Crossing Barriers:

How Exotic Arboviruses Journey Through the Placenta

Judith Oymans

Propositions

1. Shuni virus has the potential to become the next Zika virus.
   (this thesis)

2. In humans RVFV infection will most likely result in miscarriage and not in congenital malformations.
   (this thesis)

3. The increasing need for a COVID-19 vaccine will result in decreasing vaccine acceptance.

4. Negative results are an important part of scientific progress, and should be published as such to prevent scientists from repeating unnecessary experiments.

5. Everybody who has been on holiday in a country where tuberculosis (TBC) is prevalent, such as South Africa, should get tested for TBC 2 months after returning home.

6. A PhD is like an escape room that takes 4 years with an escape rate of 27%.

Propositions belonging to the thesis, entitled

Crossing barriers: how exotic arboviruses journey through the placenta

Judith Oymans
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Crossing barriers:

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Crossing barriers:

How exotic arboviruses journey through the placenta

Judith Oymans

Thesis

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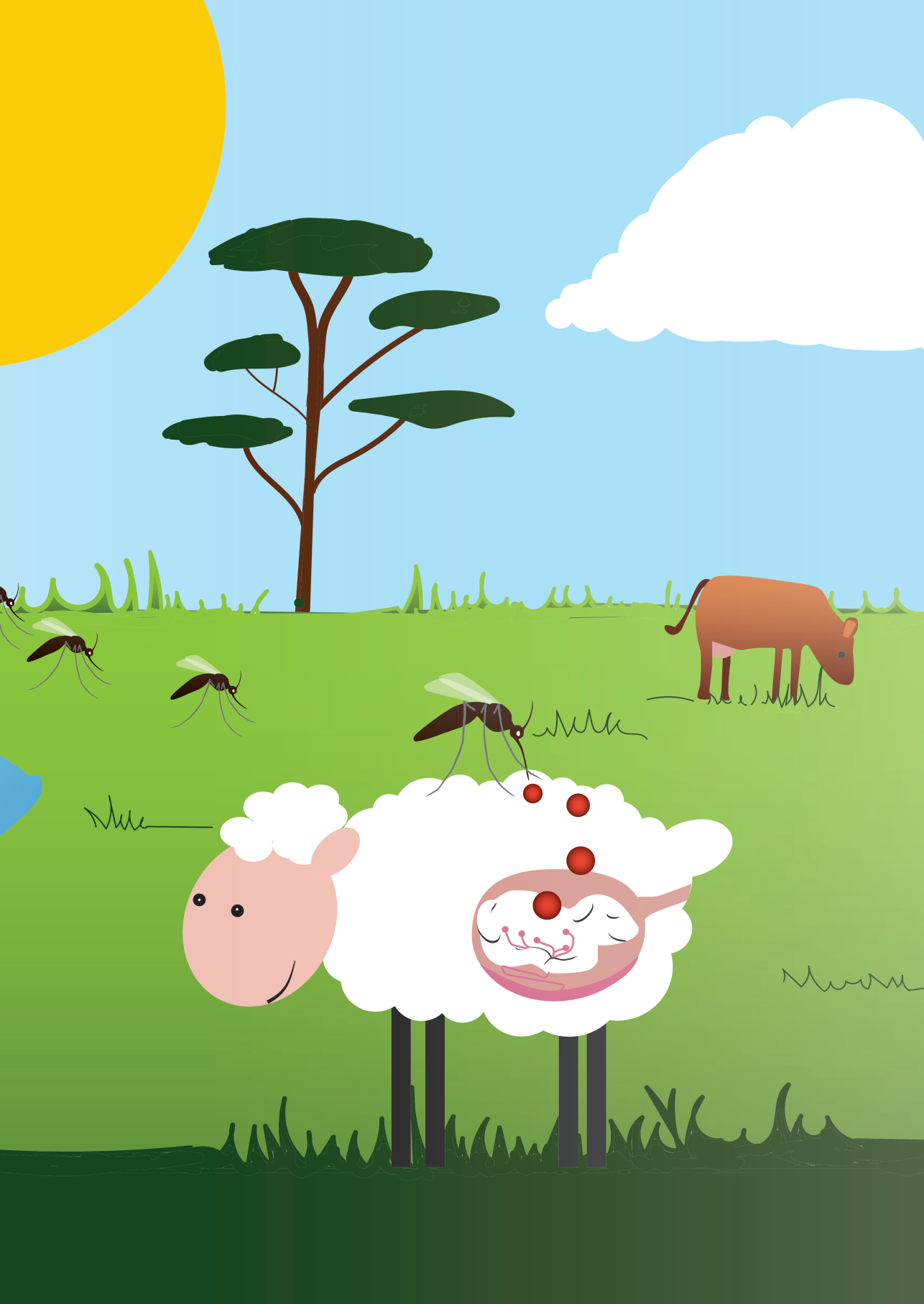

Chapter 1

General introduction

The medical importance of arboviruses

Over 500 species of viruses that are spread by arthropod vectors such as mosquitoes, midges, ticks, and sandflies are known. These viruses, belonging to 14 different virus families, are called arthropod-borne viruses, or arboviruses[1]. Most arboviruses are not pathogenic to humans or animals, however more than 100 species have been described to cause human or animal disease[1,2]. Virtually all viruses that are pathogenic to humans or animals belong to the families *Flaviviridae, Togaviridae, Reoviridae* and multiple families within the order *Bunyavirales*. Although these viruses differ greatly genetically and phenotypically, there are striking similarities in their lifecycles.

Throughout the lifecycle of an arbovirus, the virus needs to successfully infect both an insect's vector and a vertebrate host. An arbovirus is taken up by an insect vector during a blood meal on an infected vertebrate, after which the virus needs to replicate in the insect midgut and subsequently escape from the midgut to disseminate to other organs including the salivary glands[3,4]. When an infected insect feeds on a vertebrate, the arbovirus is injected with the insect saliva and initiates infection in the new host. In both hosts the viruses need to evade or overcome the immune system, and cross different barriers to reach their (intended) target cells[3,4].

The medical importance of arboviruses lies in their ability to cause severe disease, which generally is a consequence of an infection of the central nervous system (CNS), liver, cells of the innate immune system, or the placenta and foetus during pregnancy[5]. Arbovirus epidemics have become more frequent over the past decades, and these outbreaks have an increasing impact on human and veterinary health. In the US the most common cause of epidemic meningoencephalitis is a flavivirus called West Nile virus (WNV). WNV first emerged in 1999 in North America and has since remained endemic in indigenous bird species, occasionally affecting humans and horses[6]. After its discovery in 1937 in Uganda, WNV spread via migratory birds to Europe, Asia and Australia where sporadic cases of human WNV infection were observed[7,8]. Since 2008, WNV emerged more frequently in southern Europe, causing outbreaks in Italy and Greece[9,10]. WNV was detected for the first time in Germany in 2018[11] and in the Netherlands in 2020[12]. The most recent flavivirus epidemic was caused by Zika virus (ZIKV), which emerged in South America in 2015[13,14]. Although ZIKV generally causes a mild febrile illness, infection during pregnancy can result in vertical transmission, inducing congenital brain abnormalities such as microcephaly[15]. Moreover, a small percentage of infected individuals develop Guillan-Barré syndrome, a neurological disorder of the peripheral nervous system with significant morbidity and mortality[16]. Whereas the impact of WNV and ZIKV on animal and human health is currently well recognized, it is important to note that these viruses were largely neglected until two decades ago.

The (re)emergence of arboviruses is stimulated by rapidly growing human and animal populations, changes in land use, intensified travel and trade, and climate change. Particularly, increases in temperature and humidity may stimulate arthropod vectors to slowly move into new territories[17]. However, it is important to recognize that in most 'virgin soil epidemics' an indigenous mosquito species was found to be competent in transmitting a newly introduced arbovirus among immunologically naive individuals[18,19]. It is therefore important to study arboviruses and their interaction with indigenous vectors, before they emerge in new areas. Due to their ability to affect both animals and humans, arboviruses with zoonotic potential require special attention.

The ZIKV outbreak of 2015 has demonstrated how poorly the world is prepared for epidemic (arbo)viral diseases. The many studies that have been published in the wake of the outbreak have highlighted how little was known about the virus and the disease it causes, in particular about the pathogenesis that leads to congenital malformations. To be better prepared for future outbreaks, it is imperative that we gain more knowledge on viruses, that have, like Zika virus, the potential to cause congenital disease in humans and/or animals. Therefore, in this thesis I will focus on several species belonging to the order *Bunyavirales* and the family *Flaviviridae* as several members have the ability to transmit vertically[20].

Bunyavirus genome organisation and replication cycle

The order *Bunyavirales*, formely the family *Bunyaviridae*, is a large order of negative-sense, single-stranded RNA viruses that infect humans, animals (including insects), and plants. The order was established in 2017 to accommodate the large number of viruses and currently contains 12 virus families and 48 genera[21]. In this thesis, viruses of 2 genera are discussed: the genus *Phlebovirus* of the family *Phenuiviridae* and genus *Orthobunyavirus* of the family *Peribunyaviridae*. Phleboviruses generally cause mild infections, however Rift Valley fever virus (RVFV), is unique among phleboviruses as it is able to cause lethal disease in several species and is able to cross the placenta[22]. The genus orthobunyaviruses is the largest genus within the order *Bunyavirales* comprising over 170 viruses, several of which are able to compromise human and/or veterinary health[21]. Generally the viruses of veterinary importance, such as Akabane virus (AKAV) and Schmallenberg virus (SBV), are able to cross the placenta[23].

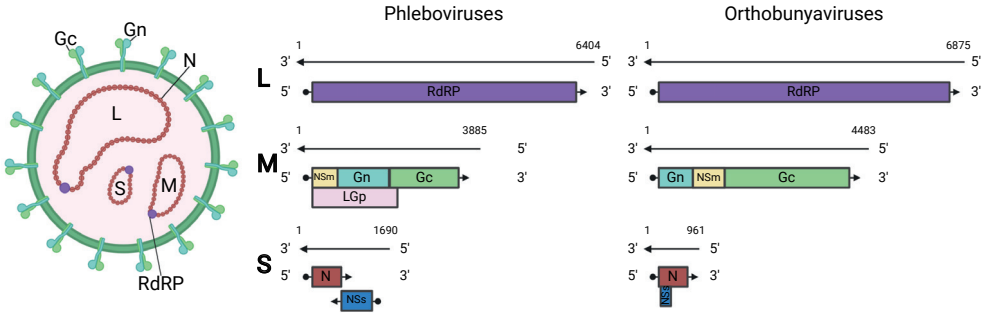

Figure 1.1. Virion structure and coding strategy of phlebo- and orthobunyaviruses. Adapted from Hughes 2020[24].

Both phleboviruses and orthobunyaviruses have a tripartite genome of which the segment names are based on their size: large (L), medium (M) and small (S) (Figure 1.1). The L segment encodes the RNA-dependent RNA polymerase (RdRp), which is crucial for the replication of the genome segments[25]. The M segment encodes the glycoproteins Gn and Gc and a non-structural protein named NSm. The exact functions of most NSm proteins have not yet been elucidated. In Rift Valley fever virus (RVFV), a phlebovirus, NSm has been shown to be dispensable for replication, infection and maturation[26] and it was reported to have an anti-apoptotic function[27]. The RVFV M segment additionally encodes a minor structural protein that was named after its molecular weight, the large 78-kD glycoprotein (LGp), which was shown to be important for virus dissemination in mosquitoes[28]. In orthobunyaviruses, NSm is thought to function as a scaffold for virion assembly[29]. The S segment encodes the nucleocapsid protein (N) and a non-structural protein named NSs. The N protein coats the genomic RNA and together with the RNA and RdRp forms the ribonucleoprotein (RNP) complex[30]. The NSs protein is considered to be the major virulence factor for bunyaviruses, and plays an important role in viral evasion from host innate immunity[31]. In phleboviruses, NSs is encoded in an ambisense manner and has several mechanisms to antagonise the innate immune system[32]. RVFV NSs has been shown to induce DNA damage, to suppress general host transcription and to trigger host translational shut-off[32-34]. Moreover, RVFV NSs directly antagonises IFN-β expression by suppression of IFN-β production at the transcriptional level[35]. In orthobunyaviruses NSs is encoded by an alternate open reading frame[24]. NSs of Schmallenberg virus (SBV), a well-studied orthobunyavirus, is able to suppress innate immunity by host transcriptional shut-off[36,37]. Additionally, SBV has been shown to induce apoptosis which may play a role in viral pathogenesis[38].

Both viruses of the families *Peribunyaviridae* and *Phenuiviridae* enter host cells by the binding of the glycoproteins Gn or Gc to attachment factors and/or receptors on the cell surface. Subsequently, the viruses enter the cell via clathrin-dependent endocytosis[39,40]. The low pH in the endosome then triggers the activation of the class II fusion protein Gc, which fuses the viral envelope with the endosomal membrane, thereby releasing the

viral RNPs (vRNPs) into the cytosol[41-43]. At the cytoplasm the vRNPs direct synthesis of mRNA and of complementary viral RNA (cRNA)[44]. Progeny vRNPs, traffic by yet unknown mechanisms to the Trans-*golgi*-Network where newly produced glycoproteins assemble into virions and the RNPs are packaged[43,45].

Rift Valley fever virus

RVFV, a phlebovirus from the family *Phenuiviridae*, was first isolated during an outbreak among sheep on the shores of lake Naivasha in the Rift Valley of Kenya[46]. The virus caused heavy mortality among the newborn lambs on the farm. Moreover, a high mortality rate of adult animals was observed as well as an increased rate of abortions[47]. RVFV proved to be a zoonotic virus as laboratory workers became ill after exposure[48]. Since the discovery of RVFV it has become apparent that RVFV is widespread in Africa, with occasionally major epidemics such as in 1951 in several states in South Africa[49]. Many farmers lost a large proportion of their sheep and nearly all of their lambs, while the losses of cattle were less significant[50,51]. Since then RVFV has remained endemic to South Africa, occasionally causing major epidemics with severe losses of sheep and cattle as a result, but also affecting humans[52,53]. RVFV was reported in Egypt for the first time in 1977 after completion of the Aswan dam, which created a new breeding ground for mosquitoes[54]. This outbreak was unique due to the large number of humans that were infected, which was estimated at 200,000 with 598 reported deaths[55-57]. In 1987, RVFV outbreaks were reported for the first time in East Africa in Mauritania and Senegal[58,59]. In the year 2000, the first incursion of RVFV outside of the African continent was reported, when two simultaneous outbreaks occurred on the Arabian Peninsula, one in northern Yemen and the other in Gazan[60]. Nowadays, RVFV is endemic throughout the whole African continent and the Arabian Peninsula, occasionally causing major epidemics and epizootics[57]. The history of these RVFV outbreaks, together with genomic analysis of RVFV strains from different regions suggest that RVFV is able to spread over long distances and is able to cause epizootics and epidemics after a single introduction[61].

RVFV has a very broad host range: apart from infecting domesticated ruminants like sheep, cattle and goats, the virus also infects several antelope species, camelids and African buffaloes[62]. Since its discovery RVFV has been isolated from more than 53 mosquito species from 8 genera within the *Culicidae* family[63]. The most important vectors for RVFV transmission belong to the genera *Aedes* and *Culex*. African, European and US breeds of mosquitoes were shown to be competent vectors under laboratory conditions[64-67], and it has recently been shown that RVFV can be efficiently transmitted from viremic European sheep to mosquitoes and *vice versa*[68].

Sheep, the most susceptible species, develop severe clinical signs after RVFV infection. Newborn lambs seldomly survive RVFV infection, whereas in adults the mortality rate is generally below 30%[69]. Clinical signs include fever, listlessness, loss of appetite, abdominal pain and diarrhoea. The most typical pathological feature is severe necrosis of the liver,

often accompanied by jaundice and oedema. Haemorrhages can occur in the heart, spleen, kidneys and intestines[70]. A hallmark of a RVFV outbreak in sheep herds, however, is the so-called 'abortion storm', in which all pregnant ewes abort almost simultaneously[71].

Goats are also highly susceptible to RVFV, however there is more variation in the onset of signs, and viremia levels are lower in goat kids as compared to lambs[69]. Cattle are less susceptible to the disease, in calves the mortality rate is around 10% and like goats the onset and duration of clinical signs varies[72]. Infection of adult cattle is less severe than in sheep[62]. Pseudo-ruminants like camels are likely less susceptible to RVFV infection as few clinical cases have been documented. Nevertheless, both in cattle and pseudo-ruminants RVFV can lead to severe disease and death[62,73].

Human infections generally occur via contact with contaminated tissues and blood released during the slaughter of diseased animals, however humans can also become infected via mosquito bites[52,74]. RVFV infection in humans generally develops as a self-limited febrile illness[75]. Clinical signs include headache, fever and muscle pain, although vomiting, nausea and general malaise are also reported[54,70,75]. In 1-2% of the cases RVFV can lead to severe disease, such as encephalitis or haemorrhagic fever, of which the latter is fatal in up to 50% of cases[50,76,77]. Moreover, infection of the retina can lead to temporary or permanent loss of vision[78].

A correlation was found between RVFV infection and miscarriage in a cross-sectional study in Sudanese women, and a recent study with human placental explants showed that human trophoblasts are highly susceptible to RVFV, indicating that in humans RVFV infection may also lead to congenital malformations or miscarriage in pregnant women[79,80]. However, the causal relationship of human RVFV infection with miscarriage is less clear than in animals.

Schmallenberg virus

In the spring of 2011, a novel virus was discovered on a farm near a small town in Germany called Schmallenberg[81]. In the first few months, the Schmallenberg virus (SBV) quickly spread throughout Europe infecting domesticated ruminants in a large part of Europe, including Belgium, France, Luxembourg, Italy and the southern part of the United Kingdom[82]. In the next year, SBV spread even further to Scandinavian and Eastern European countries, ultimately being reported in 27 countries[82]. Genetic analysis of this new virus revealed that it is an orthobunyavirus that is part of the Simbu serogroup, making SBV the first virus of this serogroup to emerge in Europe[83,84].

SBV is spread by biting midges of the *Culicoides* family[85-88]. The major target species are domesticated ruminants: cattle, sheep and goats[82]. However, SBV has also been detected in zoo ruminants, such as giraffes, camelids and Asian elephants[89,90], and also in wild ungulates in the Alps, Germany, Belgium and Poland[91-94].

SBV infection of adult ruminants can present with mild unspecific clinical signs such as reduced milk yields, fever and diarrhoea, however in most cases infections remain asymptomatic[81,95]. SBV susceptibility and permissibility of the placenta and foetus differs for the different stages of gestation, in which SBV infection during a critical phase, generally the early phase of pregnancy, may lead to severe congenital malformations whereas infection at a later phase does not. Based on available data of a closely related virus, Akabane virus (AKAV), this critical period of gestation in cattle is between 3 and 6 months[96], and in sheep between 30 and 50 days of gestation[82,97]. The congenital malformations that are caused by SBV in ruminants include varying degrees of skeletal-muscular and neurological defects that include arthrogryposis, scoliosis, hypoplasia of the central nervous system (CNS) and porencephaly. The syndrome comprising these manifestations is known as arthrogryposis-hydranencephaly syndrome[98-102]. Foetuses with these malformations are generally stillborn, however, abortions, or embryonic death as a result of SBV infections also occurs[103,104]. SBV was found to be a strictly animal virus as no SBV-specific antibodies were found in a serological study including 301 participants that live in SBV affected areas[105].

Shuni virus

Shuni virus (SHUV), an orthobunyavirus of the Simbu serogroup, is closely related to SBV[106]. It was first isolated in 1966, from a cow in a slaughterhouse and from the blood of a febrile child in Ibadan, Nigeria[107]. In 1977, SHUV was found again in two horses that had died as a result of neurological disease in Zimbabwe and South Africa[108,109]. SHUV re-emerged in 2009 in South Africa where again a correlation was observed between SHUV infection and neurological disease in horses[110]. In 2014, SHUV was detected for the first time outside of the African continent, in Israel where it was discovered in domesticated ruminants, specifically in sheep, goats and cattle[111]. Genomic analysis of the S-segment of different Israeli SHUV strains revealed a very high nucleotide similarity (99-100%), whereas the nucleotide similarity between the 'prototype' virus strain that was originally isolated in Nigeria and the Israeli strains was found to be 90-94%[112].

SHUV has been isolated from pools of field-collected *Culex theileri* mosquitoes and *Culicoides* biting midges[113-115]. A recent study was performed to test the vector competence of laboratory-reared biting midges and mosquitoes that are known vectors of other arboviruses. It was found that SHUV efficiently disseminates in the tested midges, whereas this was not the case in the tested mosquito species[116]. These results indicate a possible role for biting midges in the dissemination of SHUV virus. However, as most orthobunyaviruses are spread by mosquito vectors, more research is warranted into the vector competence of mosquitoes for SHUV.

SHUV is able to infect a wide range of mammalian host species. Next to domesticated ruminants and horses, several wildlife species, such as rhinos, giraffes, warthogs, crocodiles and several antelope species have been shown to be susceptible by detection

of viral RNA or SHUV-specific antibodies in collected sera[110,111,117]. There are indications that SHUV has zoonotic potential[118]. It has been isolated from a febrile child, and in a survey performed amongst South African veterinarians it was found that 4% of the veterinarians had antibodies against SHUV[119]. In horses, SHUV causes severe neurological disease that is often fatal[110]. Several cases of neurological disease have also been described in young cattle[120], while in adult ruminants the disease seems generally asymptomatic[121]. However, infection of pregnant animals can lead to severe congenital malformations such as arthrogryposis-hydrancephaly syndrome[111].

Flavivirus genome organisation and replication cycle

Flaviviruses are RNA viruses with positive-strand, non-segmented genomes, belonging to the genus *Flavivirus* and family *Flaviviridae*[122]. Most flaviviruses are arthropod-borne and the genus includes several important human pathogens such as West Nile virus, dengue virus, yellow fever virus and Zika virus[123].

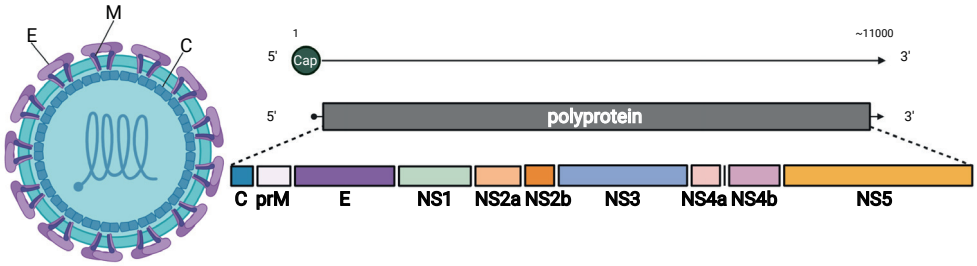

Figure 1.2. Virion structure of a mature flavivirus particle and coding strategy of flaviviruses. Adapted from Simmonds et al 2017 [122].

The flavivirus genome is approximately 11,000 nucleotides (nt) long and contains a 7-methylguanosine (m7GpppN) cap structure attached to the 5' UTR that is essential for translation of the viral genome[124]. During translation a large polyprotein is formed that is cleaved to form the structural proteins: capsid protein (C), precursor membrane (prM), envelope protein (E), and the non-structural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Figure 1.2)[122]. The NS1 protein is essential for RNA replication, particularly in the very early stage of infection[125]. NS1 is also important for evasion of the innate immune system as it binds to specific proteins of the complement system[126]. Interestingly, soluble NS1 secreted into the bloodstream of vertebrates contributes to virus transmission by increasing the infectivity of the virus, possibly contributing to more severe disease[127]. NS2a, which is a small hydrophobic protein, appears to play a role in virion assembly[128,129]. NS2b is a protease co-factor and forms a complex with NS3 that is responsible for processing of the polyprotein into the separate proteins[125]. The roles of NS4a and NS4b are not fully understood, however they have been shown to inhibit IFN signalling[130]. NS5 is vital for the

replication of the genome as it encodes the RdRp and a methyltransferase domain that caps the viral RNA[125,131].

After binding of receptors or attachment factors on the membrane of the host cell, flaviviruses enter the cell via clathrin-dependent endocytosis[132]. The low pH in the endosome triggers a conformational change in the E protein, inducing fusion of the viral membrane with the endosomal membrane, resulting in the release of the viral genome into the cytosol of the host cell[133]. Replication takes place in a membrane-associated replication complex that is assembled at the cytosolic side of the endoplasmic reticulum[134,135]. Immature virus particles are assembled on the membrane of the ER where the newly synthesised proteins and viral genomes come together. These immature particles bud into the ER, and are transported through the Golgi complex where host furin proteases cleave the prM proteins, leading to maturation of the virus particles[136]. The mature viruses then leave the host cell via exocytosis.

Wesselsbron virus

In 1955 an outbreak was reported on a sheep farm in the Wesselsbron region in South Africa. New-born lambs were dying during their first week of life and many ewes were aborting at full term. After RVFV was excluded as the causative agent of this outbreak a new virus was isolated from a decomposed lamb, which was named Wesselsbron virus (WSLV) after the region where it was found[137]. Since then WSLV has been detected throughout the African continent, although most cases were reported in Zimbabwe, Nigeria, Botswana and Senegal[138-141].

WSLV is primarily spread by mosquito vectors belonging to the *Aedes* genus, however it was also found in a tick species once[141,142]. Sheep and goats are most severely affected by WSLV infection, however other ruminants, such as camelids and horses are also susceptible to the virus[141]. WSLV has also been isolated from a black rat in Senegal, indicating that small rodents may also play a small role in the epidemiology of WSLV[139]. Moreover, WSLV has been shown to be a zoonotic virus, as several human cases have been described in different countries[139,141,143]. Most of the described cases were the result of laboratory-acquired infection, however prevalence in humans is likely underestimated as there is little to no surveillance of WSLV in hospitals.

In humans WSLV infection causes fever, headaches, myalgia and arthralgia[139,141,143]. More severe disease, manifesting with encephalitis, has only been described once, when a laboratory worker accidentally sprayed virus suspension in the eye[141]. In adult sheep and goats WSLV causes a febrile illness with mild lesions in the liver and the mortality rate of adult animals is very low[144]. Contrarily, in new-born animals the mortality rate reported is approximately 30%[145]. In pregnant animals WSLV infection leads to congenital malformations or abortion[138,141]. In pregnant sheep congenital malformations were observed in >95% of experimentally infected ewes[146]. Pathological manifestations

of WSLV infection of the foetal CNS range from anencephaly (absence of the brain) to hydranencephaly and was generally associated with arthrogryposis and muscular atrophy[146]. In experimentally infected pregnant cows congenital malformations were observed in 17% of the cases, with similar pathology as observed in sheep foetuses[147].

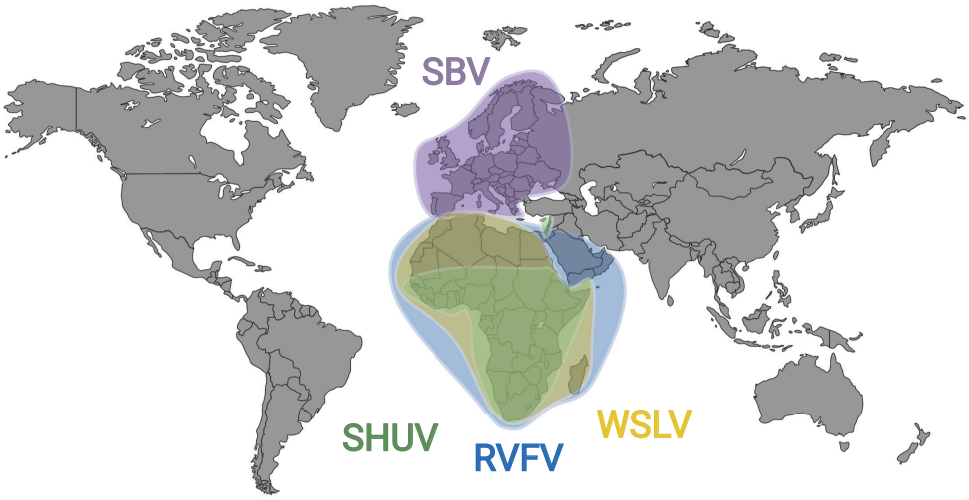

Figure 1.3. Predicted geographical distribution of SHUV, SBV, WSLV and RVFV around the world.

Biology and classification of placentas

WSLV, SHUV, SBV and RVFV are all able to transmit vertically resulting in congenital malformations or abortion in pregnant animals. To understand mechanisms of vertical transmission it is important to identify target cells and tissues that are susceptible and permissive to the respective viruses. The organ that is responsible for keeping the foetus safe from (viral) infection is the placenta. This versatile organ has multiple core functions and continues to develop and remodel during pregnancy to adapt to the specific foetal needs. The core functions can be divided into three main roles: transport of nutrients and waste products, endocrine functions, and protection from pathogens[148]. The regulation of the blood supply is essential as this enables the correct functioning of the placenta. The placenta is a unique organ as it receives blood supplies from the mother and the foetus and thus has two separate circulatory systems: the maternal-placenta circulation and the foetal-placenta circulation[149]. These separate systems become fully functional around the end of the first trimester of pregnancy[149]. The placenta provides the foetus with oxygen, water, carbohydrates, amino acids, lipids, vitamins and minerals while also removing carbon dioxide and other waste products. Transport occurs via diffusion, protein-mediated diffusion or transporter proteins[148]. The placenta is also able to metabolise amino acids,

lipids and carbohydrates to adapt them specifically to the need of the foetus[148,150]. Moreover, the placenta synthesizes important hormones that play a role in establishing and maintaining pregnancy, foetal development and the induction of labour[150,151].

The placental barrier primarily consists of foetal trophoblast cells, that form the tissue layer that determines what is allowed to cross the placenta[150]. A careful balance has to be maintained between immune responses and tolerance, and disruption of this balance can lead to complications during pregnancy. The regulation of the immune system at the placental barrier is extremely complex. It was long thought that the immune system is suppressed in the placenta, however new insights suggest that this is incorrect and that the immune system is highly active, functional and carefully controlled[152]. It has been suggested that while the maternal immune system may be repressed locally, the foetal trophoblasts and the maternal immune system collaborate to protect the foetus[50]. This involves modulation of the innate immune system, in particular IFN-β and λ[153] and invasion of macrophages, NK cells and regulatory T cells[152].

There is great variation in morphology of the placenta among species. There are two different methods of classifying the different types of placentas: by morphology or by histology [154,155]. When classifying placentas by morphology, four different types can be distinguished (Figure 1.4). The first is the diffuse placenta, in which foetal villi cover the whole surface of the uterine epithelium, as is the case for horses and camelids [154,156]. Ruminants carry a placenta comprised of placentomes, in which discrete patches of foetal villi, named the cotyledones, interact with the maternal tissue of the placenta, called the caruncles[157]. Zonary placentas comprise a complete or incomplete band of tissue surrounding the foetus, which is common in carnivores[155]. Lastly, the discoid placenta consists of a single disc and is most commonly found in humans, primates and small rodents such as mice and rats[148,154].

Classification by histology focuses on the different cell layers in the placenta and in particular on the number of cell layers that are present between the foetal and maternal blood (Figure 1.4). In the epitheliochorial placenta the foetal cell layers consist of foetal endothelial cells, which form the foetal blood vessels, followed by the foetal connective tissue, and finally the foetal epithelial cell layer called trophoblasts. The maternal part of the epitheliochorial placenta is formed by maternal epithelial cells, followed by the maternal mesenchyme and the maternal endothelial cells, which form the maternal blood vessels. The synepitheliochorial placenta is similar except for the foetomaternal syncytial cells in the maternal epithelium, which are formed by fusion of migrating trophoblasts and maternal epithelium cells. In an endotheliochorial placenta there is no maternal epithelial cell layer. The endothelial cells of the maternal blood vessels are in direct contact with the foetal trophoblasts. In the haemochorial placenta no cell layers separate the maternal blood from foetal tissue, meaning that the maternal blood is in direct contact with foetal trophoblasts[154,155].

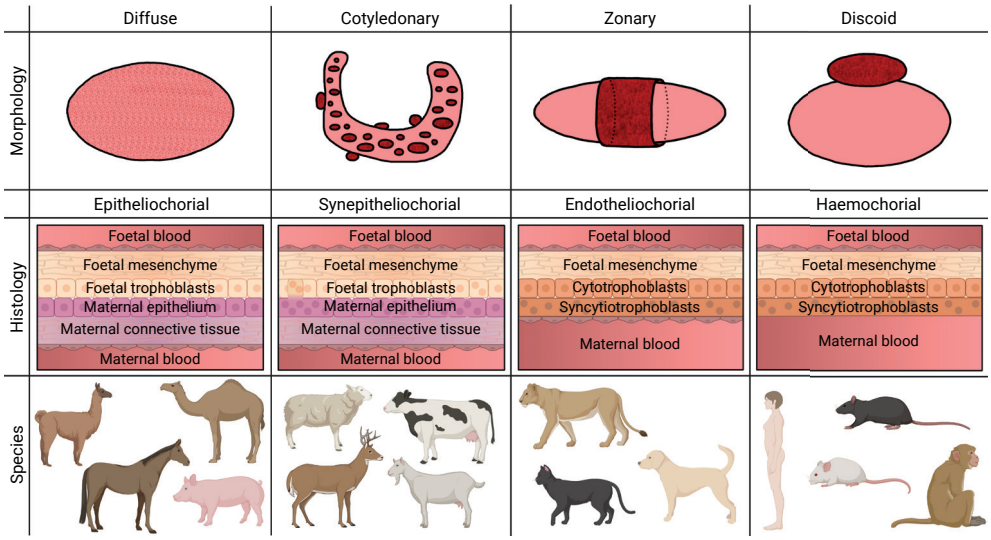

Figure 1.4. Classification of placentas by morphology, histology and species. Generally both classifications (histology, morphology) are used to describe a species' placenta. For example humans have a discoid haemochorial placenta and horses a diffuse epitheliochorial placenta.

The most descriptive manner to classify placentas is to use both classification systems together. In this thesis I studied arbovirus infection in the ovine and human placenta, therefore these placentas will be described in more detail below.

The ovine placenta

Sheep carry a cotelydonary synepitheliochorial placenta (Figure 1.5, top panel). A typical ovine placenta consist of 30-80 placentomes that are concave in shape, comprising a maternal part, the caruncle, and a foetal part, the cotyledon[157]. All placentomes are connected by a complicated network of blood vessels held together by the intercotyledonary membrane. As the placenta develops, the foetal villi invade the maternal epithelium. An average sheep pregnancy takes 150 days and the development of foetal villi starts at gestation day (GD) 24-26, while at mid-gestation, GD 70-90, the foetal villi reach their maximum length and the placentomes their maximum weight[154]. At that moment there is a considerable amount of foetal connective tissue, however as the foetus develops and grows it will require more nutrients, therefore the surface area of nutrient exchange will keep expanding accordingly until at term there is very little connective tissue left[157].

The synepitheliochorial placenta has three defining characteristics. First the cotyledonary shape of the placenta, second the binucleate cells (BNCs) and lastly the syncytial cells that are formed by the BNCs[154]. Early in pregnancy, around 15-20% of the foetal trophoblasts are BNCs, which differentiate from the mononuclear trophoblasts by nuclear division

without cytokinesis[154]. It is generally accepted that these cells then migrate to the maternal epithelium where they fuse with maternal epithelial cells to form trinucleate syncytial cells[158]. As BNCs continue to develop and fuse with maternal epithelial cells, syncytial plaques are characteristically formed with an uneven number of nuclei[158]. Recent research has shown that mononuclear trophoblasts also fuse with each other, leading to trophoblast giant cells (TGCs) which are also able to migrate and fuse with the maternal epithelium[159]. The main function of these cells is to modify the maternal environment to favour the foetus both metabolically and immunologically[154,159,160]. TGCs can do this by producing important foetal hormones and effectors which are carried in vesicles and are released upon fusion with the maternal epithelium[154]. An important hormone is progesterone, which is crucial for maintaining pregnancy[161]. At the start of the pregnancy this hormone is produced by the corpus luteum, however as the placenta continues to develop, from GD 50 the placenta becomes the main source of progesterone production[53]. Of note, in sheep about 90% of the maternal epithelium is eventually modified to foetomaternal syncytial plaques during pregnancy[154].

The villous shape of the foetal and maternal parts of the placenta is essential for the exchange of nutrients and gasses as this increases the surface area considerably[154]. At the crypts of the foetal villi haemophagous zones are formed where intermittent vascular leakage forms pools of stagnant maternal blood[154,157]. The haemophagous zone is important for uptake of iron for the developing foetus. Whole and fragmented erythrocytes are taken up by foetal trophoblasts lining the haemophagous zones[162]. During pregnancy the mass of the haemophagous zones increases as the foetuses and the placentas grow and need more iron. The blood pools become larger and the trophoblasts become more specialised, enabling them to phagocytose more erythrocytes as pregnancy progresses[162]. These trophoblasts are characteristically very large and contain large vacuoles with phagocytosed blood cells[154].

Little information is known about the immune responses within the ovine placenta. The foetal trophoblasts are negative for MHC molecules, as are the syncytial and maternal cells of the placenta, however stromal cells in the foetal mesenchyme do contain MHC molecules[157]. Although there are no lymphocytes at the foetal side of the placenta and immunoglobins do not cross the placental barrier, the ovine foetus becomes immunocompetent as pregnancy advances[157]. More research is warranted to study the role of the innate immune system in the ovine placenta including the role of cytokines.

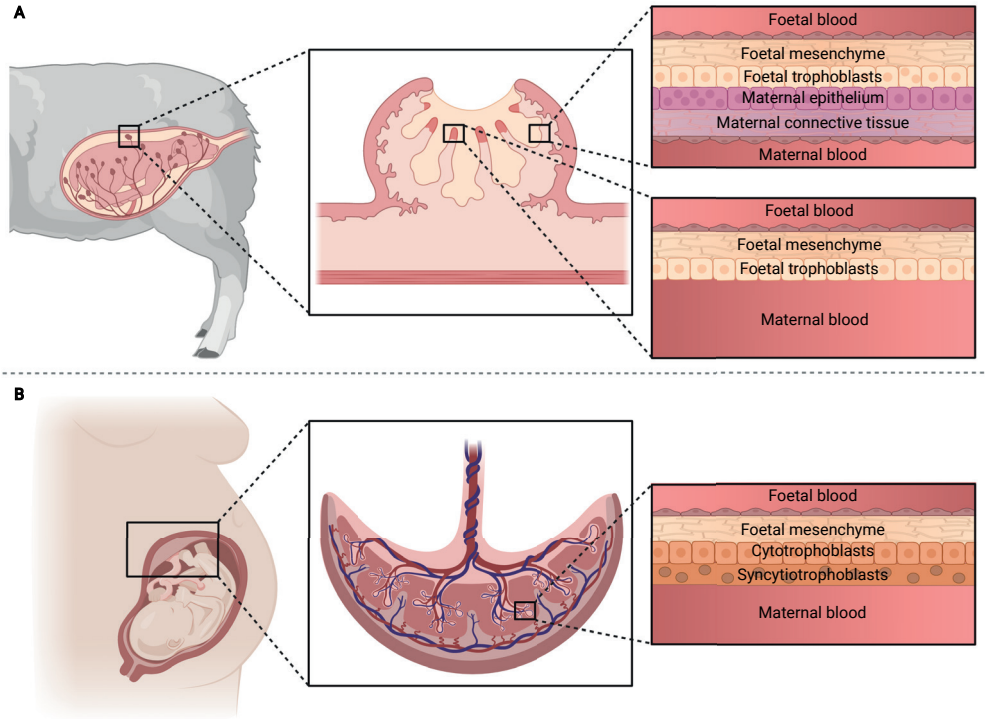

Figure 1.5. Schematic overview of ovine (top panel) and human placenta (bottom panel), highlighting the differences in morphology and histology.

Human placenta

The human placenta is a discoid haemochorial placenta composed of foetal tissues and maternal tissues, which is characterised by the direct contact of maternal blood with foetal trophoblasts (Figure 1.5, bottom panel)[148]. The foetal tissue of the placenta is called the chorionic plate, whereas the maternal tissue is called the basal plate. In between these regions is the intervillous space which contains extensively branched foetal villi which carry the foetal blood vessels[148]. This is the main region where maternal-foetal exchange of nutrients and waste products occurs[148]. Maternal blood enters the intervillous space via spiral arteries in the uterus, and although much is unclear about the venous return of maternal blood, this blood flows at a low pressure, low velocity and high-volume through the intervillous space[163]. The foetal blood flow is slowed by extensive capillary loops at the tips of the villi, carefully regulating blood flow for maternal-foetal exchange[163].

