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Short communication

Sindbis virus- a wild bird associated zoonotic arbovirus circulates in Germany

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ABSTRACT

Sindbis virus (SINV) is an arbovirus causing clinical symptoms such as arthritis, rash and fever following human infections in Fennoscandia. Its transmission cycle involves mosquito species as vectors as well as wild birds that act as natural reservoir hosts. In Germany, SINV was first time observed in 2009 in different mosquito species in the Upper Rhine valley and one year later in a hooded crow in Berlin. Recently, SINV was also detected repeatedly at various locations in Germany in the context of a mosquitoes monitoring program for arboviruses.

In this study, we detected for just the second time a SINV infection in a diseased wild bird (common wood pigeon) from Central Europe. SINV was isolated by cell culture and the complete SINV genome sequence was determined. Phylogenetic analyses revealed a close affiliation to SINV genotype I with a high similarity to human isolate sequences from Finland, Sweden and Russia. The isolate was genetically distinct from the first avian isolate suggesting the circulation of at least two different SINV strains in Germany.

In order to reveal the infection frequency in SINV positive mosquito regions 749 bird blood samples were assayed serologically and SINV antibodies found primarily in resident birds. SINV is therefore endemically circulating in mosquitoes in Germany, which results in occasional bird infections. No data are yet available on zoonotic transmission to humans.

1. Introduction

Sindbis virus (SINV) (family *Togaviridae*, genus *Alphavirus*) is a mosquito-borne and bird-associated zoonotic virus widely distributed in Africa, Eurasia, Australia, and New Zealand. SINV infections in humans are associated with fever, rash, myalgia, and arthralgia/arthritis (Adouchief et al., 2016). In northern Europe, symptomatic human SINV infections are known as Pogosta disease in Finland, Ockelbo disease in Sweden and Karelian fever in Russia (Kurkela et al., 2008; Lundstrom and Pfeffer, 2010; Adouchief et al., 2016). Human cases in Finland seem to peak presumably every seventh year (Brummer-Korvenkontio et al., 2002; Kurkela et al., 2008). SINV is mainly transmitted by ornithophilic mosquitoes of the genus *Culex* but also by *Culiseta*, *Aedes* and *Anopheles* species [for review see (Lwande et al., 2015; Adouchief et al., 2016)].

Wild birds represent important reservoir hosts and vectors of endemic or re-emerging zoonotic pathogens and are an essential part of transmission cycles in Central Europe (Hubalek, 2004). Regarding SINV, birds are the natural and main amplifying hosts (Lundstrom, 1999; Hubalek, 2008) and are also crucially involved in the spread of SINV to non-endemic areas. Phylogenetic analyses of SINV partial E2 glycoprotein as well as full-length nucleotide sequences originating from Finland and Sweden demonstrated that these viruses are closely related to SINV strains from Africa. Therefore, Lundstrom and Pfeffer

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(2010) proposed that SINV was introduced to northern Europe by migratory birds, which can act even as intercontinental carriers. A wide variety of bird species are naturally infected by SINV (Lundstrom et al., 1992, 2001), and in experimental studies birds from the orders Passeriformes, Galliformes and Anseriformes became highly viremic and are all capable of transmitting SINV to bridging vectors (Lundstrom et al., 1993).

In Germany, SINV was first isolated in 2009 in mosquito species (*Culex* spp., *Anopheles maculipennis* sensu lato) in the Upper Rhine valley (Jost et al., 2010), and one year later in a hooded crow (*Corvus corone cornix*) in Berlin (Eiden et al., 2014). Until now, autochthonous human SINV infections have not been reported in Germany. However, SINV-specific antibodies were detected in blood donors from southwest Germany in 2010/2011 (Jost et al., 2011).

In this study, we are reporting SINV infection in a common wood pigeon (*Columba palumbus*). Live virus was recovered from cell culture and complete genome sequences were subsequently obtained by next generation sequencing (NGS). Furthermore, an extensive serological investigation in more than 700 bird blood samples prior from SINV mosquitoes and bird positive regions was carried out.

