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IN VIVO AND IN VITRO MODELS TO STUDY THE MOSQUITO-BORNE USUTU VIRUS

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Abstract

Usutu virus (USUV), a mosquito-borne zoonotic flavivirus discovered in South Africa in 1959, has spread to many European countries over the last 20 years. The virus is currently a major concern for animal health due to its expanding host range and the growing number of avian mass mortality events. Although human infections with USUV are often asymptomatic, they are occasionally accompanied by neurological complications reminiscent of those due to West Nile virus (another flavivirus closely related to USUV). The knowledge about the various study models is a helpful tools for scientific to identify the best methos for different scientific questions.

Keywords: virus, zoonotic, in vitro and in vivo models

INTRODUCTION

Usutu virus (USUV) is an arthropod-borne virus (arbovirus) belonging to the genus Flavivirus within the Flaviviridae family. As a member of the Japanese encephalitis virus (JEV) antigenic complex, USUV is closely related to numerous human and animal pathogens including West Nile virus (WNV), Murray Valley Encephalitis virus (MVEV), and St Louis encephalitis virus (SLEV). USUV is maintained in the environment through a typical enzootic cycle involving mosquitoes and birds. Since first identification in South Africa in the middle of 20th century, widespread circulation of USUV was observed in several countries. In Europe, USUV emerged in 1996 causing high numbers of bird deaths. Five years later, USUV was responsible for high mortality rate among Eurosian Blackbirds (Turdus merula) in the surrounding area of Vienna, Austria. Currently, USUV is endemic in several countries in Europe.

The clinical relevance of USUV as human pathogen has been hypothesized since the first descriptions of USUV-related infection in humans. The first report of USUV infection in humans was described in Africa at the beginning of 1980s. Thirty years later, two cases of USUV-related neuro- invasive diseases were reported in immune-compromised pa- tients in Europe (Italy). Although different human cases of USUV infection have been reported until today, the effective role of the USUV as a human pathogen has yet to be clarified.

Virus genome and structure

USUV is a small and spherical virus with a lipid envelope derived from host cell membrane. The virion is 40e60 nm in diameter and contains a positive-sense RNA genome of 11 Kb in length with no 30 poly(A) tail. Genomic organization shows a similar structure comparable to other flaviviruses. The genome consists of a single-stranded RNA genome with a 5’ cap structure, a unique open reading frame (ORF) and two untranslated regions (UTRs). The 5’ and 3’ UTRs varied respectively between 95 to 96 nt and 631 to 664 nt in length among different strains. The UTRs are involved for the translation and replication of the viral genome. The predicted ORF is translated in a unique polyprotein of 3434 amino acids that is

post-translationally processed into three structural (capsid, envelope, and pre-membrane) and eight non- structural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5). Like other mosquito-borne flavivirus, genes encoding the structural proteins are located on the 5' end of viral genome and form the virion particle. The capsid protein (C) forms the central core of the virion and is asso-ciated to the viral RNA. The envelope glicoprotein (E) mediates binding to the host cells and promotes viral entry into the host cells. The pre-membrane protein (prM) are necessary for virion assembly and maturation by assisting envelope folding. The nonstructural proteins serve to different functions during infection and their functions are deduced on the basis of the similarity with other flavivirus genomes. NS1 exists in distinct forms (i.e. cellular and secreted) and is necessary on the replication of viral genome and virion maturation. The NS2A, NS2B, NS4A and NS4B are small, hydrophobic proteins that are required for virus as- sembly and play a role in the inhibition of the IFN response. NS3 and NS5 are two proteins with different enzymatic activities: NS3 protein encodes for viral serine protease (active only with NS2B cofactor), helicase, nucleoside tri- phosphatase and RNA triphosphatase. NS5 protein encodes for a methyltransferase (MTase) at the N-terminal, while C- terminal encodes for the RNA-dependent RNA polymerase.

Life cycle, hosts, vectors

USUV was isolated for the first time from a *Culex neavei* mosquito captured near the Usutu river in Ndumu, South Africa, in 1959. Subsequently, USUV circulation in the African continent has been detected in several countries: Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Ivory Coast, Tunisia, Morocco, and Algeria. Until 2001, USUV was considered as exclusively African, non-fatal for wild birds or domestic animals, and exceptionally zoonotic. In 2001, USUV was isolated from blackbirds (*Turdus merula*) found dead during an epizootic that affected the resident passerines and *Strigiformes* in Austria. Retrospective analyses have shown that the high mortality of blackbirds in Tuscany (Italy) in 1996 was also attributed to this virus. In the following years, USUV circulation was identified in many countries in western, southern, and central Europe: United Kingdom (2001–2002), Czech Republic (2005), Hungary (2005), Poland (2006), Spain (2006) [29], Switzerland (2006), Serbia (2009–2010), Greece (2010), Germany (2011), Slovakia (2012–2014), Belgium (2012), France (2015), and The Netherlands (2016). In many of these countries, USUV has managed to establish an endemic mosquito–bird life cycle and to co-circulate with WNV.