Several different types of trophoblasts can be distinguished in the human placenta: cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts[151]. Cytotrophoblasts are stem cells that proliferate and differentiate into syncytio- or extravillous trophoblasts according to the placenta's needs[151]. Syncytiotrophoblasts result from fusion and

biochemical differentiation of a layer of cytotrophoblasts[151]. This cell layer keeps expanding throughout pregnancy through continuous invasion of cytotrophoblasts until the cytotrophoblasts layer has almost disappeared, when pregnancy reaches full term[164]. Syncytiotrophoblasts form the placental barrier by forming the cell layer separating the foetal part of the placenta from the maternal blood, and therefore are crucial not only for the foeto-maternal exchange, but also for the endocrine activity of the placenta[165]. Similar to the ovine placenta, progesterone is initially produced by the corpus luteum, however at 6-8 weeks of pregnancy, one fifth of gestation, the syncytiotrophoblasts take over production of this hormone[166,167]. Endovascular extravillous trophoblasts differentiate from cytotrophoblasts and invade the basal plate to encircle the spiral arteries, thereby determining the blood flow in the intervillous space[151,168]. Interstitial extravillous trophoblasts invade the intervillous space as well as the basal plate to promote the expansion of the placenta, by recruiting more maternal arteries as well as regulating expansion of the intervillous space[148]. After the maximum size of the placenta is reached, interstitial extravillous trophoblasts fuse to become giant trophoblast cells, which is an end stage for these cells as the giant size prevents further invasion[148].

Trophoblasts also contribute to the immune system of the placenta, by eliciting an innate immune response after sensing of pathogen-associated molecular patterns (PAMPs) by Toll like receptors (TLRs)[152,169]. Immune cells of the maternal immune system, macrophages, natural killer (NK) and regulatory T cells are also present in the intervillous space to protect both mother and foetus from pathogens[152]. Moreover, multiple IFN systems, type I, II and III IFNs, are active in the human placenta and act as a first line of defence[50]. It is important to note that there are multiple developmental stages during pregnancy, each with its own unique requirements and role of the immune system.

Preparing for neglected arboviruses

Vaccination is by far the most efficient and efficacious arbovirus infection prevention strategy[170]. However in order to assess the use and need for a vaccine, the impact of a virus first has to be determined. Early detection of the different arboviruses in affected regions by active surveillance of insect vectors and susceptible species is therefore of the utmost importance[171,172]. The aim and task of several global companies is to prioritise viruses that may cause future outbreaks. The Coalition for Epidemic Preparedness Innovations (CEPI) stimulate and accelerate the development of vaccines against emerging infectious diseases[173]. The World Health Organization (WHO) has created a "Research and Development Blueprint" list of prioritized viruses that are likely to cause future epidemics and for which insufficient countermeasures are available[174]. Currently 3 arboviruses are on the list: Crimean-Congo haemorrhagic fever virus (CCHFV), RVFV and ZIKV. The list also contains disease X, which represents the knowledge that a serious international epidemic could be caused by a pathogen currently unknown to cause human epidemics[174]. During the first 50 years since its discovery, ZIKV, the first disease X on the list, was known to

cause sporadic outbreaks limited to Africa and Asia and less than 10 human cases were reported in literature[175]. This is similar to the situation with WSLV and SHUV now, of which occasional outbreaks and few human cases have been reported, therefore these viruses have the potential to be the next 'Disease X'.

RVFV

Although a number of livestock vaccines are available for RVFV, none are licensed for use outside endemic areas[170]. Vaccines based on the live-attenuated Smithburn strain are the most widely used RVF vaccines in Africa[170]. The Smithburn strain was originally isolated from mosquitoes in Uganda and passaged in mouse brains which caused it to lose its tropism for hepatocytes while retaining neurotropic properties[176]. The strain was further passaged in mouse brains and embryonated chicken eggs, until 1971, when the virus was propagated in BHK-21 cells, which allowed the production of freeze-dried vaccines[177]. The vaccine is relatively inexpensive (compared to vaccines based on inactivated virus) and effective after one vaccination[178]. However, the Smithburn strain has been associated with abortion in pregnant ruminants[179]. An alternative vaccine was developed in the US called MP-12. The MP-12 strain resulted from passaging the ZH548 strain, isolated from a human case during the outbreak in Egypt in 1978, 12 times in the presence of the mutagen 5-fluorouracil, resulting in mutations in all three genome segments[180]. The candidate vaccine was shown to be attenuated in adult, young and newborn cattle and sheep[181], however in pregnant ewes the vaccine was associated with congenital malformations[182]. Another attenuated strain, Clone 13, is a plaque purified clone of strain 74HB59, found to contain a large deletion in the NSs gene[183]. Animal trials have demonstrated that this attenuated virus is highly immunogenic and safe for use in adult, young, newborn and pregnant animals [170]. However, another study has shown that administration of a high dose of Clone 13 to pregnant ewes leads to stillbirths and congenital malformations[184]. The risk of abortion or congenital malformations by vaccination of pregnant animals highlights the need for safer and equally efficacious alternative vaccines. With the aim for a next-generation vaccine that optimally combines the safety of inactivated vaccines with the efficacy of live-attenuated vaccines, numerous experimental vaccines have been developed including vector vaccines, subunit vaccines, DNA vaccines, RNA particle vaccines, virus-like particle vaccines, and live-attenuated RVFV developed by reverse-genetics. In this thesis, the efficacy of a novel live-attenuated RVFV vaccine in pregnant ewes is reported (Chapter 3)[170]. Moreover, CEPI has recently funded the development of two live-attenuated vaccines for human use[185].

WSLV

An attenuated WSLV strain was marketed from 1959 onwards[141]. Since RVFV and WSLV outbreaks would often occur together and the signs of infection are similar (congenital malformations, stillbirth and abortion), freeze-dried RVFV and WSLV vaccines were marketed as a combination vaccine. However, the attenuated WSLV was also found to cause congenital malformations in pregnant ewes and was therefore considered unsafe

for vaccination during gestation[146]. The WSLV vaccine is still commercially available with the recommendation to not vaccinate pregnant animals[186]. As pregnant ruminants are the most susceptible species to WSLV infection, there is an opportunity for the development of a vaccine of improved safety.

SHUV and SBV

No vaccines are currently being developed for SHUV, whereas for SBV several chemically inactivated vaccines have been developed and licensed for marketing in Europe[187]. However, after the first outbreak of SBV, ruminants acquired high levels of SBV specific antibodies which made vaccination redundant[187]. Although farmers in unaffected areas in the years after the first outbreak of 2011 did choose to vaccinate their sheep, this rate declined until almost no vaccinations were reported by 2016[188]. A drawback of the inactivated vaccines that are on the market, is that vaccinated animals cannot be differentiated serologically from infected animals, interfering with surveillance of SBV in Europe. Currently no vaccine that enables the differentiation between infected and vaccinated animals (DIVA) is available, however new subunit, and vector vaccines, that may be applicable as DIVA vaccines, are being developed[189-192].

Scope of the thesis

The Schmallenberg outbreak of 2011 and the Zika virus outbreak of 2015 have demonstrated the devastating consequences of epizootics and epidemics caused by new or emerging arboviruses with the ability to transmit vertically in both humans and animals. Our poor preparedness has highlighted a knowledge gap in our understanding of the mechanisms underlying vertical transmission and the pathology that leads to abortion, stillbirth or congenital malformations. It is important that we bridge this knowledge gap by studying neglected arboviruses that exhibit vertical transmission so that we are able to identify and prioritise potential threats for animal and human health in the future. Characterising the mechanisms that underlie the pathophysiology of these viruses could also aid the development of efficient countermeasures. In this thesis, I **elucidated the transmission routes of arboviruses over the placental barrier to the foetuses by identifying primary target cells and tissues.** A first step towards being able to study these viruses is to develop protocols and tools that can also be used for the development of diagnostic procedures.

The detrimental outcomes of RVFV infection in pregnant ewes have been well described since RVFV was first discovered in the 1930s, however the pathology that leads to these outcomes has not yet been elucidated. **Chapter 2** addresses the pathogenesis of RVFV in pregnant ewes at one third- and mid-gestation. Necropsies were performed at different timepoints to gain a better understanding of how RVFV crosses the placental barrier by identification of primary target cells in the ovine placenta. This chapter further elaborates on how RVFV infection results in abortion.

A safe and efficacious vaccine could protect pregnant sheep and their foetuses from the detrimental effects of RVFV infection. Therefore **Chapter 3** evaluates the efficacy of a novel live-attenuated RVF vaccine in pregnant ewes. The so called vRVFV-4s vaccine was constructed by splitting the M-segment of RVFV into two separate segments encoding either Gn or Gc. The vRVFV-4s vaccine was previously shown to be avirulent in mice, lambs, and pregnant ewes. Moreover, it has been shown that a single vaccination protects young lambs, calves and goat kids from experimental RVFV infection [193]. Young lambs are, besides pregnant animals, most susceptible to RVFV infection [69]. To ensure that this vaccine also protects pregnant ewes, a trial was performed testing several vaccination schemes.

Due to the similarity in clinical signs following RVFV and WSLV infection, WSLV is included in the differential diagnosis of RVF. Similar to infections by RVFV, WSLV infection of pregnant ruminants leads to congenital malformations, stillbirths or abortion. Importantly, the mechanisms behind pathogenesis in WSLV infection that lead to these outcomes are also poorly characterised. **Chapter 4** therefore reports primary target cells of WSLV infection in pregnant ewes, with a particular focus on foetal tissues.

SHUV is a neglected orthobunyavirus that also causes congenital malformations, abortions and stillbirths in ruminants. **Chapter 5** investigates the interaction of SHUV with the ovine placenta identifying primary target cells at the maternal-foetal interface. Additionally, to investigate the zoonotic potential of SHUV, the susceptibility and permissibility of human placental cell lines and explants was studied.

To study SHUV more in depth, **Chapter 6** describes the development of a reverse genetics system to allow directed modification of the viral genome. RNA viruses with segmented genomes are known for their ability to occasionally reassort their genome segments with related viruses, creating new viruses with potentially different tropism and virulence. The closely related SBV is present throughout Europe whereas SHUV is present on the African continent and in Israel. Their geographic proximity makes it possible that these two viruses could soon circulate in the same territory. Using our reverse genetics system, we investigated the ability of SHUV to reassort with SBV, enabling us to predict whether reassortment in nature is possible. We additionally employed the reverse genetics system to explore the role of the NSs protein. In closely related orthobunyaviruses, NSs is the major virulence factor due to its role as an antagonist to the innate immune system. A modified SHUV was created lacking a functional NSs to determine whether this is also true for SHUV NSs.

The research reported in this thesis contributes to the elucidation of the transmission routes of arboviruses across the placental barrier, identifying potential cell and tissue targets. It addresses the lack of knowledge on some neglected arboviruses which have zoonotic potential, developing tools for their study that can be further employed in future by us and others to study virus-host interactions. However, many questions remain

unanswered. **Chapter 7** describes the most pressing, interesting and important questions that still remain to be answered and places these results in a broader perspective.

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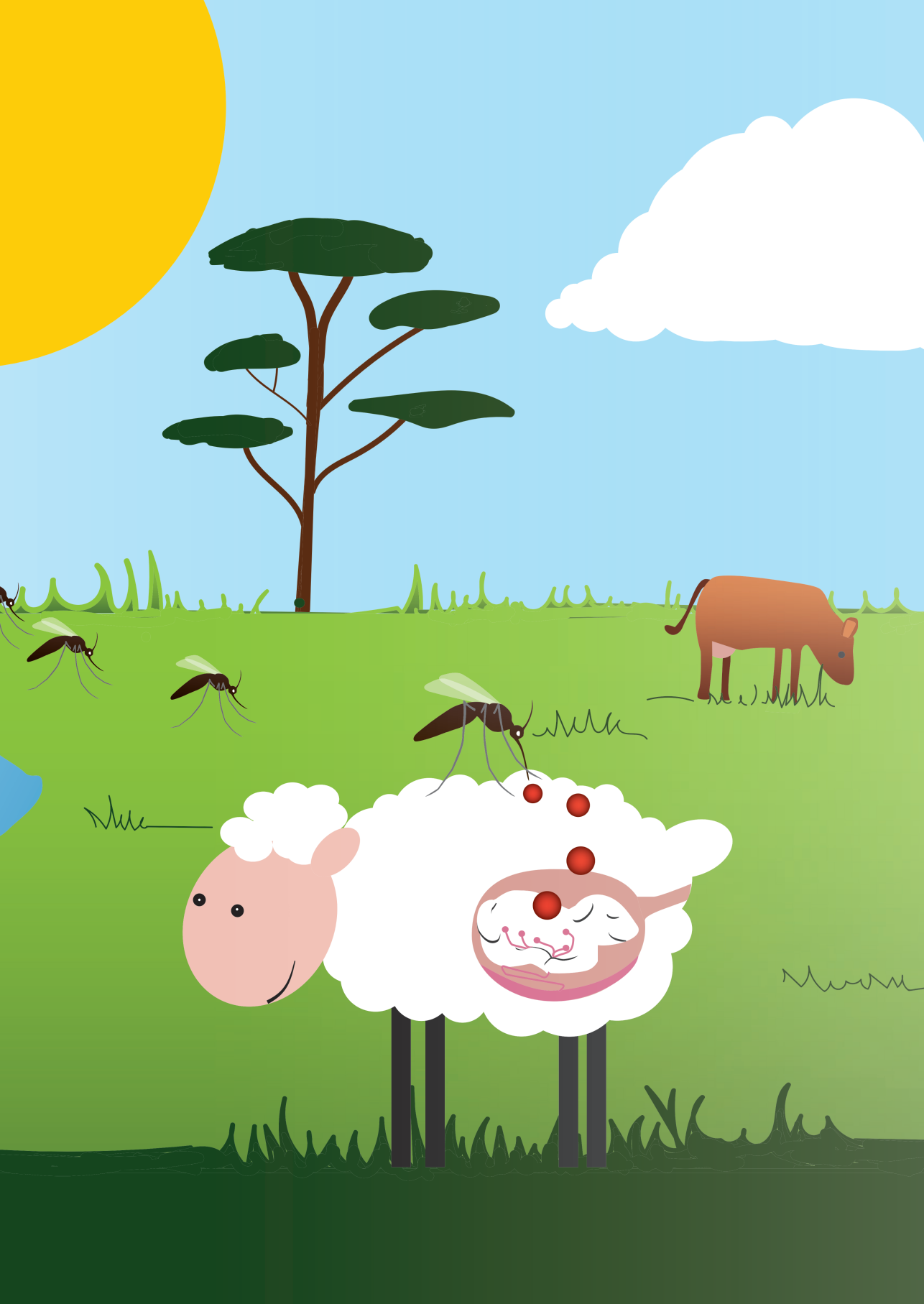

Chapter 2

Rift Valley fever virus targets the maternal-foetal interface in ovine and human placentas

Judith Oymans, Paul J. Wichgers Schreur, Lucien van Keulen, Jet Kant, Jeroen Kortekaas

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Abstract

Background

Rift Valley fever virus (RVFV) is an arbovirus of the order *Bunyavirales* that causes severe disease in ruminants and humans. Outbreaks in sheep herds are characterised by newborn fatalities and abortion storms. The association of RVFV infections with abortions of ovines and other ruminants is well recognized, whereas the pathology resulting in abortion has remained undescribed. Accumulating evidence suggests that RVFV is abortogenic in humans as well, warranting more research on the interaction of RVFV with the ruminant and human placenta.

Methodology/Principal findings

Pregnant ewes were inoculated with a highly virulent strain of RVFV and necropsied at different days post infection. Tissues were collected and analysed by PCR, virus isolation, and immunohistochemistry. The results show that RVFV replicates efficiently in maternal placental epithelial cells before the virus infects foetal trophoblasts. Moreover, the virus was shown to bypass the maternal epithelial cell layer by directly targeting foetal trophoblasts in the haemophagous zone, a region of the ovine placenta where maternal blood is in direct contact with foetal cells. Abortion was associated with widespread necrosis of placental tissues accompanied with severe haemorrhage. Experiments with human placental explants revealed that the same virus strain replicates efficiently in both cyto- and syncytiotrophoblasts.

Conclusions/Significance

This study demonstrates that RVFV targets the foetal-maternal interface in both ovine and human placentas. The virus was shown to cross the ovine placental barrier via two distinct routes, ultimately resulting in placental and foetal demise followed by abortion. Our finding that RVFV replicates efficiently in human trophoblasts underscores the risk of RVFV infection during human pregnancy.

Introduction

Rift Valley fever virus (RVFV) is a negative-strand RNA virus of the family *Phenuiviridae* (former family *Bunyaviridae*), genus *Phlebovirus*. RVFV is transmitted by mosquitoes, predominantly by species of the genera *Aedes* and *Culex*[194]. The virus is pathogenic to domesticated and wild ruminants, of which sheep are the most susceptible. Newborn lambs may succumb within hours after onset of symptoms and seldom survive the infection[46,195]. The most typical pathological feature in lambs is severe necrosis of the liver. Specifically, necropsy of fatal cases reveals a swollen, pale liver with focal to widespread necrosis of hepatocytes. Direct and indirect consequences of liver necrosis include icterus, oedema, and hydrops ascites. Other organs, such as the spleen, heart, kidney and intestines may reveal haemorrhages and congested veins. Fatal cases are generally associated with haemorrhages and signs of shock. Susceptibility decreases with age, although mortality rates in adult sheep may be as high as 60%[196]. The most characteristic feature of RVF outbreaks in sheep herds are abortion storms, in which all pregnant ewes in a herd may abort[71].

Humans can be infected via a mosquito bite, although most human cases are attributed to contact with contaminated animal tissues, particularly during the slaughtering of diseased animals[197]. The disease in humans generally follows a transient, febrile course with severe headache and muscle pains. Infection of the eye may induce temporal or permanent loss of vision resulting from retinal damage[198]. In a minority of patients, the infection progresses to encephalitis or haemorrhagic fever, of which the latter is often fatal[196,199,200]. A cross-sectional study of Sudanese women correlated RVFV infection with an elevated miscarriage rate[80]. Notably, experiments with second-trimester human foetal tissue explants recently revealed that human syncytiotrophoblasts are susceptible and permissive to RVFV replication[201]. These reports warrant further studies to determine if RVFV infections pose a perhaps underestimated risk for human pregnancy.

The high abortion rates in sheep herds suggest that RVFV crosses the ovine placenta extremely efficiently. The placenta of sheep, and that of other RVFV susceptible ruminant species, is very different from the human placenta. The human placenta has the shape of a single disque whereas the ovine placenta consists of placentomes, varying in number among the different ruminant species[202]. Placentomes are discrete areas of extensive villous interactions between the maternal epithelial cells of the uterus, the caruncle, and the foetal trophoblasts of the allantochorion, the cotelydon, enabling efficient exchange of gases and nutrients between mother and foetus. In sheep and goats, pools of maternal blood are located in the crypts of the foetal villi, directly bordering the foetal trophoblast layer. At these so-called haemophagous zones, maternal erythrocytes are phagocytosed by trophoblasts as a principle source of iron for the developing foetus. Notably, this part of the ovine placenta resembles the haemochorial placenta of humans, in that maternal blood is in direct contact with foetal trophoblasts. However, the blood pools at the

haemophagous zones in sheep and goats are filled with stagnant blood and therefore do not contribute significantly to the maternal-foetal exchange of nutrients, whereas in the human haemochorial placenta maternal blood flows along the foetal trophoblasts enabling efficient nutrient exchange.

The detrimental consequences of RVFV infection during ovine pregnancy were already reported after the first outbreak of RVF in 1930 and abortion storms have since then become a hallmark of RVF epidemics[46,47,195,203]. Nevertheless, the pathogenesis of RVFV-induced abortion in pregnant ewes was not yet described in literature. In the present study, we experimentally infected pregnant ewes at one third- or at mid-gestation and describe the pathogenic events that result in abortion of the ovine foetus. We furthermore investigated the susceptibility of human placental explants for the same RVFV strain.

Results

RVFV infection of pregnant ewes results in abortion within one week

To study the pathogenesis of RVF in pregnant ewes, two experiments were performed. Ewes were infected at either one third of gestation (experiment 1) or at mid gestation (experiment 2). In both experiments, ewes were inoculated via intravenous route with a dose of 10^5 $TCID_{50}$ of RVFV, an exposure route and dose that was used in previous studies with sheep[204-206]. In the first experiment, a group of ten ewes was distributed randomly over two groups of five animals of which the first group was inoculated with virus and the second group with medium only (mock) (Figure 2.1A). As expected, and similar as observed in lambs, all RVFV-inoculated ewes developed fever (Figure 2.1B) which correlated with viremia as determined by RT-qPCR and virus isolation on plasma samples (Figure 2.1C). At 4 days post infection (dpi), one of the RVFV inoculated ewes (animal number 1764) acutely succumbed to the infection. Necropsy revealed a swollen, discoloured liver and signs of shock. Analysis of liver and spleen samples by RT-qPCR revealed high levels of viral RNA in both organs (Figure 2.1D). Extensive haemorrhages were found in the placentas of the two foetuses carried by this ewe and in the uterine wall. The remaining ewes were euthanized and necropsied as scheduled at 6 dpi. All ewes showed a multifocal necrotizing hepatitis which was most severe in the animal that died at 4 dpi. Although no abortions had occurred, all placentas revealed extensive haemorrhages in the placentomes and all foetuses had already died (Figure S2.1, Figure S2.3 and Table 2.1).

Table 2.1. Analysis of foetuses collected from RVFV-infected ewes. Pathological findings in foetuses of necropsied ewes experimentally infected with RVFV at one third (1/3 gestation) or at mid-gestation (1/2 gestation). Ewes were necropsied at 4, 6 or 7 dpi. Viral RNA and proteins were detected by RT-qPCR and IHC, respectively. NT; not tested as foetuses were too autolytic.

	DPI	Foetuses	Alive or dead at time of dissection	Placentome		non-cotyledonary allantochorion		Liver		Brain		Amniotic fluid	Umbilical cord		Umbilical blood
				PCR	IHC	PCR	IHC	PCR	IHC	PCR	IHC	PCR	PCR	IHC	PCR
One third gestation	4	1764[1]-F1	Dead	+	+	+	+	+	-	-	-	+	+	-	NT
		1764[1]-F2	Dead	+	+	+	+	+	-	+	-	+	+	-	NT
	6	1760-F1	Dead	+	+	+	+	+	+	+	+[2]	+	+	+[2]	NT
		1761-F1	Dead	+	+	+	+	+	-	-	-	+	+	-	NT
		1761-F2	Dead	+	+	+	-	+	-	-	-	+	+	-	NT
		1762-F1	Dead	+	+	+	-	+	-	-	-	+	+	-	NT
		1762-F2	Dead	+	+	+	+	+	-	+	NT	+	+	-	NT
		1762-F3	Dead	+	+	+	+	+	+	+	NT	+	+	+[2]	NT
		1763-F1	Dead	+	+	+	+	+	+	+	+[2]	+	+	+[2]	NT
		1763-F2	Dead	+	+	+	+	+	+	+	+[2]	+	+	+[2]	NT
Mid-gestation	4	1841-F1	Alive	+	+	+	-	-	-	-	-	-	+	-	+
		1841-F2	Alive	+	+	+	-	-	-	-	-	-	+	-	+
		1842-F1	Alive	+	+	+	-	+	-	-	-	-	+	-	+
		1842–F2	Alive	+	+	+	-	+	-	-	-	+	-	-	+
		1843-F1	Alive	+	+	+	-	+	-	-	-	+	+	-	+
	7	1844-F1	Dead	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT
		1844-F2	Dead	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT
		1844-F3	Dead	+	+	+	+	+	+	NT	+[2]	NT	+	+[2]	NT
		1845-F1	Dead	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT
		1845-F2	Dead	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT
		1846-F1	Alive	+	+	+	+	+	+	+	+[2]	+	+	+[2]	+
		1846-F2	Dead	+	+	+	+	+	+	+	+[2]	+	+	+[2]	NT
		1846-F3	Dead	+	+	+	+	+	+	+	+[2]	+	+	+[2]	NT

[1]Ewe died at 4 dpi; [2]Blood vessels (blood and endothelium).

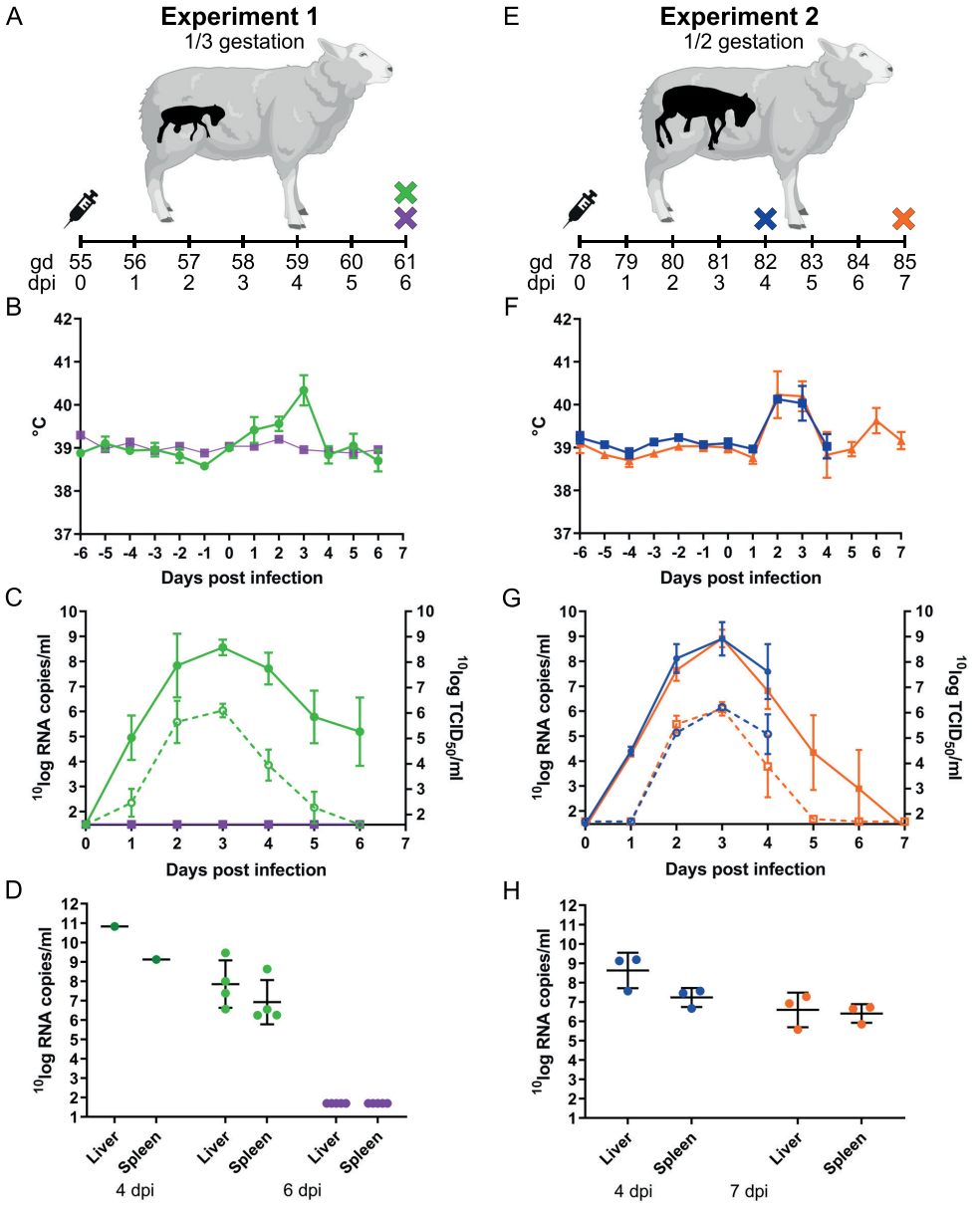


Figure 2.1. Experimental design and primary outcome parameters ewes. Ewes were inoculated intravenously with RVFV or mock inoculated at gestation day (gd) 55 (A) or day 78 (E) and euthanized at 4 (blue cross), 6 (green and purple crosses) or 7 (orange cross) days post inoculation (dpi). Purple numbers represent ewes that were mock-inoculated. Rectal temperatures (B, F), viremia by RT-qPCR (solid line, left y-axis) and virus isolation (dotted line, right y-axis) (C, G) and the presence of RVFV in the spleen and liver of the ewes (D, H) is depicted. Bars represent averages with SDs.

To study the pathology at an earlier and later stage than 6 dpi, another group of 6 ewes from the same herd was inoculated with RVFV (experiment 2). Of this group, three ewes were euthanized and necropsied at 4 dpi and the remaining three ewes when abortion was imminent (Figure 2.1E). Of note, these ewes were at the moment of inoculation at mid gestation. As expected, all ewes of this experiment developed similar rises in rectal temperatures and viremia levels as those of experiment 1 (Figure 2.1F, G). Necropsy at 4 dpi revealed multifocal necrotizing hepatitis in all ewes but no macroscopic abnormalities in placentas and unaffected, live foetuses. In the morning of day 7 post infection, one ewe had aborted 2 foetuses and a second one was is the process of aborting (2 foetuses already expelled, 1 foetus still in the uterus). Necropsy of the third ewe (#1846) revealed three foetuses that were still inside the uterus but with placentomes showing extensive haemorrhages. One of these foetuses was alive, whereas the remaining two foetuses were found dead (Table 2.1). Analysis of liver and spleen samples revealed very high viral RNA levels in the organs of ewes necropsied at 4 dpi and lower levels in ewes necropsied at 6 dpi or at abortion (Figure 2.1D, H). Viral RNA and viable virus were detected in all placentomes from both experiments (Figure 2.2). It was striking to observe that viral RNA levels increased in placentomes between days 4 and 7 (Figure 2.2), whereas viremia and viral RNA levels in spleens and livers declined in the same period (Figure 2.1D, H).

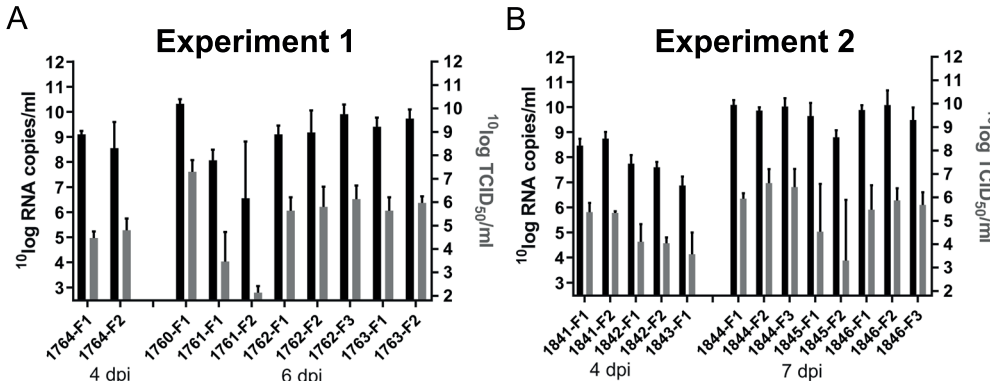


Figure 2.2. Detection of viral RNA and infectious virus in placentomes. Viral RNA copies in placentomes as determined by RT-qPCR (black columns; left y-axis) and virus titres in placentomes as determined by virus isolation (grey columns; right y-axis). Results of experiment 1 (A) and 2 (B) represent means and SDs of 3 placentomes per foetus.

RVFV infects maternal epithelial cells and foetal trophoblasts

The ovine placenta consists of multiple placentomes, in which foetal blood is separated from maternal blood by several cell layers (Figure 2.3A, B). In accordance with literature, we observed an increase in mass of the foetal and maternal villi with concomitant decrease in mesenchyme and expansion of haemophagous zones with progressing pregnancy (Figure 2.3C, D)[207].

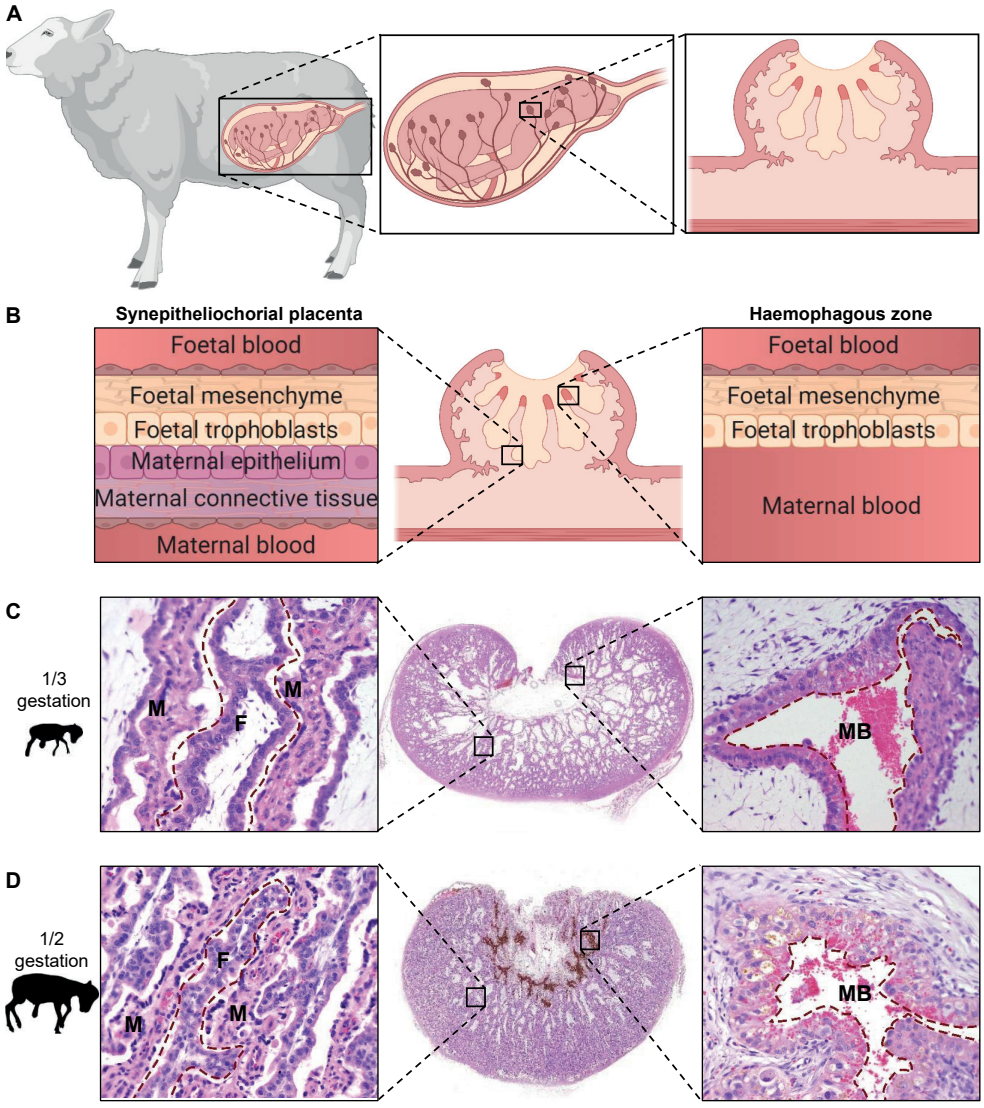


Figure 2.3. The ovine placenta at different stages of gestation. (A) Schematic presentation of an ovine foetus, the cotyledons and their blood supply. Only the foetal parts of the placenta (cotyledons) are displayed, the uterus wall and maternal part of the placenta (caruncles) are not depicted. At the right, a cross section of a placentome is depicted, showing the maternal tissues in shades of pink, and the foetal villi in orange. Haemophagous zones at the base of the foetal villi are depicted in red. (B) A schematic overview of the cotyledon (center) with the different cell layers of the synepitheliochorial placenta at the left and the haemophagous zone at the right. In the synepitheliochorial placenta, the foetal blood is separated from maternal blood by several maternal and foetal cell layers. In the haemophagous zone maternal blood is in direct contact with the foetal trophoblasts. (C, D) HE staining of placentomes, the haemophagous zones and synepitheliochorial placenta at 1/3 gestation (C) and 1/2 gestation (D). Red interrupted lines indicate the boundaries

between maternal and foetal tissues. F= foetal villus, M = maternal villus, MB= maternal blood. Notice the increase in the foetal/maternal villous interface in the synepitheliochorial placenta and the increase in size and erythrophagous activity of the trophoblasts of the haemophagous zone between 1/3 and 1/2 of the gestation period.

Necropsy of the ewe of experiment 1 that died at 4 dpi (#1764) revealed macroscopic abnormalities: extensive haemorrhages in the uterine wall, at the base of the placentomes and within the placentomes. Necropsy of ewes that were euthanized at 4 dpi in experiment 2 revealed placentomes that did not show any macroscopic abnormalities.

Immunohistochemistry showed the presence of RVFV antigen as small foci spread throughout the placentomes collected at 4 dpi in experiments 1 and 2 (Figure 2.4A). These foci consisted of a cluster of strongly stained epithelial/syncytial cells of the maternal villus with occasionally a single positive trophoblast in the epithelial lining of the opposite foetal villus (Figure 2.4B). Clusters of RVFV-positive cells were also found in the trophoblast epithelium lining the haemophagous zone (Figure 2.4C).

At 6 dpi, extensive haemorrhages were observed in the placentomes of the RVFV infected ewes (Figure S2.1). In addition, in some placentomes maternal and foetal villi were starting to separate. Both maternal epithelial cells (viable and necrotic) and foetal trophoblasts were strongly positive for RVFV antigen throughout the entire placentome (Figure 2.4F). Compared to the placentomes at 4 dpi, the area of RVFV positive maternal epithelial cells and foetal trophoblasts was greatly increased covering almost the entire maternal and foetal epithelial lining of the placentome including the haemophagous zone (Figure 2.4G, H). H&E staining revealed haemorrhages in the maternal villi with extensive necrosis of the maternal epithelium but only limited areas of necrosis of foetal trophoblasts (Figure 2.5A). In addition, a heavy influx of neutrophils was present mainly in the stratum compactum of the lamina propria and at the base of the maternal villi (Figure 2.5B).

At imminent abortion at 7 dpi, placentomes showed extensive haemorrhages with varying degrees of separation of maternal and foetal parts. Histology and immunohistochemistry revealed similar results as those obtained from analysis of the 6 dpi group with haemorrhages and a neutrophilic inflammatory response in maternal villi, necrosis of maternal epithelium and strong positive staining for RVFV in maternal epithelial cells and foetal trophoblasts. In placentomes where foetal villi had already separated from the maternal caruncle, large areas of denuded maternal villi were seen. In addition in some maternal villi, blood vessels were found that stained positively for RVFV in the endothelium and/or smooth muscle cells in the blood vessel wall (Figure S2.2). Notably, endothelial cells did not reveal signs of apoptosis or necrosis.