2. Material and methods

2.1. Sample collection

In the framework of the German nation-wide wild bird surveillance network for zoonotic arthropod-borne virus infections migratory and resident birds were monitored annually. More than 1900 blood samples from 20 bird orders and 136 different bird species were collected and investigated between 2014 and 2016 in the frame of Usutu virus and West Nile virus monitoring (Michel et al., 2018). We used RNA extracted from all bird blood samples of this sample panel for virological investigations and selected a total of 382 serum samples from 2016 for specific serology. Furthermore, 367 serum samples collected in 2017, also coming from this network and originating from seven different regions in Germany, were included in this serological study. The selected samples for SINV serology were mainly from areas with RNA detection in birds and mosquitoes, but also adjacent regions and northern areas were included.

2.2. Quantitative real-time RT-PCR (qRT-PCR)

Viral RNA was extracted from deep frozen (−70 °C) cruor of the bird blood samples with an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as well as described by Michel et al., 2018, and was analyzed by qRT-PCR with SINV-specific primers and a probe targeting a 134-nucleotide region of the non-structural protein 1 (Jost et al., 2010).

2.3. Sequencing analysis

Viral RNA (bird sample V9002) was isolated from cell culture supernatant with TRIzol reagent (Invitrogen Carlsbad, CA, USA) and prepared for a sequencing library according to a recently published protocol (Steglich et al., 2013) but using Illumina adaptors (Illumina, San Diego, CA, USA). The resulting library was sequenced on an Illumina MiSeq sequencer with v2 chemistry. The obtained full-length recovered genome sequence of the virus was submitted to GenBank under the accession number MF543016.

2.4. Phylogenetic analysis

For phylogenetic analyses, full genome SINV sequences from GenBank and own next generation sequencing (NGS) data were aligned by Geneious software package R11 (version 11.1.5). A Maximum Clade Credibility tree was reconstructed using a Markov Chain Monte Carlo (MCMC) approach as implemented by the program BEAST v1.8, under a HKY + Γ + I model and otherwise default parameters. The MCMC chains were run for 10 million generations, and were sampled every 1000 steps. A maximum clade credibility tree was generated using Tree Annotator (BEAST package) with the initial 10% of steps removed as burn-in (Drummond et al., 2012).

2.5. Virus isolation in cell culture

Isolation studies employed African green monkey derived Vero 76 kidney cells and *Aedes albopictus* derived insect cells C6/36 (both obtained from Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Germany). Clarified supernatants of the SINV qRT-PCR-positive bird cruor sample homogenates were used to inoculate cell monolayers as described before by Eiden et al. (2014). Additionally, also 50 µl serum from the bird was used for cell inoculation.

2.6. Serological investigations

In the absence of a suitable ELISA for detection of SINV antibodies in birds all serum samples from wild birds were analysed in a specific virus neutralization test (VNT) by using SINV strain V6222 (acc. no. JX570540). VNT was performed as already described by Ziegler et al. (2015), only the virus strain (SINV), the applied cells (Vero 76) and the incubation time of 6 days were modified. All samples were run in duplicate and starting with a serum dilution of 1:10. The virus concentration was 100 TCID50/well. Cytopathic effects were seen 4–6 days post infection, and the neutralizing antibody titers (ND_{50}) were expressed as the reciprocal of the serum dilution that still inhibited > 50% of cytopathogenic effect, calculated according to the Behrens-Kaerber method (Mayr et al., 1977). Serum samples with ND_{50} values above 10 were judged as positive and samples with lower titers as negative.

2.7. Ethical statement

Blood samples were taken during routine clinical examination of injured, diseased or orphaned wild birds which had been admitted to different bird clinics, bird veterinarians or wild bird rescue-stations and were made available to the German nation-wide wild bird surveillance network for zoonotic arthropod-borne virus infections. The supernumerary blood material from the birds was used for this project.

3. Results

A total of 1962 blood samples from migratory and resident birds were collected in the three-year period (2014-2016) and RNA could be extracted from 1902 of these samples (Michel et al., 2018). By this approach we were able to detect SINV RNA by qRT-PCR with a ct-value of about 17.8 from a common wood pigeon (*Columba palumbus*) sampled in the city of Giessen in August 2016. This juvenile bird was suffering from clinical symptoms such as head tilt to one side, internal bleedings into the anterior chamber of the right eye (a so-called hyphema) and neurological symptoms such as convulsions suggesting a history of severe collision trauma. The animal was well nourished and did not show any external injuries (Fig. 1). Blood was drawn before it had to be euthanized.

