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Review article

Mosquito borne virus USUTU as potential threat to human health

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ABSTRACT. USUV in Europe is detected in vectors (mosquitoes) and has a reservoir in vertebrates. There are known fatal epidemics among birds, especially blackbirds. Currently, USUV also causes rare infections in humans. However, the emergence of clinical cases, including severe neurological symptoms, and the finding of seroprevalence in asymptomatic people (e.g. blood donors, forest workers), indicate that USUV, due to its neurotropism, may become a potential public health problem. Therefore, it is very important to monitor cases infections in humans, migratory and resident birds and other animals that may constitute a reservoir of the virus, but also detection of the virus in mosquitoes (vectors), including alien and invasive species, as well as the impact of climatic factors on the ability to spread the virus in the Europe. There is currently no evidence of virus transmission during transfusion or transplantation, but the potential risk of virus transmission from an asymptomatic blood donor to an immunocompromised recipient must be considered. Although the occurrence of USUV in European countries is currently not a significant threat, surveillance and screening of blood donors for USUV should be carried out during the period of vector activity and during WNV epidemics, as well as in patients with symptoms of meningitis and encephalitis.

Keywords: USUV, human vector borne diseases, vectors, reservoir

Introduction

The climate change and the increased international travelling, trade, and transport raise the risk of introduction and/or settlement in a new places a new species of vectors (i.e. exotic mosquito species) and new pathogens (i.e. exotic viruses) into Europe. One of the alien pathogen transferred from Africa to Europe is the Usutu virus (USUV). USUV is an arbovirus (arthropod borne virus), which means that invertebrates i.e. mosquitoes, ticks, serve as a vectors to transmit them to vertebrates hosts [1]. Because the principal vertebrate hosts of USUV are birds, migratory species of birds may play an important role in the geographic spread of this virus and climate changes associated with milder winters can allow the virus to survive and create new local spread cycles in temperate climates areas with the local vector, host and reservoir species [2]. Especially, in a new area of occurrence, in Europe, the zoonotic potential of USUV is revealed in an increasing number of human cases, ranging from asymptomatic infections, through mild symptoms, to severe cases of neuroinvasion [3–5]. Due to the zoonotic nature of USUV, it is very important not only to monitor cases of infection in humans, but also to detect viruses in mosquitoes (vectors), including invasive alien species. In addition, monitoring through screening of potential local hosts and reservoirs like birds, both migratory and resident, and other animals, including: horses [1,3,6].

This mosquito borne virus belonging to the family Flaviviridae and genus *Flavivirus*, which currently contains 53 species (https://talk.ictvonline.org/taxonomy/accsess 24.02.2023), and is included to Japanese encephalitis virus serocomplex [7–11], closely related to Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV) and West Nile Virus (WNV) [9,12–14]. Antigenically (i.e. similar envelope antigens) and phylogenetically, the closest relative of USUV is WNV [15–17] but according to Gaibani et al. [18] genetically USUV is closer to

JEV than WNV. USUV and WNV have a similar ecology and their cocirculation in the environment has been frequently observed [16]. Moreover USUV and WNV cause similar clinical symptoms, uses the same vectors, mainly ornitophilic *Culex,* the main host are birds and mammals like horses are dead-end hosts. Mammals can become infected but do not develop sufficient viraemia to sustain transmission therefore they are unable to spread both viruses further [15].

The aim of this paper is to collect and summarize information on USUV's entry routes into Europe, as well as its presence and spread in Europe in mosquitoes as well as vertebrates, especially in humans. And also to draw attention to the need to collect information on the symptoms of infection in humans and the need to take into account and differentiate in the diagnosis of new pathogens that, due to climate change, may affect the health of humans and animals outside their typical areas of occurrence.

Structure and diversity

USUV it is small spherical, enveloped virus, with a diameter 40–60 nm. It has 11 kb single-stranded, positive sense RNA (ssRNA) genome [15,19,20]. The virion includes three structural proteins: capsid (C), pre-membrane/membrane (prM) and envelope (E) [19,21–24]. Moreover eight nonstructural proteins as NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B and NS5 are present [16,25]. As reported by Gaibani and Rossi [20], the capsid protein forms the central core of the virion and is associated with the viral RNA. The envelope glycoprotein mediates binding to the host cells and promotes viral entry into the host cells. The pro-membrane protein are involved in virion formation and maturation by assisting envelope folding [20]. The nonstructural proteins serve regulatory and enzymatic functions during infection including regulating replication, virion maturation or inhibition of interferon response [15,19,26,27]. Virus replication takes place in the cytoplasm of infected host cells [16].

Several strains of USUV was identified outside Africa, especially from Europe [13,14] but also from Israel [25]. According to Engel et al. [28] the evolution of the USUV in Africa shaped the genetic diversity of European lineages, and the genetic diversity of European lineages is shaped primarily by in situ evolution, while the African lineages have been driven by extensive gene flow [28]. As a result of phylogenetic studies eight lineages named based on analysis of the nucleotide sequence of the NS5 protein and the geographical origin were separate [17]. The last new lineage has been identified in Germany in 2016 and named Europe 5 [29,30]. Currently, the USUV Africa 1–3 and Europe 1–5 lineages are distinguished [3,15,28,29,31–33]. Seven lineages, Europe 1–5 and Africa 2–3, circulate in Europe [3,82,95]. Only Africa 1 has not been detected outside of its original African habitat [3,30].