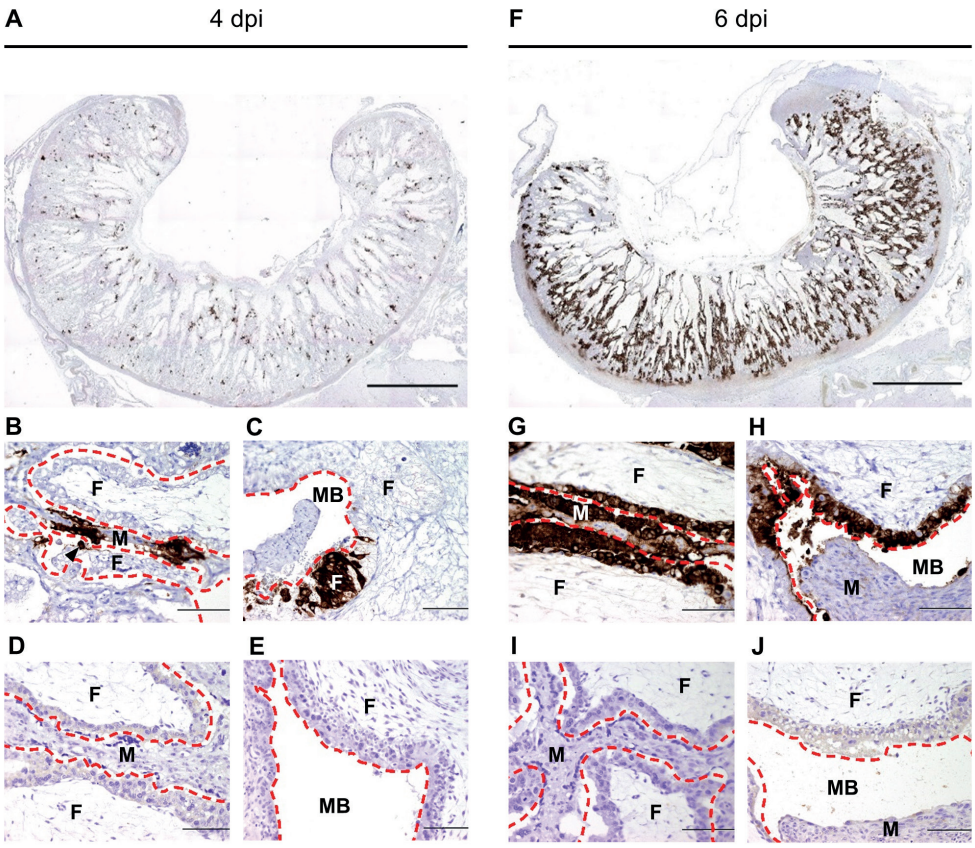


Figure 2.4. RVFV antigen in placentomes. Immunohistochemical (IHC) detection of RVFV antigen in cross sections of the placentomes at 4 dpi (experiment 2) and 6 dpi (experiment 1). At 4 dpi (A) only small foci of antigen positive cells are visible throughout the placentome while at 6 dpi (F) almost the entire placentome stains positive for RVFV antigen. Higher magnification of the synepitheliochorial placenta (SP) at 4 dpi (B) shows strong labelling of the maternal epithelial cells with only an individual positively stained foetal trophoblast (black arrowhead). At 6 dpi both maternal and foetal cell layers are strongly stained (G). In the haemophagous zone (HZ) at 4 dpi (C) only small clusters of foetal trophoblasts stain positive for RVFV antigen while at 6 dpi (H) the entire foetal trophoblast lining of the haemophagous zone stains positive. Panels D, E, I and J represent cross sections of uninfected placentomes corresponding to B, C, G and H, respectively, showing absence of background IHC staining. Red interrupted lines indicate the boundaries between maternal and foetal tissues. F; foetal villus, M; maternal villus, MB; maternal blood. Bar = 5000 µm (A, F) or 100 µm (B-J).

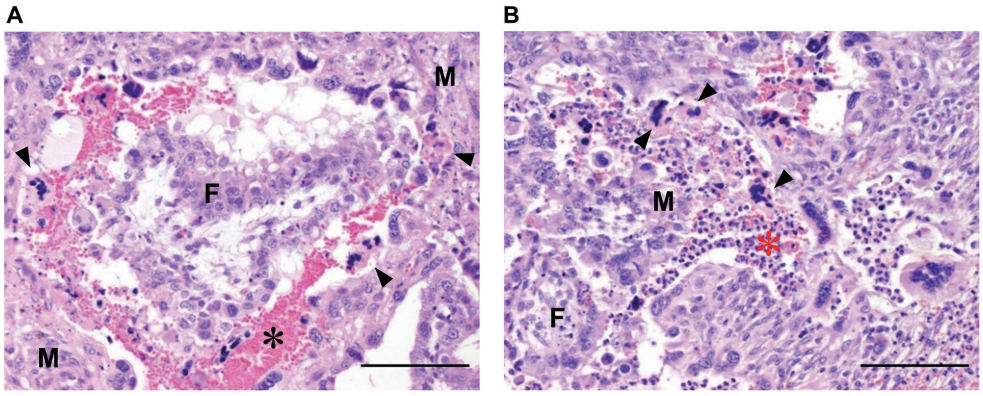


Figure 2.5. Histopathology of the placentomes at imminent abortion. HE staining of placentomes at 6 dpi. (A) Haemorrhages (black asterisk) and necrosis of maternal epithelial cells (arrowheads). Notice the relatively intact foetal epithelium. (B) Influx of neutrophils (red asterisk) and necrosis of maternal epithelium (arrowheads). F= foetal villus, M= maternal villus, Bar = 100 µm.

Detection of RVFV in foetal tissues

Ewe 1764 that acutely succumbed to the infection in experiment 1 carried two foetuses. RVFV RT-qPCR revealed viral RNA in the livers of both foetuses (Table 2.1 and Figure 2.6A). The foetuses of the ewes necropsied at 4 dpi in experiment 2 were all alive and appeared to be normal at the moment of necropsy (Figure S2.3). However, RT-qPCR showed the presence of viral RNA in the blood of all foetuses and in the livers of 3 out of 5 foetuses (Figure 2.6B). These results show that RVFV is able to reach the foetus within 4 days.

All 8 foetuses of the ewes necropsied at 6 dpi were found dead within the uterus (Table 2.1). The livers of these foetuses were also positive for viral RNA and in 4 out of 8 foetuses viral antigen was detected by immunohistochemistry. In these 4 foetuses, the liver showed massive necrosis with only a few viable hepatocytes left (Figure 2.6C). Brain samples collected from 5 of the 8 foetuses were also found to contain viral RNA. Immunohistochemical staining of brain tissues from these foetuses revealed viral antigen in the blood and the endothelium of the blood vessels throughout the brain and arachnoidea (Figure 2.6D). No viral antigen was detected in neurons or glial cells. RVFV antigen was also detected in endothelial cells of blood vessels of the umbilical cord (Figure 2.6E) and muscle tissues (Figure 2.6F).

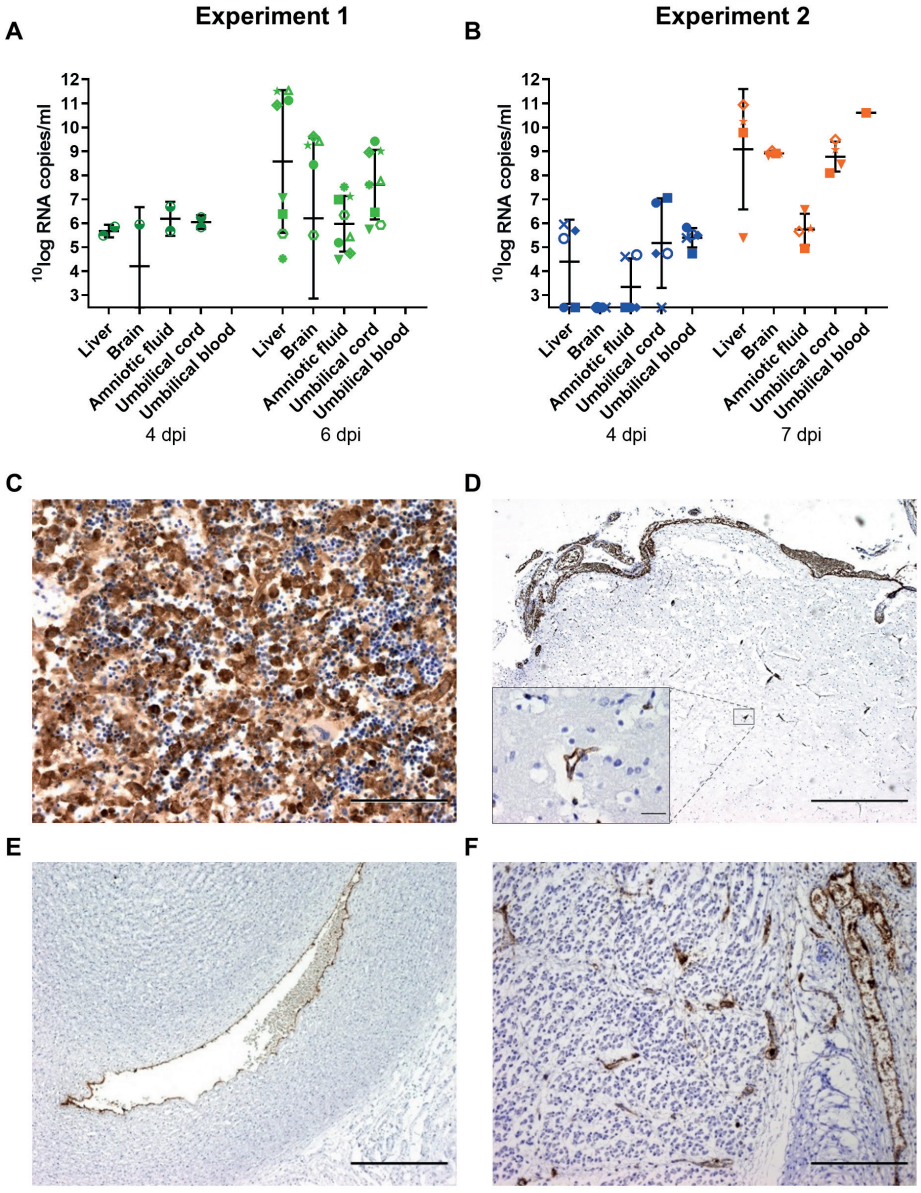


Figure 2.6. Detection of viral RNA and viral antigen in foetal organs. Detection of RVFV RNA by RT-qPCR in foetal organs collected in experiment 1 (A) and experiment 2 (B). Bars represent averages with SDs. Staining of RVFV antigen in samples collected from liver (C), brain (D) umbilical cord (E) and leg muscle (F). Notice the strong staining of endothelial cells in the blood vessels within the various organs. Bar = 500 µm (D, E, F), 100 µm (C) or 20 µm (inset D).

Most of the aborted foetuses in experiment 2 were severely autolytic and unsuited for further analysis (Figure S2.3). The foetuses that were suitable for further analysis contained severely necrotic livers and contained high levels of viral RNA in liver and brain samples. Hepatocytes and endothelial cells were strongly positive for RVFV antigen.

RVFV strain 35/74 infects cytotrophoblasts and syncytiotrophoblasts in human placental explants

To study if RVFV strain 35/74, which was originally isolated from sheep, replicates in human placentas, explants of human term placentas were inoculated with the virus. The results show that the virus replicates efficiently in cytotrophoblasts and syncytiotrophoblasts (Figure 2.7), as revealed by RT-qPCR, virus isolation and IHC. In some areas of the placenta, viral antigen was detected in cytotrophoblasts but appeared to be absent in syncytiotrophoblasts, suggesting that the former are more permissive to RVFV replication (Figure 2.7C). The differences and similarities between the ovine and human placenta are depicted in Figure S2.4.

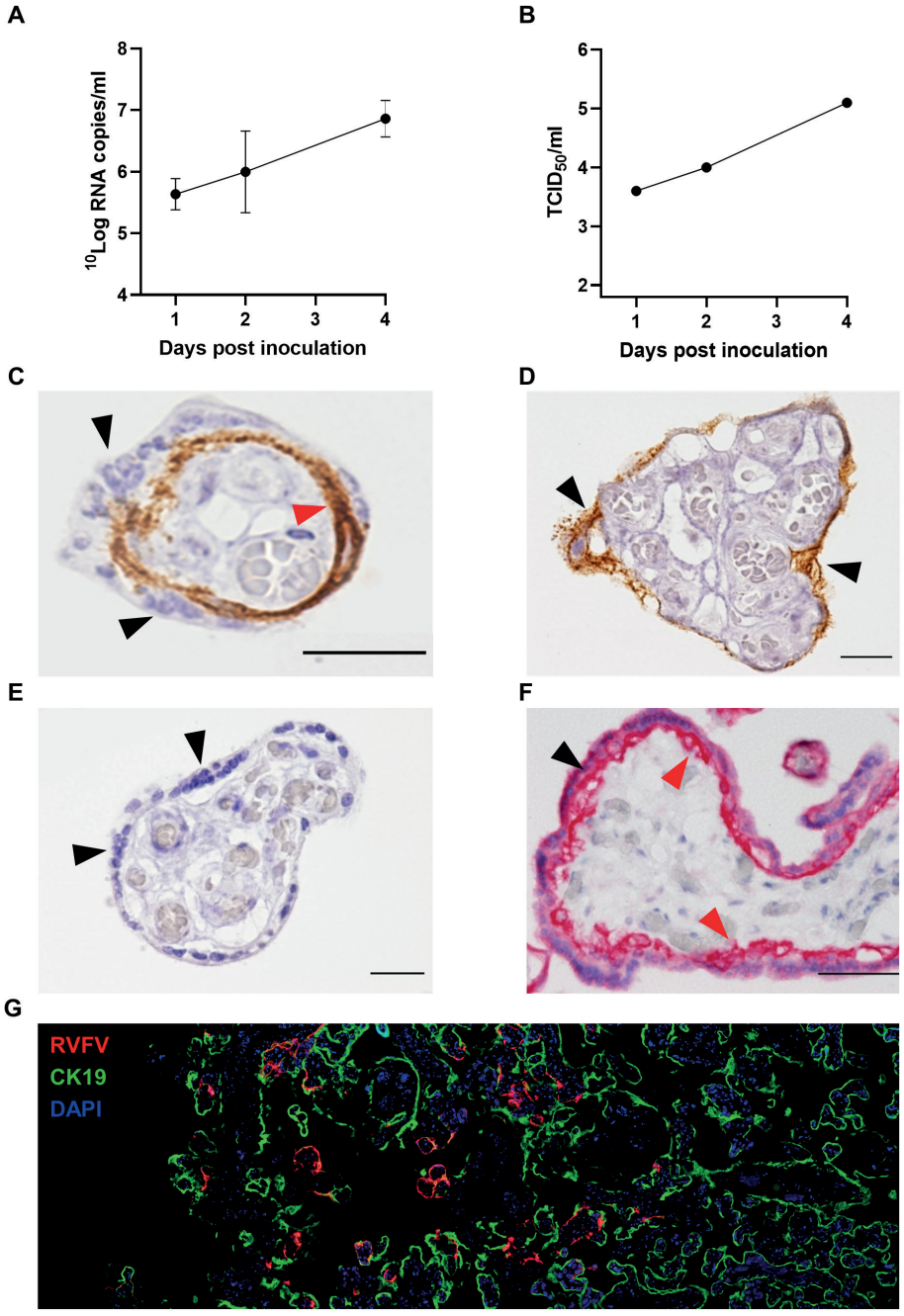


Figure 2.7. RVFV in human placental explants. Detection of viral RNA (A) and infectious virus (B) in human full term placental explants at different timepoints post infection. Viral RNA was detected by RT-qPCR and infectious virus by virus isolation. (C, D, E) Immunohistochemical staining of RVFV with mAb 4-D4, counterstained with haematoxylin. (C) Single villus with syncytiotrophoblasts

(black arrowheads) staining negative for RVFV antigen with cytotrophoblasts (brown staining, red arrowhead) staining positive. (D) Single villus in which no cytotrophoblasts are present, with positive staining of syncytiotrophoblasts (black arrowheads). (E) Single villus showing absence of background IHC staining in non-infected control placental explant. Syncytiotrophoblasts are indicated with black arrowheads. (F) Immunohistochemical staining of epithelial cells in a non-infected placental villus with a mAb to cytokeratin showing both the syncytiotrophoblast layer (black arrowheads) and the cytotrophoblast layer (red arrowheads). (G) Detection of RVFV-infected trophoblasts (red) by immunofluorescence at 4 dpi. Trophoblasts were visualised using an antibody specific for cytokeratin-19 (CK19; green). Bar = 20 µm (C, D, E) or 50 µm (F).

Discussion

The most characteristic feature of RVFV epizootics are abortion storms in sheep herds. During these events, all pregnant ewes in an affected herd may abort their foetuses. Although the detrimental effects of RVFV infection on ruminant pregnancy are well-recognized, the route that the virus uses to cross the placenta and the pathogenic events that result in abortion have remained undescribed. Here, we report that maternal and foetal epithelial cells in the ovine placenta are highly susceptible and permissive for RVFV and that abortion results from severe pathology of the placenta. In some ewes, the rapid progression of placental demise caused foetal mortality before foetuses could be infected.

During gestation, the progesterone hormone keeps the uterus and myometrium in a quiescent state to allow successful foetal development until parturition[161]. During the first trimester of gestation in sheep, progesterone is produced by the corpus luteum which resides in the ovaries. During the second and third trimester, the developing placenta becomes an additional source of progesterone. Placental progesterone is produced by binucleate trophoblasts that migrate to the maternal epithelium to fuse with uterine epithelial cells to form so-called syncytial cells. At about 90 days of pregnancy, half of the progesterone in the pregnant ewe is produced by the corpus luteum and half by the placenta. Our study has revealed that both trophoblasts and (syncytial) epithelial cells are major target cells of RVFV. Widespread necrosis of these cells likely results in a drop of systemic progesterone levels. In addition, virus-induced necrosis of placental cells leads to the production of pro-inflammatory chemokines and cytokines like TNF-α, IFN-γ and IL-8, which stimulate prostaglandin (E2/F2α) excretion by the placental epithelium. Prostaglandins bring the corpus luteum in regression, resulting in a further drop in progesterone levels. In the absence of the inhibitory effect of progesterone, prostaglandins induce uterine contractions and cervical effacement resulting in abortion. Inflammatory cytokines, most notably TNF-α, are also known to reduce vascular integrity. In the face of lowered coagulation factors in maternal blood resulting from liver necrosis, this could explain the observed placental haemorrhages.

Our study has revealed that RVFV uses at least two routes to cross the ovine placenta. The first route involves the haemophagous zones of the placenta, where foetal trophoblasts

are in direct contact with maternal blood. Trophoblast cells in the haemophagous zone are specialised in phagocytosis of erythrocytes from the blood to provide the foetus with iron. The presence of RVFV in maternal pools of blood in the haemophagous zone may therefore result in direct infection of trophoblasts or via co-uptake of the virus during erythrophagy. The second route initiates with infection of maternal epithelial cells of the placenta. Virus released from these cells exposes foetal trophoblasts. Progeny virus produced by infected trophoblasts is subsequently released into the foetal mesenchyme exposing endothelial cells of foetal bloodvessels, which were also identified as target cells of RVFV.

The finding that foetal endothelial cells and endothelial cells in the maternal caruncle are target cells of RVFV is notable, as most of our previous studies with juvenile and adult sheep did not reveal endothelial cell infection. However, in one of our studies, RVFV-positive endothelial cells were detected in lymphoid organs of a lamb that peracutely succumbed after developing exceptionally high viremia[208]. In another study, endothelial cells of the skin became infected after feeding of mosquitoes on a viremic lamb[67]. These findings, together with the haemorrhagic manifestations that were seen in RVFV infected placentomes in this study, calls for further research into the interaction of RVFV with endothelial cells.

In a recently published study by McMillen and co-workers, RVFV infection of human placental explants was shown for the first time, using a strain that was originally isolated from humans[201]. Our experiments with human placental explants corroborate this research and highlight the potential risks of RVFV infection during human pregnancy. In the same study, the pathogenesis of RVFV infection in pregnant Sprague-Dawley rats was described. This work demonstrated that vertical transmission in these rats occurs through direct placental infection and that viral loads in the placenta were higher than in the liver and other maternal organs, similar as observed in the present study. In our study, the ovine placenta was not only found to contain the highest viral loads but was also the only organ still containing high levels of infectious virus at the moment of necropsy. These findings suggest that RVFV efficiently counteracts innate immune responses in placental cells.

RVFV counteracts host innate immunity through several functions of the nonstructural NSs protein[35,209-213]. One of the major functions of NSs is the downregulation of type I interferon (IFN) responses, which occurs through downregulation of general host gene transcription and the direct inhibition of IFN-β mRNA production[35,209,213]. Whereas in most cell types, type I IFNs play a major role in innate immunity, in cells of epithelial origin, such as the cells of the placenta, innate immunity is regulated by type III IFNs, referred to as IFN-λ[214]. Importantly, both type I and type III IFNs trigger the JAK/STAT pathway, which was shown to be targeted by NSs[215]. We therefore hypothesize that RVFV NSs facilitates replication in placental tissues by downregulating JAK/STAT signalling. On the other hand, previous studies have demonstrated that RVFV can also cross the ovine placental barrier

without NSs[184]. These infections did not result in abortions but instead in stillbirths and congenital malformations, including arthrogryposis and hydranencephaly, resembling pathology in ovine foetuses infected by members of the genus *Orthobunyavirus*, such as Schmallenberg virus.

In conclusion, the present work has revealed how RVFV crosses the ovine placental barrier and has provided novel insights into the pathology that results in abortion in the most susceptible target species. The sheep isolate that was used was shown to replicate efficiently in human placental explants as well, calling for further research on the risk of RVFV infection during human pregnancy.

Materials and Methods

Cells and viruses

Culture media and supplements were obtained from Gibco unless indicated otherwise. Baby Hamster Kidney (BHK-21) cells were maintained in Glasgow minimum essential medium (GMEM) supplemented with 4% tryptose phosphate broth, 1% minimum essential medium nonessential amino acids (MEM NEAA), 1% antibiotic/antimycotic (a/a) and 5% foetal bovine serum (FBS), at 37°C with 5% CO_2. Vero-E6 cells were maintained in minimum essential medium (MEM) supplemented with 1% a/a, 5% FBS, 1% glutamine and 1% MEM NEAA, at 37°C with 5% CO_2. BHK-21 and Vero-E6 cells were purchased from ATCC.

A recombinant version of RVFV strain 35/74, originally isolated from the liver of a sheep during a RVFV outbreak in 1974 in the Free State province of South Africa, was used in the present work as described [216]. The titre was determined on BHK-21 cells as 50% Tissue Culture Infective Dose ($TCID_{50}$) according to the Spearman-Kärber algorithm[216].

Ethics statements

Animal experiments were conducted in accordance with the Dutch Law on Animal Experiments (Wet op de Dierproeven, ID number BWBR0003081) and the European regulations (EU directive 2010/63/EU) on the protection of animals used for scientific purposes. The procedures were approved by the animal ethics committee of Wageningen Bioveterinary Research (WBVR) and the Dutch Central Authority for Scientific Procedures on Animals (permit number AVD401002017894).

Human placentas were obtained after caesarean section of healthy women. This material is regarded as medical waste and therefore does not fall under the scientific medical research law of the Netherlands and does not need approval from an institutional review board. All donors have given written consent and consent forms are stored in accordance with the Dutch privacy law.

Experimental design pregnant ewe trial

At a conventional Dutch sheep farm, 30 Texel-Swifter mix breed ewes that had delivered healthy lambs before, were treated with progesterone sponges to synchronise pregnancies. After removal of the sponges, ewes were naturally mated. Ultrasounds were performed at 6-7 weeks after mating to confirm gestation. Pregnant animals to be enrolled in the studies were subsequently transported to WBVR and allowed to acclimatize for one week before the start of the experimental period under biosafety level 3 (BSL-3) conditions.

At 55 days of pregnancy (experiment 1) or 78 days of pregnancy (experiment 2), animals were inoculated via intravenous (IV) route with 10^5 $TCID_{50}$ of RVFV in 1 ml medium, or 1 ml medium (negative control animals). Following challenge, animals were closely monitored for clinical signs, body temperatures were recorded, and EDTA blood samples were collected (Figure 2.1A). The ewes of experiment 1 were euthanized and necropsied at 6 days post infection and the ewes of experiment 2 were euthanized on day 4 or at abortion (day 7). Ewes and their foetuses were euthanized by intravenous administration of 50 mg/kg sodium pentobarbital (Euthasol, ASTfarma) and subsequent exsanguination. Foetuses were exsanguinated by severing of the umbilical cord after which foetal blood was collected in EDTA tubes. From the ewes, samples were taken from the liver and spleen. From the foetuses, samples were taken from the brain, spleen, leg muscle tissue and liver. Samples were also taken from the amniotic fluid, umbilical cord and the placentomes (3 per foetus). Samples were placed on dry ice and subsequently stored at -80°C. Samples for histology and immunohistochemistry were fixed in 10% phosphate-buffered formalin for at least 48 h followed by routine processing into paraffin blocks.

Infection of human placental explants

Full term placentas were obtained by caesarean section from healthy donors at the Isala hospital in Zwolle, the Netherlands. The placentas were transported on ice and placed in large petridishes with complete medium (40% Dulbecco's Modified Eagle Medium [DMEM], 40% F12 nutrient mixture, 10% FCS supplemented with 1% a/a). Chorionic villi were separated from the placenta and cut in 4x4 mm pieces, after which the samples were washed 3x with PBS + a/a. Placental explants were incubated with $2{,}5x10^5$ $TCID_{50}$ of RVFV in 1 ml complete medium, or in 1 ml complete medium (negative controls) in a 24-wells plate. Medium was removed 16 hpi, after which explants were washed 3x with PBS + a/a. Samples were collected at 1, 2 or 4 days post inoculation (dpi). Samples for quantitative reverse-transcription PCR (RT-qPCR) were stored at -80°C. For virus isolation, 200 µl supernatant samples from each timepoint were pooled. For IHC, explants were fixed in 10% phosphate-buffered formalin for 48 h followed by routine processing in paraffin blocks. Each sample was analysed in quadruplicate. The 1 dpi timepoint was taken at 16 hpi after washing the explants. At 3 dpi, 1 ml of fresh medium was added to the 4 dpi samples.

Detection of viral RNA

RNA was extracted from ovine plasma and organ samples. Briefly, organ suspensions were prepared by homogenising 0,3-1 g of tissue in an IKA Ultra Turrax Tube DT-20 containing 7 ml CO_2-Independent Medium (CIM) supplemented with 1% a/a. The suspensions were transferred to 15 ml Falcon tubes and cell debris was removed by centrifugation for 15 min at 4952 *x g*. Fifty µl Proteinase K (5 µg/ml, Sigma) was added to 200 µl of the plasma samples or organ suspensions. Next, 200 µl AL buffer (Qiagen), supplemented with 2 µl polyadenylic acid A (5 mg/ml, Sigma) was added, the samples were thoroughly mixed and incubated at 56°C for 15 min. Subsequently, 250 µl 99% ethanol was added and RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's protocol.

RNA was extracted from human placental explants by first homogenising the explants in 1 ml TRIzol Reagent (Invitrogen) in Lysing Matrix D tubes (MP Biomedicals) using the TeSeE™ Precess 24 bead beater for 2x23 s at 6500 RPM. Supernatant (350 µl) was used for RNA isolation using the Direct-zol RNA miniprep kit (Zymo Research) according the manufacturer's protocol.

Five µl of the RNA was used in a RT-qPCR using the The LightCycler RNA Amplification Kit HybProbe (Roche, Almere, the Netherlands). Primers and probes were purchased from IDT. Forward primer: 5'-AAAGGAACAATGGACTCTGGT CA-3', reverse primer: 5'-CACTTCTTACTACCATGTCCTCCA AT-3'; Probe: 5'-6FAM-AAA GCT TTG ATA TCT CTC AGT GCC CCA A TMR-3'. Cycling conditions were as follows: 45°C for 30 min, 95°C for 5 min, 45 cycles of 5 s at 95°C and 35 s at 57°C, followed by cooling down to 30°C.

Virus isolation

Virus isolations from ovine samples were performed by serial dilution of either plasma in complete CIM supplemented with 3,5 IU/ml heparin, or organ suspension in CIM, followed by incubation with BHK-21 cells. After 1.5 h incubation at RT, the inoculum was replaced by fresh medium and after 5 days of culturing the cells at 37°C, the cytopathic effect was scored.

Virus isolations from the supernatant of the human placenta explants were performed by a 10x serial dilution series of the supernatant in complete medium on Vero cells. At 24 hpi, infection was visualized with an immunoperoxidase monolayer assay (IPMA). Briefly, cells were fixed and permeabilized 24 hpi with 4% paraformaldehyde (10 min) and ice-cold methanol (5 min). After permeabilization the plates were incubated with RVFV specific monoclonal antibody 4-D4, which recognizes the Gn protein [217]. A polyclonal rabbit-α-mouse immunoglobin/HRP antibody (Dako, Denmark) was used as a secondary antibody and 3-Amino-9-ethylcarbazole (AEC; Sigma-Aldrich) was used as a substrate. The titre was expressed as $TCID_{50}$/ml according to the Spearman-Kärber algorithm [218,219].

Histology and immunohistochemistry

Paraffin embedded tissue was cut into 4 µm sections, collected on silane-coated glass slides and dried for at least 48 h in a 37°C incubator. After deparaffinization and rehydration in graded alcohols, sections were stained routinely with haematoxylin and eosin (H&E) or immunostained for RVFV antigen. For immunostaining, endogenous peroxidase was blocked for 30 min in methanol/H_2O_2 followed by antigen retrieval through 15 min autoclaving at 121°C in pH 6 citrate buffer (Antigen unmasking solution, Vector Laboratories). As RVFV Gn-specific primary antibody, monoclonal antibody 4-D4 was used. Specificity of the immunostaining was confirmed with 2 other mAbs directed against different proteins of RVFV. Mouse envision peroxidase (Dako, Denmark) was used as secondary antibody and diaminobenzidine (DAB; Dako, Denmark) as the substrate, according to the manufacturer's instructions. Immunostaining for cytokeratin was performed using a rabbit mAb to cytokeratin 19 (Abcam 52625, USA) followed by α-rabbit-ImmPRESS-AP and Vector Red as substrate (Vector Laboratories, USA). Hematoxylin was used to counterstain the slides.

Acknowledgements

We thank Pieter Roskam and Corry Dolstra (Wageningen Bioveterinary Research) for assisting with the necropsies and immunohistochemistry, respectively. We thank Dr. Schmaljohn (USAMRIID, Fort Detrick, MD) for providing the 4-D4 mAb. We also thank Dr. Guus Vermeulen for providing us with the human placentas.

Supporting information

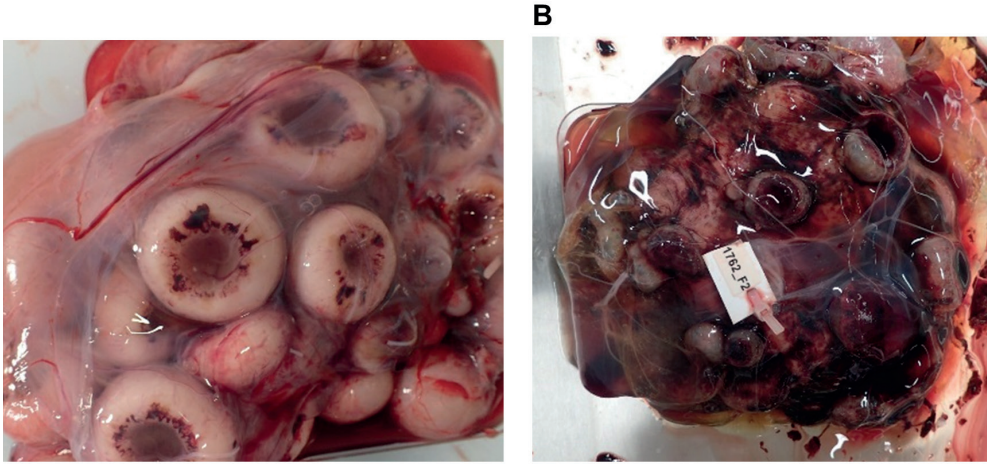


Figure S2.1. RVFV infection results in extensive placental haemorrhages. Placenta from a healthy ewe (A) and from an ewe inoculated with RVFV, necropsied six days after inoculation (B). Placentas were collected during experiment 1 at one third of gestation.

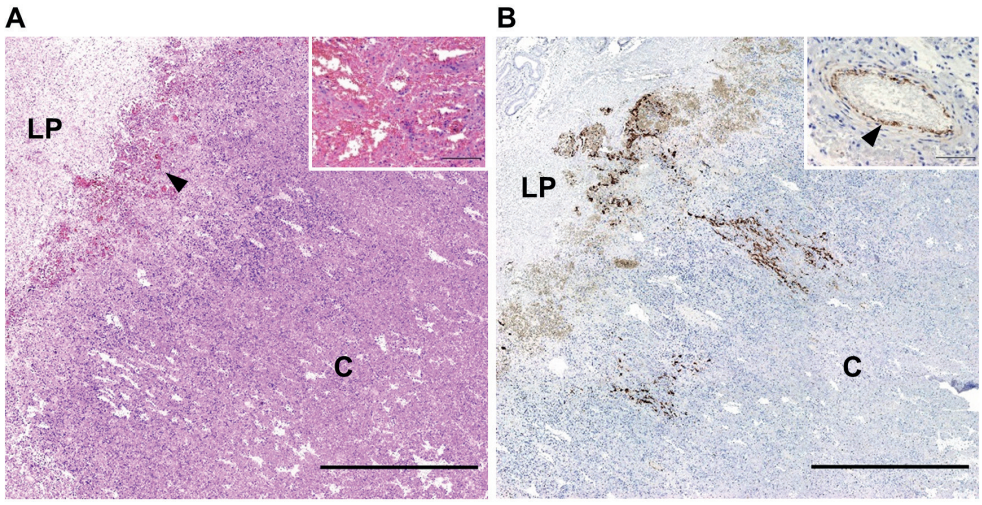


Figure S2.2. Replication of RVFV in caruncles is associated with bleeding and infection of maternal endothelial cells. (A) Micrograph of HE-stained caruncle tissue of ewe 1845 euthanized at 7 dpi (experiment 2). Notice the extensive haemorrhages at the base of the caruncle (arrowhead and inset). (B) Immunohistochemical staining of RVFV antigen. Positive staining is only seen in those areas where necrotic maternal epithelium is still present. Some maternal blood vessels are also stained (arrowhead and inset) and show the presence of RVFV antigen in the endothelial cells and the smooth muscle cells of the tunica media (inset). Bar = 1000 µm (A, B), 100 µm (inset A), or 50 µm (inset B). LP; lamina propria, C; caruncle.

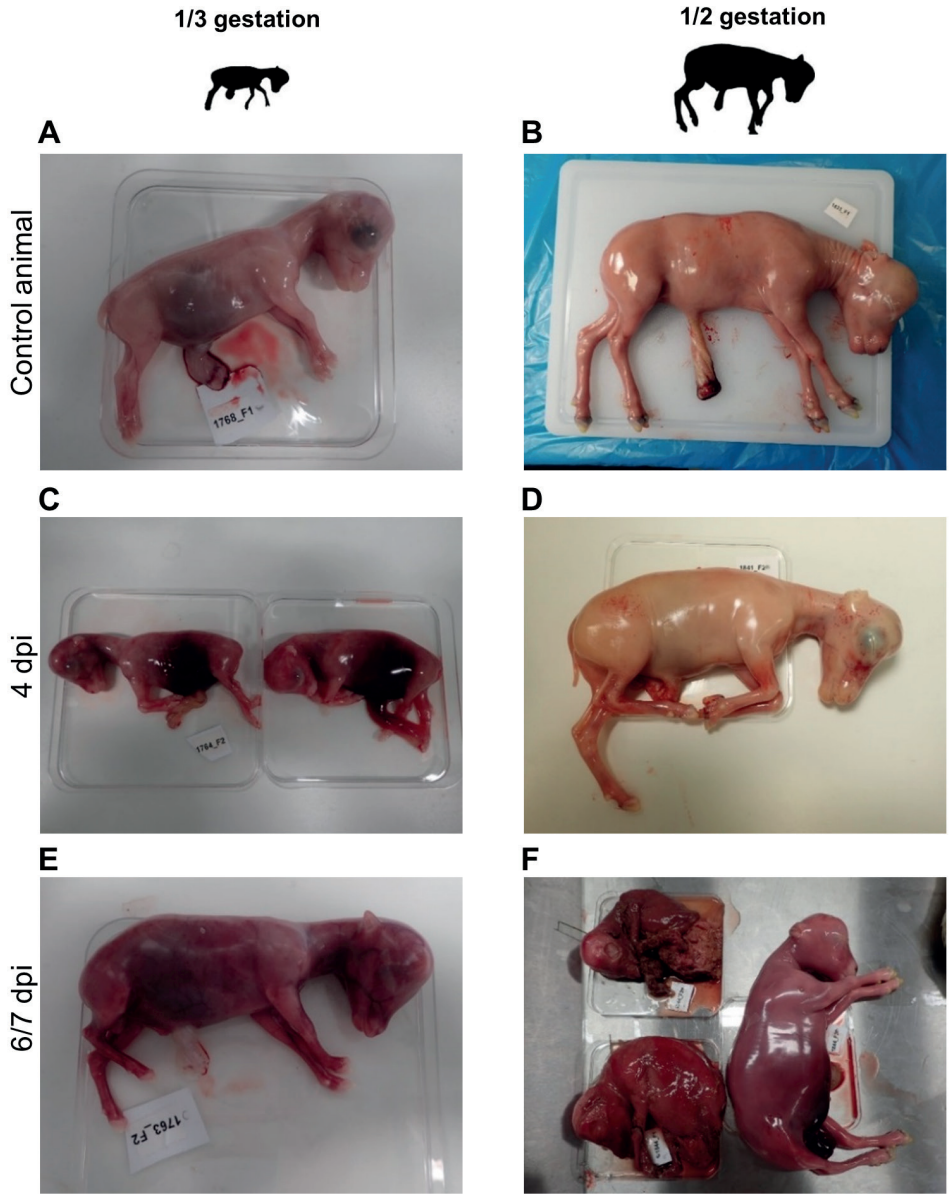


Figure S2.3. Pathological manifestations in foetuses collected from RVFV infected ewes. Healthy foetuses collected from ewes necropsied at one third (A) or at mid-gestation (B). (C) Foetuses carried by ewe 1764 that succumbed 4 days after inoculation with RVFV in experiment 1. (D) Live foetus collected from an ewe that was necropsied at 4 dpi in experiment 2. (E) Autolytic foetus collected from an ewe necropsied at 6 dpi in experiment 1. (F) Two aborted foetuses from experiment 2 (left) and one foetus (right) that was still inside the uterus at the moment of necropsy.