To date, USUV has been detected in mosquitoes belonging to seven genera (*Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, and *Ochlerotatus*). However, it seems to be most often associated with *Culex pipiens*. The main natural reservoir hosts of USUV are birds; the virus presence was demonstrated to date in 101 bird species belonging to 18 orders and 38 families. However, the natural virulence spectrum of USUV seems rather limited, with a marked virulence in the European blackbird (*Turdus merula*), house sparrow (*Passer domesticus*), grey owl (*Strix nebulosa*), and common scoter (*Melanitta nigra*). In these species, prostration, disorientation, locomotor disorders, and death may occur. The two macroscopic lesions most commonly observed at autopsy are splenomegaly and hepatomegaly. Pathohistological analysis revealed inflammatory and necrotic lesions, with histiocytic and lymphoplasmacytic infiltrates, have been described in the heart, lung, liver, kidney, spleen, and brain of the infected birds. Although the virus was isolated from mammalian species, namely rodents (*Mastomys natalensis*, *Crocidura spp.*, and *Rattus rattus*) and Chiroptera (*Rousettus aegyptiacus* and *Pipistrellus pipistrellus*), no pathological signs could be observed in these hosts and their potential role as a reservoir for this arbovirus is still questionable. Other mammals, such as equids , dog, wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), tree squirrel (*Sciurus vulgaris*), Malayan tapir (*Tapirus indicus*), chimpanzee (*Pan troglodytes*), giant panda (*Ailuropoda melanoleuca*), common eland (*Taurotragus oryx*), and white rhinoceros (*Ceratotherium simum*), as well as reptiles (green lizards (*Lacerta viridis*), presented neutralizing antibodies specific for USUV and may act as incidental hosts.

In humans, USUV infection (like WNV) is usually asymptomatic. More than 80 cases of subclinical infections have been described in blood donors or healthy patients in Italy, Serbia, the Netherlands, and Germany during the surveillance of WNV circulation. Clinical disease with moderate flu-like (rash, fever, and headache) manifestations may also occur. The neurotropism of USUV represents a growing concern for human health. In more than 32 cases to date, severe neurological disorders, including facial paralysis, encephalitis, meningitis, and meningoencephalitis, in both immunocompromised and immunocompetent patients have been observed.

These severe acute human cases, along with the avian mass mortality induced by this virus in Europe and numerous similarities with WNV biology and clinical manifestations, have prompted the development of experimental models to clarify the mechanisms underlying USUV pathogenesis and transmission. Besides, given that no approved effective therapeutics and no licensed vaccines against USUV exist so far for humans or birds, some of these models were used for their development. This is the first review to focus on in vitro and in vivo models of infection with USUV and summarize their contribution to clarify USUV pathogenesis and potential countermeasures.

USUSV Cellular Tropism and In vitro models

To date, the virus has been shown to infect a large spectrum of cells from 23 mammalian species, two avian species, and one reptile (turtle, *Terrapene carolina*). The first USUV in vitro replication assay was performed in porcine kidney (PK) cells in 1969. Later, Bakonyi et al. (2005) demonstrated USUV replication in a wide range of cells. However, only African green monkey kidney cells (Vero), PK-15 pig epithelial cells, and goose embryo fibroblasts have developed cytopathic effects (CPE). Like other flaviviruses, USUV replicates efficiently in Vero and mosquito (*Aedes albopictus*) C6/36 cells, which are commonly used for virus isolation from both clinical and animal (birds/rodents/mosquito) samples and often after replication in these cells, other cellular or animal models are used. The particular susceptibility and the extent of CPE observed in Vero cells explain their use for virus culture and viral titer studies, such as 50% tissue culture infectious dose, $TCID_{50}$, and plaque reduction neutralization tests [5]. In these cells lacking the interferon (IFN)-α and IFN-β genes, USUV infection activates cellular stress and autophagy, promoting viral replication. Further, USUV can establish a persistent infection for at least 80 days and present full-length and defective viral genomes (DVGs), containing truncations at the 5′ end, which may be a key determinant in the survival and persistence of the infection. Multiple cellular systems were used primarily to investigate USUV tropism. Mammalian cells were further used to explore USUV infection neuropathogenesis, the cell-intrinsic immune response, and/or the effect of antivirals on USUV replication.

