HOW DANGEROUS IS USUTU VIRUS FOR BIRDS AND HUMANS: SYMPTOMS, EPIDEMIOLOGY, DIAGNOSIS AND POSSIBLE TREATMENTS

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PREFACE

I have always had the dream to become a veterinarian, and in the past years a developed a specific passion for birds. My heart gets filled with joy and love if I see or hear one. And when I found a dead Eurasian blackbird in my backyard a couple of years ago at the time Usutu was circulating, I was worried for this beautiful kind of species and I wanted to know more about it.

Therefore, I like to thank my promotor Prof. dr. Niek Sanders for making this interesting phenomenon a subject to investigate and putting his trust in my copromotor and me. I would also like to thank my copromotor Dr. João Paulo Portela Catani for the advice and trust he gave me. It was a pleasant collaboration, with respect and kindness. At last, I would like to thank my mother, brother and partner in life. They have always supported me and believed in me through these six years of veterinary medicine. Without them I would not be the person I am today.
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Het Usutu virus (USUV) is een door mug overgedragen Afrikaans virus dat behoort tot het genus Flavivirus binnen de Flaviviridae familie. Het is een lid van het Japans encefalitis virus (JEV) complex. Daardoor is het verwant aan enkele gevaarlijke humane pathogenen zoals het West Nile virus (WNV). Het is een positief enkelstrengig RNA virus met envelop dat voornamelijk door ornithofiele muggen wordt overgedragen (voornamelijk van het Culex pipiens complex) met als voornaamste eindgastheer de wilde vogel.


ABSTRACT

The Eurasian blackbird population decreased by 15.7% during an Usutu Virus (USUV) outbreak in Germany in 2011 and continued to decline afterwards. Since the outbreak, above 167,119 birds died due to this virus (Lühken, 2017). This large-scale impact on population levels is a threat for birds in Europe and demands investigation. So far, USUV was never associated with fatal diseases in humans (Vázquez, 2011). In Africa, however, two cases of human USUV infections have been reported in 1981 and 2004. These were benign infections and no further notice was given. Nonetheless, these cases show that USUV can infect humans (Nikolay, 2011). In May 2009, the first European human case of Usutu virus neuroinvasive infection was reported in Italy in a woman with diffuse large B-cell lymphoma and was diagnosed with meningoencephalitis (Pecorari, 2009). Until now, multiple cases of USUV infection in humans have been reported both in African and European countries (Nikolay, 2011). USUV infection in European birds and its transmission to humans is a recent observation with a scarce number of studies. Many questions are still open for debate. By looking to similar viruses and examples of pathogens that crossed the interspecies barrier we discuss in this review the risk of Usutu virus for both bird and human health.

INTRODUCTION

Usutu virus (USUV) is an African mosquito-borne virus that belongs to the genus Flavivirus within the Flaviviridae family. It is a member of the Japanese encephalitis virus (JEV) antigenic complex. It is an enveloped positive single-stranded RNA virus that is primarily transmitted by ornithophilic mosquitoes (mainly of the Culex pipiens complex) among avian reservoir hosts (Nikolay, 2011). In 1959, the virus was first discovered and isolated from a mosquito (Culex neavei) in South Africa (McIntosch, 1985). In the past, USUV was never observed outside Africa and avian infections were not considered fatal (Vázquez, 2011). However, in the early fall of 1996, USUV caused for the first time an episode of mortality amongst mostly Eurasian blackbirds (Turdus merula) in Italy. Five years later in the summer of 2001, similar deaths of the free-living Eurasian blackbird occurred in Vienna (Austria) (Weissenböck, 2002). During the summer of 2016, a comparable episode started in the northwestern region of Europe including Belgium, Germany and the Netherlands (Garigliany, 2017). Currently, USUV is endemic in several countries in Europe (Gaibani, 2017). Different human cases of USUV infection have been reported until today but the effective role of USUV as a human pathogen has yet to be clarified. The future implication of USUTU infection in birds and humans is still unclear. This review focuses on general aspects of USUTU biology and tries to access its future risks. Why is USUV mainly transmitted by mosquitoes of the Culex pipiens complex? And why is the Eurasian blackbird most affected? Could USUV evolve and become a threat to human health?
LITERATURE STUDY

1 Emerging diseases

Emerging diseases are an important concern in human health. The pathogens responsible for these diseases have been (re)emerging for centuries (Gould, 2017). The most severe pandemic event in recent history was caused by an avian resistant flu virus, causing the death of 50 million people in 1918 (Taubenberger, 2006). Recently, Ebola reemergence frightened the world causing 764 deaths in central Africa (ECDC, 2019). Arthropod-borne viruses (arboviruses) have been causing disease for several decades. (Kilpatrick, 2012). Their opportunistic emergence is thought to be created by globalization and human invasiveness. This in combination with the rapidly evolving and adapting viruses and their evolving hosts makes the arboviruses a risk for global health.

1.1 Emerging arboviruses

Over 130 arboviruses are known to cause human disease and are responsible for some of the most explosive epidemics of infectious diseases over the past decade. Arboviral diseases such as dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV), Japanese encephalitis virus (JEV), are on the rise and are spreading more rapid and geographically extensive than before (Dash, 2013). These viruses are using mosquitoes as their blood-sucking vectors and transmits the virus to the primary vertebrate host. The mosquito-borne clade revealed two epidemiological groups: the Culex-species-associated viruses and the Aedes-species-associated viruses (Gaunt, 2001). The Culex-species-associated viruses typically cause encephalitic infections and Aedes-species-associated viruses tend to cause feverish, flu-like illness and/or hemorrhagic disease in humans and animals (Gould, 2017).