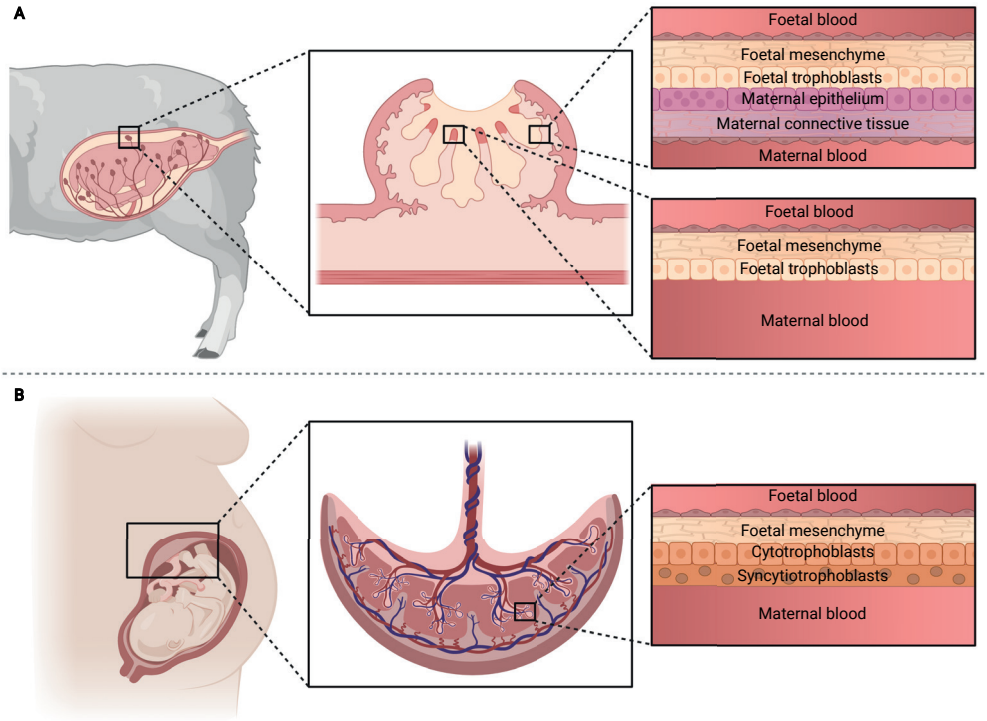
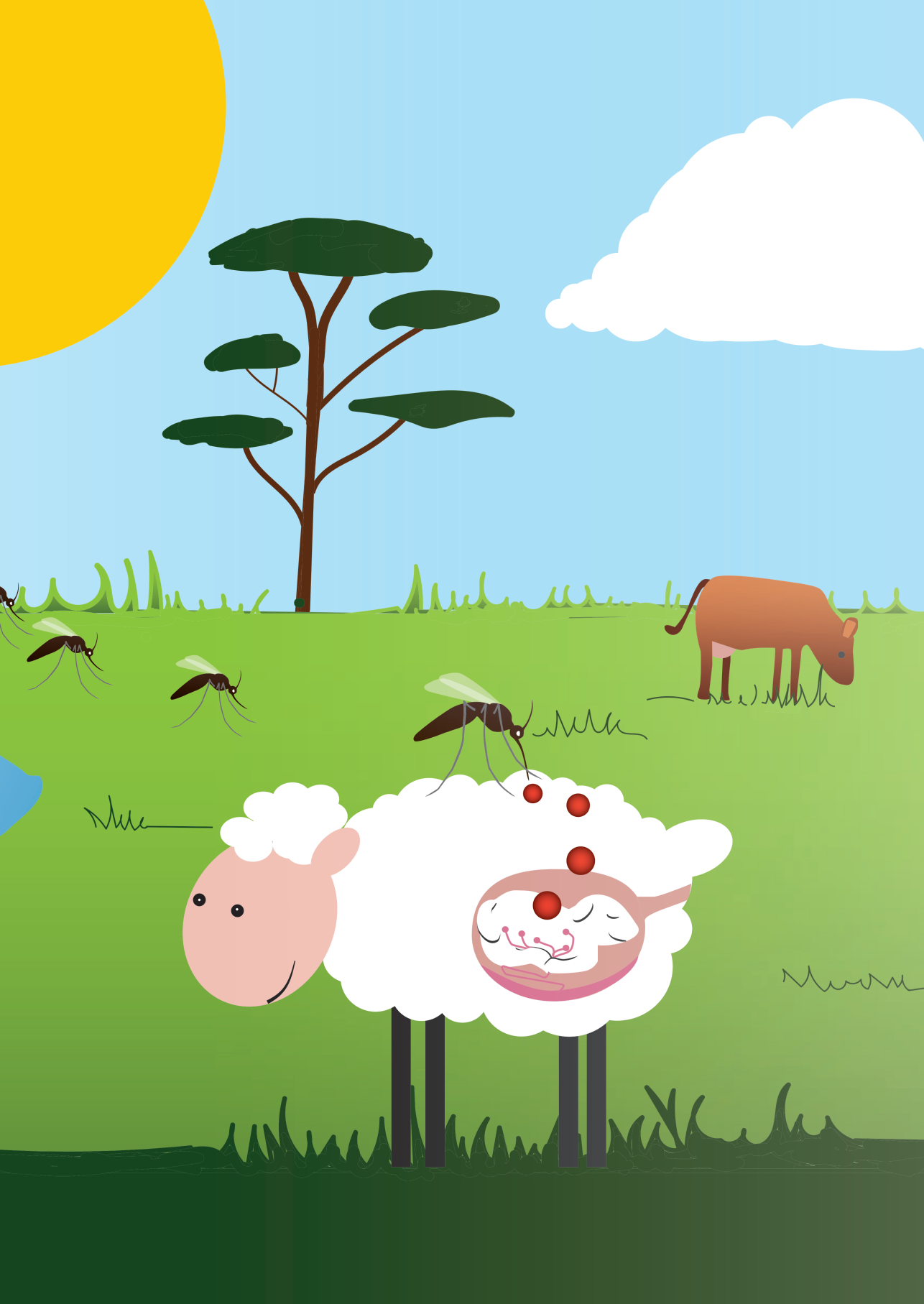


Figure S2.4. Schematic presentation of the ovine and human placenta. A human placenta consists of a single discoid plaque whereas an ovine placenta consists of placentomes (A). A cross section of both placentas is depicted, showing the maternal tissues in shades of pink, and the foetal villi in orange. Blood and arteries are depicted in red, veins are depicted in blue (B). In the synepitheliochorial placenta (C, left panel), the foetal blood is separated from maternal blood by several maternal and foetal cell layers. In the haemophagous zone (C, middle panel) maternal blood is in direct contact with the foetal trophoblasts, which is similar to the human haemochorial placenta (C, right panel).



Chapter 3

A single vaccination with four-segmented Rift Valley fever virus prevents vertical transmission of the wild-type virus in pregnant ewes

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Abstract

Rift Valley fever virus (RVFV) is a mosquito-transmitted bunyavirus that causes severe outbreaks among wild and domesticated ruminants, of which sheep are the most susceptible. Outbreaks are characterized by high mortality rates among new-born lambs and abortion storms, in which all pregnant ewes in a flock may abort their foetuses. In endemic areas, Rift Valley fever (RVF) can be controlled by vaccination with either inactivated or live-attenuated vaccines. Inactivated vaccines are safe for animals during all physiological stages, including pregnancy. However, optimal efficacy of these vaccines depends on multiple vaccinations and yearly re-vaccination. Live-attenuated vaccines are generally highly efficacious after a single vaccination, but currently available live-attenuated vaccines may transmit to the ovine foetus, resulting in stillbirths, congenital malformations or abortion. We have previously reported the development of a novel live-attenuated RVFV vaccine, named RVFV-4s. This vaccine virus was created by splitting the M genome segment and deleting the major virulence determinant NSs, and was shown to be safe even for the most susceptible species, including pregnant ewes. The demonstrated efficacy and safety profile suggests that RVFV-4s holds promise for veterinary and human application. The RVFV-4s vaccine for veterinary application, here referred to as vRVFV-4s, was shown to provide complete protection after a single vaccination of lambs, goats and cattle. In the present work, we evaluated the efficacy of the vRVFV-4s vaccine in pregnant ewes. Anticipating on the extremely high susceptibility of pregnant ewes for RVFV, both a single vaccination and double vaccination were evaluated in two independent experiments. The combined results suggest that a single vaccination with vRVFV-4s is sufficient to protect pregnant ewes and to prevent transmission to the ovine foetus.

Introduction

Rift Valley fever is a disease of ruminants and humans that is caused by Rift Valley fever virus (RVFV), a mosquito-borne virus of the order *Bunyavirales* (family *Phenuiviridae*, genus *Phlebovirus*). The RVFV genome is divided into three RNA genome segments of negative polarity. The large (L) segment encodes the viral RNA-dependent RNA polymerase, the medium (M) segment encodes a polyprotein precursor that is co-translationally cleaved by host proteases into the structural glycoproteins Gn and Gc, which are involved in attachment to target cells and fusion of the viral and endosomal membranes, respectively. The M segment additionally encodes a small 14-kDa protein, named NSm, which was shown to counteract apoptosis[220], and a large glycoprotein (LGp, 78-kDa), that comprises the NSm and Gn coding regions[28]. The latter was shown to be important for dissemination of the virus in mosquitoes[221]. The S segment encodes the nucleocapsid protein and a non-structural protein named NSs. NSs efficiently counteracts several signalling pathways of the host immune system and is considered the major virulence determinant of the virus[32].

RVFV is pathogenic to wild- and domesticated ruminants including goats, cattle, buffalo and camelids, with sheep being the most susceptible and the most severely affected[62]. Necropsy of fatal cases may reveal widespread liver necrosis, hydrops ascites and haemorrhagic manifestations. Human infections are generally attributed to contact with contaminated animal products, predominantly blood released during the slaughtering of diseased animals, however humans may also become infected after the bite of an infected mosquito. Most infected humans develop a self-limiting febrile illness, while a small fraction develops neurological disorders or haemorrhagic fever[70].

Apart from massive mortality among new-born lambs, abortion storms are a hallmark of RVF epizootics. A recent study on the pathogenesis of RVFV in pregnant ewes demonstrated that RVFV replicates efficiently in the ovine placenta, targeting maternal epithelial cells and foetal trophoblasts, resulting in placental demise and abortion[222]. Considering that the ovine placenta is the main source of progesterone from mid-gestation until term, destruction of the placenta is considered the primary cause of abortion. Although the risk of RVFV infection during human pregnancies is unclear, the infection has been associated with miscarriage and the virus was shown to replicate in human placental explants[201,222,223].

Veterinary vaccines based on inactivated- or live-attenuated virus have been marketed in several African countries. Inactivated vaccines can be applied safely during all physiological stages, including pregnancy, but require repeated dosing and yearly re-vaccination for optimal efficacy. These vaccines are therefore not ideal for emergency vaccination. Live-attenuated vaccines are either based on the Smithburn or Clone 13 strain. The Smithburn strain was derived from a mosquito isolate that was attenuated by intracerebral passage in mice[176]. Although this vaccine is efficacious after a single vaccination, its residual virulence makes it unsafe for pregnant animals[179]. Clone 13 is an alternative live-attenuated vaccine

that is prescribed for use also in pregnant animals. Clone 13 is a plaque-purified clone of strain 74HB59 found to contain a 69% deletion in the NSs gene[183,209]. This deletion was shown to render the virus avirulent for sheep lambs[184], goats[224], cattle[225] and pregnant ewes[226]. Due to its high efficacy and safety profile, Clone 13 was more recently also evaluated for use in Europe according to the guidelines of the European Pharmacopeia which involves safety evaluation at an overdose. In this study, the vaccine was confirmed to be completely safe for lambs, however inoculation of an overdose in pregnant ewes was associated with stillbirths and congenital malformations[184].

We previously reported the development of a novel live-attenuated RVF vaccine, that was constructed by splitting the M segment into two M-type segments, one encoding NSm, Gn and LGp and one encoding Gc[227]. To optimize the safety profile, the NSs gene was deleted from the S segment. The resulting four-segmented RVFV candidate vaccine was shown to be safe for pregnant ewes[228] and young lambs[229], even after application of an overdose, and to induce protective immunity in young sheep, goats and cattle[229]. Moreover, additional safety studies demonstrated that the vRVFV-4s vaccine does not induce viremia and is not shed or spread to the environment[229]. In the present study, the efficacy of two independent batches of vRVFV-4s (referring to the RVFV-4s vaccine for veterinary application) were evaluated in pregnant ewes. Considering the very high susceptibility of pregnant ewes, both a single vaccination and a double vaccination with different doses were evaluated. The results demonstrate that both a single vaccination and a double vaccination at a lower dose with vRVFV-4s prevents vertical transmission of a highly virulent challenge strain.

Results

Efficacy of vRVFV-4s in pregnant ewes (Experiment 1)

The first experiment with pregnant ewes was performed with an investigational batch of vRVFV-4s[227]. Eighteen pregnant ewes (synchronized pregnancy) were divided over three groups of 6 animals. Group "1x vac" (#1835-1840) was vaccinated with a dose of 10^6 $TCID_{50}$ via intramuscular (IM) route on gestation day (GD) 58. On the same day, the Mock group (#1841-1846) was inoculated with culture medium. Group "2x vac" (#1829-1834) was vaccinated twice, on days 51 and 65 of gestation, with a dose of 10^5 $TCID_{50}$. Three weeks post single vaccination and two weeks post double vaccination, all ewes were challenged via intravenous (IV) route with 10^5 $TCID_{50}$ of RVFV strain 35/74, previously rescued in BSR-T7 cells and amplified in BHK-21 cells[216]. Three of the mock-vaccinated ewes (#1841-1843) were euthanized on day 4 post challenge (DPC), whereas the remaining three ewes were euthanized at imminent abortion. The outline of the study is presented in Figure 3.1A. No untoward effects or other clinical signs were observed following vaccination. All mock-vaccinated ewes manifested with elevated rectal temperatures (Figure 3.1B) and viremia (Figure 3.1C) following challenge infection. Necropsy of mock-vaccinated ewes on DPC 4 revealed multifocal necrotizing hepatitis, although placentas revealed no macroscopic

abnormalities and all foetuses were still alive. In the morning of DPC 7, one mock-vaccinated ewe (#1844) had expelled 2 foetuses and a second ewe (#1845) was in the process of aborting, with two foetuses already expelled and one foetus still in the uterus. Necropsy of the third ewe (#1846) revealed three foetuses that were still inside the uterus and placentomes showing extensive haemorrhages, and varying degrees of cotyledonal detachment (Figure 3.2). One of these foetuses was alive, whereas the remaining two foetuses were found dead (Figure S3.1). Analysis of liver and spleen samples revealed very high viral RNA levels in the organs of ewes necropsied on 4 DPC and lower levels in ewes necropsied at 7 DPC (Figure 3.1D). High levels of viral RNA were detected in all placentomes and most of the foetal livers (Figure 3.1E). Details about virological and (histo)pathological findings in ewes and foetuses were reported previously[222] as the mock-vaccinated group was also part of an experiment in which the pathology of RVFV for pregnant ewes was assessed.

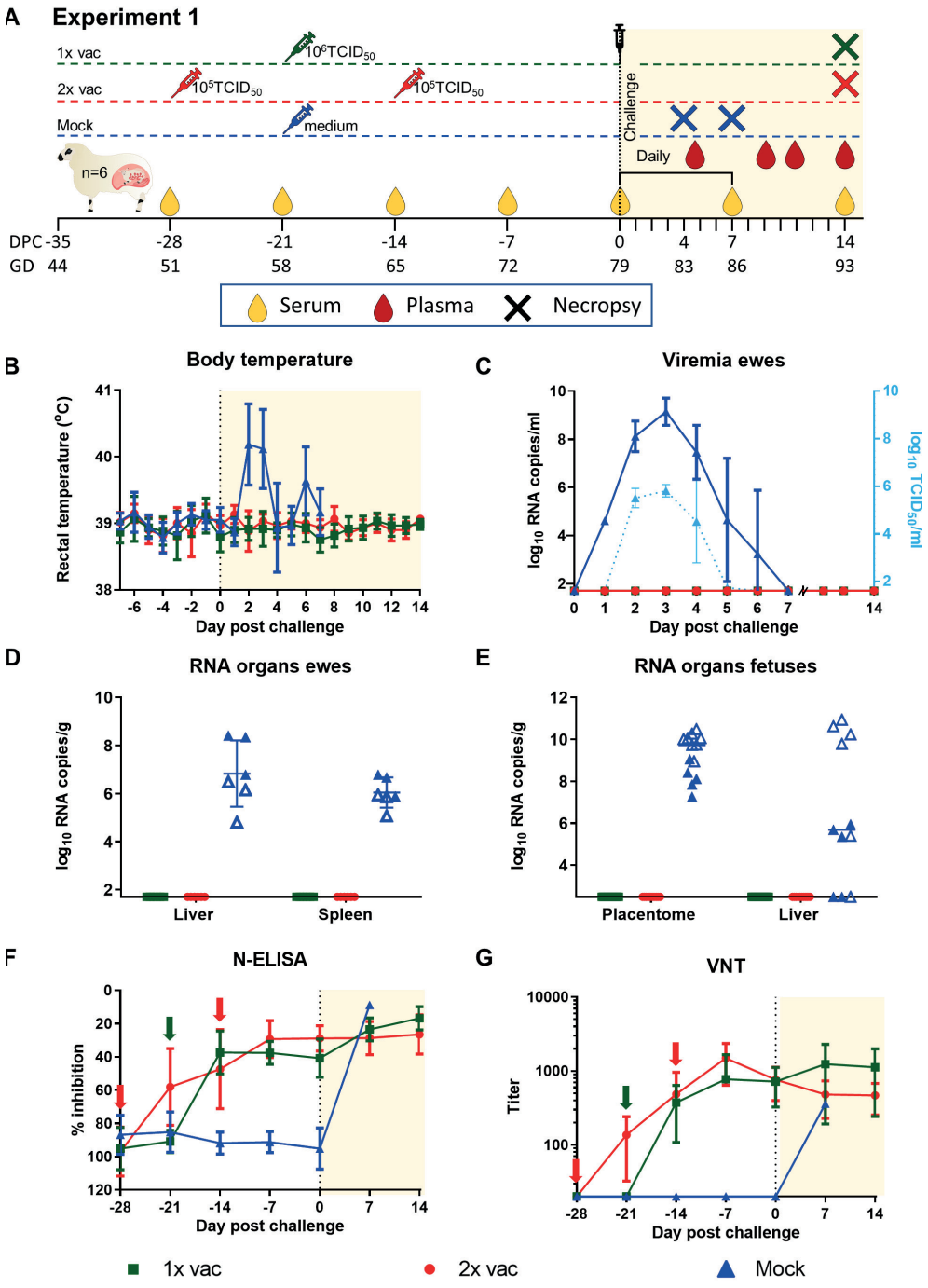

A
Experiment 1
1x vac
2x vac
Mock
10^6TCID50
10^5TCID50
10^5TCID50
medium
n=6
Challenge
Daily
DPC -35 -28 -21 -14 -7 0 4 7 14
GD 44 51 58 65 72 79 83 86 93
Serum
Plasma
Necropsy
B
Body temperature
Rectal temperature (°C)
Day post challenge
C
Viremia ewes
log10 RNA copies/ml
log10 TCID50/ml
Day post challenge
D
RNA organs ewes
log10 RNA copies/g
Liver
Spleen
E
RNA organs fetuses
log10 RNA copies/g
Placentome
Liver
F
N-ELISA
% inhibition
Day post challenge
G
VNT
Titer
Day post challenge
1x vac
2x vac
Mock

Figure 3.1. (opposite) Primary outcome parameters of vaccination challenge Experiment 1. (A) Graphical representation of the experiment. Pregnant ewes were vaccinated either once or twice at the time points indicated and challenged two- (double vaccination) or three weeks (single vaccination) after vaccination with virulent RVFV strain 35/74. Vaccinated/challenged ewes were euthanized 14 days post challenge. Ewes in the mock group were euthanized on day 4 post challenge (n=3) or at imminent abortion (n=3). (B) Rectal temperatures in °C with SD. (C) Monitoring of viral RNA by RT-qPCR (detection limit 1.3 $\log_{10}$ RNA copies/ml). Samples with an RNA copy number of >5 $\log_{10}$/ml were assayed for infectious virus by virus isolation on BHK cells (detection limit of 1.55 $\log_{10}$ $TCID_{50}$/ml). (D) Detection of viral RNA in liver and spleen samples of the ewes (detection limit 2.3 $\log_{10}$ RNA copies/gram). Closed symbols represent samples collected on DPC 4, open symbols represent samples collected on DPC 7. (E) Detection of viral RNA in placentomes and foetal livers (detection limit 2.3 $\log_{10}$ RNA copies/gram). Of each placenta, one placentome was tested. (F) Detection of anti-N antibodies by competition ELISA in weekly obtained sera. Competition is expressed as percentage inhibition ratio of the optical densities (OD) of the sample and the OD of the negative control (% S/N). All values below 40% are considered positive, between 40 and 50% are considered doubtful and above 50% are considered negative. (G) Detection of neutralizing antibody responses by VNT30. Moments of vaccination are indicated in panels F and G by arrows. Measurements were taken from distinct samples.

Ewes that had received a single vaccination and were challenged three weeks later did not present with increased rectal temperatures (Figure 3.1B) and no viremia (Figure 3.1C) was detected. At necropsy on 14 DPC, the ewes were found to carry a total of 18 live, apparently healthy foetuses with crown-rump lengths of about 25 cm (as expected for their gestation stage). It was interesting to find that in two ewes (#1838 and #1840) some placentas revealed not only concave, type A/B placentomes but also convex, type C and D placentomes (Figure 3.2D, Figure S3.2, and Table S3.1A, B).

Ewes that had received a double vaccination and were challenged two weeks later also did not develop signs of disease. Also in these ewes, no rises in rectal temperatures (Figure 3.1B) or viremia Figure 3.1C) were detected. At necropsy (15 DPC), these ewes were found to carry a total of 10 live, and apparently healthy foetuses with crown-rump lengths of about 26 cm (also as expected for their gestation stage, Figure S3.1). No viral RNA was detected in maternal or foetal organs of any of the vaccinated animals (Figure 3.1D and E).

All vaccinated ewes developed anti-nucleocapsid (N) antibodies, as determined by ELISA (Figure 3.1F), as well as virus-neutralizing antibodies as determined by VNT (Figure 3.1G). No significant differences in the antibody levels at the time of challenge were observed between the vaccinated groups.

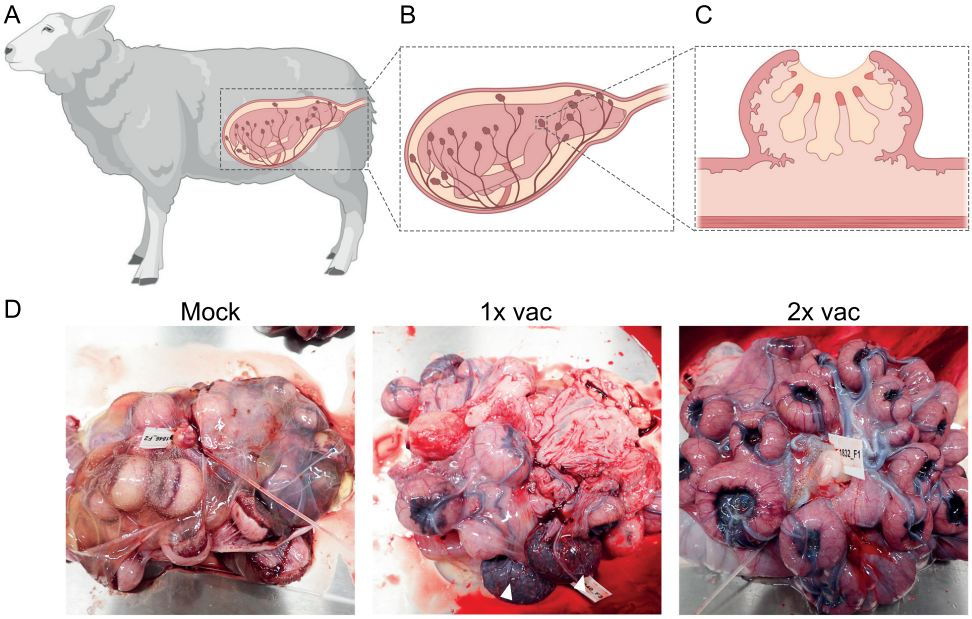


Figure 3.2. Macroscopic assessment of placentas from mock-vaccinated ewes and ewes vaccinated with vRVFV-4s followed by challenged with wild-type RVFV. (A) Cartoon of a pregnant ewe including a magnification of the ovine placenta (B) with placentomes and a magnification of a single placentome (C), revealing the foetal (beige) and maternal (pink) villi and haemophagous zone (red). (D) Representative pictures of placentas from a mock-vaccinated challenged ewe (left panel; ewe #1846, foetus #F2), a challenged ewe that was vaccinated once (middle panel; ewe #1840, foetus #F3), and a challenged ewe that was vaccinated twice (right panel; ewe #1832, foetus #F1). Note the two type D placentomes in the placenta of the 1 x vaccinated ewe (white arrowheads in middle panel, see also Figure S3.2).

Efficacy of a MSV+5 of vRVFV-4s in pregnant ewes (Experiment 2)

After the promising results obtained from the first study with the investigational batch of vRVFV-4s, a master seed virus (MSV) was developed by passage of the virus in BSR-T7 cells[229], and subsequently passaging 5 times in the same cells as presented in the present work. This MSV+5 batch was subsequently evaluated in a second study with pregnant ewes in full compliance with the European Pharmacopeia (EP) monograph 5.2.7 (Evaluation of efficacy of veterinary vaccines and immunosera). Moreover, even lower doses for the 1x vac and 2x vac groups were assessed, and necropsies were scheduled at 3 weeks, instead of 2 weeks after challenge.

Twenty four ewes were divided over 3 groups of 8 animals. The first group "1x vac" (#207-214) was vaccinated with a dose of $10^{5.5}$ $TCID_{50}$ via IM route on GD 53. The Mock group (#223-230) was inoculated with PBS on the same day. Group "2x vac" (#199-206) was vaccinated twice with a dose of $10^{4.5}$ $TCID_{50}$ on GDs 46 and 60. Three weeks post single vaccination and two weeks post double vaccination, all ewes were challenged, via IV

route, with 10^5 $TCID_{50}$ of RVFV strain 35/74. The outline of the study is presented in Figure 3.3A. In line with experiment 1, no clinical signs or other untoward events were noted after vaccination. One ewe from the control group (#226) developed laryngeal chondritis prior to challenge and had to be euthanized and removed from the experiment. After challenge, 6 of 7 mock-vaccinated ewes developed increased rectal temperatures with onset on day 3 (Figure 3.3B), associated with high viremia levels as determined by RT-qPCR and virus isolation (Figure 3.3C). One ewe acutely died on DPC 5 (#228). Necropsy of this ewe revealed a necrotic liver, haemorrhagic placentas and two dead foetuses. One ewe aborted one foetus on DPC 7. To prevent unnecessary animal discomfort, all remaining ewes were necropsied on this day as well. In total, the mock-vaccinated ewes were found to carry 14 foetuses, which had all succumbed to the infection.

Analysis of samples from mock-vaccinated ewes and their foetuses revealed very high viral RNA levels in maternal liver and spleen samples (Figure 3.3D), and high levels of viral RNA in all placentomes, foetal brains, livers, and spleens (Figure 3.3E). Of note, the mock-vaccinated group was shared with another experiment in which the Chimpanzee Adenovirus Oxford-based RVF (ChAdOx1-RVF) vaccine was evaluated[230].

In contrast to the mock-vaccinated ewes, all vRVFV-4s-vaccinated ewes did not show any clinical symptoms that could be attributed to the RVFV challenge. Ewes that were vaccinated once with $10^{5.5}$ $TCID_{50}$ were found to carry a total of 16 foetuses, whereas ewes vaccinated twice with $10^{4.5}$ $TCID_{50}$ were found to carry a total of 18 foetuses. All 34 foetuses appeared healthy and were of the expected crown-rump size (between 26-28 cm). All placentas of vaccinated ewes appeared healthy. Nevertheless, in two ewes (#207 and #214), vaccinated once with $10^{5.5}$ $TCID_{50}$, the placentas of 4 foetuses revealed not only concave, type A/B placentomes but also convex, type D placentomes (Figure S3.2, Table S3.2A, B). Similar as in experiment 1, this was observed only in 1x vaccinated ewes, and not in 2x vaccinated ewes. Again in line with experiment 1, no viral RNA was observed in the organs (livers and spleens) of vaccinated ewes except for 1 out of 32 tested placentomes in the 1x vac group in which a low level of viral RNA was detected by RT-qPCR. Importantly, no viral RNA was detected in any tested foetal tissue sample.

Similar as in the first experiment, all vaccinated ewes seroconverted, as determined by ELISA (Figure 3.3F) and VNT (Figure 3.3G), and again no significant differences at the time of challenge were observed between the vaccinated groups.

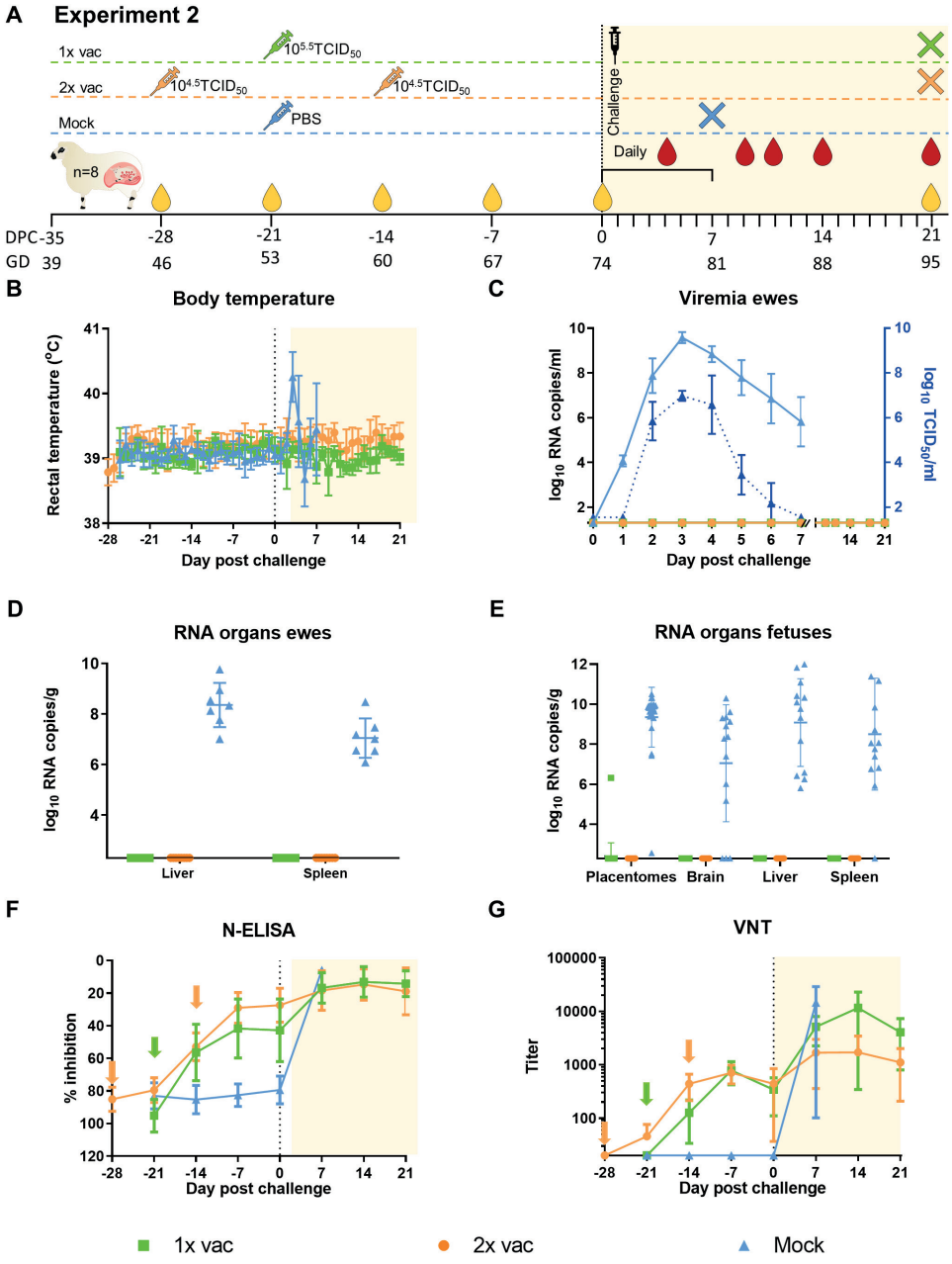


Figure 3.3. Primary outcome parameters of vaccination challenge Experiment 2. (A) Experimental design. Pregnant ewes were vaccinated either once or twice at the time points indicated and challenged two- (double vaccination) or three weeks (single vaccination) after vaccination with highly virulent RVFV strain 35/74. Vaccinated/challenged ewes were euthanized 21 days post challenge. One ewe in the mock group succumbed on day 5, whereas the other ewes were euthanized at 7 DPC. (B) Rectal temperatures in °C with SD. (C) Monitoring of viral RNA by RT-qPCR (detection limit 1.3 $\log_{10}$ RNA copies/ml). Samples with an RNA copy number of >10^5/ml

were assayed for viremia by virus isolation on BHK cells (detection limit of 1.55 $\log_{10}$ $TCID_{50}$/ml). (D) Detection of viral RNA in liver and spleen samples of the ewes (detection limit 2.3 $\log_{10}$ RNA copies/gram). (E) Detection of viral RNA in placentomes, brains, liver and spleens of foetuses (detection limit 2.3 $\log_{10}$ RNA copies/gram). Of each placenta, two placentomes were tested. (F) Detection of anti-N antibodies by competition ELISA in weekly obtained sera. Competition is expressed as percentage inhibition ratio of the optical densities (OD) of the sample and the OD of the negative control (% S/N). All values below 40% are considered positive, between 40 and 50% are considered doubtful and above 50% are considered negative. (G) Detection of neutralizing antibody responses by VNT. Moments of vaccination are indicated in panels f and g by arrows. Measurements were taken from distinct samples.

Macroscopic assessment and histopathology of placental tissues

Similar as in Experiment 1, placentas from mock-vaccinated ewes were severely affected showing haemorrhages, necrosis and cotyledonal detachment, whereas placentas from vaccinated ewes appeared healthy (Figure 3.4A). However, the observed morphological changes of some placentomes and the detection of viral RNA in one placentome in challenged ewes that had received a single vaccination prompted a more detailed analysis of the placentas by (immuno)histochemistry. H&E staining of placentomes from mock-vaccinated and subsequently challenged ewes revealed extensive haemorrhages and necrosis of maternal epithelium (Figure 3.4B, left panel) associated with RVFV antigen both in maternal and foetal epithelial cells (Figure 3.4C, left panel). Dystrophic calcification of necrotic maternal epithelial cells was visualized by Alizarin Red staining (Figure 3.4D, left panel).

Placentomes from vaccinated ewes appeared unaltered by H&E staining (Figure 3.4B, centre and right panels), and absence of viral antigen, in line with the PCR results, was confirmed by IHC (Figure 3.4C, middle and right panels), although foci with calcium deposits in the maternal epithelium were detected in some ewes that had received a single vaccination (Figure 3.4D, middle panel, see also Table S3.2A, B). This suggests that in a few ewes that had received a single vaccination some local virus infection/replication had taken place following challenge. However, the virus was apparently cleared rapidly as low-level viral RNA was detected in only 1 out of 32 placentomes tested at three weeks post challenge (1x vac group, Figure 3.3E). Importantly, no viral antigen was detected in foetal tissues, suggesting that a single vaccination was sufficient to prevent vertical transmission.

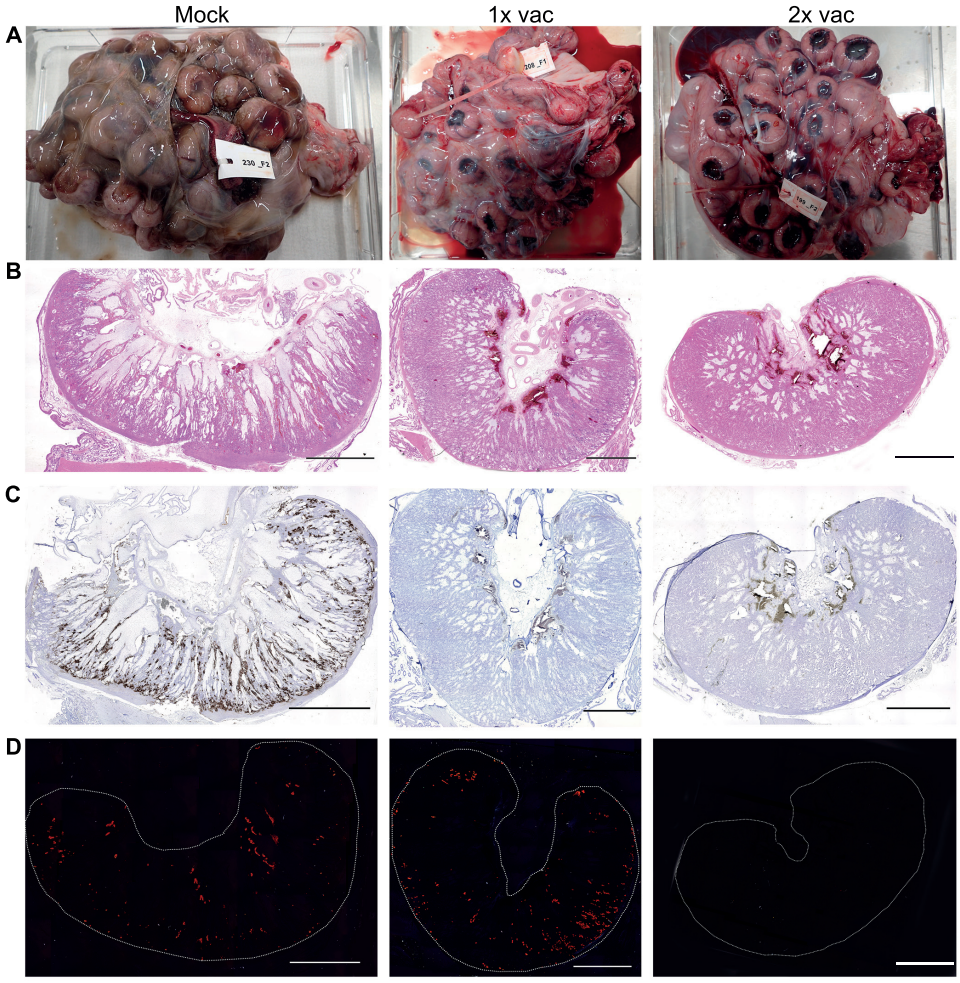


Figure 3.4. Macroscopic assessment of placentas and histopathology of placentomes from mock-vaccinated ewes and ewes vaccinated with vRVFV-4s MSV+5 and challenged with RVFV strain 35/74. (A) Placenta from a mock-vaccinated, challenged ewe (ewe #230, foetus #F2), euthanized at 7 days post challenge (left panel). Placenta from a 1x vaccinated ewe (ewe #208, foetus #F1), euthanized at 24 days post challenge (centre panel). Placenta from a 2x vaccinated ewe (ewe #199, foetus #F2) euthanized at 21 days post challenge (right panel). (B) H&E staining of representative placentome sections, (C) IHC staining of placentome sections with RVFV-specific mAb 4-D4, (D) Alizarin Red staining of placentome sections visualized using polarized light. Bars represent 5,000 μm.

Discussion

We previously reported that a single vaccination with vRVFV-4s protects young sheep, goats and cattle from virulent RVFV challenge[229]. Here, we show that a single vaccination protects pregnant ewes and their foetuses against wild-type RVFV challenge in two independent experiments. Anticipating on the high susceptibility of pregnant ewes for wild-type RVFV, we evaluated both a single vaccination and a double vaccination at two different doses. Whereas neutralizing antibody levels did not differ significantly among the groups, challenge infection resulted in a higher increase in antibody responses in ewes that had received a single vaccination with a dose of $10^{5.5}$ $TCID_{50}$, suggesting that some challenge virus may have replicated in the placentas of these ewes. This is supported by the detection of a low level of viral RNA in one placentome at necropsy.

Sheep placentomes can be classified into four types: A, B, C, and D, with type A and B being predominant under normal conditions (Figure S3.2)[231]. Type C and D placentomes represent eversions of the haemophagous zone, which are associated with physical adaptations to stressors, such as hypoxia or malnutrition, considered to facilitate exchange of oxygen and nutrients[232]. However, it is important to note that eversion of the haemophagous zone may also occur at late pregnancy and is not associated with pathological changes[233]. In ewes that received a double vaccination, only type A and B placentomes were observed, whereas in some challenged ewes that had received a single vaccination, type C and D placentomes were found. These placentome eversions may have resulted from localized placental lesions that also explain the observed calcifications[234]. Similar calcifications were previously observed in ewes that were vaccinated with the ChAdOx1-RVF vaccine candidate following challenge[230]. If indeed limited and transient challenge virus replication occurred in maternal epithelium of the placenta, it is quite remarkable that maternal immunity prevented transmission of the challenge virus to foetal trophoblasts, directly lining the maternal epithelium and previously shown to be highly susceptible to RVFV[222].

In conclusion, our results suggest that a single vaccination of pregnant ewes provides complete protection from vertical transmission and abortion. Considering that placentas and foetuses expelled from RVFV infected ewes pose a serious risk for farmers and veterinarians handling these materials, vaccination with vRVFV-4s could additionally prevent human morbidity and mortality.

Materials and Methods

Cells and Viruses

Culture media and supplements were obtained from Gibco unless indicated otherwise. Baby Hamster Kidney (BHK-21) and BSR-T7 cells were maintained in Glasgow minimum essential medium (GMEM) supplemented with 4% tryptose phosphate broth, 1% minimum essential medium nonessential amino acids (MEM NEAA), 1% antibiotic/antimycotic (a/a) and 5% foetal bovine serum (FBS), at 37°C with 5% CO_2.

The investigational batch of vRVFV-4s was described before (at that time referred as RVFV-4s)[227]. The MSV stock was prepared by infecting BSR-T7 cells with plaque-purified seed virus, cultured in complete medium (GMEM supplemented with 5% FCS (SAFC), 4% TPB, 0.001% Gentamycin and 1% NeAA) at a multiplicity of infection (MOI) of 0.002. BSR-T7 cells were used, as amplification of the vaccine virus in these cells yields high titers. Virus was harvested at 3 days post infection (DPI). To produce the MSV+5 stock the virus was passaged 5 times in BSR-T7 cells using an MOI of 0.002 and harvesting at 3 DPI.

At 2 DPI, immunoperoxidase monolayer assays were performed and titres calculated using the Spearman-Kärber algorithm as described[222]. Recombinant RVFV strain 35/74, amplified in BHK-21 cells, was used in the present work as challenge virus as described previously[216].