Occurrence and distribution

USUV is originated from sub-Saharan Africa and have been introduced into Europe [7,8] and Middle East [34]. It was first isolated by McIntosh from mosquito *Culex neavei*, originally described as *Cx. univitattus*, in the South Africa in 1959 as part of a study on the prevalence of viruses in arthropods and named for nearby the Usutu River in Eswatini, formerly Swaziland [3,16,35,36]. The Usutu virus is suspected to have emerged in Africa some 500 years ago and is gradually spreading [28,35].

Because the USUV strains from Europe and from Africa share high identity, undoubtedly the major migration routes of migratory birds play an important role in the continental and intercontinental spread of USUV and their introducing to the Europe [1,6,14,33,37,38] or Israel [25,34,39]. Engel et al. [28] suggest that the introduction of the virus from Africa into Europe has been taking place regularly over the past 50 years. Migratory birds have been infected in endemic areas in Africa and transmitted USUV along an eastern Atlantic migratory route and Black Sea/Mediterranean route [16,36,40,41]. Similarly, the introduction of USUV from Africa to Israel may be linked to bird migration via the Black Sea-Mediterranean or West Asia/East Africa migratory routes [25]. The results of phylogeographic analysis conducted by Siljic et al. [25] confirm that the evolution of USUV has been shaped by long-distance migration routes of birds (hosts and reservoir) between continents. Nikolay et al. [35] points out species which may participate in the transfer of USUV from Africa to Europe, such as the kestrel (*Falco tinnunculus*), the reed warbler (*Acrocephalus scirpaceus*), the marsh harrier (*Circus aeruginosus*), the barn-swallow (*Hirundo rustica*), the lesser whitethroat (*Sylvia curruca*), the common whitethroat (*Sylvia communis*), the garden warbler (*Sylvia borin*), the house martin (*Delichon urbica*), and the pied flycatcher (*Ficedula hypoleuca*). After reaching Europe the virus has adapted to a new environment where it can spread and overwinter in local mosquito species and reservoir host [6,38,42,43]. Llopis et al. [38] indicate that the presence of USUV antibodies in a resident species like a rock pigeon confirms local circulation of the virus. They also point out that short-distance migrating birds spread USUV to other areas of the Europe, including neighboring countries. Moreover increasing circulation European and African USUV lineages indicates the possibility of multiple introduction through migrating birds returning from African wintering grounds and still happening because African lineages was newly detected e.g. in Germany or the Camargue region in France [3,29,44].

In Africa, USUV RNA or antibodies have been found in mosquitoes, wild and domestic birds and mammals, including humans. Data come from South Africa, Senegal, Central African Republic, Uganda, Burkina Faso, Ivory Coast [29], Nigeria [44,45], Kenya [46], Morocco [16,35], Tunisia, Mali and Madagascar [47].

In 2001, USUV was first detected in Europe in Austria when caused the mass death of birds inhabiting the urban environment [16,48], especially common blackbirds. Although subsequent analysis of the stored samples indicated that the USUV appeared in Europe earlier, causing the death of blackbirds in Italian Tuscany in 1996 [15,49]. The USUV has shown the ability to spread across a large part of Europe, with circulation over the years in new areas [32,50]. In the following years USUV has been isolated or detected antibodies by serologic analysis in mosquitoes, birds, bats and rarely in other mammals, also humans. Data comes from, among others, European countries, such as Hungary [51–54], Switzerland [55], Italy [12,32,56], Spain [4,57,58], Germany [59–63], Poland [64–66], Czech Republic [67,68], United Kingdom [69,70], Belgium [31,71], the Netherlands [72], Greece [73], France [40,74–77], Croatia [78–80], Serbia [81] and Slovakia [82]. Outside of Africa and Europe this virus has been detected also in Israel [34,39].`

Some researchers indicate that there is a risk of spreading USUV to Asia and North America as well. Martín-Acebes et al. [83] suggest that there is a possibility of USUV spreading to East Asian countries as a result of the migration of infected birds. Although Usutu virus has not been detected in North America, there is a potential risk of introduction and circulation because of similar vectors, amplifying bird hosts, and climates to Europe [84]. Especially since another Flavivirus, the West Nile virus, with which co-occurrence of USUV was observed in Europe [85], was introduced to United States in 1999, and spread rapidly across continent till Canada but also to the South America. In Americas WNV caused massive bird mortality, human cases of encephalitis and horses cases was also noted [86].