For example, WNV is a Culex-species-associated virus that is widely distributed across the world. An explanation is the bird-mosquito natural cycle, in which the virus emerged in Africa and was dispersed by birds into Europe (Gould, 2017). Other underlying factors for the WNV invasion are thought to be the globalization, land use and development of transportation systems (Dash, 2013). Many humans and a wide variety of different animal species have antibodies against WNV in geographical areas where WNV circulates and do not show any clinical symptoms but at times of the year when ornithophilic Culex-species mosquitoes are numerous, WNV may also cause epidemics with symptoms varying from asymptomatic infection to viral syndrome to neurologic disease (encephalitis, meningitis and flaccid paralysis) which occurs mostly in elderly or immunocompromised people (Gubler, 2007). Why WNV is successful is due to its capacity to infect and be transmitted by an abundant variety of birds and mosquito species. Since 1999, the awareness raised new heights because of the emergence of WNV encephalitis in humans and birds throughout mainland USA, Canada and Mexico (Gould, 2017). However, it was already a known arbovirus epidemic in Africa, the Middle East, Europe and Asia/Australasia. By August 2016, 44,000 human cases of clinical WNV infections were recorded in the United States and with the milder cases not diagnosed, the estimates of infections will be much higher (Gould, 2017). This raises important questions. Why is WNV so strongly emerging? Can other related viruses be as emerging and what is its risk for global health?

WNV shares common ancestry with a series of viruses, including the Usutu virus (USUV). USUV attracted little attention until it emerged in Austria (August 2001) causing an outbreak of bird deaths.
So far, multiple cases of USUV infection in humans have been reported both in Africa and Europe and it appears to be emerging as a significant avian and human pathogen (Gould, 2017). Could a more virulent USUTU strain emerge and constitute a threat to human health? All together, we should consider its risk.

2 Usutu virus infection in birds

2.1 Etiology

Usutu virus is an African mosquito-borne virus that belongs to the genus Flavivirus within the Flaviviridae family. It is a member of the Japanese encephalitis virus (JEV) antigenic complex.

The Flaviviridae family beholds over more than 70 viruses, many of them are important pathogens such as Japanese encephalitis virus (JEV), yellow fever (YF) and West Nile virus (WNV). Virus particles belonging to this family are small (40 - 60 nm in diameter), spherical particles that contain an electron-dense core (approximately 30 nm) and are surrounded by a lipid envelope. The surface of the particles contains two viral proteins: E (envelope) and M (membrane). The E glycoprotein mediates the binding and the fusion during virus entry (Chambers, 2003).

The viruses are positive single-stranded RNA viruses. The genome contains a unique open reading frame that encodes a polyprotein precursor (Figure 1). This precursor undergoes proteolytic cleavage by viral and cellular proteases to produce three structural proteins (capsid C, the earlier mentioned envelope E and membrane M) and eight non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NSK, NS4B and NS5), all of them needed in the replication (Bakonyi, 2004). Phylogenetic analysis of the NS3 and NS5 coding regions as well as full-length genome nucleotide sequences of the USUV reference strain from South Africa (where it was first discovered) allowed its classification close to the Japanese encephalitis virus (Nikolay, 2011).

Figure 1. USUV model with its gene structure and the encoded proteins (Ashraf, 2015).

2.2 Origin and epidemiology

In January 1959, USUV was first discovered and isolated from a mosquito (Culex neavei) in South Africa, near the Usutu river (Mcintosh, 1985). Later, the strain from South Africa was called SAAR-1776 and was from then on considered as the reference strain. After this, the virus was isolated in Senegal, Central African Republic, Burkina Faso, Cote d’Ivoire, Nigeria, Uganda and Morocco (Nikolay, 2011). It is unknown if the virus originally came from Africa or was introduced from another continent and its striking that the discovery of USUV was not associated with pathology in birds nor mammals. The isolation of USUV from insects, only occurred in African countries where there were entomological surveillance programs active (e.g. Senegal) (Nikolay, 2011). Suggesting that the geographic distribution of USUV may be much wider than it appears to be.
Although, a study by Engel et al. (2016) hypothesized that USUV emerged in Africa at the beginning of the 16th century and its introduction in Europe probably started in the last 50 years. But USUV was never observed outside Africa until 1996 were the virus emerged in a pathogenic form for the first time and caused an episode of mortality amongst mostly Eurasian blackbirds (*Turdus merula*) in Italy (Tuscany region) (Weissenböck, 2002). This provided the earliest evidence that the virus was introduced in Europe and five years later, USUV emerged in Austria (2001) causing similar deaths of the free-living Eurasian blackbird. In the following years, the virus was found in other European countries such as Hungary (2005-2006) (Bakonyi, 2007), Spain (2006-2009) (Vázquez, 2011), Germany (2011-2013) (Becker, 2012, Cadar,2014) and Belgium (2012) (Gariglianiy, 2014) with virus isolation from mosquitoes, birds and bats. During the summer of 2016, a large epizootic episode like the one in Austria (2001) started in the northwestern region of Europe with activity in Belgium, Germany, France and the Netherlands (Garigliany, 2017). It was the first time that USUV was isolated in the Netherlands. This highlights the fact that the virus is continuously geographically spreading. 

Engel et al. (2016) performed a study to understand the evolutionary mechanisms of USUV and did this by obtaining the complete genome sequences of 77 USUV strains sampled from mainly mosquitoes and birds out of different countries. Phylogenetic analysis revealed that all European strains (except the Spanish strains) emanate from a common ancestor. This indicates that there was a single introduction into European countries, probably by migratory birds. Senegal was most likely the major source of the single introduction into Europe, whereas Austria represented as the primary source in Europe. Italy was the source that caused total diffusion in to Europe. Afterwards, the spread of USUV infection throughout Europe showed to be westward, that matches the chronological and geographical incidences.

**Figure 2.** Migration patterns of USUV between or within Africa and Europa (Engel, 2016).

However, the strain from South Africa and Austria are closely related, the Austrian strain may have evolved separately after introduction into Europe (Bankonyi, 2004). There is a differentiation between European and African USUV that result in phylogeographic clustering of 6 distinct lineages. This is likely due to the *in situ* evolution, which is the result of adaptation to the local environment. This phenomenon was also observed for West Nile virus in the United States. A star-like structure, in the European lineages, in which the viral strains assemble changes during the adaptation to the local environmental conditions, is evidence of the *in situ* evolution (Engel, 2016).