SINV was isolated from blood of this bird in Vero 76 cells displaying a distinctive cytopathogenic effect (CPE) 2-4 days post infection (dpi) with characteristic massive cell detachment and lysis. In contrast, C6/36 cells developed no CPE, but successfully amplified SINV (starting at 2d p.i.) as determined by qRT-PCR (on tissue culture supernatant) and subsequent passage to Vero cells (inducing a characteristic CPE within 2 days). NGS sequencing of these isolates from the common wood pigeon produced a full-length virus genome sequence (acc.nr. MF543016) encompassing 11,710 nucleotides. Phylogenetic analysis of the complete

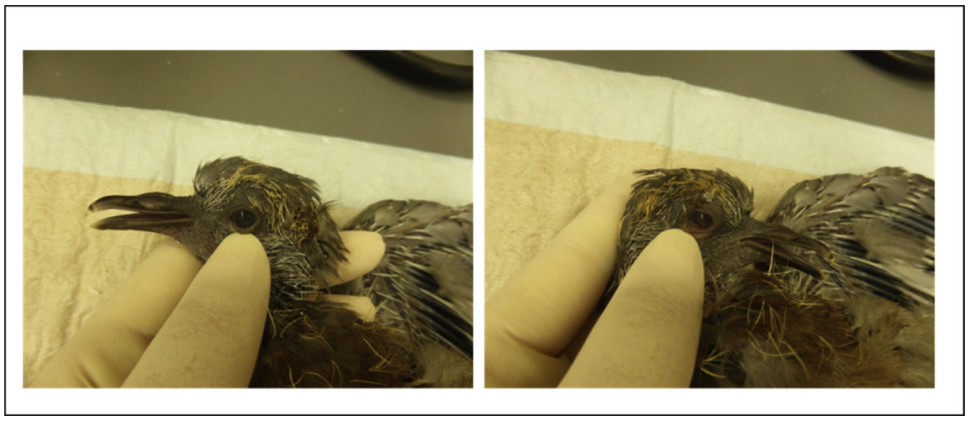

Fig. 1. This juvenile common wood pigeon (*Columba palumbus*) was found diseased in the city of Giessen in August 2016, characterized by head tilt to one side, hyphema of the right eye and further neurological symptoms such as convulsions. The animal was well nourished and did not show any external injuries otherwise. Blood was drawn from it before euthanasia.