Life cycle of USUV

A natural enzootic transmission cycle of USUV involves ornithophilic mosquitoes *i.e. Culex pipiens* complex as vectors and birds as host reservoir. In Europe, they are mainly Eurasian blackbirds (*Turdus merula*), Eurasian magpies (*Pica pica*), house sparrows (*Passer domesticus*), and Strigiformes, such as the Great Gray Owl (*Strix nebulosa*) as amplifying hosts [2,16,36]. Just like the West Nile Virus (WNV) it can also infect mammals, including humans [8] who act as dead-end hosts. The dynamics of the mosquito population and their activity as well as the virus reproduction rate depend strongly on the environmental factors like temperature and humidity. Efficient USUV transmission requires warm temperatures because at high temperature increase the reproduction and biting rates of mosquitoes and decrease the extrinsic incubation period [2]. In African countries USUV was isolated from vectors especially in the rainy season [35]. *Cx. pipiens* is the main vector in both tropical and temperate zones [87].

In temperate regions, USUV showed a high correlation with temperature. In regions were USUV and WNV cooccurred, transmission is more effectively with increase of temperature for USUV. Froz et al. [88] showed that in northwestern Europe, mosquitoes are very effective vectors for USUV and WNV, with similar infection rates of 11% at 18°C and 53% at 23°C. With further temperature increase, efficiency increases only for USUV. At 28°C *C. pipiens* is more competent for USUV than for WNV, respectively 90% and only 58% [15,29,88]. Moreover Froz et al. [88] showed in their study that USUV not only infects *C. pipiens*, but also efficiently distributes and accumulates in its saliva. Holicki et al. [36] reported that *Culex pipiens* biotyp *molestus* and *Cx. torrentium* are competetnt for USUV and viable virus was observed in their saliva 21 days after infection. While *Ae. aegypti* have low infection rates and lack of virus in saliva. They also concluded that because of high abundances of *Cx. pipiens* biotype molestus and *Cx. torrentium* in Central and Northern Europe, these species play an important role in the spread, maintenance, and introduction of USUV [36].

Mosquito vector

USUV was isolated first of all from ornithophilic mosquitoes from genus *CuIex* [50]. They bite mostly birds, but also other hosts like reptiles, amphibians, humans or other mammals.

In African countries viruses were mainly isolated from *Cx. neavei* [28,35,87,89,90] and a lesser extent from *Cx. perfuscus, Cx. quinquefasciatus, Aedes minutus, Mansonia africana* and *Coquillettidia aurites* [9,28,87,89]. In Senegal USUV was isolated also from *Cx. perfuscus* and *Aedes minutus*, especially in the rainy season. *Cx. perfuscus* was noted as host of Usutu virus also in Central African Republic [35,90]. In Senegal virus occur also in *C. antennatus* [47]. USUV was also reported also from *Cx. quinquefasciatus* in Ivory Coast [35,90], the ornitophilic *Coquillettidia aurites* in Uganda and *Mansonia africana* preferring mammals, including humans, in Central African Republic [35]. In Kenya a single isolated was recovered in *Cx. pipiens* [46,90]. USUV also was detected in *Manasonia aurites* in Uganda [8,16].

In Europe *Cx. pipiens* is the main vector of this virus and natural infections of both *Culex* biotypes, *molestus* and *pipiens*, were reported in Europe during the Usutu epidemic [9,87,91]. USUV was isolated from *Cx. pipiens* in Germany, Spain, Italy [3,6,40,46,92–94], Greece [73], Serbia [3,81,95], France [3,40,76,96], Croatia, Czech Republic, Slovakia [82], Switzerland [3,96]. USUV-positive *Cx. pipiens s.l.* have also been collected in Austria, France, Italy, Serbia, Spain, and Switzerland [36]. Virus was also found in other species of mosquitoes as well *Culex perexiguus* from Spain [3,46,97], *Culex modestus* in Czech Republic [3,19,46,60,98], France [96], Germany [36], Serbia [36], *Cx. torrentium* in Germany [34,36], Austria, Switzerland [3], invasive *Aedes albopictus* in Italy [3,6,9,19,32,35,89,92,93,95,99], Croatia [3,95], France [96]. In Austria [91,100,101] and France [96] USUV was found in invasive species *Aedes japonicus japonicus* [95]. RNA of USUV was detected in *Culex hortensis, Culex territans, Culiseta annulata, Aedes vexans*, and *Aedes rossicus* from Austria [1,41], *Ochlerotatus caspius*, *Ochlerotatus detritus*, *Anopheles maculipennis s.l.* from France [96] and Italy [3,93], *Culiseta annulata* from Italy [3,93]. USUV was also isolated in species as *Anopheles plumbeus, Coquillettidia richiardii*, and some *Ochlerotatus* species in Austria and Italy [29]. USUV was not detected in any species of mosquito from Poland [3].

Outside Africa and Europe, in three species of mosquitoes: *Cx. perexigus*, *Cx. pipiens*, and *Ae. albopictus* USUV RNA was found in Israel [16,34,39].

Due to the wide spread of *Cx. neavei* on the African continent, it may play a key role in the spread and endemic transmission of USUV, especially since its role in transmitting other flaviviruses such as WNV is known [89]. *Cx. neavei* may play a major role in a mosquito-bird transmission cycle in Africa because of their biting activity at night, and its high abundance in the tree canopy, which coincides with roost and nest behavior of birds [89].