The viral adaption and the continuously introduction of the virus through migratory bird flyways are considered to be the main reasons for the diffusion and persistence of USUV. Although, climate change and the increased use of human transportation (mainly ship – and aircrafts) are also determinants to consider in the long-distance spread of USUV. It is known that the environmental temperature influences the magnitude of the mosquito population and the reproduction of the virus.
Warm temperatures increase the reproduction and biting rates of mosquitoes increasing dissemination of the virus (Brugger, 2009).

2.3 Life cycle

The enzootic life cycle of USUV is similar to that of other flaviviruses of the JEV serocomplex. The virus is primarily transmitted by ornithophilic mosquitoes (mainly of the Culex pipiens complex) among avian reservoir hosts (Nikolay, 2011). Thus, its lifecycle involves mosquito-bird-mosquito cycles, in which mosquitoes act as blood-sucking vectors and transmits the virus to birds which act as the primary vertebrate host. Many different species of birds may be infected by USUV and some of them develop symptoms of disease. Humans are considered to be incidental or “dead-end” hosts, but also bats, rodents and horses can be incidentally infected (Ashraf, 2015).

![Figure 4. The natural life cycle of USUV](from www.mayoclinic.com, 2012).

2.3.1 Mosquitoes as vectors

In the mosquito-bird-mosquito cycle, the mosquito behaves as the vector that consumes the blood of the primary vertebrate host and infects itself with the virus. After the bloodmeal, the virus passes into the lumen of the midgut of the arthropod and nestles itself in the epithelial cells. The virus then invades the salivary gland and when the mosquito feeds on a new (not infected) host, the virus is transmitted through the saliva and infects the new host (Mellor, 2000). So far, USUV was found in eight different species that belong to different genus Aedes, Anopheles, Culex, Culiseta, Ochlerotatus, Coquillettidia and Mansonia. Although its isolated from many different species, the virus is majorly found in the Culex species in which Culex pipiens acts as the main common vector (Gaibani, 2017).

2.3.1.1 Culex pipiens complex

Culex mosquitoes are geographically distributed in all regions over the world. As we said before, they are known as ornithophilic vectors for arboviruses such as USUV and WNV. The host range of many species has been found to be larger than birds. Many of these species not only feed on birds, but also on amphibians, reptiles, humans, and other mammals. More specific, the Cx. pipiens complex has been showed to be involved in epidemic USUV transmission in Europe (Nikolay, 2011). A study of Fros et al. (2015) showed that Cx. pipiens is a highly competent vector for USUV, even more than for WNV. They demonstrated this by offering the female mosquitoes a bloodmeal that contained similar dose of USUV or WNV and evaluating its infection. The mosquitoes were then maintained at a

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1 [www.mayoclinic.com](http://www.mayoclinic.com) (last consulted in May 2019)
temperature of 28°C. For USUV, 80% of the mosquitoes were infected after the bloodmeal. Of these infected mosquitoes, 69% had infected saliva which is a prerequisite for infecting the primary and incidental hosts. In contrast to USUTU, only 46% of the female mosquitoes were infected with WNV. This indicates that USUV infects a large percentage of \textit{Cx. p.\textit{pipiens}} mosquitoes and these are potentially an even more effective vector for USUV than WNV.

Other factors also indicate the USUV transmission by \textit{Culex}. These mosquitoes are found in all zoogeographic regions. In Europe, \textit{Cx. p.\textit{pipiens}} is even the most numerous mosquito species (Fros, 2015). Thus, the chances that they get in contact with infected hosts are much higher. USUV is endemic in parts of Mediterranean Europe, as a result, \textit{Cx. p.\textit{pipiens}} is significantly more competent for USUV at higher temperatures. In the study of Fros et al. that was mentioned before, they used a mean summer temperature at all times to maintain the female mosquitoes. Yet, USUV activity is also found in more temperate regions. This can explain that the virus also distributes into central and northwestern parts of Europe. These daily temperature fluctuations around the mean can additionally increase the vector competence because the infectivity in \textit{Cx. p.\textit{pipiens}} depends strongly on the temperature. Other \textit{Culex} species have been isolated such as \textit{Cx. Quinquefasciatus} which belongs to the \textit{Cx. p.\textit{pipiens}} complex. This species was directly involved in the emergence of USUV in Europe (Saiz, 2017). Its appearance is majorly in the domestic environment and it is the most anthropophilic vector for USUV. This species has a dynamic population because of its steady presence (Nikolay, 2011). Other factors that can explain the USUV specificity for the \textit{Cx. p.\textit{pipiens}} is population density of vectors and amplifying hosts (birds), mosquito survival and host feeding behavior of \textit{Cx. p.\textit{pipiens}} (Fros, 2015).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Global distribution of \textit{Cx. p.\textit{pipiens}} complex mosquitoes (Ciota, 2013).}
\end{figure}

One other factor is the production of viral small-interfering RNAs (siRNA) by the virus in infected \textit{Cx. p.\textit{pipiens}}. The siRNA pathway is the major small RNA pathway that targets the virus in \textit{Cx. p.\textit{pipiens}} mosquitoes upon infection. The activation of this pathway was investigated by isolating small RNAs from pools of USUV infected mosquitoes that was analyzed using deep-sequencing. Although, Fros et al. (2015) did not identify viral piwi-interacting RNAs (vpiRNA) derived from USUV. These are an animal-specific class of small silencing RNAs, distinct from microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Ozata, 2018). Therefore, the lack of vpiRNAs in the infection could be due to an inability of Culex mosquitoes to process viral RNA into vpiRNAs or to flaviviral RNA. This pathway is the key to antiviral immunity in arthropods (Fros, 2015).
2.3.2 Birds as primary vertebrate host