Experimental design pregnant ewe trials

Two experiments were performed to determine the efficacy of the vRVFV-4s vaccine in pregnant ewes. For both experiments, ewes were treated with progesterone sponges to synchronise pregnancy after which the ewes were naturally mated. At 6-7 weeks post conception, pregnancy was confirmed via ultrasound and the general health of the ewes was assessed by a veterinarian.

Efficacy of an investigational batch of vRVFV-4s in pregnant ewes (Experiment 1)

Eighteen pregnant ewes were transported to the BSL-3 facility of WBVR, where they were allowed to acclimatise for 7 days. Serum was collected weekly from that moment onwards (Figure 3.1A). At day 51 and 65 of gestation, the group that was to be vaccinated twice was vaccinated with 10^5 $TCID_{50}$ of the RVFV-4s vaccine. At GD 58, the 1x vac group was vaccinated with 10^6 $TCID_{50}$ and the Mock group with culture medium. Three weeks later, the ewes were challenged by IV inoculation of 10^5 $TCID_{50}$ of recombinant RVFV[235]. Ewes were observed twice per day for general health and signs of abortion and rectal temperatures were recorded daily. EDTA blood samples were collected daily for the first seven days and at specific timepoints thereafter (9, 11, and 14 days DPC). At 14 DPC, or when a humane endpoint (HEP) was reached, the ewes and their foetuses were euthanized by IV administration of 50 mg/kg sodium pentobarbital (Euthasol, ASTfarma)

and subsequent exsanguination. From the ewes samples were taken from the liver and spleen. From the foetus, samples were taken from the liver and from one placentome. Samples were either placed on dry ice and stored at -80°C for virus isolation and RNA extraction or samples were fixed in 10% phosphate-buffered formalin for at least 48 hours before routine processing into paraffin blocks.

Efficacy of MSV+5 in pregnant ewes (Experiment 2)

To study efficacy of the MSV+5 in pregnant ewes, twenty-four ewes were transported to WBVR at day 39 of pregnancy, after which the animals were allowed to acclimatize for 1 week. Ewes that received a single vaccination (1x vac), were inoculated via IM route with a dose of $10^{5.5}$ $TCID_{50}$ on GD 53. On the same day, the Mock group was inoculated with PBS. Ewes that received two vaccinations, were inoculated with a dose of $10^{4.5}$ $TCID_{50}$ on GD 46 and 60 (2x vac; Figure 3.3A). On GD 74, all groups were challenged via IV route with 10^{5} $TCID_{50}$ of recombinant RVFV. Rectal temperatures were measured daily and each day the ewes were observed twice for general health and signs of abortion. Serum samples were taken weekly from GD 46 and plasma was collected daily after challenge for the first week and at intervals thereafter (9, 11, 14 and 21 DPC). All ewes were euthanized at 21 DPC, or when a HEP was reached, by IV administration of 50 mg/kg sodium pentobarbital (Euthasol, ASTfarma) and subsequent exsanguination. Samples were collected from the liver and spleen from the ewes. Two placentomes were collected from each placenta and foetal samples were taken from the brain, liver and spleen. Samples were either placed on dry-ice and stored at -80°C for virus isolation or used for RNA extraction, or samples were fixed in 10% phosphate-buffered formalin for at least 48 hours before routine processing into paraffin blocks. Experimental details are noted in Table 3.1 according to the ARRIVE guidelines[236].

Table 3.1

	Investigational batch	MSV+5
Animals		
Species	Sheep	
Breed and/or strain	Texel cross breed	
Source	Conventional Dutch sheep farm	
Sex	Female	
Developmental stage (age)	Between 1.5 and 5 years	
Health and physiological status	Healthy and pregnant	
Weight	60 kg	
Identification	By ear tag and by non-irritating colored spray on the back of the animal.	
Housing and Husbandry		
Type of facility	BSL-3 facility	
Type of housing	Stables of 18m2	
Bedding material	Wood shavings	
Number of animals per stable	6	
Light/dark cycle	12/12	
Temperature	21°C	23°C
Quality of water	Tap water, quality checked daily	
Type of food	Hay, grass pellets and sheep grain	
Access to water and food	Water ad libitum, Food once per day	
Acclimatization time	7 days	
Experimental Procedure		
Number experimental groups	3	
Number of animals per group	6	8
Statistical support	In the unprotected control group, all ewes were expected to abort or to carry infected foetuses. To show protection of at least 80% in the vaccinated groups (a = 5% and β = 90%) Using the Fisher's exact test, one-sided testing, with the software G*Power 3.0.10., we calculated that we needed at least 6 animals per group.	
Randomisation procedure	Animals were sorted by age and subsequently divided over experimental groups	
Experimental unit	Group	
Route of administration	Vaccination: IM injection right gluteus maximus muscle Challenge: IV injection in jugular vein	
Anaesthesia and analgesia	Not applicable	
Method of euthanasia	Intravenous injection with sodium pentobarbital	
Humane endpoints	-The animal is recumbent and does not rise even after stimulation -The animal is unable to drink -The animal is lethargic (listless, apathic, non-responsive to stimuli) -Signs of abortion	
Observations	The animals were checked for clinical signs once per day, with an intensification of twice per day after challenge	

Detection of viral RNA

Organ samples were homogenised by adding 0.3–1 g of tissue to an IKA Ultra Turrax Tube DT-20 containing 7 ml CO_2-Independent Medium (CIM) supplemented with 1% a/a. The suspensions were transferred to 15 ml Falcon tubes and cell debris was removed by centrifugation for 15 min at 4952 *x g*.

Organ suspensions or plasma samples (200 µl) obtained in Experiment 1 were added to 50 µl Proteinase K (5 µg/ml, Sigma). Next, 200 µl AL buffer (Qiagen), supplemented with 2 µl polyadenylic acid A (5 mg/ml, Sigma), after which the samples were thoroughly mixed and incubated at 56°C for 15 min. Subsequently, 250 µl 99% ethanol was added and RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's protocol.

Organ suspensions or plasma samples (500 µL) obtained in Experiment 2 were added to 2.5 ml NucliSENS easyMAG Lysis Buffer (Biomérieux, Marcy-l'Étoile, France), after which RNA was extracted using the NucliSENS easyMAG (Biomérieux) according to manufacturer's protocol.

Five µl of the RNA was used in a RT-qPCR using the The LightCycler RNA Amplification Kit HybProbe (Roche, Almere, the Netherlands). Primers and probes were purchased from IDT. Forward primer: 5'-AAAGGAACAATGGACTCTGGTCA-3', reverse primer: 5'-CACTTCTTACTACCATGTCCTCCAAT-3'; Probe: 5'-6FAM-AAAGCTTTGATATCTCTCAGTGCCCCAA-TMR-3'. Cycling conditions were as follows: 45°C for 30 min, 95°C for 5 min, 45 cycles of 5 s at 95°C and 35 s at 57°C, followed by cooling down to 30°C.

Virus isolation

Virus isolations were performed on RT-qPCR positive samples with a threshold above 10^5 RNA copies/ml as this has been previously shown to be a cut-off point below which no live virus can be detected[228]. Virus isolations of plasma were performed by serial dilution in complete CO_2-indepentent medium (CIM; supplemented with 5% FBS and 1% a/a) supplemented with 3.5 IU/ml heparin, and virus isolation of organ suspensions were serially diluted in complete CIM. Subsequently, the virus dilutions were incubated with BHK-21 cells. After 1.5 h incubation at RT, the inocula were replaced by fresh medium and after 5 days of culturing the cells at 37°C and 5% CO_2 cytopathic effects were scored.

Virus neutralisation test and ELISA

Serum RVFV neutralising antibodies were measured using a virus neutralization test (VNT) as described earlier[237]. Briefly, serial dilutions (50 µl) of heat-inactivated sera (2 h, 56 °C) were incubated with 50 µl of RVFV-4s_{eGFP} ($10^{3.6}$ $TCID_{50}$/ml) for 2 h at room temperature. Subsequently, 20,000 BHK-21 cells (in 50 µl) were added to each well. Plates were incubated for 2 days at 37 °C and 5% CO_2 and scored using an EVOS-FL microscope (Life Technologies). VNT_{50} titres were calculated using the Spearman–Kärber algorithm.

Presence of RVFV nucleoprotein-specific antibodies in sera was determined using the ID Screen® Rift Valley Fever Competition ELISA (ID-Vet, Montpellier, France).

Histology and immunohistochemistry

Paraffin-embedded tissues were cut into 4 µm sections, collected on silane-coated glass slides and dried for at least 48 h in a 37°C incubator. After deparaffinization and rehydration in graded alcohols, sections were stained routinely with haematoxylin and eosin (H&E) or immunostained for RVFV antigen. For immunostaining, endogenous peroxidase was blocked for 30 min in methanol/H_2O_2 followed by antigen retrieval through 15 min autoclaving at 121°C in pH 6 citrate buffer (Antigen unmasking solution, Vector Laboratories). As RVFV Gn-specific primary antibody, monoclonal antibody 4-D4 was used. Specificity of the immunostaining was confirmed with 2 other mAbs directed against different proteins of RVFV. Mouse envision peroxidase (Dako, Denmark) was used as secondary antibody and diaminobenzidine (DAB; Dako, Denmark) as the substrate, according to the manufacturer's instructions. Hematoxylin was used to counterstain the slides. Calcium deposits were stained with Alizarin Red (Merck, Darmstadt, Germany).

Ethics statement

Animal trials were conducted in accordance with European regulations (EU directive 2010/63/EU) and the Dutch Law on Animal Experiments (Wod, ID number BWBR0003081). Permissions were granted by the Dutch Central Authority for Scientific Procedures on Animals (Permit Number: AVD401002017816). Specific procedures were approved by the Animal Ethics Committees of Wageningen Research. The following HEPs were applied: 1) the animal is recumbent and does not rise even after stimulation, 2) the animal is unable to drink, 3) the animal is lethargic (listless, apathic, non-responsive to stimuli), 4) Signs of abortion.

Acknowledgements

We thank the animal caretakers for providing assistance in the animal trials and Pieter Roskam for assisting with the necropsies. We thank Dr. Schmaljohn (USAMRIID, Fort Detrick, MD) for providing the 4-D4 mAb and Prof. Klaus Conzelmann (Ludwich-Maximilians-Universität, München) for providing the BSR-T7 cells. The first study was performed within the scope of the Castellum program, funded by the former Ministry of Economic Affairs, with additional support of the Ministry of Agriculture, Nature and Food Quality of the Netherlands project code: WOT-01-001-003. The second study was commissioned by Ceva Animal Health, Ceva-Phylaxia. Cartoons in this manuscript were created by BioRender.com.

Supporting information

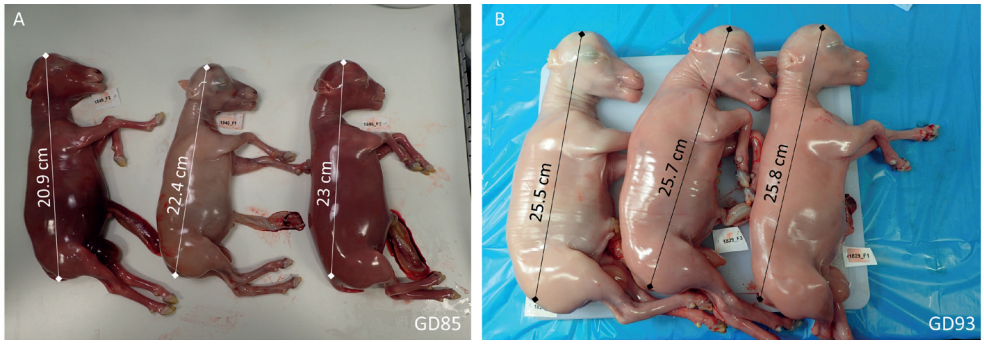


Figure S3.1. Representative pictures of foetuses from a mock-vaccinated ewe and a vaccinated ewe. (A) Foetuses collected at imminent abortion from mock-vaccinated ewe #1846 on GD85 (Experiment 1). The central foetus was alive at necropsy, whereas the two siblings were found dead. (B) Foetuses collected from ewe #1829 on GD93 (Experiment 1, 2x vac group). All thee foetuses were alive at necropsy.

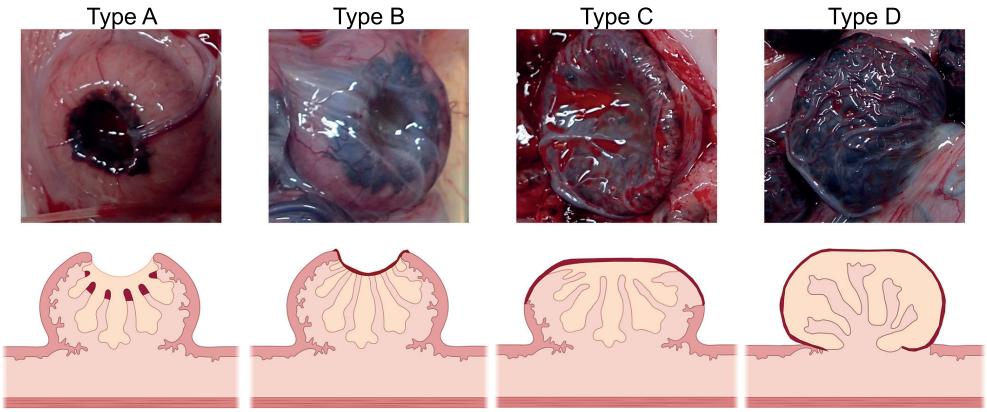


Figure S3.2. Morphology of type A, B, C and D placentomes. The haemophagous zone, indicated in red in the cartoons, is an area where maternal blood extravasates between the maternal crypts (pink) and fetal villi (beige). In type A placentomes, the haemophagous zone is for the most part positioned on the inside of the placentome. In type B placentomes, the haemophagous zone is partially everted, revealing a larger external surface area. The placentome further transits to type D placentomes via the intermediate, and characteristically flat, type C placentome.

Table S3.1A. Experiment 1, Group 1x vac

Ewe number #	Foetus number #	Type placentomes	Calcifications +/-
1835	F1	A	+
	F2	A	+
1836	F1	A, B	-
	F2	A, B	-
	F3	A, B	-
	F4	A, B	-
1837	F1	B	-
	F2	A, B	-
1838	F1	B, D	-
	F2	B, C, D	+
	F3	B	-
1839	F1	A, B	-
	F2	A, B	-
	F3	A, B	-
1840	F1	A	-
	F2	B, D	-
	F3	A, B, D	-
	F4	A, B, D	-

Table S3.1B. Experiment 1, Group 2x vac

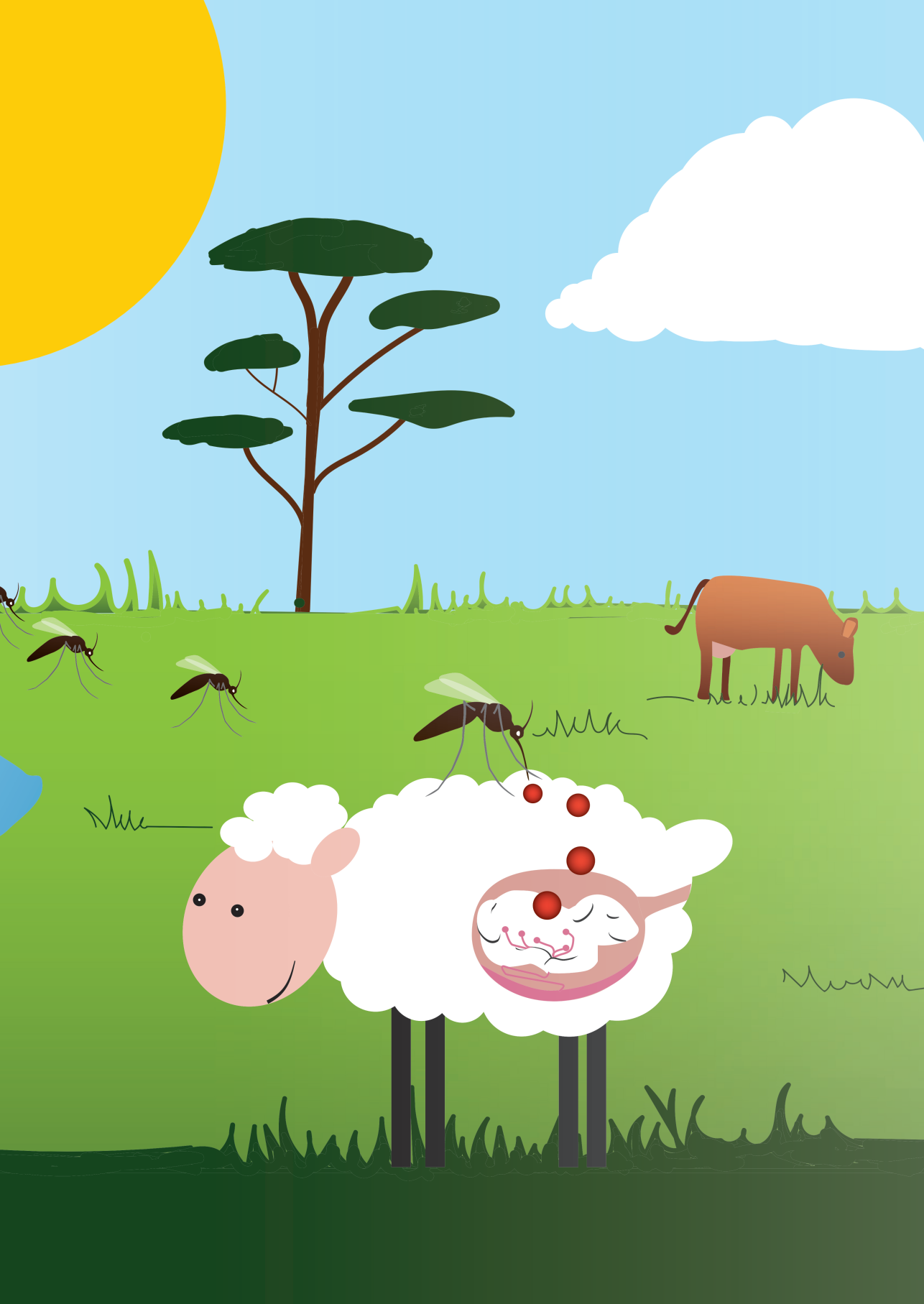
Ewe number #[a]	Foetus number #	Type placentomes	Calcifications +/-
1829	F1	A,B	-
	F2	A	-
	F3	A	-
1830	F1	A	-
	F2	A	-
1831	F1	A	-
1832	F1	A	-
1833	F1	A	-
1834	F1	A	-
	F2	A	-

Table S3.2A. Experiment 2, Group 1x vac.

Ewe number #	Foetus number #	Type placentomes	Calcifications +/-
207	F1	A, B, D	-
	F2	A, B, D	-
	F3	D	-
208	F1	A	+
	F2	A	+
209	F1	A, B	-
	F2	A, B	-
210	F1	A, B	-
	F2	A, B	-
211	F1	B	+
	F2	B	-
212	F1	A	-
213	F1	A, B	-
	F2	A, B	-
214	F1	A, B, D	-
	F2	B	-

Table S3.2B. Experiment 2, Group 2x vac.

Ewe number #	Foetus number #	Type placentomes	Calcifications +/-
199	F1	A	-
	F2	A	-
200	F1	A, B	-
201	F1	A	-
	F2	A	-
	F3	A	-
202	F1	A	-
	F2	A	-
203	F1	A, B	-
	F2	A, B	-
204	F1	A	-
	F2	A	-
205	F1	A, B	-
	F2	A	-
	F3	A	-
206	F1	B	-
	F2	B	-
	F3	B	-



Chapter 4

Early pathogenesis of Wesselsbron disease in pregnant ewes

Judith Oymans, Lucien van Keulen, Paul J. Wichgers Schreur, Jeroen Kortekaas

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Abstract

Wesselsbron virus (WSLV) is a neglected, mosquito-borne flavivirus that is endemic to the African continent. The virus is teratogenic to ruminants and causes a self-limiting febrile illness in humans. Wesselsbron disease manifests with similar clinical signs and occurs in the same areas under the same climatic conditions as Rift Valley fever, which is therefore included in the differential diagnosis. Although the gross pathology of WSLV infection in pregnant ewes is reported in literature, the pathogenesis that leads to stillbirths, congenital malformations and abortion has remained undescribed. In the present study, pregnant ewes were inoculated with WSLV and subjected to detailed clinical- and histopathology 8 days later. The virus was mainly detected in foetal trophoblasts of the placenta and in neural progenitor cells, differentiated neurons, oligodendrocytes, microglia and astrocytes. Our study demonstrates that WSLV efficiently crosses the maternal–foetal interface and is highly neuroinvasive in the ovine foetus.

Introduction

In the past two decades, the world has experienced a remarkable increase in the emergence and re-emergence of arthropod-borne viruses (arboviruses), several of which belong to the family *Flaviviridae*, genus *Flavivirus*. Examples include West Nile virus (WNV), which emerged in New York city in 1999, and has become the leading cause of epidemic meningoencephalitis in the US. WNV is also (re)emerging and spreading northwards in Europe, being detected for the first time in Germany in 2018[6,9,11]. In 2015, Zika virus (ZIKV) emerged in South America, resulting in an unprecedented outbreak, manifesting with congenital brain abnormalities[15]. Whereas the impact of WNV and ZIKV on human health is currently well recognized, it is important to remember that both viruses were largely neglected until two decades ago. The (re)emergence of arboviruses is stimulated by rapidly growing human and animal populations, intensified travel, trade and climate change. Particularly, increase in temperature and humidity may stimulate arthropod vectors to slowly move into new territories[17]. Due to their ability to affect both animals and humans, arboviruses with zoonotic potential require special attention.

A neglected zoonotic arbovirus is Wesselsbron virus (WSLV). WSLV was first isolated from the brain and liver of a decomposed lamb in the Wesselsbron district in South Africa in 1955[137]. The next month, the same virus was isolated from a human and mosquitoes of the *Aedes* genus, also in South Africa[238]. Since then, WSLV has been detected throughout the African continent either by virus isolation from vertebrates and mosquitoes or through detection of antibodies[137,141,142]. WLSV infects a wide range of domesticated animals like sheep, goats, cattle, camels and horses[138,140,141]. In 2013, WSLV was isolated from a black rat in Senegal, indicating that small rodents may also play a role in the maintenance of the virus[139]. Since the first isolation of WSLV from a human case in 1955, 33 human cases have been described, more than half of which were associated with laboratory exposure. These infections were associated with fever, headaches, myalgia and arthralgia[139,141,143]. Encephalitis as a result of WSLV infection was recorded once, when a person became infected after accidentally spraying a virus suspension into the eye[141]. Considering that there is little to no surveillance of WSLV in hospitals, prevalence of the infection in humans is almost certainly underestimated.

Sheep seem to be the most susceptible to WSLV infection[141]. The infection in adult sheep remains asymptomatic or manifests with a mild-to-moderate fever[144,239]. In newborn lambs, the disease is more severe and can lead to death, within 3 days in 35% of cases, while older lamb are less susceptible[141,240]. In pregnant ewes, the infection may result in abortion or congenital malformations[146]. Developmental abnormalities include various malformations of the central nervous system (CNS), including hydranencephaly and muscular malformations (arthrogryposis). In goats and calves, congenital malformations and abortions seem to be less common[147].

Although gross pathology resulting from WSLV infection during ovine gestation was already reported in literature[141,146], the pathogenic events that result in vertical transmission and congenital malformations have remained undescribed. Insight into the pathogenesis of WSLV disease may facilitate the development of control tools, including vaccines, and may also improve our understanding of the pathology of related (zoonotic) neuroteratogenic flaviviruses.

In the present study, ewes were inoculated with WSLV at one-third of gestation. With the aim to identify primary and secondary target cells and tissues, ewes were euthanized and necropsied 8 days after inoculation. Organs of the ewes and foetuses were evaluated by (histo)pathology, and the presence of WSLV was evaluated by reverse transcription quantitative PCR (RT-qPCR) and immunohistochemistry (IHC). Inoculation resulted in viremia in all inoculated ewes. Importantly, whereas no virus was detected in liver and spleen samples collected at necropsy, 8 days post inoculation, WSLV was shown to replicate efficiently in placental and foetal tissues. Immunohistochemistry illustrated that WSLV is highly neurotropic, neuroinvasive and neurovirulent in the ovine foetal CNS, targeting both neurons and neuroglial cells.

Results

Clinical Manifestation after Experimental WSLV Inoculation

To identify primary target cells of WSLV in pregnant ewes, ten ewes at 54 days of gestation were randomly divided over two groups. After a week of acclimatisation, at day 61 of gestation, one group was inoculated with WSLV ($10^{6,7}$ $TCID_{50}$) and the other group was mock-inoculated with medium. Rectal temperatures were measured and plasma samples were taken daily (Figure 4.1A). Surprisingly, no fever was measured in the WSLV-inoculated ewes (Figure 4.1B). However, viremia, as determined by detection of viral RNA, was observed during the first five days following infection (Figure 4.1C). At 8 days post inoculation, the ewes were euthanized and necropsies were performed. Samples were taken from the liver, spleen and the iliac and inguinal lymph nodes (LN), which drain the placenta. No macroscopic abnormalities were observed during necropsy, and all organ samples were negative for WSLV RNA (Figure 4.1D), suggesting WSLV is cleared rapidly from the blood and organs of the ewes.

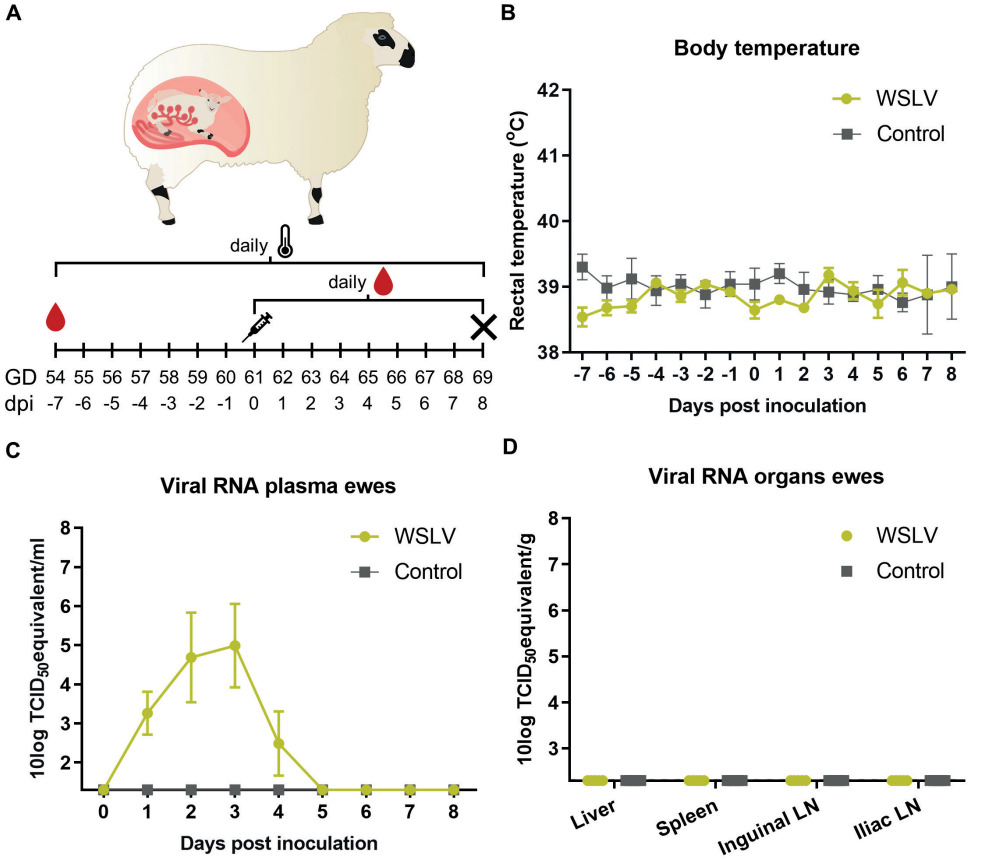


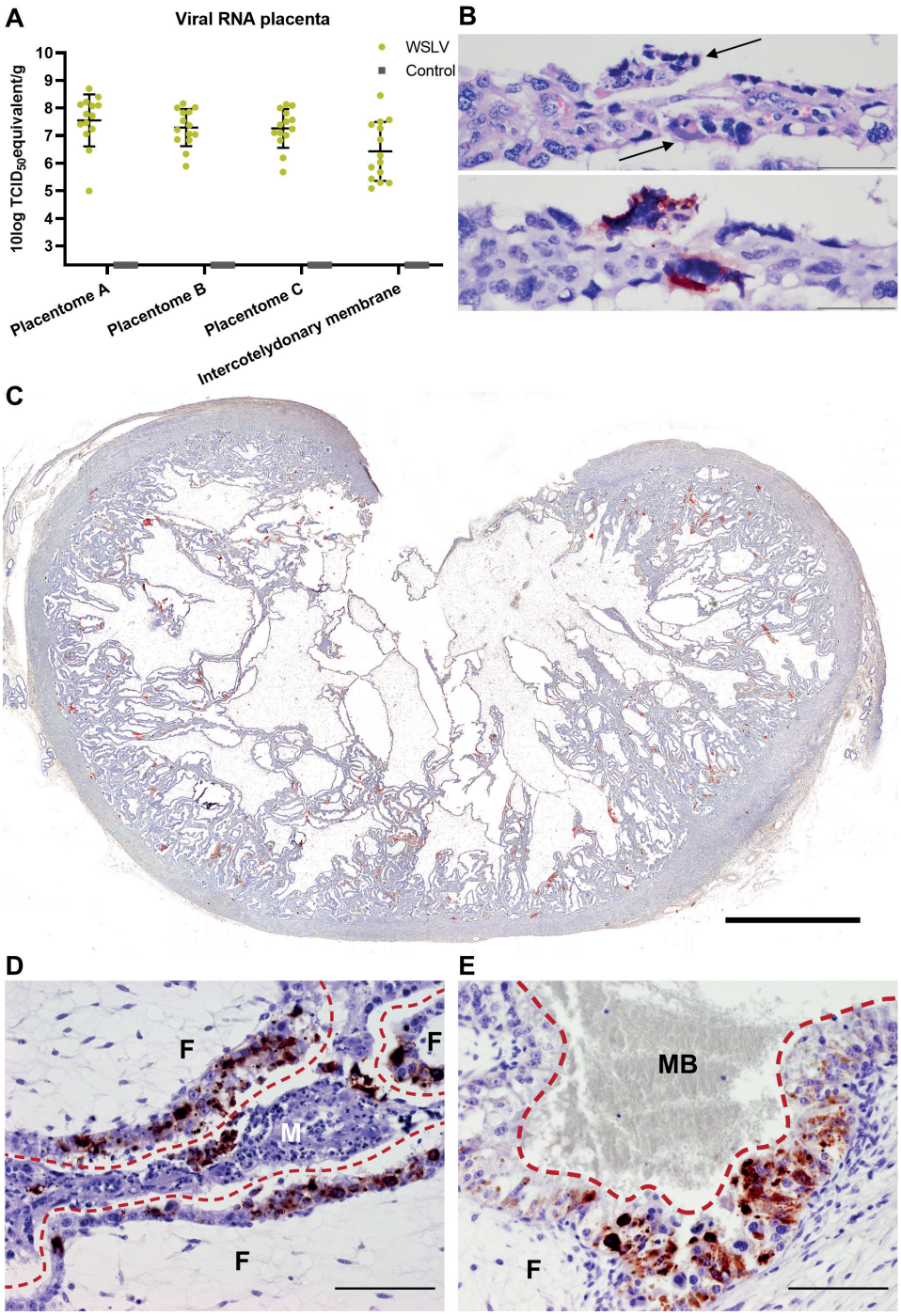
Figure 4.1. Wesselsbron virus (WSLV) infection in pregnant ewes. (A) Experimental set-up of pregnant ewe trial. Ewes were inoculated at gestation day (GD) 61. At 8 days post inoculation (dpi), the ewes were euthanized and necropsied. Rectal temperatures (B), viremia (C) and viral RNA in the organs of the ewe (D) are depicted. Error bars represent averages with standard deviation (SD).

WSLV Replicates in the Ovine Placenta

The ovine placenta comprises around 30–60 placentomes connected by an intercotelydonary membrane. Each placentome contains maternal and foetal tissues, which are separated by several cell layers. The maternal epithelium and foetal trophoblasts form the actual barrier between mother and foetus in the synepitheliochorial part of the placenta, whereas in the so-called haemophagous zones, foetal trophoblasts are in direct contact with stagnant pools of maternal blood[222].

During necropsy, samples were taken from three placentomes from different parts of the placenta and from the intercotelydonary membrane of each foetus. Both the placentomes and the intercotelydonary membrane were shown to contain high levels of viral RNA (Figure 4.2A). Haematoxylin and eosin (HE) staining of the placentomes showed small areas with necrosis of the maternal epithelial cells (Figure 4.2B top). To detect WSLV by immunohistochemistry, a polyclonal antiserum was raised by immunizing rabbits with baculovirus-produced WSLV NS1 protein. Immunohistochemistry with this antiserum revealed positive staining of the necrotic areas (Figure 4.2B bottom). Foci of WSLV antigen were distributed across the whole placentome in all tested placentomes (Figure 4.2C) and consisted of infected foetal trophoblasts surrounding infected maternal epithelial cells (Figure 4.2D). WSLV antigen was also detected in foetal trophoblasts of the haemophagous zones (Figure 4.2E). The presence of high viral RNA loads in the placenta and absence of viral RNA in other organ samples collected at 8 days post inoculation demonstrates that the ovine placenta is a primary target organ of WSLV. The observed necrosis in the maternal epithelium suggests WSLV first replicates in maternal epithelial cells, after which foetal trophoblasts are targeted in the foetal part of the placenta.

Figure 4.2. (opposite) WSLV infection in the ovine placenta. (A) Detection of viral RNA in the placentas of WSLV-inoculated and control ewes. Samples were collected from three placentomes and the intercotelydonary membrane from each foetus. Bars represent averages with SD. (B) Top: HE (haematoxylin and eosin) staining of a maternal villus with necrotic syncytial epithelial cells (arrows). Bottom: Serial section showing the same cells staining positively for WSLV. (C) Overview of a WSLV infected placentome showing the distribution of WSLV-positive foci across the whole placentome. (D) Synepitheliochorial placenta showing positive staining for WSLV of the maternal epithelium and adjacent foetal trophoblasts. (E) Haemophagous zone of the placenta also showing strong staining of the foetal trophoblasts which are in direct contact with maternal blood (MB). The red interrupted lines indicate the barrier between the maternal (M) and foetal (F) part of the placenta. Bars are 50 µm (B), 5000 µm (C) or 100 µm (D,E).


A
Viral RNA placenta
WSLV
Control
10log TCID50equivalent/g
10
9
8
7
6
5
4
3
Placentome A
Placentome B
Placentome C
Intercotelydonary membrane
B
C
D
F
F
M
F
E
MB
F

WLSV is Highly Neurotropic and Neuroinvasive in the Ovine Foetus

Necropsy of the foetuses revealed no macroscopic differences between the foetuses of the WSLV-infected group and the control group. RT-qPCR analysis of foetal liver, brain, amniotic fluid, umbilical cord and plasma samples revealed the presence of high levels of viral RNA, except for the amniotic fluid samples (Figure 4.3A). WSLV antigen was not detected by IHC in the umbilical cord, suggesting that the viral RNA that was detected in umbilical cord homogenates originated from the foetal blood. WSLV was detected in foetal hepatocytes (Figure 4.3B) and sporadically in striated muscle fibres and osteoblasts (Figure 4.3C,D).

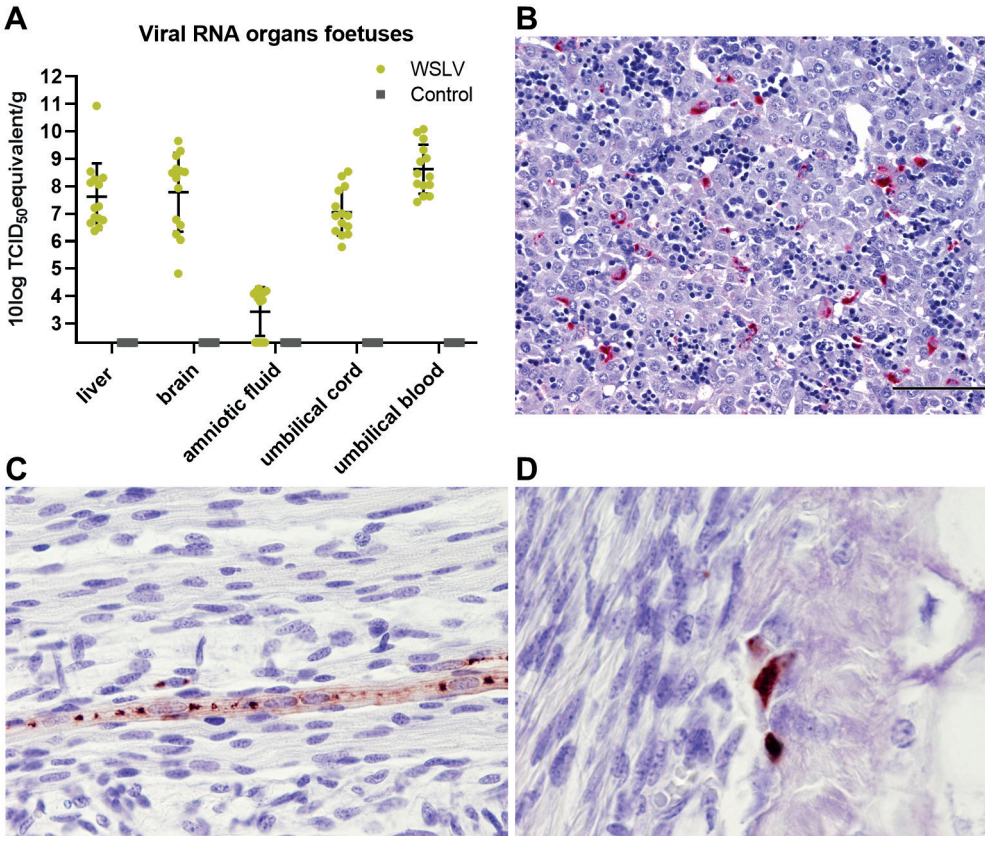


Figure 4.3. WSLV infection in foetal organs. (A) Viral RNA detected by PCR in the liver, brain, amniotic fluid, umbilical cord and plasma of the foetuses. Bars represent averages with SD. (B) Immunohistochemical staining for WSLV antigen in the foetal liver showing scattered infected hepatocytes. Notice the extramedullary haematopoiesis in the hepatic sinusoids, which is normal in the foetal liver at this stage. (C) WSLV-positive myocyte in the striated muscle of the hind leg. (D) WSLV-positive osteoblasts in the cortical bone tissue of the femur. Bars are 100 µm (B), 50 µm (C) or 20 µm (D).