As for Europe studies of Mancini et al. [93] showing that *Cx. pipiens* s.l. is most involved in the circulation of WNV and USUV in Italy and retains the viruses over the winter. This creates the possibility of local circulation of the virus. Based on other experimental research by Puggioli et al. [9] *Ae. albopictus*, an invasive species colonizing Europe and known to be highly anthropophilic and being a vector of several arboviruses, has a low vector competence for USUV as yet, though a replication in the mosquito body can occur. *Cx. pipiens,* one of the most abundant species in the northern hemisphere and the main vector for USUV in Europe because of urban occurrence has been known as a bridge vector between birds and humans for another flavivirus, West Nile Virus [2,11,89,93,95]. *Cx. modestus* and *Cx. perexiguus* are an important vector in the wetlands of southern Europe [95]. C*x. quinquefasciatus* belonging to the *Cx. pipiens* species complex is the most anthropophilic among African USUV vectors [46]. It occurs in the vicinity of human settlements and their population dynamic not related to the rainy season because it can colonize artificial containers [35]. Since *Cx. pipiens*, like *Cx. quinquefasciatus*, are found in an urbanized environment, they can play a key role in the transmission of the virus also

Table 1. Confirmed presence of USUV or antibodies in birds (wild, farmed and captive from zoo aviary) by European country based on literature data

Country	Birds
Austria	Blackbird *Turdus merula*, Barn swallow *Hirundo rustica*, Black redstart *Phoenicurus ochruros*, bearded reedlings *Panurus biarmicus,* Blue tit *Cyanistes caeruleus,* Common reed bunting *Emberiza schoeniclus*, Eurasian blackcap *Sylvia atricapilla*, Eurasian jay *Garrulus glandarius*, Eurasian nuthatch *Sitta europaea*, European pied flycatcher *Ficedula hypoleuca*, European robin *Erithacus rubecula*, Garden warbler *Sylvia borin*, Great tit *Parus major*, Hooded crow *Corvus cornix*, House martin *Delichon urbica*, house sparrow *Passer domesticus,* Jackdaw *Corvus monedula*, Lesser whitethroat *Sylvia curruca*, Reed warbler *Acrocephalus scirpaceus*, Song thrushes *Turdus philomelos*, Whitethroat *Sylvia communis*, Barn owl *Tyto alba*, Eagle owl *Bubo bubo*, great grey owls *Strix nebulosa* Long-eared owl *Asio otus*, great grey owl *Strix nebulosa,* Tawny owl *Strix aluco*, Ural owl *Strix uralensis*, Common pheasants *Phasianus colchicus*, Indian peafowl *Pavo cristatus*, Beared vulture *Gypaetus barbatus*, Marsh harrier *Circus aeruginosus*, Eurasian collared dove *Streptopelia decaocto*, Krestel *Falco tinnunculus*, Great spotted woodpecker *Dendrocopos major*, azure-winged magpies *Cyanopica cyanus*
Belgium	Blackbird *Turdus merula*, European robin *Erythacus rubecula*, house sparrow *Passer domesticus*, Eurasian bullfinch *Pyrrhula pyrrhula*, Great spotted woodpecker *Dendrocopos major*, chaffinch *Fringilla coelebs*, common scoters *Melanitta nigra*
Croatia	Blackbird *Turdus merula*
Czech Republic	Blackbird *Turdus merula*, Eurasian coot *Fulica atra*
France	Blackbirdm *Turdus merula*, Magpie *Pica pica*, greater rhea *Rhea americana,* common peafowl *Pavo cristatus*. Abyssinian ground hornbill *Bucorvus abyssinicus*, scarlet ibis *Eudocimus ruber*, emu *Dromaius novaehollandiae,* marabou stork *Leptoptilos crumenifer*
Germany	Blackbird *Turdus merula*, Blue tit *Cyanistes caeruleus*, Canary *Serinus canaria domestica*, Common starling *Sturnus vulgaris*, House sparrow *Passer domesticus*, Redstart *Phoenicurus phoenicurus*, Song thrushes *Turdus philomelos*, Great gray owl *Strix nebulosa lapponica*, Long-eared owl *Asio otus*, Northern hawk-owl *Surnia ulula*, Osprey *Pandion haliaetus*, Inca tern *Larosterna inca*, White stork *Ciconia ciconia*, Common kingfisher *Alcedo atthis*, European green woodpecker *Picus viridis*
Greece	Domestic pigeon *Columba livia domestica*
Hungary	Blackbird *Turdus merula*, Eurasian tree sparrow *Passer montanus,* Eurasian jay *Garrulus glandarius*, Common starling *Sturnus vulgaris*
Italy	Blackbird *Turdus merula*, Common starling *Sturnus vulgaris*, Eurasian jay *Garrulus glandarius*, Magpie *Pica pica*, Long-eared owl *Asio otus*, domestic chicken *Gallus gallus domesticus*, Mallard duck *Anas platyrhynchos*, Eurasian collared dove *Streptopelia decaocto*, Rock pigeon *Columba livia*, Nightjar *Caprimulgus europaeus*, Charadriiformes (*Larus* sp.) Great grey owl *Strix nebulosa*, Northern hawk owl *Surnia ulula*, little owl *Athene noctua*, Partridge *Alectoris rufa*
Netherlands	Blackbird *Turdus merula*, great gray owl *Strix nebulosa lapponica*, Eurasian coot *Fulica atra*
Poland	Black-headed gull *Larus ridibundus*, goshawk *Accipiter gentilis*
Serbia	Mute swan *Cygnus olor*
Slovakia	Eurasian great tit *Parus major*
Spain	Song thrushes *Turdus philomelos*, Common pheasants *Phasianus colchicus*, Red-legged partridges *Alectoris rufa*, pigeons *Columba livia* var. *domestica*), Greylag goose *Anser anser*, mallard duck *Anas platyrhynchos,* yearling eagle owl *Bubo bubo*, booted eagle *Hieraaetus pennatus*, ostrich *Rhea americana*