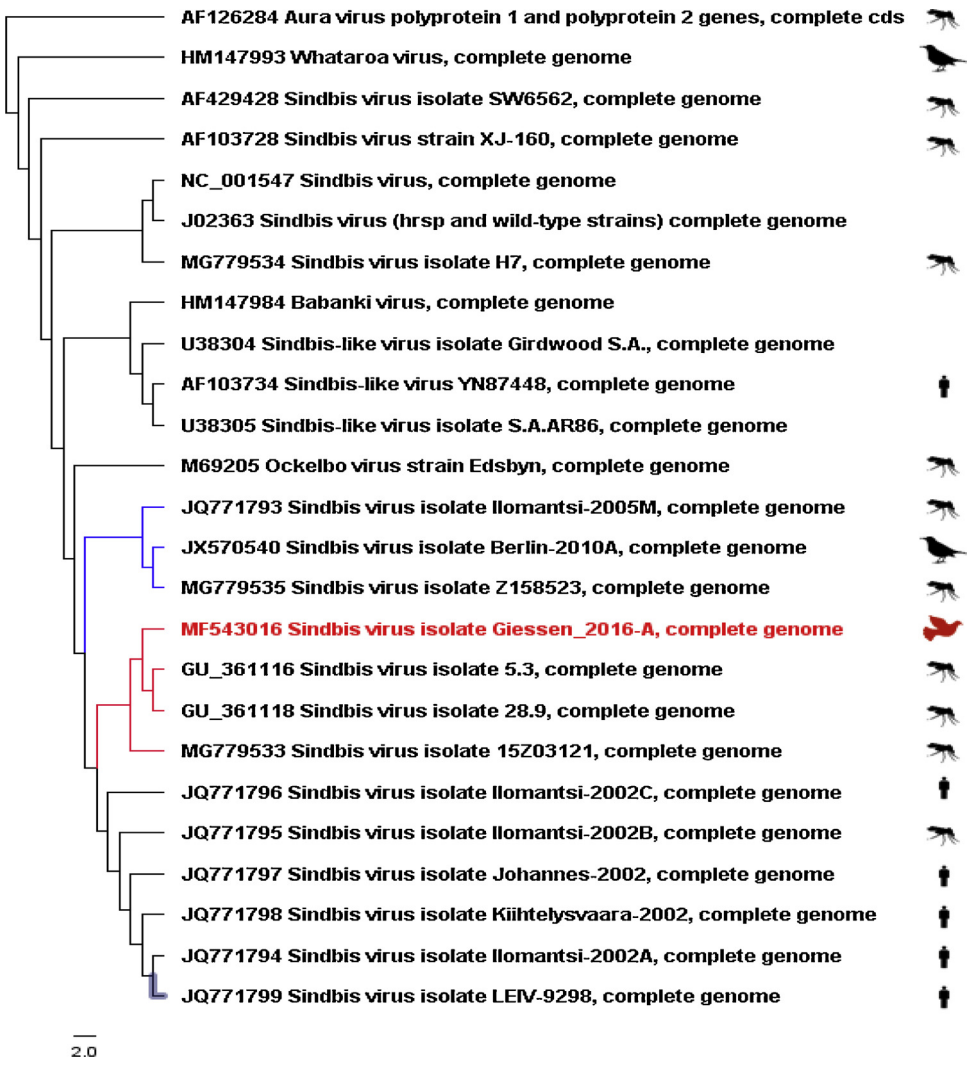

Fig. 2. Phylogenetic tree based on full-genome sequences of SINV. The tree is based on full genome sequences of Sindbis virus isolates obtained from European samples (human, bird, mosquito). Icons = original source of the virus isolate. Red text marks the isolate of this study. For better visualization, the branches of the phylogenetic groups of the SINV isolated from a hooded crow (blue) and a common wood pigeon (red) are colored.

SINV genome revealed a close similarity of this bird isolate to viruses found in mosquitoes in the Upper Rhine valley and differences to the SINV sequence from the hooded crow and from mosquitoes found in Berlin before. Furthermore, phylogenetic analyses demonstrated that the SINV isolate MF543016 clusters within the SINV genotype I as found in Finland, Sweden and Russia (for more details see Fig. 2).

Serological studies were performed with 749 bird serum samples collected in 2016 and 2017, which originated from seven different regions in Germany (Fig. 3), but were not equally distributed in both year. The serological evaluation of the wild bird samples was carried out annually and for the individual local areas of their collection (Fig. 3). Ten out of 186 serum samples from collection sites in/near Giessen (region no. 6) and collected in 2016 contained specific neutralizing antibodies against SINV. The titres ranged from 1/10 to 1/60 and were