For our competent vector *Cx. pipiens* to be effective, vertebrate species that serve as amplifying hosts are required. In the natural life cycle of USUV, free-living birds have the role as primary vertebrate host. This is consistent with the fact that most of the *Culex* species are ornithophilic. Therefore, the virus is highly pathogenic for multiple different avian species. Both migratory and indigenous wild birds are susceptible for infection which corresponds with the introduction, diffusion and persistence of the virus into Europe. For example, infection has been demonstrated serologically in birds in Austria (Eurasian blackbird, Snowy owl, etc.), Germany (Eurasian blackbird, Canary, House sparrow, etc.), Italy (Eurasian blackbird, Eurasian magpie, Nightjar, etc.), Belgium (Great spotted woodpecker, Bullfinch, etc.), Greece (Domestic pigeon), Switzerland (Greater flamingo, chicken, etc.), Hungary (Eurasian blackbird) and many more European countries (Ashraf, 2015). In captivity, exotic Passeriformes are as susceptible to USUV (Steinmetz, 2011). There were many investigations on several different bird species in different countries to demonstrate USUV infection and the results show that the Eurasian blackbird is the most severe affected species.

\[
\text{Table 1. USUV-infected avian species in Europe.}
\]

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dendrocopos major</em></td>
<td>Great spotted woodpecker</td>
<td>Belgium (2014, 2017)</td>
</tr>
<tr>
<td><em>Pyrrhula pyrrhula</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Turdus merula</em></td>
<td>Eurasian blackbird</td>
<td>Italy (2010-2013)</td>
</tr>
<tr>
<td><em>Erythrus rubecula</em></td>
<td>Robin</td>
<td></td>
</tr>
<tr>
<td><em>Passer domesticus</em></td>
<td>House sparrow</td>
<td></td>
</tr>
<tr>
<td><em>Sturnus vulgaris</em></td>
<td>Common starling</td>
<td></td>
</tr>
<tr>
<td><em>Turdus merula</em></td>
<td>Eurasian blackbird</td>
<td></td>
</tr>
<tr>
<td><em>Turdus pilaris</em></td>
<td>Fieldfare</td>
<td></td>
</tr>
<tr>
<td><em>Turdus iliacus</em></td>
<td>Redwing</td>
<td></td>
</tr>
<tr>
<td><em>Aegolius funerius</em></td>
<td>Boreal owl</td>
<td></td>
</tr>
<tr>
<td><em>Strix nebulosa</em></td>
<td>Great grey owl</td>
<td></td>
</tr>
<tr>
<td><em>Turdus merula</em></td>
<td>Eurasian blackbird</td>
<td>Austria (2002-2003)</td>
</tr>
<tr>
<td><em>Strix nebulosa</em></td>
<td>Great grey owl</td>
<td></td>
</tr>
<tr>
<td><em>Hirundo rustica</em></td>
<td>Barn swallows</td>
<td></td>
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<tr>
<td><em>Turdus merula</em></td>
<td>Eurasian blackbird</td>
<td>Hungary (2007)</td>
</tr>
<tr>
<td><em>Strix nebulosa</em></td>
<td>Great grey owl</td>
<td>The Netherlands (2016)</td>
</tr>
<tr>
<td><em>Hirundo rustica</em></td>
<td>Barn swallows</td>
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<tr>
<td><em>Turdus merula</em></td>
<td>Eurasian blackbird</td>
<td>France (2016)</td>
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<td><em>Strix nebulosa</em></td>
<td>Great grey owl</td>
<td></td>
</tr>
<tr>
<td><em>Sterna hirundo</em></td>
<td>Barn swallows</td>
<td>Spain (2012)</td>
</tr>
<tr>
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<td>Eurasian blackbird</td>
<td></td>
</tr>
<tr>
<td><em>Strix nebulosa</em></td>
<td>Great grey owl</td>
<td>Germany (2011)</td>
</tr>
<tr>
<td><em>Serina canaria domestica</em></td>
<td>Eurasian blackbird</td>
<td></td>
</tr>
<tr>
<td><em>Ardea cinerea</em></td>
<td>Canary</td>
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<tr>
<td><em>Passer domesticus</em></td>
<td>Grey heron</td>
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<tr>
<td><em>Picus viridis</em></td>
<td>House sparrow</td>
<td></td>
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<tr>
<td></td>
<td>Eurasian green woodpecker</td>
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</tbody>
</table>
2.3.2.1 Eurasian blackbird

Among avian species, Eurasian blackbirds (*Turdus merula*) showed the highest mortality due to USUV infection (Ashraf, 2015). This higher frequency of USUV infection in blackbirds in comparison to other birds may be because of the wide distribution of the species, its size and color and its close association with humans (Lühken, 2017). The Eurasian blackbird is a medium-sized thrush and is distributed all over Europe and found in every possible location from forests to city gardens. It’s one of the most famous birds because of its close association with humans. On the other hand, the higher sensitivity for USUV infection may be due to a higher virus susceptibility (genetically), behavioral traits, or different spatial-temporal distribution in relation to the infected vector distribution. Females of the *Culex* species prefer to canopy where there is a great abundant number of birds and they bite at night (Nikolay, 2011).

For example, whilst the USUV outbreak in Germany, the Eurasian blackbird population decreased by 15.7% in 2011 and continued to decline afterwards (Lühken, 2017). In other countries as well, massive die-offs occurred such as Hungary, Switzerland, Belgium, Italy and the Netherlands (Garigliany, 2016). These massive die-offs can be explained by the fact that the virus was introduced for the first time in a naïve and highly sensitive population. Therefore, it was suggested that the Eurasian blackbird populations were almost extinct in some areas (Becker, 2012). This can be explained by local high virus transmission such as a favorable distribution of vectors and hosts (Lühken, 2017). Due to the persistent presence of USUV in Europe, we can expect a long-term decline of Eurasian blackbird populations, leading to an alteration of the bird communities in the areas where the outbreaks take place.