Table 1. Confirmed presence of USUV or antibodies in birds (wild, farmed and captive from zoo aviary) by European country based on literature data

Country	Birds
Switzerland	Blackbird *Turdus merula*, Blue tit *Cyanistes caeruleus*, European greenfinch *Carduelis chloris*, European robin *Erithacus rubecula*, House sparrow *Passer domesticus*, Northern Hawk owl *Surnia ulula*, Great gray owl *Strix nebulosa lapponica*, Pygmy owl *Glaucidium passerinum*, Snowy owl *Nyctea scandiaca*, Tengmalm's owl *Aegolius funereus*, domestic chicken *Gallus gallus domesticus*, marabou stork *Leptoptilos crumeriiferus*, ruddy shellduck *Tadorna ferruginea*, red-breasted goose *Branta ruficollis*, Humboldt penguin *Spheniscus humboldti*, laughing kookaburra *Dacelo novaeguineae*, steamer duck *Tachyeres pteneres*, greater flamingos *Phoenicopterus ruber*
United Kingdom	Blackbird *Turdus merula*, carrion crow *Corvus corone*, European magpie *Pica pica*, turkey *Meleagris gallopavo*, domestic chicken *Gallus gallus domesticus*

to humans and other mammals.

In summary, the mosquitoes belonging to 7 genera such as *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, and *Ochlerotatus* are competent vector for USUV [7,15,20]. In Europe the most important is ornithophilic *Cx. pipiens* complex [15] and in Africa is *Cx. neavei* [90] and also genera *Coquillettidia* and *Mansonia* [7]. Mosquitoes from genus *Coquillettidia*, like *Culex*, are mainly ornitophilic, whereas *Mansonia africana*, is mainly mammalophilic species [35]. In genus *Culex* also occurred mammalophilic mosquitoes like *Cx. pipiens* biotype *molestus* [36]. *Culex pipiens* biotype *molestus* and *Cx. torrentium* from Germany proved competent for USUV, as indicated by harboring viable virus in their saliva 21 days post infection, while *Ae. aegypti* are relatively refractory to an USUV infection, have low infection rates and lacking virus in their saliva [36]. *Ae. albopictus* also showed a low vector competence for USUV [9,29].

Animals with USUV RNA or antibodies detected

Birds are the natural host and reservoir of USUV which become infected when bitten by mosquitoes [29]. Currently, this virus is found in both African and European bird species, mostly in Strigiformes and Passeriformes [40]. The USUV occurs less frequently in mammals, especially horses, bats, canids, rodents, and also infects humans [3,29,33,61].

Birds

In Africa virus was detected in piping hornbill (*Bycanistes sharpii*), little greenbul (*Andropadus virens*), and Kurrichane thrush (*Turdus libonyanus*) from Nigeria [13,19,35]. As they are not migratory species therefore, they could not have contributed to the spread of USUV to Europe [13], but they constitute a local reservoir of the virus. Antibodies have been detected in e blackbird (*Turdus merula*) in Morocco [19,35] and laughing doves (*Spilopelia senegalensis*) in Tunisia [19]. Seroprevalence was also observed in white-faced whistling-duck (*Dendrocygna viduata*) and Hottentot teal (*Spatula hottentota*) from Madagascar and in domestic birds in Mali and Madagascar [47].

In European countries USUV first of all was isolated from a blackbird *Turdus merula*. Infected blackbirds were detected in Austria, Hungary [48, 51,100], Czech Republic [3,67,102], Switzerland, Italy, Germany [3,19], and the Netherlands [3]. The virus has contributed to the high mortality of blackbirds in different regions of Europe, *eg.* the epidemic in 2011 caused a 74% decline in breeding pairs of blackbirds near Heidelberg (Germany) [31,59]. USUV or antibodies have also been isolated from many other bird species, wild, farm and kept in aviaries and zoos, first of all Passeriformes and Strigiformes [33,103], but also Galliformes, Accipitriformes, Falconiformes, Piciformes, Columbiformes [19]. The list of species by country is provided in Table 1. In total, 58 European bird species belonging to 13 orders and 26 families are mentioned in literature [91] and some exotic from zoos [74].