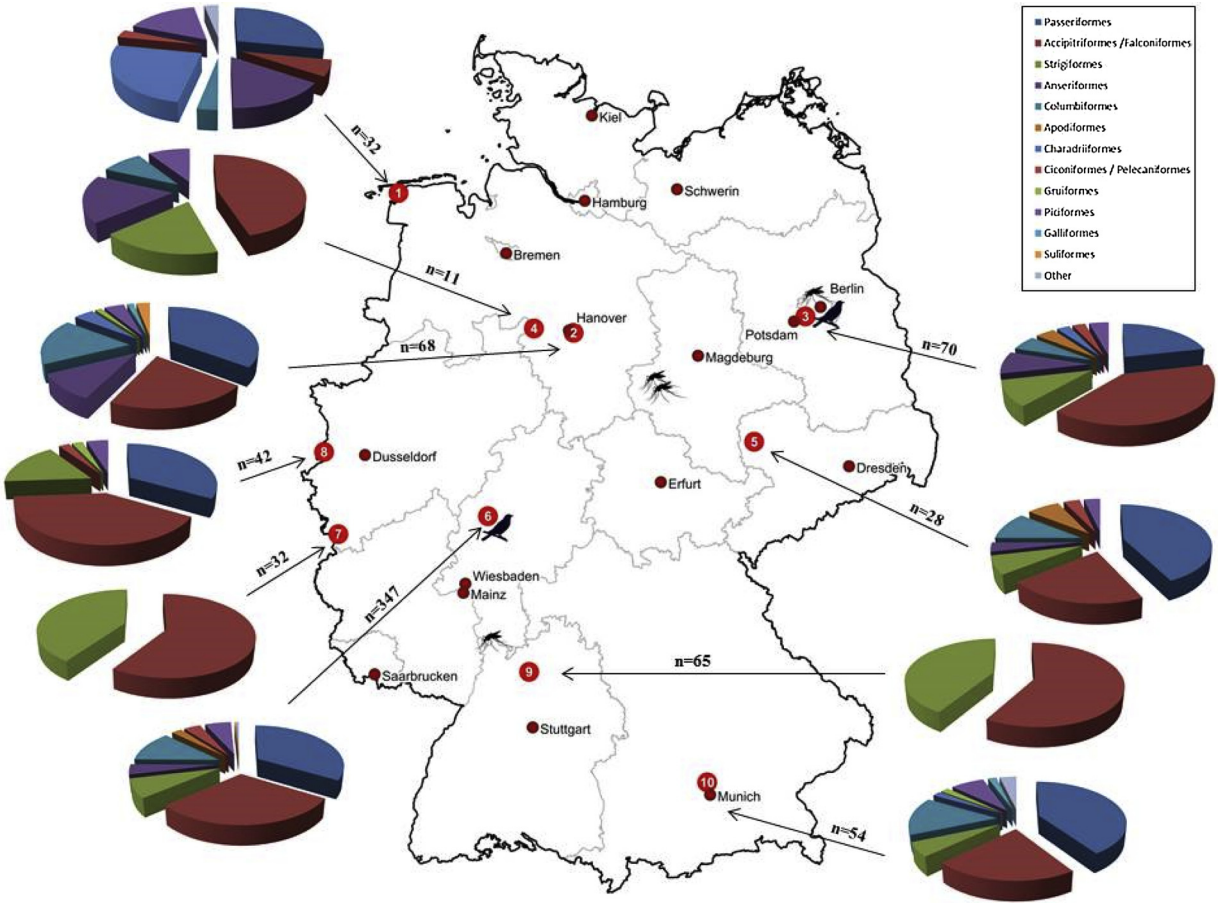

Fig. 3. Sampling sites (sorted by numbers 1–10) and zoological orders of wild birds from which blood samples were collected from 2016 to 2017 (n = number of individuals tested per sites). From the sites with numbers 4, 7 and 9 blood samples were only collected in 2016, whereas at the sites with numbers 1, 5 and 8 sample collection was done only in 2017. Furthermore, the detection sites of SINV positive mosquitoes (mosquito-icon) and birds (bird-icon) are embedded in the map. The location of the SINV positive mosquitoes was described by Jöst et al. (2010) and Scheuch et al. (2018). The SINV positive bird in Berlin was described by Eiden et al. (2014) and the SINV positive bird in Giessen is part of this current report.

detected for example in one common buzzard (*Buteo buteo*), one Northern goshawk (*Accipiter gentilis*), two Eurasian magpies (*Pica pica*) and two carrion crows (*Corvus corone*). Furthermore, in a falconry in western Germany (region no. 7) three of 32 birds of prey had SINV specific neutralizing antibodies. A similar picture was found in a falconry in Baden-Württemberg (region no. 9), where eight of 65 birds of prey and owls were tested serologically positive. Their specific neutralizing antibody titres ranged from 1/20 to 1/160. More SINV-antibody positive birds were found in Berlin (region no. 3, one common buzzard of 65 examined birds) and in Munich (region no. 10, one feral pigeon of 10 examined birds). Serological results for birds tested in 2017 were similar: two of 161 serum samples from Giessen (no. 6) carried SINV specific neutralizing antibodies, five of 44 sera from the Munich area (no. 10, titres ranging from 1/10 to 1/40) and one (European kestrel) of 28 birds coming from the Leipzig area (no. 5).

Unfortunately, it was not possible to obtain samples from the two falconries with seropositive birds in 2017 again. Details on sample number per region, bird species and ND_{50} titres are summarized in Tables 1 and 2. These results for primarily resident bird species reveal an enzootic circulation of SINV in mosquitoes or birds in these particular regions of Germany.