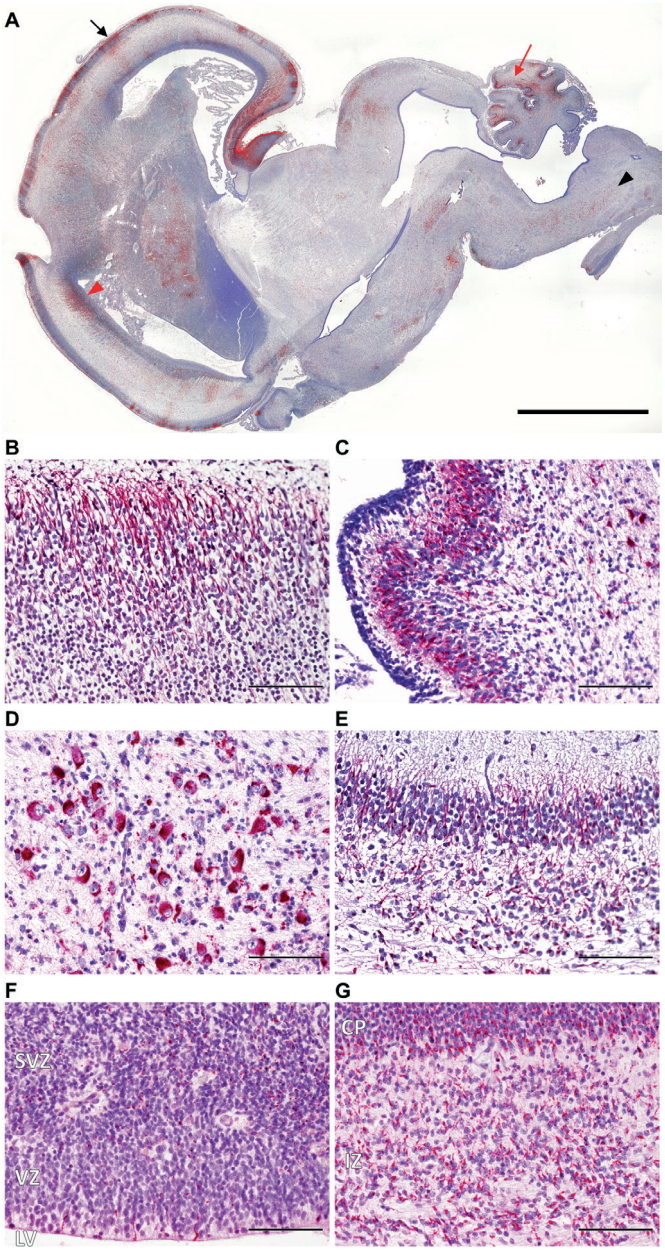


Figure 4.4. WSLV infection in the foetal brain. (A) Immunohistochemical detection of WSLV antigen in a longitudinal section of the foetal brain. WSLV antigen is present in all areas of the brain, including the cerebrum (black arrow), cerebellum (red arrow), brain stem (black arrowhead) and (sub) ventricular zone (red arrowhead). Detailed staining of WSLV antigen in the neurons of the cerebral cortical plate (B), cerebellar cortex (C), brain stem (D), hippocampus (E), (sub)ventricular zone (F) and intermediate zone (G). SVZ: subventricular zone, VZ: ventricular zone, LV: lateral ventricle, CP: cortical plate and IZ: intermediate zone. Bars are 5000 µm (A) or 100 µm (B–G).

Most interestingly, WSLV antigen was immunohistochemically detected throughout the foetal brain, including the telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon (Figure 4.4A). Infected differentiated neurons were observed in all parts of the brain, such as the pyramidal cells in the cortical plate, purkinje/granule cells in the cerebellar cortex, motoric neurons in the brain stem and pyramidal neurons of the hippocampus (Figure 4.4B–E). In addition, WSLV antigen was found in the ventricular and subventricular zone where radial glial cells and neural progenitor cells divide and proliferate (Figure 4.4F). Infection of nondifferentiated neuroblasts and glial precursor cells migrating from the (sub)ventricular zone to the cortical plate caused an abundant staining for WSLV in the intermediate zone (Figure 4.4G).

To specifically identify the different cell types in the brain that are targeted by WLSV, double immunofluorescent stainings were set up in which various cell markers and WSLV antigen can be detected simultaneously. These experiments confirmed that foetal neurons are the primary target cells of WSLV (Figure 4.5). In addition, WSLV antigen was detected in all types of neuroglial cells: oligodendrocytes, microglia and astrocytes (Figure 4.5). Microglial cells in WSLV-infected foetuses had adopted a rounded, amoeboid shape in contrast to the ramified microglia found in mock-inoculated foetuses, suggesting activation of microglia following neuroinvasion by WSLV (Figure 4.6A,B). Activated microglia were seen invading sites of WSLV infection in the brain (Figure 4.6C,D), leading to the formation of microglial nodules throughout the brain.

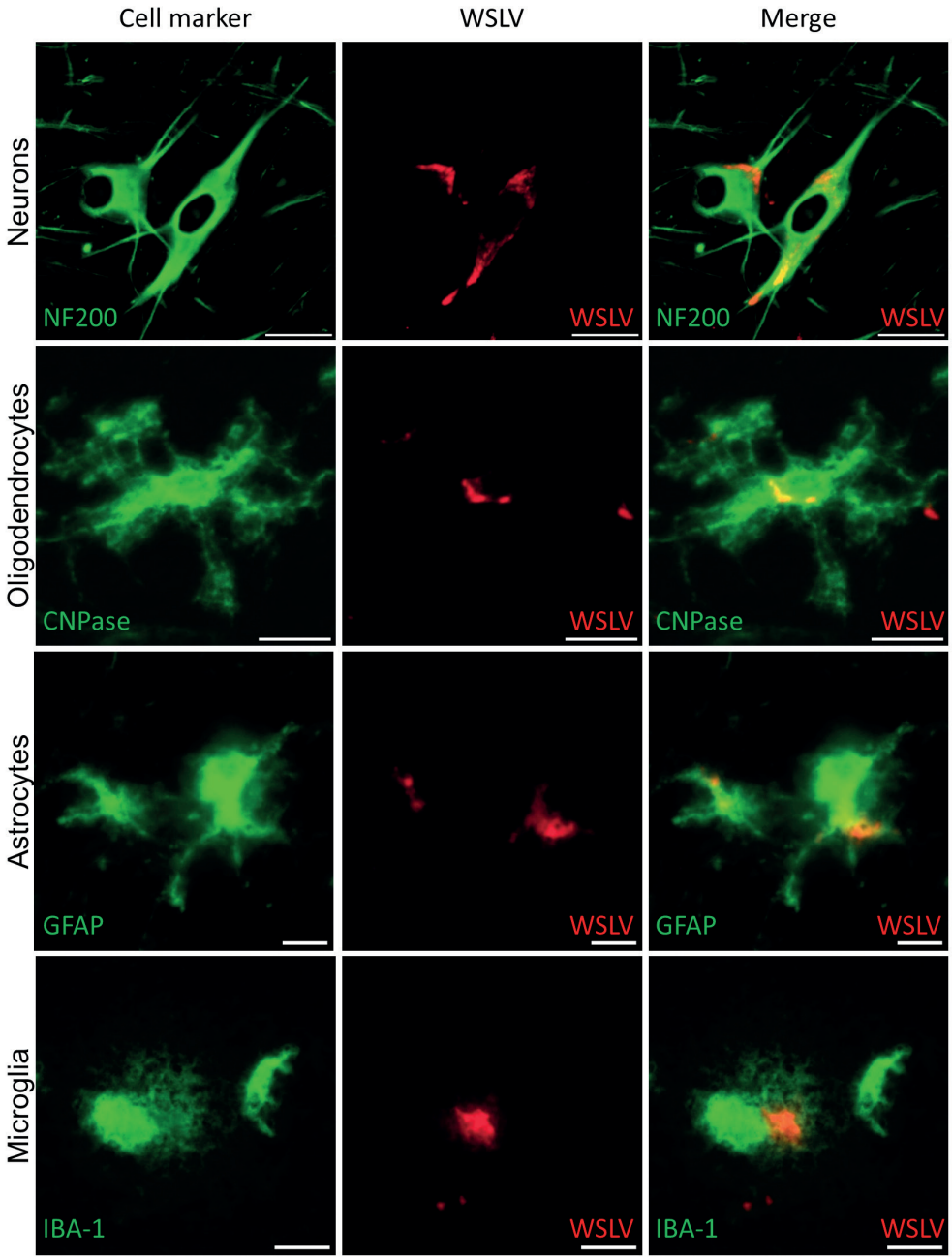


Figure 4.5. Identification of WSLV-infected foetal brain cells. Double immunofluorescent microscopy images of foetal brains to identify WSLV-infected cells. Cell markers are indicated in green, WSLV antigen in red. Neurons were visualised using anti-Neurofilament 200 (NF200), oligodendrocytes by anti CNPase, astrocytes using anti Glial Fibrillary Acidic Protein (GFAP) and microglia using anti IBA-1. Bars are 50 µm (neurons), 20µm (oligodendrocytes) or 10 µm (microglia and astrocytes).

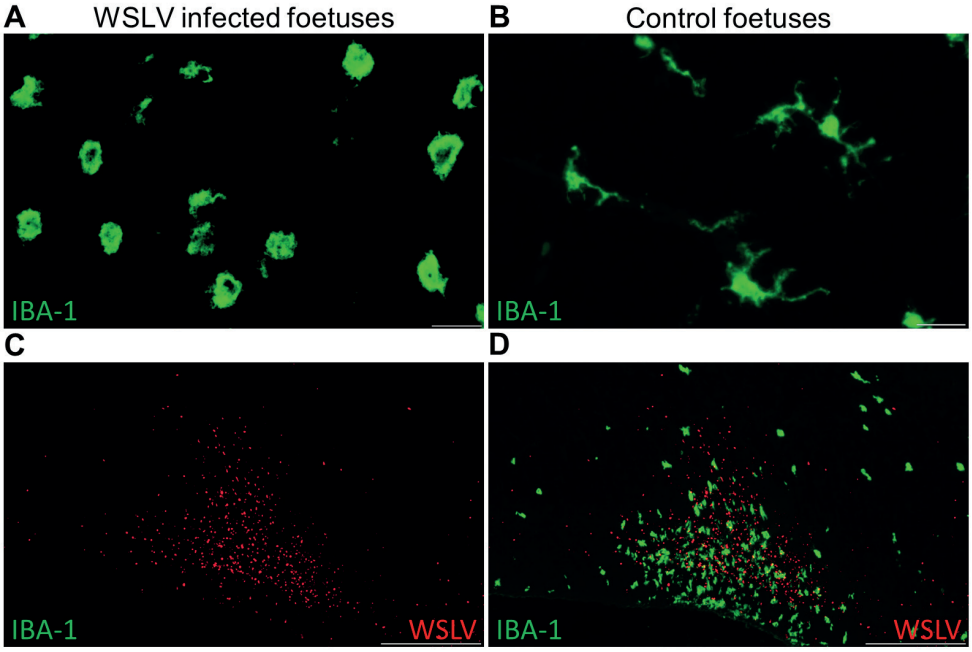


Figure 4.6. Activated microglia after WSLV infection. Specific staining of microglia (anti IBA-1) in the brain of WSLV-infected foetuses (A) and control foetuses (B). Note the morphological change of the microglia in the WSLV-infected brain from a resting ramified appearance to a more rounded shape. Foci of WSLV replication in the brain (C) were actively invaded by amoeboid microglia, leading to the formation of microglial nodules (D). Bars are 20 µm (A,B) or 500 µm (C,D).

No Evidence of WSLV Replication in Human Term Placental Explants

As WSLV is a zoonotic virus with the ability to transmit vertically in ruminants, we were interested in the ability of WSLV to infect human placental explants. To validate our protocol, ZIKV was taken along as a positive control, as it has been described in literature that ZIKV is able to infect term placental explants[241]. Term placental explants were incubated with WSLV, ZIKV or culture medium only, and samples were taken at 2, 4 and 6 days post inoculation. Although ZIKV was able to infect the placenta explants at all time points and showed slight growth over time, no evidence of WSLV infection was observed (Figure 4.7).

As WSLV showed to be neurotropic, we further studied its zoonotic potential by investigating the possible capability of WSLV to infect human brain cells. Three human glioblastoma cell lines and an astrocyte cell line were incubated with WSLV, after which the monolayers were fixed and stained for WSLV antigen (Figure S3.1). The glioblastoma cell lines were highly permissive for WSLV, however, infection of astrocytes appeared to be limited.

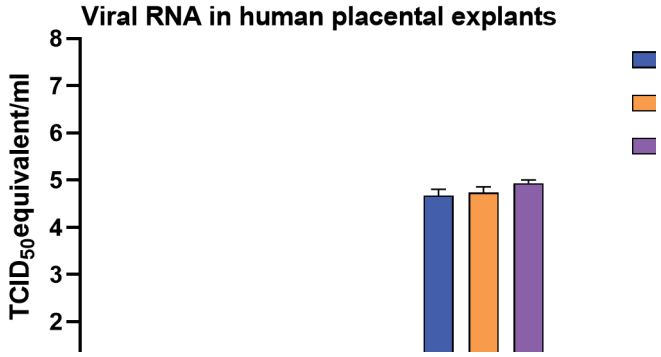


Figure 4.7. Susceptibility of human term placental explants for WSLV. Human term placentas were incubated with Zika virus (ZIKV), WSLV or culture medium only. At 2, 4 and 6 days post inoculation (dpi), samples were collected for RNA extraction (n = 4), after which viral RNA was measured by RT-qPCR.

Discussion

WSL disease was first described in 1956 by Weiss, Haig and Alexander[137]. These authors reported the association of WSLV infection with congenital malformations, stillbirths and abortions in sheep flocks and the isolation of the virus from the brain of a dead lamb. Although further observations from the field and from controlled animal experiments have confirmed the teratogenic potential of WSLV in ruminants, the pathogenesis of the disease has remained undescribed. The present study was performed to identify primary target cells and tissues with the aim to understand how WSLV crosses the ovine placental barrier and how the virus invades the foetal brain.

Inoculation of European-breed ewes at one-third of gestation did not result in febrile reactions or clinical signs within 8 days post inoculation, suggesting that WSLV infections in the field, at least in this breed of sheep, may remain unapparent until pregnancy is compromised. Moreover, at necropsy, no gross pathology in both the ewes and foetuses was observed. Despite the absence of macroscopic lesions or viral RNA in maternal liver and spleen samples at 8 days post inoculation, high levels of viral RNA were detected in placental samples. Immunohistochemical analysis revealed WSLV antigen in maternal epithelial cells, although staining was much more pronounced in foetal trophoblasts. The observation that areas of necrosis in the maternal epithelium were surrounded by infected foetal trophoblasts suggests that WSLV infects the maternal epithelium first, followed by infection of foetal trophoblasts. In addition to foetal trophoblasts lining the maternal epithelium, we found that WSLV infects trophoblasts in the haemophagous zones as well, representing a second route of vertical transmission. Remarkably, the presence of widespread, albeit focal, viral replication in the placenta was not associated with inflammation, and placentas appeared healthy.

Immunohistochemical analysis of the foetal organs revealed WSLV antigen in the liver and, sporadically, in foetal striated muscle fibres and osteoblasts. Although no cytopathic effect was observed, viral replication in these cells may compromise muscle and bone development. The most striking finding of this study was the strong staining of WSLV antigen throughout the foetal brain, including the cerebral cortex, cerebellum and brain stem, with antigen detection in neurons, glial cells and neural progenitor cells. Whereas WSLV antigen in microglia may have resulted from infection, staining of these cells may also represent phagocytosed virus. The latter is supported by our finding that microglia in WSLV-affected foetal brains had adopted the activated, amoeboid phenotype and were found in large numbers in proximity to WSLV-affected areas. Notably, our finding that not only neural progenitor cells but also differentiated cells are targeted by WSLV suggests that the foetal brain is also susceptible at later time points of gestation. The apparent absence of lesions could suggest that WSLV replicates in the foetal brain without causing significant pathology. However, considering the previous reports of WSLV-associated embryonic, foetal or neonatal death and teratogenic defects, we consider it unlikely that the foetuses of the present work would have developed normally, had pregnancy been allowed to continue.

Despite the severe consequences of infection during pregnancy and the widespread distribution of WSLV in Africa, the virus has not been associated with significant outbreaks in endemic areas. This is possibly explained by herd immunity resulting from exposure to the virus outside gestation and protection of newborns via maternally-derived antibodies. An introduction of WSLV into an immunologically naive population, particularly during the breeding season, could however result in rapid dissemination and unprecedented disease manifestation. Such "virgin soil" arbovirus outbreaks tend to be severe, as previously exemplified by the Bluetongue and Schmallenberg outbreaks in Europe and the West Nile virus (WNV) and ZIKV outbreaks in the Americas[82,242-244]. It is therefore important to further assess WSLV virulence for animals and humans and its ability to be disseminated by mosquito vectors indigenous to currently unaffected areas.

Materials and Methods

Viruses and Cells

WSLV, strain SAH177 passage 14 on Vero cells and originally isolated from a human in 1955 in South Africa[238], was kindly provided by prof. Janusz Paweska of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS), Sandringham, South Africa. ZIKV strain PRVABC59, originally isolated from human serum in Puerto Rico in 2015, was obtained from American Type Culture Collection (ATCC; VR-1843). Virus stocks were prepared by inoculating Vero-E6 cells at low multiplicity of infection (MOI; 0.01). Vero-E6 cells were obtained from ATCC (CRL-1586) and maintained in minimal essential medium (MEM; Gibco, Thermo Fischer Scientific, Breda, The Netherlands) supplemented with 5% foetal bovine serum (FBS; Gibco), 1% antibiotic/antimycotic (a/a;

Gibco), 1% nonessential amino acids (Gibco) and 1% L-glutamine (Gibco) (complete medium) at 37 °C with 5% CO_2. Virus stocks were titrated by incubating Vero E6 cells with serial dilutions of the virus for 6 days, after which cytopathic effect (CPE) was observed and titres were calculated using the Spearman–Kärber algorithm [218,219]. Both WSLV and ZIKV were passaged twice on Vero-E6 cells at our institute, after which they were used in experiments described below.

Sf9ET cells (ATCC® CRL-3357™) were cultured in Insect-XPRESS medium (Lonza, Maastricht, the Netherlands) supplemented with 1% a/a. High Five cells were maintained in Express Five medium (Gibco) supplemented with 1% glutamine and 1% a/a. Both insects' cell lines were cultured in suspension at 28 °C in a shaking incubator.

Experiments with Explants from Human Term Placentas

Term placentas (n = 2), obtained after caesarean section, were received from the Isala hospital (Zwolle, the Netherlands). Placentas were transported on ice, after which explants of 4 × 4 mm were cut from the chorionic villi of the placenta. Each explant was washed three times in PBS supplemented with 1% a/a and placed in a well in a 24-well plate with 1 mL complete medium (40% Dulbecco's modified Eagle medium (DMEM; Gibco), 40% F12 nutrient mixture (Gibco), 10% FBS and 1% a/a). Explants (n = 4) were subsequently inoculated with 2.5×10^5 $TCID_{50}$/mL ZIKV or WSLV. At 16 hours post infection, medium was refreshed, and at 2, 4 and 6 days post infection, explants were frozen at −80 °C until further processing.

Ethics Statement

The pregnant ewe trial was conducted in accordance with the Dutch Law on Animal Experiments (Wet op de Dierproeven, ID number BWBR0003081) and the European regulations on the protection of animals used for scientific purposes (EU directive 2010/63/EU). The procedures were approved by the animal ethics committee of Wageningen Bioveterinary Research (WBVR) and the Dutch Central Authority for Scientific Procedures on Animals (permit number AVD401002017894).

Human placentas were obtained after caesarean section of healthy women. Placentas are regarded as medical waste and therefore do not fall under the scientific medical research law of the Netherlands, and the experiments described in this manuscript do not require approval from an institutional review board. All donors have given written consent, and consent forms are stored in accordance with the Dutch privacy law.

Pregnant Ewe Trial

The pregnancies of 10 Texel–Swifter mix-breed ewes were synchronised by progesterone sponge treatment and natural mating at a conventional Dutch sheep farm. To confirm pregnancy, ultrasounds were performed at 6–7 weeks after mating. At day 54 of gestation, the ewes were transported to WBVR to acclimatise for 7 days until the start of the trial.

At 61 days of gestation, the ewes were inoculated intravenously with $10^{6.7}$ $TCID_{50}$ WSLV in 1 mL medium or with 1 mL medium (negative control animals). During the whole study, animals were monitored for clinical signs twice a day, and rectal body temperatures were measured once per day. EDTA blood samples were collected daily from the day of inoculation. At 8 days post inoculation, the ewes were euthanised by intravenous administration of 50 mg/kg sodium pentobarbital (Euthasol®, ASTfarma, Oudewater, Netherlands) and subsequent exsanguination. Foetuses were exsanguinated as well by cutting the umbilical cord, and foetal blood was collected in EDTA tubes. At the necropsy of each ewe, samples were taken from the liver, spleen, iliac lymph node and inguinal lymph node. From the foetuses, samples were taken from the liver, brain and leg muscle/bone marrow of the femur. Samples from three placentomes and the intercotelydonary membrane were taken per placenta, and samples were taken from the umbilical cord and amniotic fluid. All samples were collected in duplicate, one sample was placed on dry ice and stored at −80 °C, while the other sample was fixed in 10% neutral buffered formalin for 48 h for histology and immunohistochemistry. Formalin-fixed samples were processed routinely into paraffin blocks.

Detection of Viral RNA

Organ samples from the pregnant ewe trial were homogenised by mixing 0.3–1 g of tissue with 7 mL complete medium in IKA Ultra Turrax DT-20 tubes. Cell debris was removed by centrifugation for 15 min at 4952 x g in 15 mL Falcon tubes. Organ suspension or plasma (500 µL) was added to 2.5 mL NucliSENS easyMAG Lysis Buffer (Biomérieux, Marcy-l'Étoile, France), after which RNA was extracted using the NucliSENS easyMAG (Biomérieux) according to manufacturer's protocol.

To extract RNA from the human placental explants, the explants were lysed in Lysing Matric D tubes (MP Biomedicals) in 1 mL TRIzol Reagent (Invitrogen). Homogenised suspensions were made using the TeSeE Precess 24 bead beater for 2 × 23 s at 6500 RPM. RNA was isolated from 350 µL suspension using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's protocol.

Primers and probes were designed using the PrimerQuest Tool for Integrated DNA technologies (IDT, Leuven, Belgium) that target the NS5 protein of WSLV. Primers and probes with the following sequences were purchased from IDT: forward primer: 5'-GGA CCA TGA AAG TGT TGG-3', reverse primer: 5'-CAA TCA CAT CTG GAT AGG-3'; Probe: 5'-6FAM- TGA ACG ATG GAA ACA CGT GAA CAC AGA TMR-3'. ZIKV primers and probes were used as described by Lanciotti et al. [245]. Five µL of the RNA was used in a RT-qPCR using the LightCycler RNA Amplification Kit HybProbe (Roche, Almere, the Netherlands). Cycling conditions were as follows: 45 °C for 30 min, 95 °C for 5 min, 45 cycles of 5 s at 95 °C and 35 s at 57 °C, followed by cooling down to 30 °C.

WSLV and ZIKV standards for quantification were made by isolating RNA of a 100× diluted virus stock. Virus was lysed in Trizol, and RNA was isolated using the Direct-zol RNA miniprep kit (Zymo Research) according to the manufacturer's protocol. A 10x dilution series was made of the isolated RNA in H_2O, and the dilution series was aliquoted for PCR runs. As the $TCID_{50}$ titre of the virus stock was known, this standard was used to quantify the RNA as $TCID_{50}$ equivalent/mL.

Production of Polyclonal Rabbit Sera against WSLV NS1

The sequence of the NS1 protein of WSLV, NCBI GenBank number: JN226796.1, was used to develop a DNA construct, encoding this gene, flanked by a N-terminal GP64 signal sequence and a C-terminal twin Strep-Tag. The resulting construct was synthesised by GenScript (Piscataway, NJ, USA). The gene with flanking sequences was subsequently cloned into a pBAC-3 baculovirus vector, after which a recombinant baculovirus was produced using the flashBAC ULTRA baculovirus expression system (Oxford Expression Technologies, Oxford, UK). Transfection mixtures containing the pBAC-3 vector, a bacmid and celfectin II were added to wells of a 6-well plate, each containing 1,000,000 SF-9 ET cells. After successful rescue of the baculovirus, stocks were prepared by inoculating SF-9 ET suspension cultures at low MOI (<0.1). The NS1 protein was produced by infecting High Five cells at high MOI (>5) according to manufacturer's protocol (Thermoscientific, Rockferd, IL, USA). Strep-Tactin resin (IBA) columns were used to purify the proteins from the supernatants, after which buffers were exchanged to Tris-buffered saline in Amicon Ultra centrifugal filters (Merck). Purity of the NS1 protein was assessed in a 4–12% SDS gel stained with GelCode Blue stain reagent (Thermoscientific).

Polyclonal rabbit sera against WSLV NS1 were produced by GenScript (Piscataway, NJ, USA) by immunizing two New Zealand rabbits with 1 mg of purified NS1 following their standard protocol for polyclonal antibody production.

Histology and Immunohistochemistry

Paraffin-embedded tissues were cut into 4 µm sections, collected on silane-coated glass slides and dried for at least 48 h at 37 °C. After deparaffinisation and rehydration in graded alcohols, sections were either stained routinely with haematoxylin and eosin (HE) or immunostained. For immunostaining, endogenous peroxidase was blocked for 30 min in methanol/H_2O_2, followed by antigen retrieval by autoclaving the slides in pH 6 citrate buffer (Antigen unmasking solution, Vector Laboratories) for 15 min. The WSLV NS1 polyclonal antiserum that was produced as described above was used at a dilution of 1:1000 to detect WSLV NS1. Antibodies to cell markers were used to visualize neurons (anti-Neurofilament 200, Sigma-Aldrich, St. Louis, MO, USA), astrocytes (anti Glial Fibrillary Acidic Protein, Sigma-Aldrich), oligodendrocytes (anti CNPase, Sigma Aldrich) and microglia (anti IBA-1, ITK Diagnostics, Uithoorn, Netherlands). Horseradish peroxidase (HRP; Invitrogen, Carlsbad, CA, USA) or Alkaline phosphatase (AP; Vector laboratories, Peterborough, UK) conjugated anti-mouse or anti-rabbit polymers were

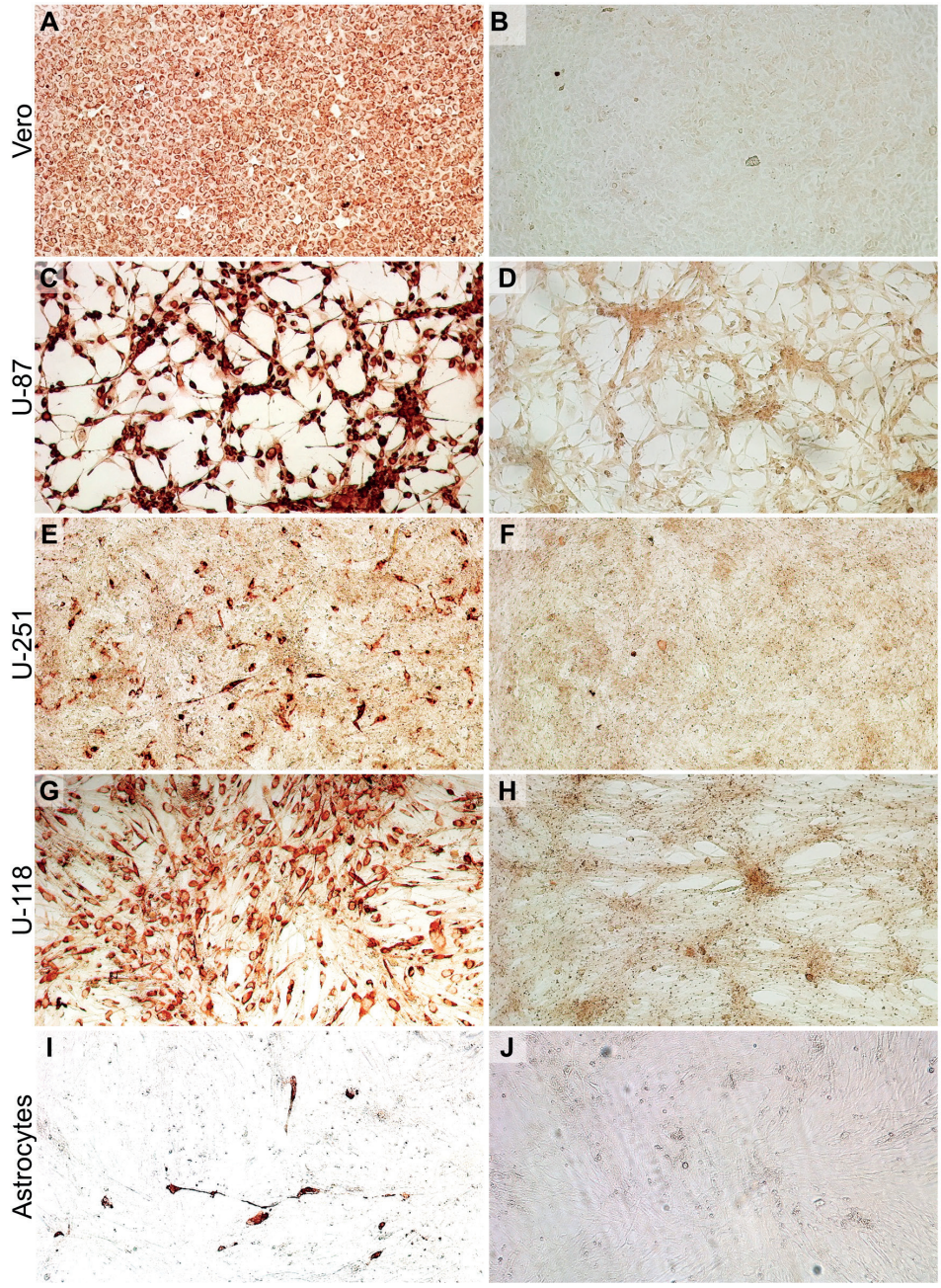
used as secondary antibodies, followed by incubation with NovaRed or VectorRed substrate respectively (Vector Laboratories). Sections were briefly counterstained with haematoxylin, dehydrated and mounted permanently. For immunofluorescent double staining, the HRP substrate was replaced by Alexa Fluor 488 or 546 tyramide reagent (Invitrogen) and sections were mounted in antifading mounting medium containing DAPI (Vector laboratories). Sections were photographed with an Olympus BX51 (fluorescence) microscope equipped with Cell D/Cell Sense software and a high-resolution digital camera. Overview images were constructed with the multiple image alignment tool of the Cell D software, and immunostaining was coloured red using the phase colour coding tool. Monochromatic digital photographs for immunofluorescence were false coloured in green for the Alex Fluor 488 dye and in red for the Alexa Fluor 546 dye.

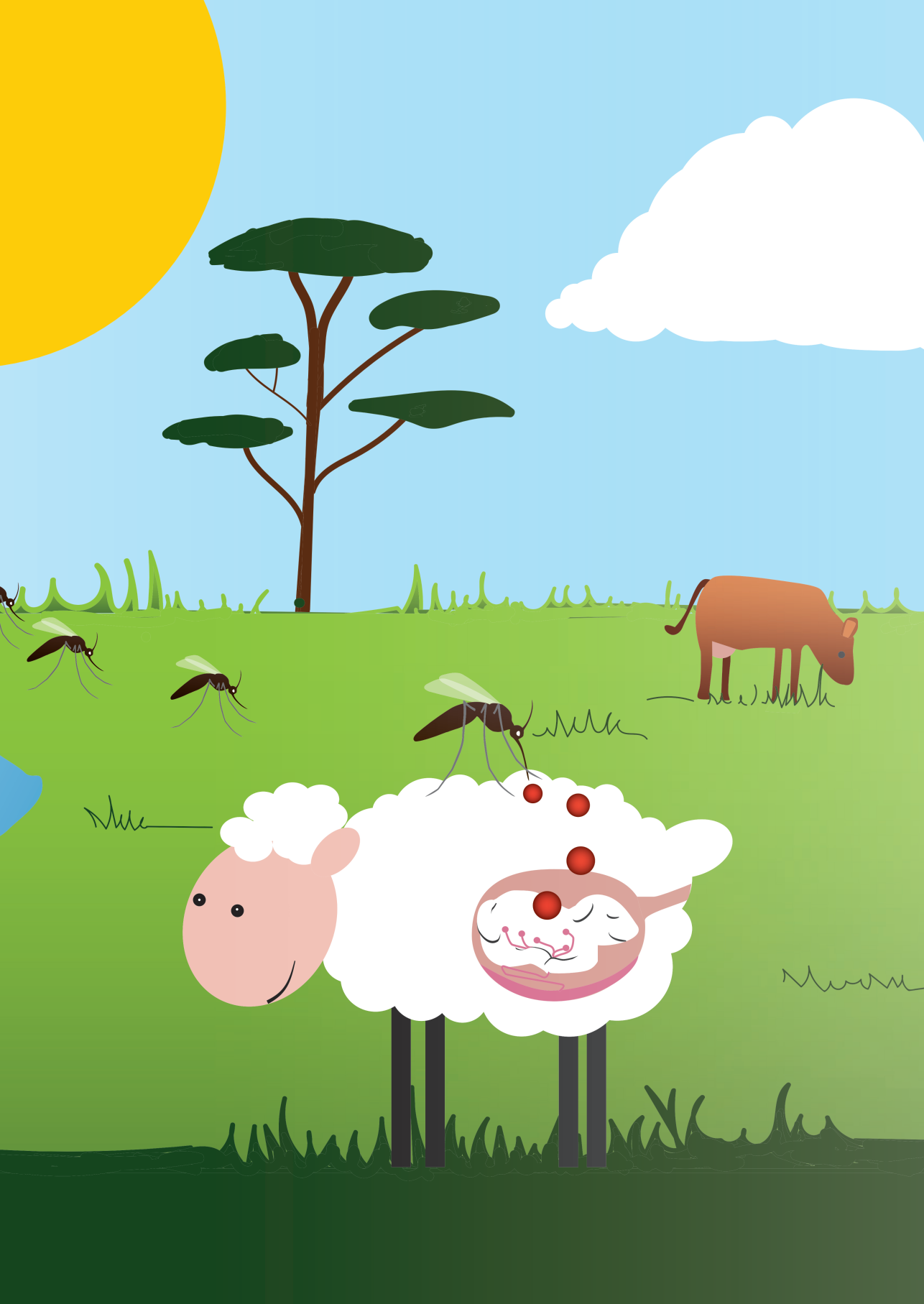
Acknowledgements

We thank Pieter Roskam and Corry Dolstra (Wageningen Bioveterinary Research) for assisting with the necropsies and histology, respectively. We thank prof. Janusz Paweska from the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) for providing us with WSLV.

Figure S4.1. (opposite) WSLV infection in human brain cell lines. WSLV and mock infection of Vero cells (A,B), human glioma cells (U-87, C and D; U251, E and F; U118, G and H) and human astrocytes (I and J). All cells were infected with a MOI of 0.1 (based on WSLV titre determined on Vero cells) and incubated for 3 days. An immunoperoxidase monolayer assay (IPMA) was subsequently performed to visualise infected cells. Briefly, cells were fixed and permeabilised at 3 dpi with 4% paraformaldehyde (10 min) and ice-cold methanol (10 min). After permeabilisation, the plates were incubated with rabbit polyclonal antibodies recognizing WSLV NS1, followed by incubation with polyclonal rabbit-α-mouse immunoglobin/HRP antibody (Dako, Denmark) as secondary antibody and 3-Amino-9-ethylcarbazole (AEC; Sigma-Aldrich) as substrate.

Supporting information





Chapter 5

Shuni virus replicates at the maternal-foetal interface of the ovine and human placenta

Judith Oymans, Lucien van Keulen, Guus M. Vermeulen, Paul J. Wichgers Schreur, Jeroen Kortekaas

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Abstract

Shuni virus (SHUV) is a neglected teratogenic and neurotropic orthobunyavirus that was discovered in the 1960s in Nigeria and was subsequently detected in South Africa, Zimbabwe and Israel. The virus was isolated from field-collected biting midges and mosquitoes and shown to disseminate efficiently in laboratory-reared biting midges, suggesting that members of the families *Culicidae* and *Ceratopogonidae* may function as vectors. SHUV infections have been associated with severe neurological disease in horses, a variety of wildlife species and domesticated ruminants. SHUV infection of ruminants is additionally associated with abortion, stillbirth and congenital malformations. The detection of antibodies in human sera also suggests that the virus may have zoonotic potential. To understand how SHUV crosses the ruminant placenta, we here infected pregnant ewes and subsequently performed detailed clinical- and histopathological examination of placental tissue. We found that SHUV targets both maternal epithelial cells and foetal trophoblasts that together form the maternal-foetal interface of the ovine placenta. Experiments with human placental explants furthermore revealed replication of SHUV in syncytiotrophoblasts, which are generally highly resistant to virus infections. Our findings provide novel insights into vertical transmission of SHUV in sheep and call for research on the potential risk of SHUV infection during human pregnancies.

Introduction

SHUV is an orthobunyavirus of the family *Peribunyaviridae* that was first discovered in 1966 in Ibadan, Nigeria, where it was isolated from the serum of an adult cow and a febrile child [244,245]. In subsequent years, SHUV was isolated from cattle, goats and horses in South Africa and Zimbabwe. In 2014, SHUV emerged in Israel, representing its first detection outside the African continent [112]. SHUV was isolated from various field-collected *Culicoides* biting midges and *Culex theileri* mosquitoes, suggesting that both midges and mosquitoes may act as vectors [113,114]. A recent study has demonstrated efficient dissemination of SHUV in laboratory reared *Culicoides nubeculosus and Culicoides sonorensis* biting midges, but not in laboratory-reared *Culex pipiens* and *Aedes aegypti* mosquitoes [116].

SHUV affects a wide variety of vertebrate host species. The virus infects domesticated ruminants, horses and a wide variety of wildlife species such as rhinos, crocodiles and giraffes [110,117,246]. In horses, cattle and wildlife, SHUV causes severe neurological disease manifesting with tremors and ataxia [110,117,121]. In domesticated ruminants, SHUV is capable of crossing the placental barrier, resulting in congenital malformations, stillbirth or abortion [111]. Importantly, SHUV-specific antibodies were detected in 5 (3.9%) of 123 veterinarians from South Africa in a serological survey, warranting further research into its zoonotic potential [119].

Congenital malformations, stillbirths and abortions of ruminant livestock are characteristic outcomes of infections caused by orthobunyaviruses of the Simbu serogroup. The most widespread orthobunyavirus of the Simbu serogroup affecting ruminants is Akabane virus (AKAV), which is endemic to Africa, the Middle East, Asia and Australia [247,248]. Additional Simbu serogroup orthobunyaviruses of veterinary importance include Schmallenberg virus (SBV), Aino virus, Peaton virus and Shamonda virus [249-252]. Examples of orthobunyaviruses pathogenic to humans include La Crosse virus, a major cause of encephalitis in children in the US, Oropouche virus, which generally causes a febrile illness, and Ngari virus, associated with haemorrhagic fever [253-255]. Orthobunyaviruses infecting humans have not been associated with congenital malformations.

Our understanding of vertical transmission of teratogenic arboviruses remains rudimentary. Improved understanding of the mechanisms involved in vertical transmission of arboviruses in both animals and humans may facilitate prioritization of arbovirus research and the development of countermeasures. To further our understanding of vertical transmission of SHUV, we experimentally inoculated pregnant ewes at one third of gestation and identified maternal epithelial cells and foetal trophoblasts as target cells of SHUV. Moreover, as SHUV is the only known teratogenic orthobunyavirus with zoonotic potential, we examined the interaction of SHUV with human placental explants. The results of these experiments demonstrate that SHUV replicates efficiently in human

syncytiotrophoblasts, the placental cells that form the primary barrier between mother and foetus.

Results and Discussion

Vertical transmission of SHUV in pregnant ewes

To study the interaction of SHUV with the ovine placenta early after exposure, 4 pregnant ewes were inoculated intravenously with a dose of $10^{5.6}$ $TCID_{50}$ at 48 days of gestation (Figure 5.1A). No increases in rectal temperatures were measured after inoculation in any of the ewes (Figure 5.1B). The duration and levels of viremia varied significantly among animals. No viremia was measured in sheep number #198, whereas in sheep #195 and #196 low levels of viral RNA were detected. In animal #197, higher RNA levels were measured (Figure 5.1C).

When ewes were necropsied at 7 dpi, all foetuses were alive without visible pathological changes. No abnormalities were noted to the organs of both ewes and foetuses. Analysis by PCR revealed high levels of viral RNA in the spleen and lymph nodes of one ewe, #197 (Figure 5.1D). No viral RNA was detected in the liver of this animal. High levels of viral RNA were also detected in all 3 of the tested placentomes from each placenta (n=2) of ewe #197 (Figure 5.1E). Low amounts of viral RNA were detected in 2 out of 3 placentomes from the single foetus of ewe #196. In the other two animals no evidence of virus replication was observed. Virus was isolated from plasma collected on day 5 from ewe #197 and from one placentome of the placenta of foetus F2. Viral RNA was detected in the liver of one of the two foetuses of ewe #197 (Figure 5.1F), confirming that SHUV crossed the placental barrier.

In order to identify the primary target cells of SHUV in the ovine placenta, tissue sections were analysed with IHC. Whereas no lesions were detected in the studied placentomes (Figure 5.1G), small foci of SHUV-positive maternal epithelial cells were detected throughout the placentomes, with occasional detection of infected trophoblasts (Figure 5.1H). Interestingly, inoculation of pregnant ewes with AKAV at gd 32, revealed infection of trophoblasts at 5 dpi, whereas infected maternal epithelial cells were not detected [256].