According to Bakonyi et al. [1] blackbirds, sparrows as well as representatives of the Strigiformes are particularly susceptible to USUV neuroinvasions and mass mortality. The prevalence of USUV in Austria has decreased over time, possibly due to establish herd immunity in Austrian bird populations, but has spread to neighboring countries causing further local enzooties in wild urban bird populations [1]. Nikolay [97] points out that the high infection rates of blackbirds should be associated with the preferences of main vector – *Cx. pipiens*. This mosquito species feeds on variety of vertebrates as birds, humans or horses, but prefers birds and especially blackbirds and Eurasian magpies [97].

As stated Ashraf et al. [13] migrating birds as *Sylvia communis* (whitethroat), S*ylvia curruca* (lesser whitethroat), *Sylvia borin* (garden warbler), *Falco tinnunculus* (kestrel), *Circus aeruginosus* (marsh harrier), *Delichon urbica* (house martin), *Acrocephalus scirpaceus* (reed warbler), *Ficedula hypoleuca* (pied flycatcher), and *Hirundo rustica* (barn-swallow) because of occurrence of USUV-specific antibodies may have played a role in the introduction of USUV into Europe. Because USUV has been isolated from resident, short-distance and migratory bird species in Europe [16,59], the virus must have a local avian reservoir and be transmitted by local mosquito species. Further, as blackbirds migration routes lead to the East Asian countries, such as eastern Russia, Eastern China, Taiwan, Korea, and Japan, where the main vector *Cx. pipiens* is also common, they may contribute to the spread of USUV in the future in Asia [13].

Reptiles

In Slovakia USUV neutralizing antibodies were found in 26.8% of serum samples from green lizards *Lacerta viridis* living in the Slovak Karst National Park [3,82]. This is most likely the result of bites by *CuIex* mosquitoes.

Mammals

In Africa USUV was isolated from small mammals [92]. In Senegal virus was detected from two species of rodent as natal multimammate mouse *Mastomys natalensis* and black rat *Rattus rattus,* as well as in specimen of shrew *Crocidura* sp. [104]. Antibodies were detected in dogs and horses [3]. USUV seropositivity was documented in horses in Tunisia [16], and in horses and dogs in Morocco [16]. A case of isolating USUV from a rodent, furred rat (*Praomys* sp.) is also described [13,35], but later it turned out to be a related flavivirus, the Yaounde virus [19,35].

In Europe USUV RNA or antibodies were found in several species of mammals. In Germany and Belgium RNA of this virus was detected from brains of bats common pipistrelle *Pipistrellus pipistrellus* [3,61,97,104]. USUV neutralizing antibodies were documented in horses in Croatia, Italy, Serbia [3,16,95,97], Spain [3,16], Poland [3,16,65], hunting dogs [16,29,95] and gray squirrels in Italy [3,95], wild boars in Serbia [3,16,95] and France [3,75], wild ruminants as red deer *Cervus elaphus*, fallow deer (*Dama dama*), mouflon (*Ovis aries musimon*), roe deer (*Capreolus capreolus*) in Spain [3,4,20,56,104,105] and roe deer in France [3,75]. Antibodies were also found in several canine species, especially in African wild dogs *Lycaon pictus*, but also in Iberian wolf *Canis lupus signatus,* Maned wolf *Chrysocyon brachyurus* and Asian lion *Panthera leo persica,* from zoo in France [74].

Outside Europe and Africa, USUV seropositivity was documented in horses from Israel [25,39].

Mammals like rodents, bats, deers, squirrels, wild boars, dogs or horses act as accidental or dead-end hosts [3,29,33,84]. Horses appear to be susceptible not only to West Nile virus, but also to USUV [29,65]. Research conducted in Poland by Bażanów et al. [65] showed a high seroprevalence of anti-USUV neutralizing antibodies in horses, amounting to 27.98%, which suggests that the virus is actively circulating in this region using native vectors and has its reservoir among native host species. In Italy highest seroprevalence in horses amouting to 89.2% was observed in 2008 but in 2009 it fell to 7.8% [3,7]. According to Diagne et al. [104], the detection of USUV in small mammals, i.e. rodents and shrews abundant in Europe and Africa, in which no disease symptoms and pathological changes were observed, indicates the possibility of the existence of a non-avian reservoir in the environment. Therefore, these authors point to the need to monitor small mammals for environmental and public health reasons.

Human infections by USUV

Unlike in Africa, in Europe, USUV causes disease mainly among birds, who show mild to severe clinical signs including necrotic lesions in the heart, spleen, liver and brain, contributing to mass deaths, especially blackbirds, gray owls and house sparrows [29,36,59]. The second group at risk of USUV infection are humans, whose course of infection may range from asymptomatic to severe meningoencephalitis [3].

Human USUV infection or antibodies were reported in Africa but most of all in Europe.

Symptomatic USUV infections in humans are relatively rare, with a total of approximately 40 cases reported worldwide, as well as cases of asymptomatic infections, e.g. among blood donors, which were detected accidentally, mainly during screening for WNV [3,5,25,56,62,106]. According to Vilibic-Cavlek et al. [3] USUV neurotropism in humans has been reported in both immunocompromised and immunocompetent patients. However, severe infection occurs mainly in immunocompromised patients [107]. Pacenti et al. [5] in their seroprevalence studies (including blood donors) conducted in Italy indicate that most cases of USUV infection in humans are asymptomatic.

