (25 mosquitoes per pool).¹⁴ The most abundant mosquito was *Aedes vexans* (46.6%) followed by *Ochlerotatus sticticus* (32.0%) and *Culex pipiens/Cx. torrentium* (15.0%) (Table 1). USUV-specific RNA was detected in one pool (pool no. 1477) of *Cx. pipiens/Cx. torrentium* captured in the middle of August 2010 at Weinheim (Figure 1). Compared with 2009, the number of collected *Cx. pipiens/Cx. torrentium* was nearly identical in 2010 (Table 1). The USUV infection rate was low because only 1 of 422 *Cx. pipiens/Cx. torrentium* pools from Weinheim was positive for USUV.

Morphologic species determination of the mosquitoes included in the pool that were positive for USUV was refined by sequence analysis of cytochrome oxidase c subunits 1 and 2 (CO1 and CO2) of mitochondrial DNA according to a published protocol.¹⁵ Sequence analysis of CO1 and CO2 demonstrated that the mosquitoes included in pool 1477 belong exclusively to *Cx. pipiens pipiens*. Inoculation of C6/36 cells with pool 1477 caused a cytopathic effect after 144 hours, and USUV-specific RNA was detected by real-time RT-PCR in the supernatant of the infected cell culture after five passages. Moreover, electron microscopy of the infected cell culture showed enveloped viral particles ≈60 nm in diameter.

For extensive phylogenetic analysis, a part of the envelope protein coding sequence (nt positions 1159–2527) was amplified by RT-PCR in two overlapping fragments with reported primers.³ Subsequent phylogenetic analysis by Bayesian inference showed a close relationship between novel USUV strain

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FIGURE 1. Locations of study sites in Germany and phylogenetic analysis of Usutu virus (USUV) strains. For each trapping site, proportions and number of collected mosquitoes are given as a pie chart. Mosquitoes in the pool that were positive for USUV were trapped at Weinheim (underlined). The Bayesian phylogenetic tree was inferred with MrBayes and is based on partial protein envelope nucleotide sequences (length = 1,215 nucleotides) of USUV. For each sequence, the GenBank accession number, strain designation, and strain origin are shown. Posterior probabilities are shown on each node, if higher than 0.50. Scale bar indicates number of nucleotide substitutions per site.

1477 and the USU338-04 strain from Austria, which detected in a dead blackbird (*Turdus merula*) in 2004 (Figure 1).

Our study demonstrates the potentially emerging USUV in southwestern Germany. The virus most likely spread from Austria to Germany rather than being independently introduced from Africa. This finding is the first direct detection of USUV in Germany, although neutralizing antibodies were detected in wild birds in Germany.¹¹ No obvious increase in deaths of birds were detected in Germany in 2009 and 2010, which suggested a low prevalence or virulence of circulating USUV. However, in Italy, USUV-related illness in humans was reported even though the USUV infection rate in mosquitoes was rather low.⁶

USUV strain 1477 was detected in a pool of *Cx. pipiens pipiens* mosquitoes in Weinheim, a small city in the upper Rhine valley and the only trapping site that represented an urban

ecosystem. The density of blackbirds and *Cx. pipiens pipiens* mosquitoes in such an urban ecosystems is much higher compared with an agro-ecosystems or forest. This might explain why USUV was not found in other regions of Germany covered by our trapping sites.

In conclusion, after detection of several arboviruses that are new to Germany in 2009^{12,13} another important arbovirus was demonstrated to circulate in Germany in 2010. Thus, mosquito-based surveillance for arboviruses in Germany is important for public health because it provides data about arbovirus activity and distribution. Further studies will be conducted to estimate the medical importance of USUV in southwestern Germany.

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TABLE 1
Mosquito species and numbers of females per species caught in Germany, 2009 and 2010

Mosquito species	No. (%) mosquitoes collected		
	2009	2010	Total
<i>Culex pipiens</i> / <i>Cx. torrentium</i> *	5,163 (32.2)	5,383 (9.9)	10,546 (15.0)
<i>Aedes vexans</i>	4,366 (27.2)	28,384 (52.3)	32,750 (46.6)
<i>Ae. cinereus</i>	311 (2.0)	2,329 (4.3)	2,640 (3.8)
<i>Ochlerotatus annulipes</i>	594 (3.7)	32 (< 0.1)	626 (0.8)
<i>Oc. cantans</i>	0 (0)	294 (0.5)	294 (0.4)
<i>Oc. communis</i>	0 (0)	82 (0.1)	82 (0.1)
<i>Oc. punctator</i>	0 (0)	41 (< 0.1)	41 (< 0.1)
<i>Oc. rusticus</i>	0 (0)	654 (1.2)	654 (0.9)
<i>Oc. sticticus</i>	1,086 (6.8)	16,280 (30.0)	17,366 (32.0)
<i>Culiseta annulata</i>	438 (2.7)	363 (0.7)	801 (1.1)
<i>Anopheles claviger</i>	3,626 (22.6)	288 (0.5)	3,914 (5.6)
<i>An. maculipennis</i>	473 (3.6)	164 (0.3)	637 (0.9)
<i>An. plumbeus</i>	0 (0)	27 (< 0.1)	27 (< 0.1)
Total	16,057	54,321	70,378

* Sibling species *Cx. pipiens* and *Cx. torrentium* were not tested separately because female adults cannot be distinguished morphologically.

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