The absence of viremia in one ewe, the low-level viremia in two ewes, and the relatively late onset of viremia in ewe #197, may suggest that the SHUV isolate used in the present work, being originally isolated from a cow, is not optimally adapted to sheep. The virus that was successfully isolated from the plasma of sheep #197 may therefore be more suitable for future experimental inoculation of the same species.

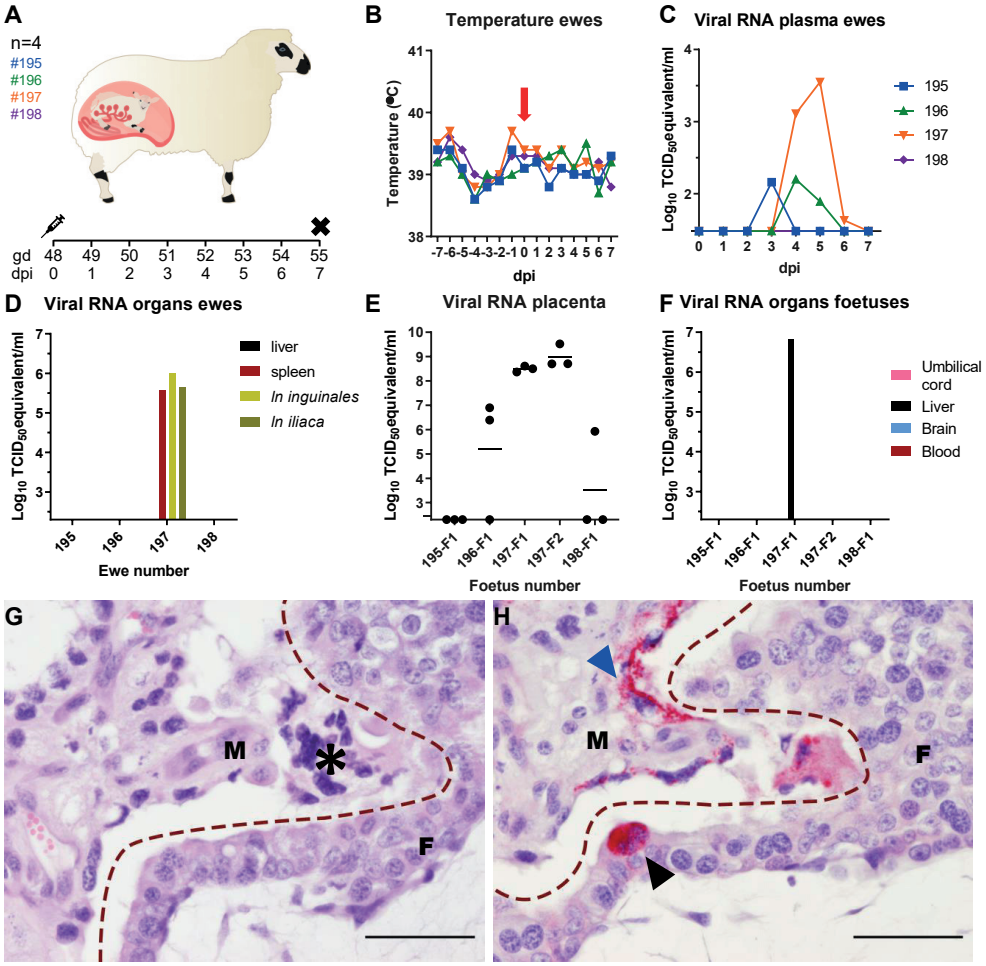


Figure 5.1. Clinical and laboratory findings following infection of pregnant ewes with SHUV. (A) Experimental set-up of the pregnant ewe trial. Ewes were inoculated at gestation day (gd) 48. EDTA blood samples were collected and rectal temperatures measured daily. At 7 dpi the ewes were euthanized and tissue samples collected. (B) Rectal temperatures. The moment of inoculation is depicted by the red arrow. (C) Viral RNA in plasma of the ewes and (D) viral RNA in maternal organs. Ln: lymph node (E) Viral RNA detected in three placentomes per placenta. Horizontal bars represent means. (F) Viral RNA detected in foetal organs. (G) H&E staining of a placentome from ewe #197, showing the boundary between a maternal villus (M) and foetal villus (F). Notice the syncytial cell in the maternal epithelium (asterisk). (H) Serial section showing the same area staining for SHUV antigen (visualized by VectorRed). SHUV-positive maternal epithelium is indicted by a blue arrowhead. A single SHUV-positive binucleate trophoblast is indicated by a black arrowhead. Dashed lines mark the placental barrier separating maternal (M) and foetal (F) villi. Bars: 50 µm.

SHUV targets human trophoblasts

To investigate if SHUV can infect cells of the human placenta, a human trophoblast cell line (HTR-8) and a human endothelial cell line, derived from an umbilical vein (HUVEC cells), were incubated with the virus. Vero E6 cells and an ovine placental cell line were taken along as positive controls. SHUV efficiently replicates in all these cell lines (Figure 5.2A).

After demonstrating that SHUV can replicate in both HTR-8 and HUVEC cells, explants from human term placentas, obtained from healthy donors after caesarean section, were incubated with SHUV (Figure 5.2B). On days 1, 2, 4, and 6 dpi, placental explants were used for RNA isolation (n=4 per placenta). At the same timepoints, supernatants were also collected for virus titration. RT-PCR on RNA extracted from the explants revealed that after removal of the inoculum, $10^{4.5}$ $TCID_{50}$ equivalents/ml viral RNA was detected (Figure 5.2C). Viral RNA levels did not increase before 2 dpi, however a 2 log_{10} increase was observed between days 2 and 4, which was maintained until day 6. Titration of the supernatants revealed a steady increase of infectious SHUV from day 1 onwards to reach a final titre of 10^6 $TCID_{50}$/ml (Figure 5.2D). These results demonstrate that human placental cells are susceptible and permissive to SHUV.

Immunohistochemical analysis of the human placental explants subsequently revealed SHUV antigen distributed throughout the placental explants. SHUV-specific staining was restricted to syncytiotrophoblasts surrounding the foetal villi, inferring that SHUV can infect this cell type (Figure 5.2E,F). The finding that human syncytiotrophoblasts are susceptible and permissive to SHUV replication is notable, as these cells form the only continuous barrier between the maternal and foetal circulation and are generally highly resistant to virus infection.

In conclusion, our results demonstrate the ability of SHUV to replicate in human placental trophoblasts. Although evidence of vertical transmission in humans is currently lacking, our findings call for increased awareness of SHUV infections in humans.

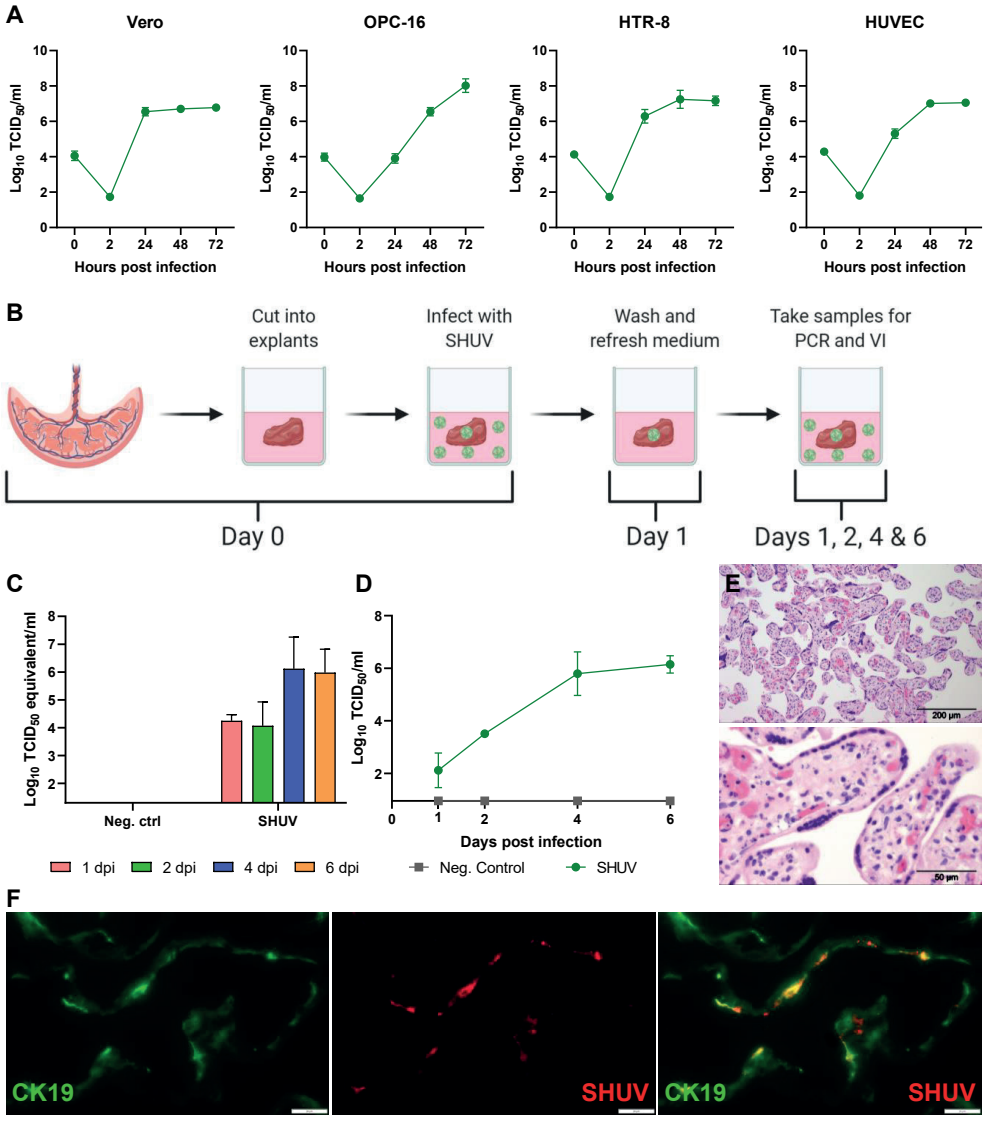


Figure 5.2. Replication of SHUV in human placental cell lines and explants. (A) Growth kinetics of SHUV in Vero cells, ovine placental cells (OPC-16), human trophoblast cells (HTR-8) and human umbilical vein endothelial cells (HUVEC). Cells were incubated with a MOI of 0.01 and samples were taken at 0, 2, 24, 48 and 72 hours post infection. (B) Procedure used to evaluate replication of SHUV in human placental explants. On day 0, explants (n=4 per timepoint) were incubated with SHUV. At 1 day post infection, medium was removed and explants were washed 3 times with PBS. Explants and superantant samples collected at 1, 2, 4 and 6 dpi were used for RNA isolation and virus titrations. (C) Viral RNA detected in organ suspensions of placental explants. (D) Virus titers detected in pooled supernatant samples collected during two separate experiments. (E) H&E staining of normal human placental explants at 4 dpi showing the foetal villi. (F) Detection of SHUV-infected syncytiotrophoblasts by immunofluorescence at 4 dpi. Syncytiotrophoblasts were visualised using an antibody specific for cytokeratin-19 (CK19). SHUV antigen was detected using an antibody recognizing the SHUV Gc ectodomain. White bars: 20 µm. Data points are means with SD.

Materials and Methods

Viruses and Cells

Culture media and supplements were obtained from Gibco (Thermo Fisher Scientific, Breda, the Netherlands) unless stated otherwise. SHUV strain Iban101007 was obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) through the University of Texas Medical Branch. This strain was originally isolated from serum of a cow in a slaughterhouse [244]. The virus was passaged twice in Vero E6 cells before use in the described experiments. Virus titre was determined by end-point dilution assay using Vero E6 cells and subsequent staining with a SHUV-specific antibody (SHUV-Gc_{head}; [257]) and calculated using the Spearman-Kärber algorithm [218,219].

Vero E6 cells, obtained from the American Type Culture Collection (ATCC), were maintained in minimal essential medium (MEM) supplemented with 5% foetal bovine serum (FBS), and 1% antibiotic/antimycotic (a/a), 1% glutamine and 1% Minimal Essential Medium Non-Essential Amino Acids (MEM NEAA). Human umbilical vein endothelial (HUVEC) cells (ATCC® CRL-1730™) were maintained in Ham's F-12K (Kaighn's) medium supplemented with 10% FBS, 0.03 mg/ml Endothelial Cell Growth Supplement (ECGS) and 0.002% heparin. Human trophoblasts cells (HTR-8) cells (ATCC® CRL-3271™) were maintained in RPMI medium supplemented with 5% FBS and 1% a/a. All cells were cultured at 37°C with 5% CO_2.

To develop immortalised ovine placental cells (OPC), the foetal and maternal parts of a placentome were separated. The maternal part (caruncle) was attached to a sterile cotton and subsequently submerged in 90% DMEM/Ham's F12 medium supplemented with 200 U/ml collagenase I and 10% Hank's Salt solution (Biochrom, Berlin, Germany). After 1 h, the tissue was discarded and the cell suspension was cultured in DMEM/Ham' F12 medium supplemented with 10% FBS and 1% a/a. After 2 passages, a heterogenous cell suspension was sent to InSCREENeX GmbH, Braunschweig, Germany to immortalise single cells. Fifteen clones were provided and cultured in DMEM/Ham's F12 medium supplemented with 10% FBS and 1% a/a. Clone #16 (OPC-16) showed best growth kinetics, therefore this cell line was selected for the present work.

Titrations were performed by end-point dilution assay on Vero cell monolayers in 96 well plates, followed by immunostaining of SHUV antigen using a rabbit polyclonal antiserum [257]. Titres were determined using the Spearman-Kärber algorithm and expressed as $TCID_{50}$/ml.

Ethics statement

The pregnant ewe trial was conducted in accordance with the Dutch Law on Animal Experiments (Wet op de Dierproeven, ID number BWBR0003081) and the European regulations on the protection of animals used for scientific purposes (EU directive 2010/63/

EU). The procedures were approved by the animal ethics committee of Wageningen Bioveterinary Research (WBVR) and the Dutch Central Authority for Scientific Procedures on Animals (permit number AVD401002017894).

Human placentas were obtained from healthy women after caesarean section. Placentas are regarded as medical waste and therefore do not fall under the scientific medical research law of the Netherlands. The experiments did not require approval from an institutional review board. All donors have given written consent, and consent forms are stored in accordance with the Dutch privacy law.

Pregnant ewe trial

At a conventional Dutch sheep farm the pregnancies of Texel-Swifter mix-breed ewes were synchronised by progesterone sponge treatment after which natural mating occurred. Ultrasounds were performed to confirm pregnancy after which 4 ewes were transported to WBVR on day 41 of gestation. The ewes were allowed to acclimatize for 7 days and the ewes were confirmed negative for SBV antibodies as this virus is closely related to SHUV and might interfere with the experiment. At day 48 of gestation, the ewes were inoculated intravenously with $10^{5.6}$ $TCID_{50}$ SHUV in 1 ml culture medium. Animals were closely monitored for clinical signs twice per day. Rectal body temperatures were recorded and EDTA blood samples were collected daily. At 7 days post inoculation, the ewes were euthanized by intravenous inoculation of 50 mg/kg sodium pentobarbitol (Euthasol, ASTgarma, Oudewater, the Netherlands) and subsequent exsanguination of both the ewe and the foetuses. From the ewes, samples were taken from the liver, spleen, and the lymph nodes (ln) that drain the placenta, namely *ln iliaca* and *ln inguinales*. From each placenta, three placentomes were collected. From the foetuses samples were taken from the liver, brain, umbilical cord and foetal blood. All samples were collected in duplicate. One organ sample was stored at -80°C for RNA extraction and virus isolation and one sample was stored in 10% neutral buffered formalin for 48h for histology and immunohistochemistry (IHC).

Infection of human cell lines: monolayers of Vero E6, HUVEC, HTR-8 or OPC-16 cells, were incubated with SHUV at a multiplicity of infection (MOI) of 0.01 in triplicate. Two hours post infection, medium was removed and cells were washed twice with PBS before adding fresh medium. Samples were collected at 0, 2, 24, 48 and 72 hpi.

For isolation of SHUV from serum, 1 ml of serum was added to 40 ml culture medium in a T150 flask containing a monolayer of $4{,}5x10^6$ Vero E6 cells, seeded 6 hrs earlier. Isolation of SHUV from placentomes was performed by the same protocol using 1 ml of a 1% placentome suspension. Inocula were maintained with the cells until cytopathogenic effect was observed at 4 days post infection (dpi), followed by collection of supernatant samples. Titers in supernatant samples were determined by end-point dilution assay as described above.

Experimental infection of human term placentas

Healthy human term placentas were obtained after caesarean section from the Isala hospital in Zwolle, the Netherlands, and processed into explants as described [222]. Explants were either incubated with $2x10^4$ $TCID_{50}$/ml SHUV or medium (mock). The placental explants were maintained at 37°C and 5% CO_2 under continuous shaking for maximum perfusion of the explant. At 1 dpi, the medium was removed, and explants washed three times with PBS before fresh medium was added. At 3 dpi, 1 ml fresh medium was added to each well to add fresh nutrients. At 1, 2, 4 and 6 dpi, 4 individual explants were collected in 1 ml Trizol (Invitrogen). On the same days, supernatant samples were collected and pooled for virus titration as described above. Samples were stored at -80°C until analysis. Furthermore, at each time point, four individual explants were fixed with 10% neutral buffered formalin for IHC. Each experiment was performed in quadruplicate and repeated with two different placentas.

Detection of viral RNA

RNA extractions of organs from the pregnant ewe trial were performed by homogenising 0.7 g of tissue in an IKA Ultra Turrax Tube DT-20 containing 7 ml of complete Vero medium. Cell debris was subsequently removed by centrifugation and 200 µl organ suspension or plasma was added to 50 µl Proteinase K (5 µg/ml, Sigma) after which 200 µl AL buffer (Qiagen) supplemented with 2 µl polyadenylic acid A (5 mg/ml, Sigma) was added. After thorough mixing, samples were incubated at 56°C for 15 min. After lysis, RNA was extracted using the Qiagen RNeasy kit according to the manufacturer's protocol.

Homogenisation of placental explants occurred in Lysing Matric D tubes (MP Biomedicals) in 1 ml Trizol using the TeSeE™ Precess 24 bead beater for 2x23 s at 6500 RPM. RNA was isolated form 350 µl supernatant using the Direct-zol RNA miniprep kit (Zymo Research) according to manufacturer's protocol.

The Lightcycler RNA Amplification kit HybProbe (Roche, Almere, the Netherlands) was used for RT-qPCRs with 5 µl of the RNA, employing the following cycling conditions: 45˚C for 30 min, 95˚C for 5 min, 45 cycles of 5 s at 95˚C and 35 s at 57˚C, followed by cooling down to 30˚C [222]. Primers and probes, targeting the SHUV S segment, were purchased from Integrated DNA technologies (IDT). Forward primer: GAAGGCCAAGATGGTACT, reverse primer: ACAGGATTTGCTGTGTATTG and probe: FAM- AGTAAGACGGCACAACCGAGTGTT-BHQ1.

A SHUV standard for quantification of RNA as $TCID_{50}$ equivalent/ml was made by isolating RNA of our virus stock with a known titre (Figure S5.1), using the method that has been described earlier for Zika and Wesselsbron virus [258].

Histology and immunohistochemistry

Formalin-embedded tissues were routinely processed into paraffin blocks, cut into 4 µm sections, collected on silane-coated glass slides and dried for at least 48 h at 37 °C.

After deparaffinisation and rehydration in graded alcohols, sections were stained with hematoxylin-eosin (HE) or immunostained. For immunostaining, endogenous peroxidase was blocked for 30 min in methanol/H_2O_2, followed by antigen retrieval by treating the cells with 0.2% trypsin in TBS for 30 min at 37°C. The SHUV polyclonal antiserum was used at a dilution of 1:250 and incubated for 1 h at 37°C. Alkaline phosphatase (AP; Vector laboratories, Peterborough, UK)-conjugated anti-rabbit IgG polymer was used as secondary antibody, which was incubated with the cells for 30 min at 37°C. This was followed by incubation with ImmPACT VectorRed substrate (Vector Laboratories) for 20 minutes at RT. Sections were briefly counterstained with hematoxylin, dehydrated and mounted permanently.

For the detection of cytokeratin 19, a marker of human trophoblasts, epitope retrieval was performed by 15 min of autoclaving at 121˚C in citrate buffer of pH 6 (Antigen unmasking solution, Vector Laboratories), followed by incubation for 1h at 37°C with a rabbit monoclonal antibody to cytokeratin 19 (Abcam 52625, USA) at a dilution of 1:400. The next steps were performed as described in the previous paragraph.

For detection of SHUV and cytokeratin 19 by immunofluorescence, a two-step immunostaining was performed to avoid interference between the different procedures. First, sections were incubated with the SHUV antiserum, then autoclaved for 15 min at 121˚C in citrate pH6 to destroy the antibodies/ enzymes of the first staining and to retrieve the epitopes for the second immunostaining for cytokeratin 19. Anti-rabbit horseradish peroxidase-conjugated polymer was used as secondary antibody (Invitrogen, Carlsbad, CA, USA) followed by incubation with Alexa Fluor 488 or 546 tyramide reagent (Invitrogen) and sections were mounted in antifading mounting medium containing DAPI (Vector laboratories).

Sections were photographed with an Olympus BX51 (fluorescence) microscope equipped with Cell D/Cell Sense software and a high-resolution digital camera. Monochromatic digital photographs for immunofluorescence were false colored in green for the Alex Fluor 488 dye and in red for the Alexa Fluor 546 dye.

Acknowledgements

We thank Prof. Dr. Scott Weaver from the University of Texas Medical Branch for providing SHUV strain Iban101007, from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). We thank Pieter Roskam for assisting with the necropsies and Corry Dolstra and Sebastiaan van den Broek (WBVR) for their assistance with histology. The ovine placental cells (OPC-16) were immortalized by InSCREENeX (Kristina Nehlsen) within the VetBioNet program of the European Union's Horizon 2020 research and innovation programme under grant agreement No 731014, led by Norbert Stockhofe at WBVR. Cartoons were created with BioRender.com.

Supporting information

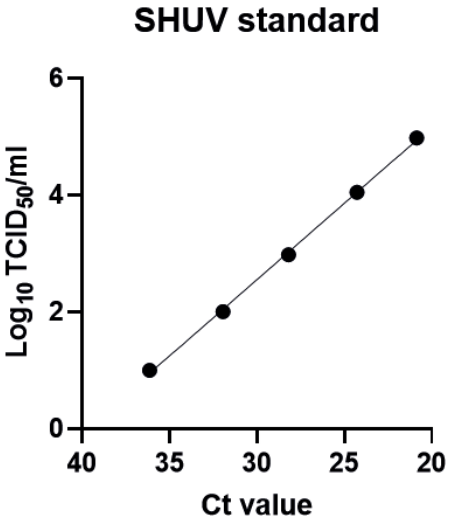
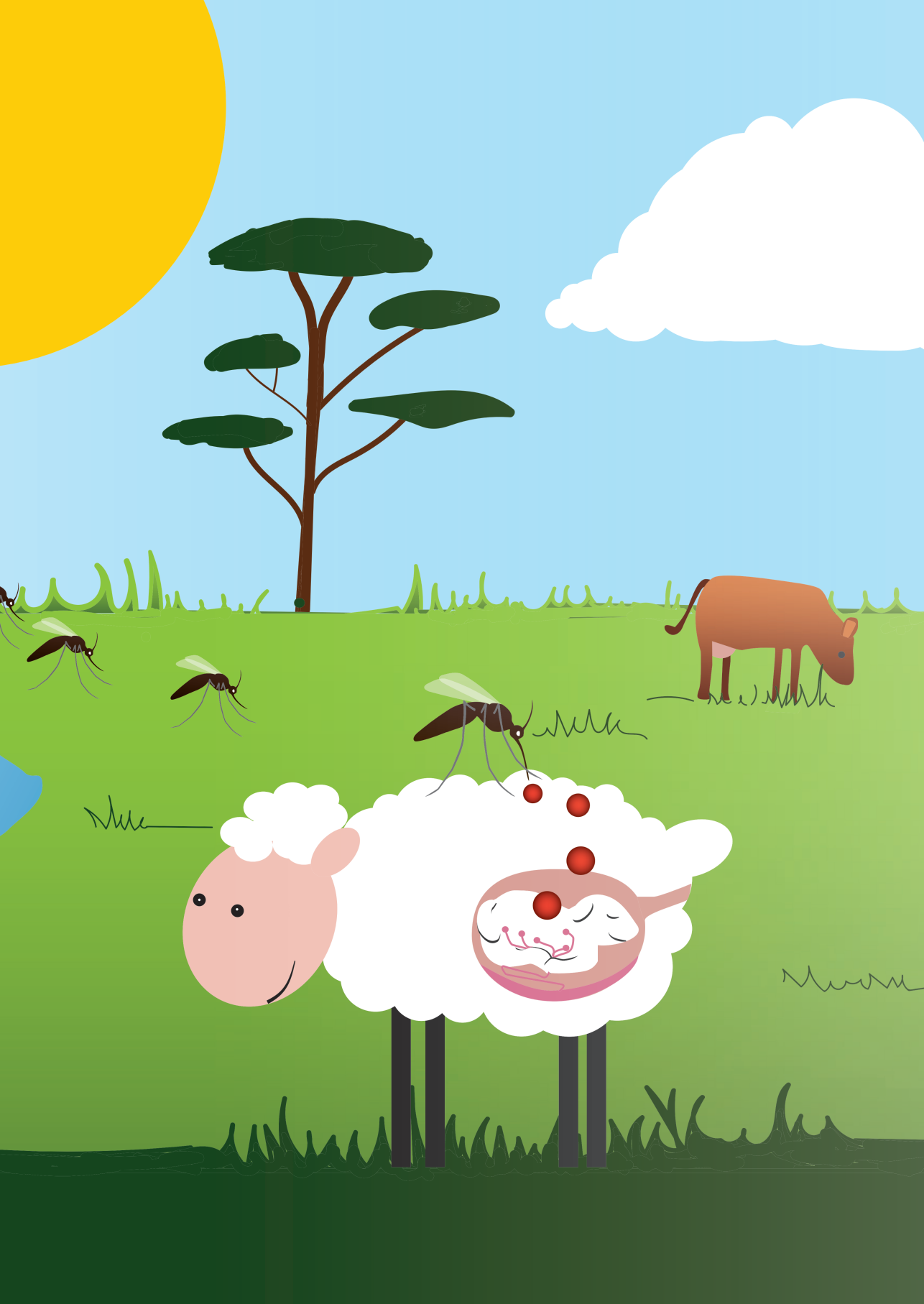


Figure S5.1. Standard curve to calculate TCID50 equivalents of SHUV. SHUV RNA of a stock of known TCID50 titer was lysed in Trizol, followed by isolation of RNA using the Direct-zol RNA miniprep kit (Zymo Research) according to the manufacturer's instructions. A 10-fold dilution series was prepared in water, and aliquoted for RT-qPCR runs.



Chapter 6

Reverse genetics system for Shuni virus, an emerging orthobunyavirus with zoonotic potential

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Abstract

The genus *Orthobunyavirus* (family *Peribunyaviridae*, order *Bunyavirales*) comprises over 170 named mosquito- and midge-borne viruses, several of which cause severe disease in animals or humans. Their three-segmented genomes enable reassortment with related viruses, which may result in novel viruses with altered host or tissue tropism and virulence. One such reassortant, Schmallenberg virus (SBV), emerged in north-western Europe in 2011. Shuni virus (SHUV) is an orthobunyavirus related to SBV that is associated with neurological disease in horses in southern Africa and recently caused an outbreak manifesting with neurological disease and birth defects among ruminants in Israel. The zoonotic potential of SHUV was recently underscored by its association with neurological disease in humans. We here report a reverse genetics system for SHUV and provide first evidence that the non-structural (NSs) protein of SHUV functions as an antagonist of host innate immune responses. We furthermore report the rescue of a reassortant containing the L and S segments of SBV and the M segment of SHUV. This novel reverse genetics system can now be used to study SHUV virulence and tropism, and to elucidate the molecular mechanisms that drive reassortment events.

Introduction

The genus *Orthobunyavirus* (family *Peribunyaviridae*) is the largest genus within the order *Bunyavirales*, comprising over 170 named arthropod-borne (arbo) viruses divided over 18 serogroups[21,24]. The most widespread orthobunyavirus is Akabane virus (AKAV), a member of the Simbu serogroup, which is endemic to Africa, Australia, and Asia and causes congenital malformations in ruminants[260]. Other veterinary pathogens of this serogroup include Aino virus, Shamonda virus, and Peaton virus[83,261]. Apart from veterinary pathogens, the genus *Orthobunyavirus*, also comprises human pathogens, such as Oropouche virus (OROV), which causes a mild, self-limiting febrile illness[257,262]. Orthobunyaviruses causing more severe human disease include LaCrosse virus (LACV), the leading cause of pediatric arboviral encephalitis in the US, and Ngari virus, which was associated with large outbreaks of haemorrhagic fever in Africa[255,256].

Orthobunyaviruses contain a negative-strand RNA genome that is divided into three segments, named after their size, large (L), medium (M), and small (S)[24]. The L segment encodes the viral polymerase. The M segment encodes two structural glycoproteins, Gn and Gc, involved in receptor binding and the fusion of the viral and endosomal membranes. The M-segment additionally encodes a non-structural protein called NSm, which was suggested to function as a scaffold for virion assembly[29]. The S segment encodes the nucleocapsid protein (N) and a non-structural protein (NSs) in overlapping open reading frames. The NSs protein is considered the major virulence determinant of orthobuyaviruses by antagonising host innate immune responses, including type I interferon responses[37,38].

On November 2011, a previously unidentified orthobunyavirus was detected in the blood of a diseased cow from a farm near the German town Schmallenberg. The so called Schmallenberg virus (SBV) belongs to the Simbu serogroup and was first associated with fever, diarrhea, and reduced milk yield, but was later found to be the causative agent of severe congenital malformations in ruminants, manifesting with arthrogryposis and hydranencephaly[81,82]. SBV was shown to be transmitted by *Culicoides* biting midges and spread rapidly across north-western Europe, ultimately being reported in 27 countries by September 2013[85]. Although the number of cases of congenital malformations have decreased since the outbreak, SBV is still circulating in Europe. Analysis of serum samples collected from over 300 people who lived at or near a farm where SBV was found revealed no SBV-specific antibodies, suggesting that SBV is not infectious to humans[105].

Shuni virus (SHUV) is another member of the Simbu serogroup, which was first isolated in the 1960s in Nigeria from a slaughtered cow and from the blood of a febrile child[246]. In 1977, SHUV was isolated from the brains of two horses that had succumbed to neurological disease, one in South Africa and one in Zimbabwe[263,264]. The virus re-emerged in 2009 in South Africa, where it was again associated with the neurological disease of horses[110]. Analysis of serum samples collected from veterinarians revealed a seroprevalence of 4%,

suggesting that SHUV, in contrast to SBV, is infectious to humans[119]. In 2014, SHUV emerged in Israel, where it was associated with congenital malformations in sheep, goat and cattle and fatal neurological disease in young cattle[111,112,121]. SHUV has been isolated from both field-collected midges and *Culex theileri* mosquitoes[113,265]. Recently, the virus was shown to infect and to disseminate in two laboratory-reared *Culicoides* species: *C. nubeculosus* and *C. sonorensis*[116]. In the same study, SHUV did not disseminate to the saliva of two mosquito species, *Culex pipiens pipiens* and *Aedes aegypti*.

An important feature of orthobunyaviruses is their ability to reassort their genome segments with related viruses[266,267]. The exchange of the M segment may lead to changes in host tropism as the glycoproteins mediate cell entry. Whereas SBV was considered a novel orthobunyavirus upon discovery, careful phylogenetic analysis has suggested that SBV is an ancestor of Shamonda virus (SHAV), which appears to contain the L and S segments of SBV, while the M segment was obtained from another, unidentified orthobunyavirus[84]. Despite belonging to the same serogroup, there is generally little cross neutralization between members of the Simbu serogroup[268]. Therefore, M segment reassortants may be capable of replicating in animals or humans that were infected previously by another member of the same serogroup. What drives the reassortment events and under which conditions segment exchange takes place is largely unknown. Of note, accumulating evidence suggests that compatibility of the viral RNA polymerase (encoded by the L-segment) and the nucleocapsid protein (encoded by the S-segment) is an important determinant of reassortant potential[269].

We here report the development of a reverse genetics system for SHUV that can be used to study virulence and tropism. The system was validated by creating a SHUV mutant lacking NSs expression and was subsequently used to evaluate viability of reassortants of SBV and SHUV, resulting in the rescue of a novel orthobunyavirus containing the S and L segment of SBV with the M segment of SHUV.

Results

Rescue of Wild-Type rSHUV and rSHUV-ΔNSs

To establish a reverse genetics system for SHUV, plasmids were designed to encode full-length S, M, and L segments in antigenomic-sense orientation, flanked by a T7 promoter at the 5' ends and a T7 terminator and hepatitis delta virus ribozyme at the 3' ends. The DNA constructs were synthesized and cloned into pUC57 plasmids by the GenScript Corporation, resulting in pUC57-SHUV-S, pUC57-SHUV-M, and pUC57-SHUV-L (Figure 6.1A). As a control, plasmids encoding SBV antigenomic-sense full-length S, M, and L were constructed. The nucleotide and amino-acid identities between SBV and SHUV are indicated in Figure 6.1B. For each virus, S plasmids with abrogated NSs genes were also constructed. Considering that the NSs and N proteins are encoded by overlapping open reading frames, we introduced four stop codons into the NSs genes without changing the amino acid sequences of the N proteins. Each stop codon was introduced downstream

of a methionine, to prevent the possible production of truncated NSs proteins Figure 6.1C). Following transfection, cytopathic effects (CPEs) were observed and supernatants were harvested and transferred to Vero cells to produce virus stocks (Figure 6.1A). Virus identities were subsequently confirmed by RT-PCR and the Sanger sequencing of the S segment amplicons.

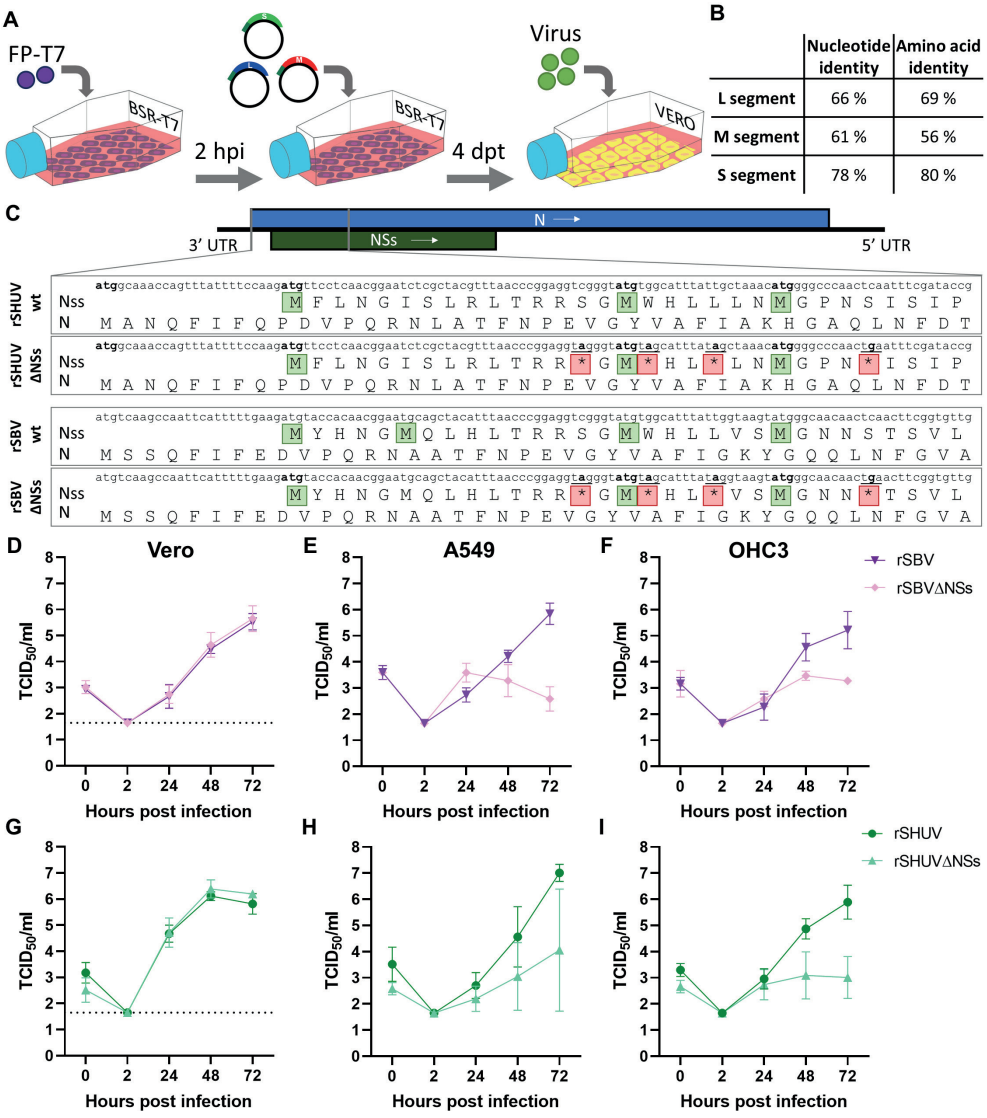

Figure 6.1. Rescue and growth characterization of recombinant Shuni virus (SHUV). (A) A schematic presentation of the reverse genetics system. Briefly, BSR-T7 cells were incubated with FP-T7. Two hours later, the medium was replaced, and the cells were transfected with pUC57 plasmids encoding each of the three genome segments. Supernatants containing recombinant virus were harvested at 4 days post transfection and transferred to Vero cells. The virus was harvested from the

Vero cells at 4 dpi. (B) Nucleotide and amino acid identity between wild-type rSBV and rSHUV. (C) Partial sequences of the S-segments of rSHUV, rSHUVΔNSs, rSBV, and rSBVΔNSs. The start codons are marked by green boxes. The four introduced stop codons are indicated by red boxes. The replication of rSBV (purple) and rSBVΔNSs (pink) in Vero cells (D), A549 (E), and OHC3 cells (F). The replication of rSHUV (light green) and rSHUVΔNSs (dark green) in Vero (G), A549 (H), and OHC3 cells (I). The cells were infected at a multiplicity of infection (MOI) of 0.01, and the samples were collected at 0, 2, 24, 48, and 72 hpi. At 2 hpi, the inocula were removed, the cells were washed, and fresh medium was added.

Replication of rSHUV and rSHUVΔNSs in Interferon-Competent and Interferon-Incompetent Cells

Previous studies have shown that SBVΔNSs replicates poorly in IFN-competent cells, whereas wild-type SBV and SBV-ΔNSs replicate equally efficiently in cells deficient in type I IFN responses[37,38]. The 80% amino acid identity of SBV and SHUV NSs (Figure 6.1B) suggests that the deletion of SHUV NSs would result in a similar phenotype. To test this hypothesis, the growth kinetics of SBV, SBV-ΔNSs, SHUV, and SHUV-ΔNSs were compared in different cell lines. In agreement with the aforementioned studies, the deletion of NSs expression had no influence on replication in Vero cells in which both wild-type and SBVΔNSs replicated to 10log 6 $TCID_{50}$/mL (Figure 6.1D). Growth was subsequently evaluated in IFN-competent cell lines, A549 cells, and an immortalized ovine hepatocyte cell line (OHC3), which was developed in the present work . Replication of SBVΔNSs was impaired in A549 cells as the final titre of SBVΔNSs was 3 logs lower than the wild type (Figure 6.1E), and, also, in OHC3 cells, a final difference of 2 logs was noted (Figure 6.1F). Next, the growth kinetics of SHUV and SHUVΔNSs were compared in the different cell lines. No difference in the replication in Vero cells were observed (Figure 6.1G). However, a 3-log difference in growth was observed in A549 and OHC-3 cells, although the attenuation of SHUVΔNSs was less clear in A549 cells due to a large variation in growth (Figure 6.1H,I).