USUV shows different replication characteristics in rodent species and rodent-derived cell types. The woodchuck (*Marmota monax)* liver cells (WCH-17, ATCC No: CRL-2082), rat (*Rattus norvegicus*) brain cell line (C6), and hamster (*Mesocricetus auratus)* kidney cell line (BHK-21) were susceptible to USUV infection but did not display CPE. However, primary astrocytes, microglial cells, and neurons of a wild-type mouse (*Mus musculus*) supported efficient USUV replication and showed CPE. While a bank vole (*Myodes glareolus*) kidney cell line (BVK168, RRID: CVCL_A014) showed CEPs following USUV infection, the virus did not replicate at all in the lung cells of this animal and did not show CPE in kidney or brain cells of the common vole (*Microtus arvalis*). Likewise, USUV could infect human cells from different origins, including the upper respiratory tract, brain, and retina, but only a few of these cells exhibited CPE.

USUSV and in vivo models

Mosquito Infection Models

Before USUV emergence in Europe, only one study registered experimental infections with USUV in mosquitoes. It showed the susceptibility of *Cx. neavei* to USUV, but no effective transmission to hamsters could be demonstrated [96]. After USUV detection in dead birds and several ornithophilic mosquito species in many European countries, the vector competence of European, African, and even American mosquito populations was addressed through experimental infections of these invertebrate hosts. *Cx pipiens* has been used as the major experimental model (in 4/7 studies). This can be justified by the abundance of this vector and the fact that USUV has been frequently detected [97] and co-circulating with WNV in biotypes of this mosquito complex collected in nature. Some North American and European populations of *Cx. pipiens pipiens*, *Cx. pipiens molestus*, *Cx. quinquefasciatus*, and/or hybrid forms have shown that both European and African strains of USUV effectively infect their bodies and accumulate in their saliva under laboratory conditions. However, two UK strains of *Cx. pipiens* infected with a USUV strain of African origin showed a very low vector competence, which could be due to the genetic variability of USUV strains or mosquito populations from the same species. Further, the infectivity of USUV in *Cx. pipiens* showed a pronounced temperature dependency. A clear relationship between the virus titer in the blood sample and the infection rate of *Cx. naevi* was demonstrated. Thus, a range of factors should be carefully considered to compare the competence of a particular mosquito species for the same virus.

The vector competence of *Cx pipiens* for USUV was compared with that for WNV and

ZIKV. While none of the tested mosquitoes accumulated ZIKV in the saliva and were considered as incompetent vectors for ZIKV, *Cx. pipiens molestus* and *Cx. pipiens pipiens* were shown to be susceptible to USUV infection and to disseminate the virus in their salivary glands. The infection and transmission rates with USUV (80% and 69%, respectively) were significantly higher than with WNV (46% and 33%, respectively) under elevated temperature (28 °C) in these mosquitoes.

Two mosquito species of the genus *Aedes* were assessed for their vector competence to USUV, namely *Ae. Albopictus*, repeatedly found infected in northern Italy, and *Ae. japonicas*, which is invading Europe and disseminating USUV in Graz (Austria). North American and European populations of *Ae. albopictus* appeared to be experimentally incompetent vectors for USUV and the detection of USUV from field-collected *Ae. albopictus* was explained by simple recent engorgement from viremic birds. In contrast, field-collected *Ae. japonicus* mosquitoes from the Netherlands showed USUV-positive saliva after 14 days at 28 °C, and, therefore, could play a role in the transmission cycle of the virus in Europe.

Bird Infection Models

USUV is highly pathogenic in some wild and captive bird species, due to its extensive tropism and virulence in various tissues and organs. Thus, these hosts are the most plausible in vivo models to characterize the pathogenesis of USUV infection. Besides, USUV has very selective pathogenicity within these hosts, including members from the same bird family. For instance, the natural USUV infection might be unapparent in domestic geese (*Anser anser f domestica*), while in another anatid, the common scoter (*Melanitta nigra*), USUV could result in fatal infection. Thus, it would be tempting to use such models to identify molecular determinants associated with virulence and host tropism, which may help anticipate key events leading to the possible emergence of USUV in new hosts and territories. However, to date, only three avian species have been used to address the susceptibility of these hosts to USUV infection. Domestic chicken (*Gallus gallus domesticus*) and geese (*Anser anser f domestica)* were reported to resist USUV infection under experimental conditions. More recently, the domestic canary (*Serinus canaria*), a passerine species, such as highly susceptible blackbirds, showed a mortality rate of 30% after infection via the intraperitoneal (IP) route with two different doses (10^3 and 10^6 $TCID_{50}$) of a European strain of USUV. In addition, USUV induced a specific humoral immune response in almost all the survivors after 15 days of infection. Chicken and goose embryos were also tested for their susceptibility to the virus. While USUV showed viral replication in goose embryos tissues, some studies showed that chicken embryos were resistant to infection, while one recent paper demonstrated that they are highly susceptible to USUV infection in a dose-dependent manner. These contradictory results could be explained by the genetic variability of the USUV strains and the differences in the genetic backbone of the eggs used, conditioning the immune response between breeds/individuals of the same bird species.