Only two human cases of USUV disease in Africa in 1981 with symptoms of fever and rash in the Central African Republic and in 2004 in child with fever and jaundice in Burkina Faso were noted [3,15,35,108]. According to Martín-Acebes et al. [83] the number of human infections in Africa may be underestimated due to the varied symptoms. This is due to the limited number of studies conducted in Africa on the seroprevalence and circulation of the virus [45]. Among the few are studies conducted in Nigeria in 2018, where serum samples from patients with fever were tested, obtaining a seroprevalence of 64% [44].

However, in Europe, for the first time USUV neurotropism has been observed in immunocompromised as well as in immunocompetent patients [3,12,78,109–111]. Cases of neuroinfection like meningitis, encephalitis, polyneuritis and facial paralysis but also other symptoms like rush, fever and Influenza-like symptoms were noted [3,5,53,110,111]. Most symptomatic human cases have been reported in Italy, about 13 cases, where it has been a notifiable disease since 2017 and the occurrence of USUV infections in humans is monitored [5]. In Europe, neurological symptoms were detected first of all in patients in Italy [3,53,97,108,110,111], but also from Croatia [3,78,97], Germany [97], Austria [107], France [3,15] and Hungary [3].

The world's first case of USUV neuroinfection was reported in 2009 in the Emilia-Romagna region of Italy in a patient with diffuse lymphoma who was diagnosed with meningoencephalitis with fever and neurological symptoms [12]. In the same year, Cavrini et al. [110] reported a case of USUV viremia manifesting itself with severe impairment of brain function in an immunocompromised patient who was a recipient of an orthotropic liver transplant. Pacenti et al. [5] reported eight cases of infection in 2018, in this one with neurological disease, six wit fever and influenza-like symptoms and one viremic blood donor who developed myalgia and arthralgia. Furthermore, in a retrospective studies conducted by Grottola et al. [108] and Cavrini et al. [111], USUV RNA and USUV antibodies were detected in cerebrospinal fluid, plasma, and serum collected from patients with meningitis and without neurological diseases. USUV RNA was noted in 1.1% and USUV antibodies in 6.57% of samples of cerebrospinal fluid and serum collected in Modena and were higher compared to data for West Nile Virus with 2.96% [108]. Cheng et al. [50] suggest that USUV infections in humans in Europe may be even more common than WNV infections in areas where the both viruses coexist. Also other works indicate that, in areas where WNV and USUV co-occur, the incidence of anti-USUV antibodies in humans is higher than that of anti-WNV antibodies [5,30,85,95,108,112,113]. Co-infections of WNV and USUV in humans would therefore be possible and probable [16]. During screening tests carried out in the period 2012-2018, several cases of seropositive, asymptomatic blood donors were detected [106,111,112,114,115]. The highest seropositivity of 46.3% was found among asymptomatic blood donors in the Lombardy region in 2016–2018 [115]. Interesting results were obtained by Percivalle et al. [112] who observed a high seroprevalence among forest workers of 18.1% compared to blood donors with 1% in the Po River Valley in the Lombardy in 2014–2015. In summary, a total of 22 clinical cases were reported in Italy, including 12 cases of meningoencephalitis, 2 cases of encephalitis and 8 cases of fever. Additionally, one case of viremia was found in a blood donor and the presence of USUV antibodies was found in 40 healthy blood donors and 6 healthy forest workers [56,112]. Moreover, the occurrence of USUV infections in humans is monitored in Italy as it is a notifiable disease since 2017 [5].

Apart from Italy, where symptomatic and asymptomatic cases detected during screening are best known, USUV in humans has also been reported in other countries. In **Austria** one clinical case of meningitis in 2021 was noted [56,116]. In **Croatia**, 6 cases of meningitis and meningoencephalitis (including one fatal) were reported during the West Nile Virus outbreaks, three cases each in 2013 and 2018 [56,78–80]. In the **Czech**

Republic, in South Moravia region the first record of a neurological human disease caused by USUV was noted during WNV outbreak in 2018 by Zelena et al. [68]. In **Hungary** infection was detected in patient with aseptic meningitis in 2018 [53]. In 2016, in **France**, an USUV infection manifested by idiopathic facial paralysis was observed in a patient from Montpellier, Occitania [76]. According to Simonin et al. [76], phylogenetic analysis of the virus isolated from the patient showed similarity with the strain detected a year earlier in common blackbirds in the Camargue region by Lecollinet et al. [77]. Also one case of fever was detected in in Nouvelle-Aquitaine, France in 2022 [56]. Usutu virus has also been detected by metagenomic next-generation sequencing in plasma samples collected from a patient transplanted for lymphoma at the University Hospitals of Geneva, **Switzerland** in 2020 [55].