4. Discussion

Surveillance data for SINV infections in Germany were hardly available until recently. The first indication of an ongoing SINV circulation was obtained, when 16,057 mosquitoes were trapped in Southwest Germany in summer 2009 and eight arthropod pools, all originated from the city Weinheim, were tested positive (Jost et al., 2010). Recently, we have reported the first SINV infection in a wild bird, a hooded crow, in Germany (Eiden et al., 2014). Until now, autochthonous SINV infections in humans have not yet been reported in Germany.

When a total of 97,648 mosquitoes were eventually collected from 2011 to 2016 throughout Germany and were assayed for arbovirus infections. SINV positive mosquitoes were detected in three different regions of Germany (in Halberstadt and Quedlinburg, cities in the German federal state of Saxony-Anhalt, and in Berlin). This allowed full genome sequencing of mosquito isolates (Scheuch et al., 2018). As a result, different full genome sequences from mosquitoes and from birds are now available for the phylogenetic studies.

SINV found so far in birds and mosquitoes from Germany all belong to the Palearctic/Ethiopian cluster and are distinct from the second known Oriental/Australian SINV cluster (Sammels et al., 1999; Liang et al., 2000). However, there seem to be two different transmission lines in Germany as the common wood pigeon isolate in combination with the Upper Rhine valley mosquito sequences (acc. no. GU361116 and GU 361118, red line in the Fig. 2) were clearly distinct from the hooded crow isolate (acc. no. JX570540) and corresponding mosquito sequences from Berlin (acc. no. MG779535, blue line in the Fig. 2).

The occurrence of two separated transmission lines and foci was further substantiated by the serological findings of this investigations, where locally limited serological reagents were found among the bird panel examined, mainly in short-distance migrants, partial migrants

Table 1
SINV positive neutralization assay results from wild bird blood samples in 2016, neutralisation titres in brackets. The classification of the numbers of the local regions corresponds to the representation in Fig. 3.

Local region	Order	Common name	Scientific name	Migration pattern	SINV antibody positive (ND50)
no. 3	Accipitriformes	Common Buzzard	*Buteo buteo*	R, P, S	1 (10)
no. 6	Anseriformes	Northern Mallard Duck	*Anas platyrhynchos*	R, P, S	1 (15)
	Accipitriformes	Common Buzzard	*Buteo buteo*	R, P, S	1 (10)
	Accipitriformes	Northern Goshawk	*Accipiter gentilis*	R, P	1 (15)
	Passeriformes	Eurasian Magpie	*Pica pica*	R	2 (15, 20)
	Passeriformes	Carrion Crow	*Corvus corone*	P, S	2 (10, 20)
	Pelecaniformes	Grey Heron	*Ardea cinerea*	R, P, S	2 (15, 60)
	Strigiformes	Eurasian Eagle Owl	*Bubo bubo*	R	1 (20)
no. 7	Accipitriformes	Golden Eagle	*Aquila chrysaetos*	zoo bird	1 (30)
	Accipitriformes	Black Kite	*Milvus migrans*	zoo bird	2 (10, 20)
no. 9	Accipitriformes	White-tailed Sea-eagle	*Haliaeetus albicilla*	zoo bird	4 (30, 50, 120, 160)
	Accipitriformes	Bald Eagle	*Haliaeetus leucocephalus*	zoo bird	1 (50)
	Accipitriformes	Steppe Eagle	*Aquila nipalensis*	zoo bird	1 (20)
	Accipitriformes	Griffon Vulture	*Gyps fulvus*	zoo bird	1 (20)
	Strigiformes	Great Grey Owl	*Strix nebulosa*	zoo bird	1 (25)
no. 10	Columbiformes	Feral Pigeon	*Columba livia f. domestica*	R, (P)	1 (10)

R = resident species, P = partial migrant, S = short distance migrant, L = long distance migrant.