But what does this decline in population mean for the ecosystem? Essential changes in ecosystem services can occur due to the emergence of USUV. These ecosystem services are natural processes that benefit humans and are provided by birds including the Eurasian blackbird. Birds contribute supporting services, these include all other ecosystem processes (e.g. soil formation, nutrient cycling and provisioning of habitat). They maintain the ecosystem throughout the world by foraging, spreading seeds and pollinating. In addition, migratory birds link ecosystem processes and fluxes that are separated by great distances and times (Whelan, 2008). The Eurasian blackbird is a partially migratory bird. It is suggested that food limitation and intraspecific competition expressed by dominance behavior is involved in the regulation of partial migration (Lundberg, 1985). All this indicates that by decline of the population or local extinction of the Eurasian blackbird, the ecosystem services could decline in places where the Eurasian blackbird is abundant and territorial. Although, once the territory is available for other species, they can provide the ecosystem services and prevent it from declining.

The question if Eurasian blackbirds will naturally evolve and become resistant to USUV, is yet to be further examined. Nevertheless, it has been described that over the years, less birds become infected and die. This can maybe be explained by the development of herd immunity (Chvala, 2007).

2.4 Symptoms and lesions

The observed clinical signs for USUV infection in birds are non-specific clinical signs such as apathy, looking for drinking water, immobility, ruffled plumage, half-closed eyes and anorexia, along with severe neurological signs (depression, incoordination, inability to fly, jerky movements, torticollis and nystagmus) (Garigliany, 2017). The birds mostly suffer for several days, because most species were in
a compromised nutritional status when found at a time when food resources seem unlimited in affected locations (Steinmetz, 2011). The most remarkable sign is that the birds stop showing any sign of mistrust towards man (Garigliany, 2017).

There have been several necropsies performed of dead birds (including Eurasian blackbirds) in several European countries. For example, during the episode of wild bird deaths due to USUV (mostly Eurasian blackbirds) in Italy, 1996, several animals were subjected to necropsy, which predominantly showed swollen livers and spleens (Weissenböck, 2013). Manarolla et al. (2010) performed necropsies of different species over a period of several years. Species like the Boreal owl, the Great grey owl and Eurasian blackbirds were used for necropsy. The lesions observed in the dead birds consisted of general congestion, hepatomegaly and splenomegaly of varying severity. Thus, the cause of death is most likely multi-organ failure (Chvala, 2004.) This corresponds to the birds with USUV infection in 1996 and with several other necropsies. During the necropsies, the majority of Eurasian blackbirds were infested with intestinal parasites, such as tapeworms, nematodes and acanthocephalids (Chvala, 2004). It is known that free living birds are mostly infested with numerous parasites which weakens the host and therefore be more vulnerable for USUV infection.

![Figure 6. Pathological observation of a USUV-infected blackbird. A. Enlarged and hyperaemic liver in an USUV-infected Eurasian blackbird (left). B. Enlarged and hyperaemic spleen (right) in an USUV-infected Eurasian blackbird (Manarolla, 2010).](image)

We have to keep in mind that only a fraction of dead wild birds was noticed and still a small proportion of those gathered and made available for diagnosis. Because of the emergence of USUV, dead bird surveillance programs were set up in areas where there were a lot of die-offs. In addition, the majority of dead wild birds with USUV infection is probably underestimated due to the lack of dead bird surveillance programs in certain smaller areas and they also rely on the participation of the general public to collect and submit dead birds to local collection centers. Although, significant virus activity is not very likely to be missed, a more widespread distribution of USUV cannot be fully excluded (Chvala, 2007).

Except for the macroscopic lesions, histological examination can be performed and microscopical signs of USUV infection can also been seen. Histologically, neuronal necrosis, myocardial lesions, and
coagulative necrosis of the liver and spleen were observed (Chvala, 2004). Also, USUV-specific antigen was demonstrated by immunohistochemistry in several organs.

2.5 Diagnosis

Necropsies of dead birds is an easy method to determine a suspected diagnose. Nonetheless, the suspicion of USUV infection requires laboratory confirmation. We can distinguish between direct methods in which we detect the virus by cell culture or genomic amplification and indirect methods in which we detect the antibody response to the infection (Vázquez, 2011).

2.5.1 Direct methods

These direct methods include RT-PCR, real time-PCR, immunohistochemistry and in-situ hybridization.

2.5.1.1 RT-PCR

RT-PCR stands for reverse transcription-polymerase chain reaction and demonstrates USUV-specific viral RNA in organs of birds, blood and mosquitoes. This technique is highly sensitive and specific. It detects a minimal amount of viral RNA and it even works on degraded RNA (e.g. dead birds) (Weissenböck, 2007). The first step is to isolate the RNA with reverse transcriptase (RNA-dependent DNA polymerase) to synthesize a cDNA (complement DNA) strand to the RNA template. This is more stable than the RNA meaning that RNA is vulnerable for ribonuclease (RNase) degradation (Bridge, 2017). The cDNA is than amplified with primers with the use of DNA polymerase that amplifies the gene that is of interest, the NS5 gene. The flavivirus NS5 gene encodes a protein with RNA-dependent RNA polymerase and methyl-transferase activity. It is therefore involved in virus replication. The nucleotide sequence of the NS5 gene has been determined for a number of flaviviruses which has permitted comparison with other flavivirus NS5 gene sequences, in particular other mosquito-borne flaviviruses (Fulop, 1995). The last step is the PCR product analysis, which can be done by several methods (e.g. agarose gel electrophoresis) (Bridge, 2017).