Positive results were also obtained among blood donors outside Italy. In **blood donors** tests conducted in **Austria** between 2016 and 2018, a total of 25 positive samples were found [107,117]. One person developed a rash, while the rest had no symptoms. Moreover, one person examined in 2018 was diagnosed with double infection with WNV and USUV [107]. In **Germany**, during screening tests for WNV carried out in 2016, an acute, asymptomatic USUV infection was detected in one blood donor. This person had no symptoms but reported previous mosquito bites [62]. Earlier in 2012, the presence of USUV-specific IgG and IgM antibodies was found in one German healthy blood donor [63]. In 2018 six samples from **Dutch** blood donors tested positive [72]. Also in **Hungary** in five blood donors USUV seropositivity was confirmed in 2019 [54]. In serological studies conducted in the South Bačka district of Vojvodina, **Serbia** in 2015 among people at risk of infection with arthropod-borne diseases, antibodies against USUV were detected in 7.5% of samples [26,118]. Previously in Vojvodina region, the genetic material of USUV was isolated from mosquitoes *Cx. pipiens* [81]. Coroian et al. [119], guided by another study that showed the presence of antibodies against USUV in a dog from the Iași region in **Romania**, decided to examine a total of 1,200 blood samples from healthy blood donors from the northwestern region of Romania in 2019–2020. However, all samples tested negative for USUV. The authors of the study indicate that the negative results may be related to the low incidence of WNV, with which USUV co-occurs. In summary, serological tests carried out among blood donors in several European countries showed positive results ranging from 0% to 46.3% of cases [85,108,115,119,120].

According to Roesch et al. [29] USUV shares several features with WNV. Both viruses are transmitted mainly by mosquitoes of the *Culex* genus, the main host are birds, and apart from them, they are found mainly in horses, less often in other animals and humans. Moreover, both viruses co-circulate in the environment, as evidenced by co-infections reported in both birds and humans [15,29,79,81,92,107]. Zannoli and Sambri [15] indicate that in the areas where both viruses occur, USUV infection is higher than WNV infection and amounts to 7% and 3%, respectively.

Based on its similarity to WNV, the incubation period is estimated at 2-14 days. According to literature data, clinical manifestation in humans are similar to West Nile fever. Most human USUV infection are asymptomatic or clinically mild [16,57,66], occasionally associated with rash [107]. Cheng et al. [50] and Engel et al. [28] list symptoms of Usutu infection as fever, rash, hepatitis, headache, nuchal rigidity, hand tremor and hyperrefexia. Also severe cases of neuroinfection like meningitics, meningoencephalitis, encephalitis, polyneuritis, or idiopathic facial paralysis was noted [8,15,33,50,90].

USUV infection is diagnosed directly by detection of viral RNA in blood and cerebrospinal fluid samples or by isolation of the virus in cell culture, or indirectly by detecting anti-USUV antibodies in serum and cerebrospinal fluid samples. Directly nucleic acid amplification methods such as real-time RT-PCR and nested RT-PCR are used. Indirect methods are based on serological tests, including: enzyme immunoassays (ELISA) or immunofluorescence tests. If a positive result is obtained, it should be confirmed with more specific tests, such as PRNT, to exclude cross-reactivity with antibodies to other flaviviruses, e.g. WNV [15].

Conclusions

Currently, USUV causes sporadic infections in humans. However, the emergence of clinical cases, including neuroinvasion, and seroprevalence in asymptomatic humans, indicates that USUV can be pathogenic to humans and become a potential public health problem [28,29].

The cross-reactivity of anti-WNV and anti-USUV antibodies, related to the similarity of envelope proteins [27], complicates the interpretation of the results [12]. Since serological tests such as ELISA do not always distinguish USUV from WNV, other tests such as NS1-based protein microarray or real-time quantitative multiplex RT-PCR have been developed to provide verification [29,55,111].

It is important to monitor the occurrence of USUV in humans, extending screening for WNV also to include USUV that co-occurs and uses similar vectors and hosts. This is important because most cases are asymptomatic, while symptomatic cases associated with neuroinfection in people, especially those with reduced immunity, may be severe. As Cadar and Simonin [56] emphasize, in order to understand the circulation of USUVs, it is also important to study the interactions between animals, people and various environmental factors, such as climatic conditions and vector population dynamics.

The presence of USUV antibodies in asymptomatic blood donors and forest workers indicates the presence of this virus in both the environment and the human population. There is currently no evidence of virus transmission during transfusion or transplantation, but the potential risk of virus transmission from an asymptomatic blood donor to an immunocompromised recipient and causing disease in the recipient must be considered [18,106,117,121]. Bakonyi et al. [117] draw attention to the need to introduce regulations regarding screening of blood donors for USUV in endemic areas. In particular, this should apply to periods of increased bird mortality and WNV outbreaks.

During the WNV epidemic, disease and seroprevalence were frequently observed among asymptomatic blood donors, as well as increased mortality among birds due to USUV. Because WNV and USUV viruses share many characteristics and co-circulate in the environment using the same reservoir and vectors, existing WNV surveillance programs should be expanded and adapted to USUV testing in birds, horses, mosquitoes and humans, and specific diagnostic methods should be developed [15,92,122,123].

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