and resident birds. In Sweden, it could be shown that passerines are the most important hosts for SINV, with thrushes as resident birds identified as the main amplifying hosts (Lundstrom et al., 2001). Furthermore, in experimental infection studies in birds of the order Passeriformes, Galliformes and Anseriformes a highly viremic phase was shown which render them capable of functioning as bridging vectors (Lundstrom et al., 1993). Resident birds were also confirmed as SINV hosts by our serologically investigations, where e.g. clearly antibody positive reagents were found among birds of prey and owls of the falconry in Baden-Württemberg (no. 9), not far away from the positive mosquito detection in the Upper Rhine Valley in 2009 (Jost et al., 2010). Similar hot spots of virus circulation were found in this study in the region of Giessen (no. 6), where serologically positive birds (prior resident birds) and the virus-positive juvenile pigeon were detected. Furthermore, local circulation of the virus is assumed in the region of Berlin (no. 3), where a SINV positive bird (Eiden et al., 2014) and mosquitoes were coevally detected (Scheuch et al., 2018), in Western Germany at the border to Belgium (no. 7) and in the area of Munich (no. 10) as well as in the region of Leipzig (no. 5). Unfortunately, no SINV RNA could be detected in the blood samples from 2017 (data not shown). Furthermore, the bird sample panel used for serological investigation of the birds in 2017 did not include the two falconries and additional samples from some local regions, partly due to restrictions imposed by ongoing avian influenza outbreaks.

Fifty different mosquito species are known to occur in Germany and several of them are competent vectors for a variety of pathogens (Becker et al., 2014). Recently, *Culex torrentium* and *Culex pipiens* mosquitoes from Northern Sweden were confirmed as potent SINV vectors by experimental challenge studies (Lwande et al., 2019). *Culex torrentium* and *Culex pipiens* mosquitoes are also present in Germany, and the SINV vector competence of other indigenous mosquitoes in Germany should be reviewed.

Knowledge about SINV infections in birds is very scant for Germany, our investigations are still in their infancy. Also, hardly any data for human infections in Germany exist, making it impossible to take conclusions about the epidemiology of SINV infections in this country, for example to judge whether there is also a 7-year periodicity as in Fennoscandia (Kurkela et al., 2008).

Therefore, further virological and serological testing of wild birds via the existing German nation-wide wild bird surveillance network for zoonotic arthropod-borne virus infections should continue for the coming years and include also SINV, because these are essential to detect a circulation of the virus and to allow a risk assessment for zoonotic pathogens. Especially the unusually hot climatic conditions in the summer of 2018 with an extremely long period of high temperatures over months may have provided favorable conditions for a further distribution of the virus between mosquitoes and birds.

5. Conclusion

Our findings give clear evidence for a broad spatial distribution of SINV in Europe. This view is also supported by SINV antibody prevalences in humans and animals from Austria, Belarus, Czech Republic, Slovakia, Poland, and the UK (Hubalek, 2008). Furthermore, a cumulative occurrence of SINV in mosquitoes in different regions of Germany has been found by mosquito monitoring programs since 2012 (Scheuch et al., 2018). The fact that the virus has been detected for the second time in a short-range migratory/particularly resident bird highlights the role of these birds for the maintenance of SINV and other zoonotic viruses. Furthermore, specific SINV-antibodies in resident birds demonstrate the currently local circulation of this virus in different regions in Germany between wild birds and mosquitoes.

Monitoring programs of wild birds as well as for mosquitoes and other hosts need to be continued to assess the human and animal

Table 2
SINV positive neutralization assay results from wild bird blood samples in 2017, neutralisation titres in brackets. The classification of the numbers of the local regions corresponds to the representation in Fig. 3.

Local region	Order	Common name	Scientific name	Migration pattern	SINV antibody positive (ND50)
no. 5	Falconiformes	European Kestrel	*Falco tinnunculus*	R, P, S	1 (10)
no. 6	Columbiformes	Common Wood Pigeon	*Columba palumbus*	R, P, S	1 (10)
	Passeriformes	Thrush	*Turdus sp.*	S, L	1 (10)
no. 10	Passeriformes	Eurasian Blackbird	*Turdus merula*	R, P	2 (10, 40)
	Passeriformes	Carrion Crow	*Corvus corone*	P, S	2 (10, 30)
	Galliformes	Common Pheasant	*Phasianus colchicus*	R	1 (10)

R = resident species, P = partial migrant, S = short distance migrant, L = long distance migrant.

infection risk for the zoonotic SINV in Germany.

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Declaration of Competing Interest

None.

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