2.5.1.2 Real time-PCR (RT-PCR)

In May 2009, the first European human case of Usutu virus neuroinvasive infection was reported in Italy (Pecorari, 2009). After this, the ability of USUV to infect and cause disease in humans has made it a necessity to develop a rapid and specific method for clinical diagnosis of USUV infection (Cavrini, 2011). Cavrini et al. (2011) developed a quantitative RT-PCR which is very sensitive and detects and distinguishes USUV from other arboviruses, particularly from JEV members. In addition, it generates highly specific quantitatively results more quickly (within four hours after the sample is received) (Weissenböck, 2007). However, the test only identifies viral isolates circulating in Europe with the genetic variability of African USUV strains not considered (Nikolay, 2013). Therefore, Nikolay et al. (2014) presented a RT-PCR based on viral isolates from Europe and Africa to ensure detection of a maximum of genetically diverse USUV strains. The first steps are the same as in the protocol of Cavrini et al. (2011), however the later and adjusted one uses USUV specific primers and fluorogenic probes which were able to detect five different USUV isolates from Africa (Nikolay, 2014). The probe is labeled with a reporter fluorescent dye at the 5’ end and a quencher fluorescent dye at the 3’ end. Emission of the reporter fluorescent is not been detected because the quencher fluorescent is keeping the fluorescence of the reporter under control. During the amplification phase, the probe is
getting cleaved by RNA dependent DNA polymerase which induces the reporter fluorescent to release from the probe. Hereby, the quencher fluorescent is not able anymore to control the emission of the reporter fluorescent. The increase in emission is being monitored in real time by a sequence detector. This fluorescence is being used in a computer system to calculate the amount of copies of the target DNA in the sample (Gibson, 1996).

2.5.1.3 **Immunohistochemistry (IHC)**

Immunohistochemistry is being performed on tissue samples that were fixed in 7% neutral buffered formalin (Chvala, 2007). These are processed into sections with a microtome and then the sections are incubated with specific antibodies. Because of the incubation with specific antibodies, the viral antigens can be visualized in infected cells by microscopy. Chvala et al. (2004) detected flavivirus antigen, the E-glycoprotein, in the brain of Eurasian blackbirds (predominantly present in the cerebral cortices, the thalamus and the metencephalon, including the cerebellum), also in the heart (abundant in myofibres), lungs (in endothelial cells of capillaries and small venules), proventriculus (in glandular epithelial cells), small intestine (crypt epithelial cells), kidneys (in tubular epithelial cells) and spleen.

![Figure 7](image1.png)

**Figure 7. Presence of flavivirus antigens in tissues from USUV-infected birds.** A (left). IHC. Section of the cerebral cortex in a Eurasian blackbird showing numerous neurons contain flavivirus antigen in their cytoplasm (Chvala, 2004). B (right). IHC. Section of the heart in a Eurasian blackbird showing flavivirus antigen within myofibers (Chvala, 2004).

2.5.1.4 **In-situ hybridization (ISH)**

This technique is used to identify the presence and distribution of USUV-specific nucleotide sequences (Chvala, 2004). It corresponds with immunohistochemistry except that this last technique identifies viral antigens. A complementary USUV-specific digoxigenin-labelled oligonucleotide probe is being used that binds with the nucleotide sequences (Weissenböck, 2007). This junction is being visualized by anti-digoxigenin antibodies accompanied with an enzyme. This enzyme catalyzes the formation of a colored substrate which reveals the location of the virus (Brown, 1998). Chvala et al. (2004) showed that the ISH and IHC findings were largely comparable. ISH gave more positive results in the epithelial cells of proventricular glands and intestinal crypts whereas with IHC the viral RNA was more abundant in certain cell populations of the spleen, kidney, lung and intestine. However, it clearly indicates the value of both IHC and ISH in the diagnosis of USUV infection (Chvala, 2004).
2.5.2 Indirect methods

These indirect methods include plaque-reduction neutralization test (PRNT) and hemagglutination inhibition test (HIT).

2.5.2.1 Plaque-reduction neutralization test (PRNT)

The biological parameter of in vitro virus neutralization measured by plaque-reduction neutralization test is the most virus-specific serologic test among flaviviruses. This test is the laboratory standard and is still used as confirmation when other tests are positive for infection (Roehrig, 2008). The concept of the PRNT allows for virus-antibody interaction to occur in a tube or microtiter plate, and then the mixture is plated on virus-susceptible cells. The cells are overlaid with a semi-solid medium that restricts spread of the virus. Each virus that initiates a productive infection produces a plaque. Antibody effects on viral infectivity is measured by counting the plaques and comparing them with the starting concentration of virus to determine the percentage reduction in total virus infectivity (Roehrig, 2008).

2.5.2.2 Hemagglutination inhibition test (HIT)

The basic idea of the hemagglutination inhibition test (HIT) is to detect USUV-specific antibody response in plasma samples (Chvala, 2005). The test uses the ability of the E-glycoprotein of the virus to bind and agglutinate avian erythrocytes so that they form a visible grillage in a U-bottom microtiter plate (=hemagglutination). Antibodies from infected individuals prevent the agglutination of the erythrocytes on to the E-glycoprotein, which afterwards forms a pellet (=no hemagglutination) (Hobson-Peters, 2012). This does not mean that there is an infection with USUV because cross-reactivity with other flaviviruses have been described. In general, the specificity of HIT is considered to be less than PRNT (Weissenböck, 2007). Although it is not considered to be highly specific, the HIT is an appropriate method for the indirect diagnosis and initial screening of USUV infections (Meister, 2008).