In addition to their susceptibility to USUV, the avian models available to date to study USUV, namely chicken and goose embryos and domestic canaries, have shed new light on USUV pathogenesis and transmission in birds. Similar to WNV, death due to USUV in domestic canaries was more likely attributed to a multi-systemic failure than to a pure neurologic disease, and the virus infected all major systems and a wide variety of cell types. The myocardial cells strongly supported viral replication, as viral antigens were systematically detected by immunohistochemistry (IHC) in the experimentally infected chicken embryos and canaries.

In all these three models, USUV displayed a particular tropism for the eyes. Visual impairment and ocular lesions have been described following infection of birds with other flaviviruses, such as WNV. A vision assessment should be performed during future experimental infections in vivo with USUV.

Immunocompetent Models

Developing an animal model relevant to human USUV infection seems to be extremely challenging because experimental infections have shown that immunocompetent mammals rarely develop severe forms of USUV disease. African fruit bats (*Eidolon helvum*) and (*Rousettus aegyptiacus*) and the Angolan free-tailed bat (*Tadarida (Mops) condylura*) were not susceptible at all to USUV injected intraperitoneally. Guinea pigs showed only an antibody response following intracerebral inoculation with the USUV SAAR-1776 strain. The Abyssinian grass rat (*Arvicanthis abyssinicus*) could exhibit a trace of viremia 1–2 days after IP inoculation of USUV (unknown strain) and developed neutralizing antibodies. Immunocompetent mouse models showed different susceptibilities to USUV infection across the studies. Intracerebral (IC) inoculation of USUV

successfully induced signs and mortalities in neonatal and 3–4 weeks-old immunocompetent mice. However, this injection route is not pertinent enough to describe USUV neuropathogenicity, as it only models viral neurovirulence. Thus, peripheral inoculation (e.g., subcutaneous SC or IP) was more commonly used to reflect both USUV neurovirulence and neuroinvasiveness. Experimentally, no mortality was observed following IP infection with USUV of Naval Medical Research Institute (NMRI) mice aged over 2 weeks with a European USUV strain. Similarly, the USUV prototype strain SAAR-1776 showed no pathogenicity in adult Swiss mice via the IP route. However, in the study of Diagne et al. , both SC and IP infections of this strain resulted in a 30% and 50% mortality, respectively, in 3–4-week-old Swiss Webster (CFW) mice after 15 days of infection. Likewise, in the same study, the IP inoculation of a mouse-derived USUV strain induced a 10% mortality 10 days after infection. USUV infection failed to elicit pathogenicity in wild-type 129/Sv mice via the IP and IN routes but induced a typical neurological disease in a single 129/Sv mouse infected via the ID route. These findings indicate that the outcome of USUV infection in immunocompetent mice depends on several factors, such as the strain of virus or mouse used. Age is also a key determinant of susceptibility to USUV and suckling mice are generally much more susceptible than older animals. NMRI suckling mice showed 100% mortality with as few as 10^3 Plaque-forming units (PFU) after 11 days of infection. Dose-dependent mortality was observed in Swiss suckling mice, as 84% and 40% survived the infection with 10^2 and 10^4 PFU, respectively. The higher predisposition of newborn neurons to apoptosis and the incomplete development of the BBB are plausible explanations for this difference in the infection outcome. Although immunocompetent models present limitations regarding their efficiency to manifest the USUV-associated disease, they are important to obtain knowledge about USUV pathogenesis under functional innate and adaptive immune responses of the host. In immunocompetent mice, USUV infection induced clinical signs, such as disorientation, depression, paraplegia, and paralysis, associated with extensive neuronal death, including both necrosis and apoptosis in the brain. Alternatively, no trace of viral infection or a simple detection of the USUV genome in brain portions of USUV-infected mice were described after 15 days post-infection, without the induction of specific clinical signs. These models reflect the infection in humans, in which most individuals show subclinical infections but rare cases can develop clinical disease.

CONCLUSIONS

USUV it can be currently considered as a leading model for the study of flaviviral pathogenesis and the development of prophylactic and therapeutic solutions against these more pathogenic flaviviruses. Indeed, it can be handled under level 2 biosafety conditions; besides, field strains are easily accessible and have a certain degree of natural genetic variation. Despite these advantages, little effort has been made so far to the development of in vitro and in vivo models for the study of this neurotropic virus, given that human infections most often remain asymptomatic, or with a benign clinical expression and only a few bird species naturally develop severe forms of USUV virus disease.

The different in vitro and in vivo models are essential to investigate the specific pathogenicity, virus transmission routes, and host tropism.

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