2.6 Possible treatments

A specific vaccination for USUV infections in birds is currently not available (Blázquez, 2013). Therefore, treatment is symptomatically (keeping them comfortable, offering drinking water, maybe forced feeding) until the birds die or the other option is euthanasia. Because wild birds are usually infected with different types of intestinal endoparasites, they may have a lower immunity and thus are more vulnerable for infection than captive birds who are treated against these endoparasites. This also applies for the ectoparasites, there USUV is being transferred by mosquitoes. Captive birds, especially endangered Passeriformes and Strigiformes, are being regular treated against these ectoparasites with a spray and it is likely that these treatments have prevented infection with USUV (Steinmetz, 2011). The treatment can help to reduce virus transmittance below the minimal virus concentration and minimal exposure can induce protective immunity against USUV infections in a range of avian species (Steinmetz, 2011). In the current European areas with USUV activity, avian mortality decreased within a few years. The reason for this is uncertain. However, experience from USUV outbreaks indicates that virus and avian host immune system adapt well to each other and a high degree of herd immunity in the affected populations species seems to develop over time.
Therefore, the infections are clinically obscure and avian mortality and pathological changes do not usually occur in previously exposed individuals (Steinmetz, 2011).

Blázquez et al. (2013) demonstrated an upregulation of the autophagic pathway in USUV-infected cells. This gives the possibility of manipulating the autophagic pathway to modulate infection with USUV. Autophagy is an important cellular pathway that helps with important parts in viral infections (Ashraf, 2015). USUV is one of the flaviviruses that likes to advantage of these autophagic processes by incorporating the components from this cellular pathway into their own replication (Blázquez, 2013). Modulation of this autophagy with pharmacological products could be an antiviral approach against USUV. Blázquez et al. (2013) did this by analyzing an inductor of autophagy (rapamycin) and two inhibitors (3-MA and wortmannin). Treatment with rapamycin induced an increase in viral titer and the inhibitors a decrease in viral titer. These results provide a basis for a new antiviral therapy and confirm that pharmacological intervention on the autophagic pathway modulates USUV infection (Ashraf, 2015).

3 Usutu virus infection in humans

3.1 History of human cases

In 1981, the first human case occurred in Central African Republic with a patient who had a fever and a rash. The second case was in a 10-year old patient with also fever and jaundice in 2004 (Burkina Faso) (Nikolay, 2011). This showed that USUV can infect humans with benign infections. Nevertheless, in May 2009, the first European human case of Usutu virus neuroinvasive infection was reported in Italy in a woman with diffuse large B-cell lymphoma and was diagnosed with meningoencephalitis (Pecorari, 2009). The patient received several courses of chemotherapy and two weeks after the last administration she was admitted in the hospital for hyperpyrexia although she was treated with antipyretics and antibiotics. The patient also received a blood transfusion. Her neurological examination was divergent, and a suspicion of meningoencephalitis was established. A cerebrospinal fluid sample was taken and examined for several viral agents such as adenoviruses, parvovirus B19, WNV, etc. which all testes negative. Except, a further analysis of the same CSF specimen revealed the presence of flaviviruses (Pecorari, 2009). To confirm an infection with USUV, Percorari et al. (2009) performed two USUV-specific RT-PCRs targeting the NS5 and premembrane (preM) regions of the USUV genome on two plasma specimens that were collected. The results shared a 99%-100% nucleotide identity with the USUV Budapest and Vienna sequences. The immunosuppressed status may have played a role in this USUV infection and pathogenicity. It is likely that the infection was transmitted by mosquitoes. The patient lives in an area where competent viral vectors are circulating and because the blood transfusion took place after the first signs of meningoencephalitis (Pecorari, 2009). In this same period of time, there was another immunocompromised patient with USUV infection in the same area who also received a blood transfusion (Vázquez, 2011). These cases were detected thanks to active surveillance program of blood and organ donations for WNV, which indicates that USUV may also be underrecognized in some other areas where the surveillance for WNV is lacking (Vázquez, 2011).

Later, in the late summer of 2013 in Croatia, three people were detected with neuroinvasive USUV infection of which two patients suffered from an underlying disease (hypertension and diabetes) and the third patient was healthy (Vilibic-Cavlek, 2014). In France 2016, a patient was reported with USUV infection and showed atypical neurological symptoms (facial palsy, eyelid ptosis and gradual
paresthesia of both right limbs). He was given corticoids and valaciclovir (antiviral treatment). The symptoms of facial palsy disappeared within a few weeks (Simonin, 2018). In Germany, 2016, an acute USUV infection of a blood donor was detected using a cross-reactive WNV screening test and further sequencing of the genome. The patient tested positive for WNV, but was actually positive for USUV infection (Cadar, 2017).

Several cases have been reported in several western European countries but in general the number of cases of human USUV infections is limited and more clinical work on infected patients is necessary to define its pathogenicity for humans (Nikolay, 2011).

3.2 Diagnosis

As previously cited, the epidemiological episodes of deaths of wild birds and the first reported human case of USUV infection made it a necessity to develop USUV-specific diagnostic tools for human infection (Gaibani, 2012). However, diagnosis can be challenging, particularly in areas where USUV co-circulates with other cross-reacting flaviviruses such as WNV (Vázquez, 2011). The Q-PCR was developed, as previously described, to detect the USUV genome in humans. However, it can only identify acute-stage infections and a serological test is required to evaluate the immune status of the population on large scale. The standard way to do this in humans is by PRNT, except this cannot be done for large screens (Gaibani, 2012). Therefore, Gaibani et al. (2012) developed a novel enzyme-linked immunosorbent assay (ELISA) to detect the specific IgG response to USUV in blood of humans.

3.2.1 Enzyme-linked immunosorbent assay (ELISA)

The principle of this test is that viral antigens are being isolated from infected cells and is being absorbed into a 96-well microtiter plate. Afterwards, the plate is being coated with a buffer solution which prevents the binding of non-specific antibodies. Now, serum of the concerned patient is being added and thereafter a peroxidase-conjugated rabbit anti-human IgG antibody. This is to evaluate if the patient has primary antibodies (IgG) against USUV. This is being visualized by a chemical light reaction in the well due to a conversion of a specific substrate by the peroxidase enzyme.

To overcome the cross-reactivity between USUV and WNV IgG antibodies, Gaibani et al. (2012) calculated the USUV-to-WNV ratio (U/W). Nevertheless, it is expected that cross-reactivity will be higher for IgG than for IgM detection and thus development of tests for USUV-specific IgM is a higher necessity (Vázquez, 2011).

Although, the availability of the ELISA, makes it possible to begin serological surveillance programs to define USUV-infection in humans, especially in areas where this virus has been demonstrated to circulate in vectors and birds (Tamba, 2011).

3.3 Human health

As USUV is so closely related to WNV and other highly pathogenetic viruses of the Japanese encephalitis virus (JEV) antigenic complex, it holds a potential risk to become an important threat to human health. A study in a disease-endemic area in Italy even showed that human USUV infection is not a sporadic event and showed a higher incidence than infection with WNV (Simonin, 2018). More virulent strains can emerge highlighting that risk must be considered. Gaibani et al. (2013) revealed several potential functional mutations in USUV, isolated of the human case in 2009. It showed two
unique amino acid substitutions (Tetro, 2017). In particular, one substitution was situated in the DIII domain of the viral Envelope protein that is recognized by flavivirus neutralizing antibodies (Gaibani, 2013). A second amino acid substitution (D3425E) that was identified in the RNA-dependent RNA polymerase (RdRp) domain of the NS5 protein which is a critical component in de the virus replication. Interestingly, a comparable amino acid substitution in the RdRp domain of the NS5 region, was observed in other flavivirus strains that are associated with meningoencephalitis in humans such as JEV and WNV. In addition, studies of WNV have shown that substitutions in these genes were associated with variation in the capacity of WNV to invade the central nervous system of laboratory mice. These two found mutations could have played a role in the tropism and neuroinvasive capacity of USUV for human neurological cells and that there is a difference comparing with the genomes of USUV isolates from mosquitoes or birds (Gaibani, 2013). The potential role of these mutations is yet to be further examined. Nonetheless, it suggests a small increase in prevalence of the virus in the European population and an increased risk of adaptation in the human host (Tetro, 2017).

**DISCUSSION**

This revision discussed general aspects of USUTU biology and pointed out the possible risks for both bird and human health. USUV has become a resident pathogen in central Europe and co-circulates with other flaviviruses like WNV which is a dangerous human pathogen. Considering the similarities of USUTU and other pathogenic flaviviruses, the risk of becoming a future threat for human heath cannot be out ruled. The virus is transmitted mostly by *Culex* species mosquitoes, which is the most abundant mosquito species in Europe. These mosquitos have showed that they cause increased oral infection rates at higher temperatures, which in association to climate changes can increase the spread of USUV (Brugger, 2009).

In addition to competent and effective USUV vectors, sufficient vertebrate species are required as amplifying hosts. Many bird species are prevalent in Europe as USUV has been detected in a large number of avian species in many different countries. Infection has been found in both migratory and non-migratory birds and both free-living and captive birds. For example, infection has been demonstrated in the Great grey owl, the Robin, the Bullfinch and in captive exotic Passeriformes. Among these avian species, the Eurasian blackbird showed the highest mortality due to USUV infection (Ashraf, 2015). Several factors can explain the massive die-offs of this species. The wide distribution of the species in Europe could be an important factor as well as the fact that the virus was introduced for the first time in a naive and highly sensitive population. Over the years, less birds become infected and die which can maybe be explained by the development of herd immunity. Nonetheless, the sensitivity of the Eurasian blackbird for infection should be further examined to predict the future impact of USUV in this species.

The wide distribution of infected wild birds is indicated by surveillance of dead birds in areas where there occurred massive die-offs. This indication is probably underestimated due to the lack of dead bird surveillance programs in certain areas. Considering that USUV is now endemic in Europe and still spreading geographically, there is an increasing need to implement and reinforce more veterinary and entomological surveillance plans, which would be very similar to surveillance systems for WNV. In addition, the first human case of USUV infection in 2009 was detected thanks to a regional WNV surveillance plan implemented to identify WNV circulation in Italy with neuroinvasive cases in humans (Vázquez, 2011). Actually, the most human USUV infections have been detected in areas where a surveillance program for WNV exists. This also suggest that USUV infections in humans may
be underrecognized in areas where such surveillance program is not available. The first reported human case of USUV infection made it a necessity to develop USUV-specific diagnostic tools for human infection. Nevertheless, the precise diagnosis can be challenging in areas where USUV co-circulates with WNV and other cross-reacting flaviviruses. Nikolay et al. (2014) presented a quantitative PCR to detect the USUV genome in humans and Gaibani et al. (2012) developed a novel ELISA to detect the specific IgG response to USUV in blood of humans. This will be crucial for precision of screening and diagnostics.

The future perspectives for USUV infection in birds and humans is yet unclear. The reported cases of human infections indicate its potential severity as a human pathogen. The similarities with the WNV infections, (the neuroinvasiveness in immunocompromised patients) emphasize the need to be cautious about its potential threat to human health. However, more clinical research is needed to estimate the risk for human health (Nikolay, 2011). This could be done by identifying the presence of antibodies against USUV in humans that live in concerned areas, to determine the prevalence of infection. Also, clinically affected humans should be closely followed to know more exactly how the virus affects people. The newly developed real-time PCR should be used on regularly base for identification of USUV in human plasma, serum and cerebrospinal fluid.

As for avian species, possible outcomes can occur. Die-offs are currently decreasing probably because of development of herd immunity. Although, it is suggested that the virus will survive in Central Europe until the end of the century and that optimal environmental conditions for USUV outbreaks due to global warming in about 10 year will occur which predicts that the outbreak frequency increases successively from the beginning to the end of the century (Brugger, 2009). In addition to this, mutations can occur and develop viral variants that are more pathogenic for humans and birds. Because of global transport, long-distance travel, population growth, environmental and climatic changes, vector borne diseases including USUV are emerging all over the world (Cheng, 2018).

In conclusion, further studies as well as surveillance systems should be encouraged worldwide to unveil other characteristics of USUV infection and to predict its future evolution.

References

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