Rescue of an SBV/SHUV Reassortant

The ability of orthobunyaviruses to reassort their genome segments was previously shown to be mediated by the panhandle sequences in the UTRs[269]. The alignment of the genome segment UTRs revealed high identity between the SBV and SHUV panhandles of each segment, with the exception of position 8 in the M and S UTRs (Figure 6.2A). We were successful in rescuing a virus containing the L and S segment of SBV, and the M segment of SHUV. However, we were unable to rescue all other possible reassortants, including the vice versa—a virus containing the S and L segments of SHUV and the M segment of SBV. The identity of the reassortant virus ($rSBV_{LS}/SHUV_{M}$) was confirmed by conventional PCR (Figure 6.2B). To further confirm the identity of the reassortant, an immunofluorescence assay was performed using antisera that specifically recognize SBV or SHUV Gc_{head}. Antisera raised against SBV Gc_{head} only recognized SBV, whereas antisera raised against SHUV Gc_{head} specifically recognized SHUV as well as the reassortant, but not SBV (Figure 6.2C).

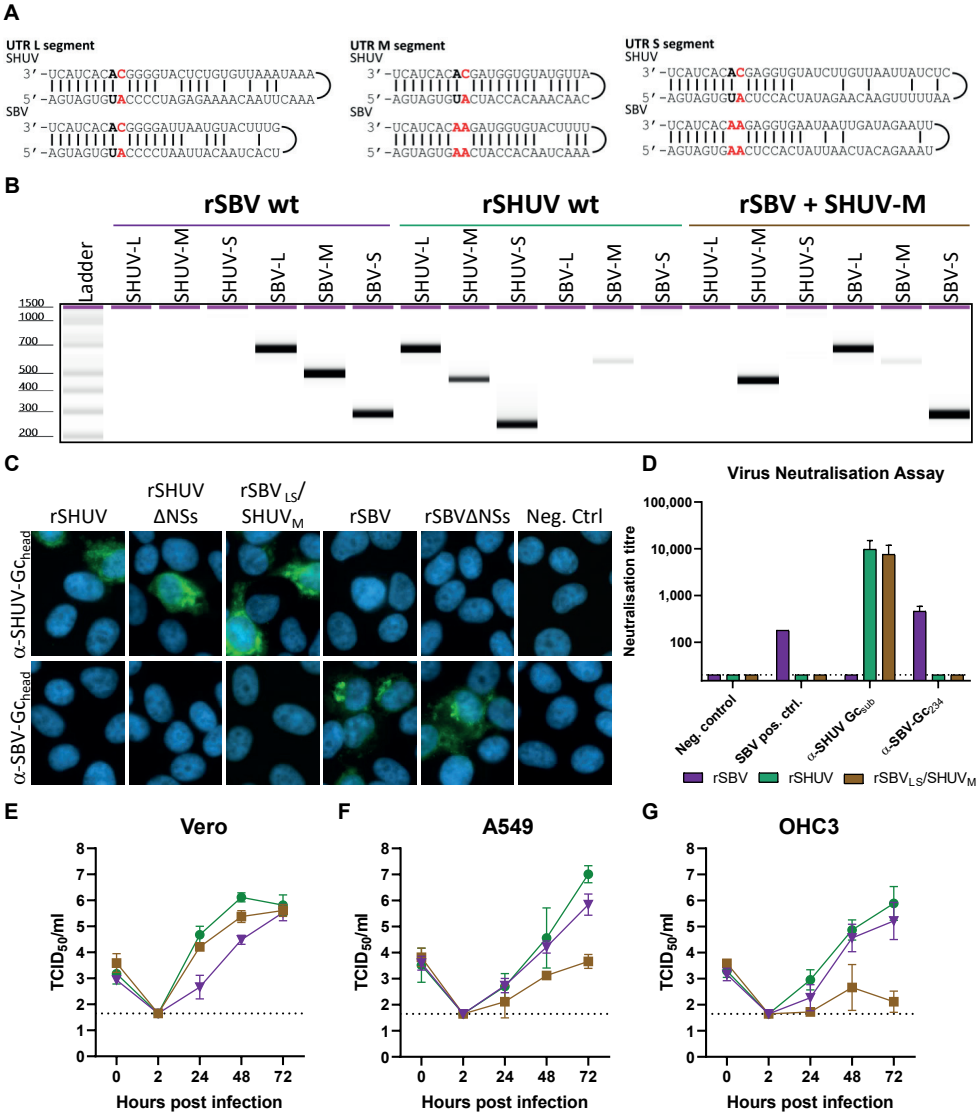

Figure 6.2. Characterization of a Schmallenberg virus (SBV)-SHUV reassortant. (A) The alignment of the UTRs of SBV and SHUV. SHUV contains a single mismatch at position 9, while SBV contains a double mismatch at positions 8 and 9 (indicated in red) in the UTR of the M and S segment. (B) The identity of the reassortant was confirmed by PCR using primers specifically recognizing SBV or SHUV L-, M-, or S-segments. PCR products were analyzed with the Tapestation system. (C) The immunofluorescence staining of Vero cells infected with rSHUV, rSHUVΔNSs, rSBV, or rSBVΔNSs at 24 hpi. The cells were infected using an MOI of 0.01. (D) Virus neutralization assay with a convalescent sheep serum against SBV, and rabbit antisera raised against the Gc head domains of SBV or SHUV. Replication of rSBV (purple), rSHUV (green) and the reassortant (rSBVLS/SHUVM), brown) in Vero (E), A549 (F) and OHC3 cells (G). The cells were infected at an MOI of 0.01, and the samples were collected at 0, 2, 24, 48, and 72 hpi. After 2 h, the medium was removed, the cells were washed, and fresh medium was added.

The successful rescue of a virus containing SBV L and S and SHUV M, suggests that such a reassortant could also emerge in the field. It was therefore interesting to investigate if this virus could be neutralized by SBV or SHUV-convalescent sera. Whereas SHUV convalescent serum was not available, SBV convalescent serum from sheep neutralized SBV, but not SHUV or the reassortant. In agreement with this result, the SBV-Gc_{head} antiserum neutralized SBV only, whereas the SHUV-Gc_{head} antiserum neutralized both SHUV and the reassortant (Figure 6.2D).

Finally, we compared the growth dynamics of the $SBV_{LS}/SHUV_{M}$ with wild-type SHUV and SBV in IFN-competent and IFN-incompetent cell lines. Interestingly, $SBV_{LS}/SHUV_{M}$ replicated equally efficiently as wild-type SHUV and SBV in Vero cells (Figure 6.2E). However, it was striking to find that the reassortant replicates poorly in the IFN competent cells A549 and OHC3 (Figure 6.2F,G).

Discussion

We report a reverse genetics system that can be used to study SHUV virulence and tropism. Recombinant SHUV was shown to replicate more efficiently than rSBV in Vero, A549 and OHC3 cells. In agreement with studies previously performed with SBV lacking NSs, both rSBVΔNSs and rSHUVΔNSs were significantly attenuated in IFN-competent cells, attributed to the IFN antagonistic function of orthobunyavirus NSs proteins[37,38].

The emergence of SHUV in Africa and Israel, its association with both congenital malformations and fatal encephalitis in ruminants[111], horses and wildlife[110,270], and its recent association with neurological disease in humans[271], calls for awareness and the development of control tools. Furthermore, areas where related orthobunyaviruses are endemic should also anticipate the emergence of reassortants. Considering that SBV is endemic to Europe, we used our SHUV and SBV reverse genetics systems to evaluate the viability of reassortants of these two viruses. All six combinations of genome segments were evaluated, whereas only the combination of SBV S and L with the M segment of SHUV was shown to be viable. These findings are strikingly similar to those of a previous study in which the possibility of SBV and OROV to reassort was evaluated, using minigenome-reporters and virus-like particles (36). Specifically, this previous study demonstrated that SBV N and L proteins can use the promoter of the M segment of OROV, whereas the vice versa did not result in genome replication. From this, it was concluded that only one viable virus may result from the reassortment of SBV and OROV, a virus containing the L and S segment of SBV and the M segment of OROV. The authors of this previous study speculated that two residues in the panhandle of the M segment, at positions 8 and 9, play a role in determining the ability to reassort. The SHUV UTRs, like the UTRs of OROV, Bunyamwera virus, Oya virus, and Perdoes virus, contain one mismatch at position 9, while the SBV UTR contains a double mismatch, which is also the case for Akabane virus (AKAV). These positions have been shown to be important for promotor activity[269,272]. Particularly

position 8 of the 5′ UTR forms highly specific protein-RNA hydrogen bonds. A difference in the promotor binding site of SBV and AKAV may enable the recognition of another nucleotide. This suggests that the polymerases of AKAV and SBV are more promiscuous to accept/recognize the genome segments of other bunyaviruses and thus are more likely to reassort. The same mismatch is found in the UTR of the S segment; however, as it seems to be the case that the L and S segments of the genome have to be from the same virus, this mismatch is probably redundant for the possible formation of a viable reassortant. This previous work and our present study underscore that further research is needed to understand the molecular basis of this apparently restricted reassortment potential.

It was striking to observe that the SBV/SHUV reassortant replicated more efficiently in Vero cells than SBV and equally efficiently as SHUV. In strong contrast, the replication of the reassortant was reduced in the IFN competent A549 and OHC3 cells. This phenotype is possibly related to the suboptimal compatibility of the SBV and SHUV genome segments and/or proteins, which may be improved upon by further passaging of the reassortant virus. The interaction between the SHUV M segment and SBV N protein could be suboptimal, resulting in a larger amount of unbound double-stranded RNA in the cytosol, which could form secondary RNA structures, normally not found in bunyaviruses, and stimulate interactions with cytoplasmic sensors, such as MDA-5[273].

Considering the limited cross-neutralization between members of the Simbu serogroup, it was plausible to assume that the M segment reassortant is not neutralized by SBV-specific antibodies[268]. Indeed, the reassortant was not neutralized by a sheep convalescent antiserum against SBV. As a convalescent serum with SHUV-neutralizing activity was not available, rabbits were immunized with baculovirus-produced Gc_{head}. The corresponding protein from SBV was previously shown to induce high levels of neutralizing antibodies, which was taken along in this experiment as a positive control for SBV neutralization and as a negative control for SHUV neutralization. The antiserum against SHUV Gc_{head} effectively neutralized rSHUV and the reassortant, whereas the cross-neutralization of both sera was not observed. This finding confirms that the Gc_{head} domain is a very powerful immunogen with potential use for vaccine applications, with the important note that protection may be species specific.

In conclusion, the SHUV reverse genetics system developed in the present work can be used to further study the interactions of SHUV with its vertebrate and insect hosts. It would additionally be interesting to evaluate if SHUV can form viable reassortants with other orthobunyaviruses and to study the virulence and tropism of these novel viruses.

Materials and Methods

Cells and Cell Culture

Culture media and supplements were obtained from Gibco (Thermo Fischer Scientific, Breda, The Netherlands) unless indicated otherwise. BSR cells constitutively expressing T7 RNA polymerase (BSR-T7 cells) were kindly provided by Prof. Klaus Conzelmann (Ludwich-Maximilians-Universität, München). These cells were maintained in Glasgow minimum essential medium (GMEM) supplemented with 1% minimum essential medium nonessential amino acids (MEM NEAA), 4% tryptose phosphate broth, 1% antibiotic/antimycotic (a/a), and 5% foetal bovine serum (FBS) (complete medium). Vero E6 cells were maintained in minimum essential medium (MEM) supplemented with 1% a/a, 5% FBS, 1% glutamine and 1% MEM NEAA. Human A549 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% a/a. A549, Vero E6 and BSR-T7 cells were cultured at 37 °C with 5% CO_2.

An immortalized sheep liver cell line was developed by isolating hepatocytes from the liver of a 4-week-old lamb. The liver was rinsed by injecting Hank's Balanced Salt Solution (HBBS) in the portal vein. Next, the liver was cut into small slices that were incubated with 0.1% collagenase IV in HBBS for 30 min at 37 °C. Liver cells were filtered through a 70-µm cell strainer and subsequently centrifuged 5 min at 300× *g*. The pellet was resuspended in Ammonium-Chloride-Potassium (ACK) lysis buffer (Thermo Fisher Scientific, Breda, The Netherlands) and incubated for 5 min at room temperature. The cells were washed twice with HBBS and cultured in collagen-coated T75 flasks. The cells were maintained in DMEM supplemented with 10% FBS, 0.04% supplement B, 0.02% epidermal growth factor (EGF), and 1% a/a at 37 °C with 5% CO_2. After 2 cell passages, the cell cultures were sent to InSCREENeX GmbH, Braunschweig, Germany, to immortalize the cells as described by Lipps et al. [274]. The cells were maintained in collagen (InSCREENex)-coated flasks with DMEM supplemented with 10% FBS and 1% a/a at 37 °C with 5% CO_2. The immortalized cell line was named Ovine Hepatocyte Cell #3 (OHC3).

Sf9ET cells (ATCC® CRL-3357™) were cultured in Insect-XPRESS medium (Lonza, Maastricht, The Netherlands) supplemented with 1% a/a. High Five cells were maintained in Express Five medium supplemented with 1% glutamine and 1% a/a. Both these insects' cell lines were cultured in suspension at 28 °C in a shaking incubator.

Rescue of SBV and SHUV from cDNA

The full-length cDNA of the L, M, and S genome segments from SBV strain BH80/11-4 (GenBank numbers: HE649912.1, HE649913.1 and HE649914.1) and SHUV strain 18/09 (GenBank numbers: NC_043699.1, NC_043698.1, NC_043697.1) were synthesized and cloned in antigenomic-sense orientation in pUC57 vectors, flanked by a T7 promoter at the 5′ end and a hepatitis δ ribozyme and T7 terminator at the 3′ end by the GenScript Corporation (Piscataway, NJ, USA), generating pUC57-SBV-S, pUC57-SBV-M, pUC57-SBV-L,

pUC57-SHUV-S, pUC57-SHUV-M, and pUC57-SHUV-L. To determine the nucleotide and amino acid identity between the SBV and SHUV segments, the NCBI BLAST sequence analysis tool was used [275]. To create SBV and SHUV NSs deletion mutants, 4 stop codons were introduced downstream of the methionine codons in the NSs genes without changing the amino acid sequences of the N proteins generating pUC57-SBV-S-ΔNSs and pUC57-SHUV-S-ΔNSs. The first three mutations in SBVΔNSs are as described by Elliott et al. [276]; however, a fourth stop codon was introduced to prevent translation from a fourth methionine further downstream.

To optimize rescue efficiency, BSR-T7 cells were infected prior to transfection with a recombinant fowlpox virus expressing T7 polymerase (fpEFLT7pol), here referred to as FP-T7, kindly provided by the Institute for Animal Health (IAH, Compton, UK). Specifically, BSR-T7 cells were seeded in T75 flasks in complete medium with 10% Opti-MEM (Gibco, Thermo Fischer Scientific, Breda, The Netherlands) 1 day before transfection. The medium was replaced by 5 mL Opti-MEM containing $10^{6.5}$ $TCID_{50}$ of FP-T7 and incubated at 37 °C and 5% CO_2 for 2 h. The medium was subsequently removed, and the cells were washed once in Opti-MEM after which 5 mL fresh Opti-MEM was added. The transfection mixture was prepared according to manufacturer's instructions (*Trans*IT®-LT1; Mirus Bio, Madison, WI, USA). Briefly, highly pure 7.5 µg pUC57-L, 7.5 µg pUC57-M and 5 µg pUC57-S (of SBV or SHUV) were added to NaCl, before adding LT1 transfection reagent according to manufacturer's protocol. With the aim to rescue reassortants, plasmids encoding SHUV L, M, or S segments were replaced for the corresponding plasmids of SBV. All 6 combinations were transfected and subsequently incubated at 37 °C and 5% CO_2. Four hours post transfection, 10 mL complete medium was added. At 5 days post transfection, supernatants were harvested and passaged on Vero-E6 cells in 6-well plates. Larger stocks were prepared by inoculating T150 flasks containing 6×10^6 Vero E6 cells at a multiplicity of infection (MOI) of 0.01. The virus was harvested at four days post infection. Each rescue experiment with the aim to rescue reassortant viruses was performed three times.

To confirm the identities of the wild type, ΔNSs mutant viruses and reassortants, reverse transcriptase (RT)-PCR and Sanger sequencing were performed. To this end, 200 µL virus stock solution was added to 2 mL EasyMAG lysis buffer (Biomérieux, Amersfoort, The Netherlands), followed by RNA isolation by the NUCLISENS® EasyMAG robot (Biomérieux, Amersfoort, The Netherlands). Next, cDNA was generated using the Superscript IV kit (Thermo Fisher Scientific, Breda, The Netherlands) with random hexamers according to manufacturer's protocol. The NSs coding region of the S-segment was amplified by PCR, using the Phusion High-Fidelity PCR Master mix (Thermo Fisher Scientific). For SHUV, a 320-bp fragment and for SBV a 545-bp fragment was amplified using primer sets 1 and 2, respectively, as described in Table 6.1. The PCR products were concentrated and purified with the PCR clean-up kit (Macherey-Nagel, Bottrop, Germany) according to manufacturer's protocol. Purified fragments were sent to Macrogen (Amsterdam, The Netherlands) for sequencing.

Table 6.1. Primers used in conventional PCR.

Primer Set	What	Primer Sequence ('5-'3)	Length Expected Product
1	S segment SHUV	ATGGCAAACCAGTTTATTTTCCA TGATCTGCAACCCATTTTGC	320 bp
2	S segment SBV	GTGAACTCCACTATTAACTACAG TCCATATTGTCCTTGAGGACCCTATGCATT	545 bp
3	L segment SHUV	AGAGAAAACAATTCAAAATGGATCCTTACC TAAGTGAGTTGTAAAACTCTTTGAATATAGGATGAGTA	654 bp
4	M segment SHUV	TGGAGAGCTGGTGAAAACTGTCA GTTTTGAGGCCACAAGTGACATC	432 bp
5	S segment SHUV	AGAACAAGTTTTTAAATGGCAAACCAGT TAACCAATGTAAATTTGATGCCACCAAATG	238 bp
6	L segment SBV	CATGGCTAGACATGACTACTTTGGTAG AAAATGTTATAATCATTGCCATATCTATTTATAACCTTTTGT	666 bp
7	M segment SBV	CCTGTTTAGCTTTTGCACTCCC CACATGTTACCTCAATGGATTCGC	483 bp
8	S segment SBV	TTGAAGATGTACCACAACGGAATGCA CGTGCTAGATATCCTGACATCCTG	286 bp

To confirm the identity of the recombinant wild type and reassortant viruses, segment- and virus-specific reverse transcriptase PCR (RT-PCR) reactions were performed using the primers listed in Table 6.1 (primer sets 3–8). The primers were ordered at Integrated DNA technologies, Leuven, Belgium. The different DNA fragments were visualised with an Agilent 2200 Tapestation system in combination with a D1000 Screentape (Agilent, Amstelveen, The Netherlands).

Production of Rabbit Antisera Against the SBV and SHUV Gc Head Domains (Gc_{head})

The N-terminal 234 amino acids of the Gc protein of SBV, here referred to as Gc_{head}, were previously shown to be highly immunogenic [190]. With the aim of developing rabbit antisera against the Gc protein of SBV and SHUV, we developed constructs encoding the Gc_{head} of both viruses. DNA fragments encoding the N-terminal 234 amino acids of the SBV Gc protein or 255 amino acids of the SHUV Gc protein flanked by a N-terminal GP64 signal sequence and a C-terminal twin Strep-Tag were synthesised by GenScript (Piscataway, NJ, USA). Following the cloning of the fragment in a pBAC-3 baculovirus vector, recombinant baculoviruses were generated with the flashBAC™ ULTRA baculovirus expression system (Oxford Expression Technologies, Oxford, UK). Briefly, transfection mixtures containing the pBAC-3 vector, a bacmid, and cellfectin II were added to 24-wells plates containing 200,000 SF9ET cells per well. Rescued viruses were subsequently amplified in SF9-ET suspension cultures infected at low MOI. For protein production, High Five cells were infected at high MOI according to the manufacturer's protocol (Thermo Fisher Scientific, Breda, The Netherlands). Proteins were purified from supernatants using Strep-Tactin® resin (IBA, Göttingen, Germany) according to the manufacturer's instructions. Buffers

were exchanged to Tris-buffered saline + 200 mM NaCl using Amicon® Ultra centrigufal filters (Merck-Millipore, Amsterdam, The Netherlands). Proteins were separated in 4–12% SDS gels (Thermo Fisher Scientific, Breda, The Netherlands) and stained with GelCode Blue Stain reagent (Thermo Fisher Scientific, Rockford, US A). Two New Zealand White rabbits per viral protein were subsequently immunised with 1 mg of protein by Genscript (Piscataway, NJ, USA) following their standard polyclonal antibody service protocols for the production of rabbit antisera.

Immunofluorescence Assays

Vero E6 cells (25,000/well) were seeded in Grace Bio-Labs CultureWell™ removable chambered cover glasses (Sigma-Aldrich, Zwijndrecht, The Netherlands) in complete medium. The cells were subsequently infected with virus at an MOI of 1. After 24 h, the cells were fixed in 4% paraformaldehyde for 30 min and permeabilized using a 1% Triton-X-100 in PBS solution. To stain the infected cells, the primary polyclonal rabbit sera against SBV-Gc_{head} or SHUV-Gc_{head} (in PBS supplemented with 5% horse serum, Gibco, Thermofisher Scientific, Breda, The Netherlands; diluted 1:4000, 2 h at 37 °C) in combination with a donkey anti-rabbit IgG Alexfluor568 (Life Technologies, Thermofisher Scientific, Breda, The Netherlands, in PBS supplemented with 5% horse serum, Gibco, Thermofisher Scientific, Breda, The Netherlands; diluted 1:500, 1 h at 37 °C) secondary antibody was used. The nuclei were visualized using 4', 6-diamidino-2-phenylindole (DAPI) and cells were submerged in Vectashield (H-1000, Vector Laboratories, Peterborough, UK) prior to imaging. The images were obtained with an inverted fluorescence wide-field ZEISS Axioskop 40 microscope with appropriate filters and a 1.3 NA 100× oil objective in combination with an Axiocam MRm CCD camera.

Virus Neutralization Test

Sera were heat-inactivated at 56 °C for 30 min and serially diluted (1:3) in 96-well plates with a starting dilution of 20. Subsequently, 50 µL of virus was added with a titre of $10^{4.7}$ $TCID_{50}$/mL, after which the samples were incubated at RT for 2 h. Next, 30,000 Vero cells in 100 µL were added to each well. The plates were incubated for 48 h at 37 °C with 5% CO_2. All dilutions and suspensions were prepared in complete medium. After 48 h, an immunoperoxidase monolayer assay (IPMA) was performed as described previously using the rabbit antisera as primary antibodies and anti-goat-HRP (Dako, Agilent, Santa Clara, CA, USA) as a secondary antibody [222]. A well was scored positive for neutralization if less than 50% of the cells were stained, the negative control (without serum) was used as 100% cell staining. All samples were tested in triplicate. Titres were defined as the average of the highest dilution that was positive for (>50%) neutralization.

Growth Curves

A549 and Vero cells (750,000) were seeded in T25 flasks and infected with virus the following day at an MOI of 0.01 in 5 mL complete medium. Directly upon infection, a 250-µL sample was taken and stored at −80 °C until analysis. At 2 h post infection, the

cells were washed and the medium was replaced and another sample was collected. At 24, 48, and 72 hpi, additional samples were collected. All growth curves were performed in triplicate and each sample was titrated in triplicate using end-point dilution assay as described previously[67]. An immunoperoxidase monolayer assay (IPMA) was performed as described using the rabbit antisera as primary antibodies and anti-rabbit-HRP (Dako, Agilent, Santa Clara, CA, USA) as a secondary antibody[222]. Titres were determined using the Spearman-Kärber algorithm and were expressed as $TCID_{50}$/mL.

Acknowledgements

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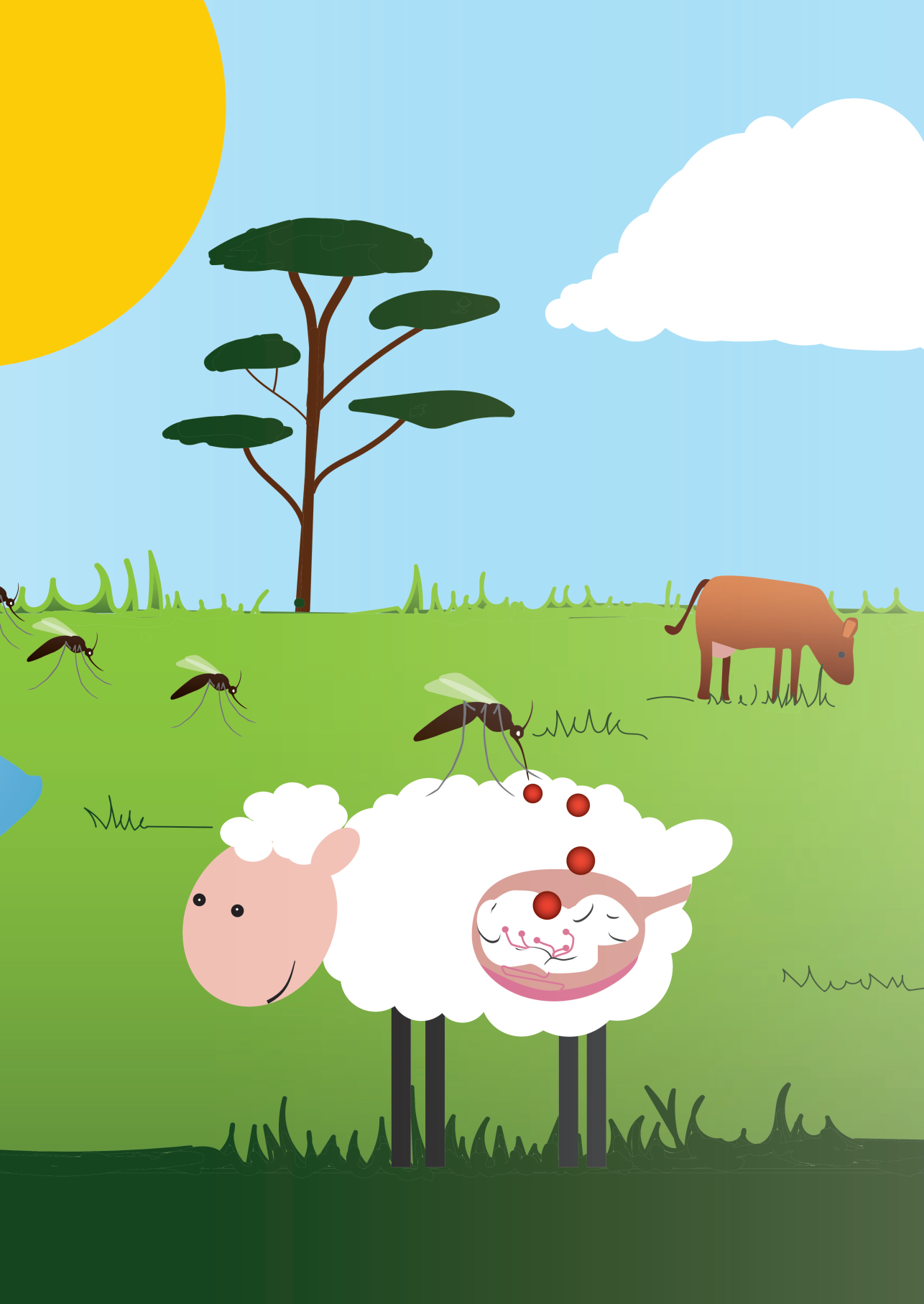

Chapter 7

General discussion

The way in which the human population currently interacts with our planet has generated an excellent breeding ground for new arbovirus outbreaks. Globalisation, climate change, extensive travel and intensive farming are all factors contributing to the increase in arbovirus outbreaks that have been observed over the last decades. Previous outbreaks, such as the Schmallenberg outbreak of 2011 and the Zika outbreak of 2015, have demonstrated our lack of preparedness when facing outbreaks of viruses that exhibit vertical transmission[82,244,277]. The pathogenesis that leads to congenital malformations, stillbirths or abortions during infection with these viruses is poorly understood. This thesis sheds light onto the transmission routes of different arboviruses over the placenta to the foetus, ultimately aimed to facilitate the development of effective countermeasures.

In this thesis, the interactions of RVFV, WSLV and SHUV with the maternal-foetal interface in the ovine placenta were studied to gain a better understanding of vertical transmission of arboviruses (Chapters 2, 4 and 5). We observed that all three viruses target both maternal epithelial cells and foetal trophoblasts to cross the ovine placenta (Figure 7.1). The ovine placenta is extremely susceptible and permissive to RVFV infection, as practically all maternal epithelial cells and trophoblasts became infected, which resulted in necrosis and ultimately in placental demise (Chapter 2). In Chapter 3, we demonstrate that a novel live-attenuated RVF vaccine, named vRVFV-4s, prevents placental demise and abortion. Whereas ewes that received a double vaccination were completely protected from challenge virus replication, viral RNA was detected in a single placentome of an ewe that had received a single vaccination. Moreover, calcifications and eversions of placentomes from type A/B to type C/D were observed in the same group, indicating that some replication of challenge virus had occurred in these placentas. This finding suggests that immunity induced by vRVFV-4s vaccination prevents vertical transmission even in placentas not completely protected from RVFV replication.

Contrary to wildtype RVFV infection, the ovine placentas infected by WSLV or SHUV appeared macroscopically healthy. Analysis by IHC showed foci of WSLV antigen in trophoblasts distributed across the whole placenta (Chapter 4). Occasionally infected maternal epithelial cells were observed surrounded by WSLV positive trophoblasts. Moreover, careful examination of maternal epithelial cells opposite WSLV-positive trophoblasts, that stained negative for WSLV antigen, showed necrosis, suggesting that WSLV first replicates in maternal epithelial cells. Small foci of SHUV-positive maternal epithelial cells were detected throughout SHUV-infected placentomes, occasionally with an infected trophoblast (Chapter 5).

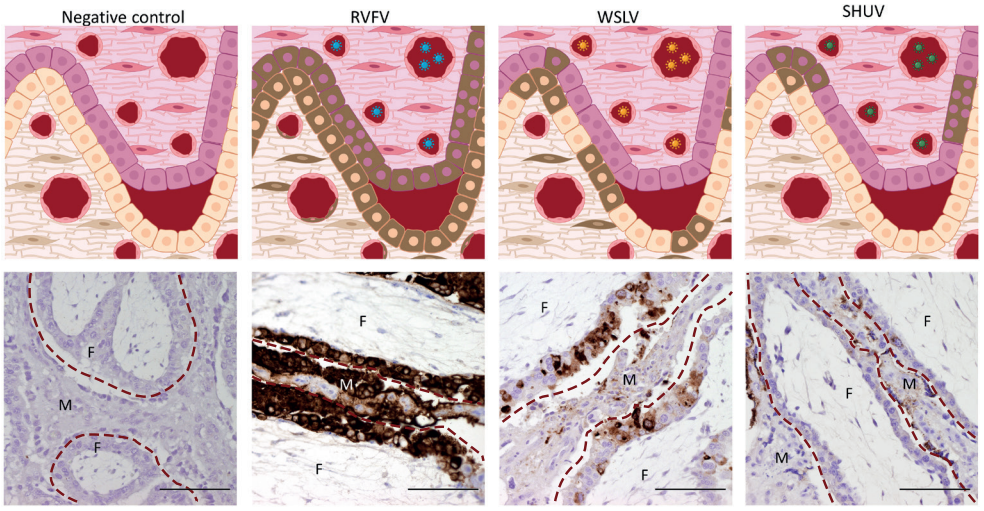

Figure 7.1. Infection of the ovine placenta by RVFV, WSLV and SHUV. The top depicts a schematic overview of infected cells in the ovine placenta at 6 (RVFV), 8 (WSLV) or 7 (SHUV) days post inoculation. The tissues depicted in pink and yellow represent the maternal and foetal parts of the placenta respectively. Brown colouring indicates infected cells. RVFV infection affected maternal epithelial cells, foetal trophoblasts, foetal endothelial cells and foetal fibroblasts. Few WSLV-infected maternal epithelial cells were detected, however small groups of infected trophoblasts and foetal fibroblasts were found, whereas SHUV infects mainly maternal epithelial cells as well as, occasionally, trophoblasts. The bottom row shows representative images of immunohistochemically (IHC) stained ovine placentas for RVFV, WSLV and SHUV antigens. The dashed line indicates the placental barrier, areas labelled F and M represent foetal and maternal parts of the placenta respectively. The scale bars represent 100 µm.

We also investigated the susceptibility of human placental explants to infection by arboviruses. No cases of WSLV in pregnant humans have been reported in literature and in our hands no WSLV replication was detected in human term placental explants (Chapter 4). However, it is possible that WSLV is capable of infecting placental tissues at earlier stages of pregnancy. Further research is needed to determine whether this is the case. RVFV and SHUV were both shown to target human trophoblasts in explants of term placentas (Chapters 2 and 5 respectively). There is limited data available that suggests vertical transmission of RVFV can lead to detrimental outcomes in infected pregnant women[80,278,279]. Two human cases of vertical transmission have been reported: in one case, an infected pregnant woman delivered a baby with a rash, enlarged liver and spleen and jaundice[278]. In the second case, a woman who developed clinical signs of RVFV infection 4 days before delivery, delivered a lethargic, jaundiced infant who died of RVF infection within one week[279]. Moreover, an association between RVFV infection of pregnant women and miscarriage was found in a cross-sectional study of Sudanese women[80]. Although vertical transmission of SHUV in humans has not been reported, our findings call for increased awareness for SHUV infections during human pregnancies. The combined results of our studies call for more awareness of zoonotic arboviruses with (neuro)teratogenic potential.

This includes the development of better diagnostics to detect viral infection for use in hospitals in affected areas when women present signs of encephalitis, abortion or other complications during pregnancy.

Although this thesis has provided important novel insights into mechanisms of vertical transmission of certain arboviruses in the ovine and human placenta, numerous questions remain. The rest of this discussion will focus on areas of arbovirus research that are still to be addressed or that require further exploration.

Question 1. How do arboviruses travel from the periphery to the placenta?

Infection of mammals by arboviruses initiates at the skin through the bite of an infected arthropod. Mosquitoes use their proboscis to probe the skin and continuously deposit saliva, which contains the virus, until a blood vessel is pierced[280]. Establishing a primary replication cycle in the skin is a critical stage of infection; the virus needs to disseminate from the skin before virus replication is controlled by the innate immune response[281]. Recent studies involving bunya- (Bunyamwera virus; BUNV) and flaviviruses (e.g. dengue virus; DENV), have shown that local immune responses to mosquito bites enhance initial virus replication and dissemination, which was associated with more severe pathology[282,283]. Inflammatory responses at the bite site include the recruitment of immune cells such as resident macrophages. While these macrophages may phagocytose and neutralize virus, some arboviruses (e.g. DENV) have evolved to replicate in these cells, generating the first virus progeny already at the bite site[281,282,284]. For DENV, it has also been shown that factors in mosquito saliva disrupt the endothelial cell layer, facilitating virus dissemination[283]. Proteases in mosquito saliva process proteins in the extracellular matrix, exposing viral attachment factors like heparan sulphate and thereby promote attachment of arboviruses to susceptible cells[285].

From the site of the mosquito bite, the virus travels either through the blood, or via the lymphatic system to target organs. For RVFV, the liver is the primary target organ[286]. Hepatocytes are easily accessible as they are in direct contact with blood due to the fenestrated endothelial cells in the liver[287]. Replication of RVFV in the liver generates high amounts of virus progeny, which leads to higher viremia levels. The virus subsequently disseminates via blood vessels to the rest of the body, including the placenta.

Trophoblasts in the human placenta are in direct contact with maternal blood, however, in the synepitheliochorial zone of the ovine placenta the maternal blood is separated from foetal trophoblasts by maternal endothelial cells, maternal connective tissue and maternal epithelial cells[154]. To infect the ovine placenta, the virus has to exit the bloodstream and cross the connective tissue to reach maternal epithelial cells. Contrary to the fenestrated endothelial cells of hepatic blood vessels, the endothelial cells in the placenta form a continuous layer, preventing the virus from moving passively from the

blood to the placenta. The virus has to either infect endothelial cells, or use lymphocytes as a vehicle to travel between the endothelial cells[288]. However, we found no evidence of infected endothelial cells or infected leukocytes in our studies with RVFV, WSLV or SHUV in the ovine placenta. Therefore, further studies to determine how these arboviruses pass through endothelial cell layers of maternal blood vessels are needed.

It is worth noting that the results described in Chapters 2, 4 and 5 indicate that the maternal epithelium of the placenta is highly susceptible to SHUV, RVFV and WSLV infection. Cell surface factors that are exploited by the virus for attachment, such as integrins and heparan sulphate, are abundantly present on maternal epithelial cells and are known to facilitate infection of some arboviruses[289,290].

At the haemophagous zone, ovine trophoblasts are in direct contact with stagnant pools of maternal blood. Here, the virus does not need to pass an endothelial layer to reach foetal trophoblasts. Both WSLV and RVFV have been shown to infect foetal trophoblasts at the haemophagous zones (Chapters 2 and 4). However, IHC analysis of the placenta revealed infected trophoblasts at the synepitheliochorial zone at the same timepoints, suggesting that transmission at the haemophagous zone is an alternative route.

Interestingly, at 7 days post inoculation (dpi), high amounts of viral RNA were detected in the ovine placentas, whereas no or limited amounts of viral RNA were detected in the plasma, liver, spleen and lymph nodes. Although no virus was detected in these organs, infection had likely occurred at an earlier timepoint and was cleared from these organs by 7 days post infection, as was demonstrated most clearly for RVFV (Chapter 2). Vertical transmission of RVFV has previously been reported without detectable viremia in another study with pregnant ewes[291]. The results of this former study demonstrated that vertical transmission can occur in the absence of detectable viremia.

Overall, the most likely route from the periphery to the placenta for RVFV, SHUV and WSLV is from the location of the mosquito bite, via the blood stream to a primary target organ (e.g. liver) after which progeny virus reaches the placenta via the bloodstream. However it remains a likely possibility that a small amount of virus could directly reach the placenta from the site of the mosquito bite via the lymphatic vessels or blood stream, as depicted in Figure 7.2.

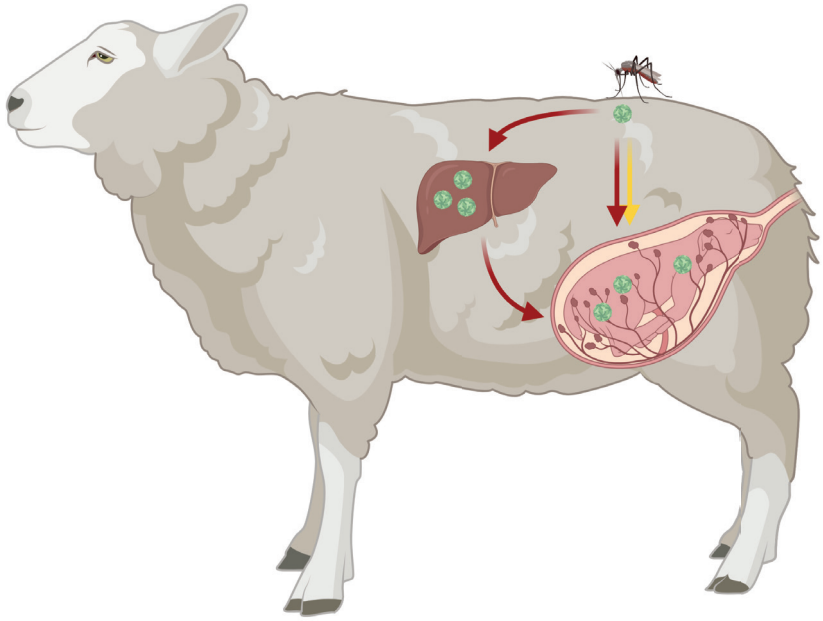

Figure 7.2. Schematic overview of potential transmission routes from the periphery to the placenta.

Question 2. How do arboviruses cross the placenta?

To cause congenital malformations or abortions, arboviruses have to cross the placental barrier. The results described in Chapters 2, and 4 suggest that WSLV and RVFV can use two routes to cross the ovine placenta. Vertical transmission via the first route starts with infection of maternal epithelial cells in the synepitheliochorial zone of the placenta. Virus progeny released from these cells is subsequently in direct contact with foetal trophoblasts that line the maternal epithelium, and which were shown to be susceptible to RVFV and WSLV. This first route was also observed for SHUV in the ovine placenta in Chapter 5. Virus released from trophoblasts is released into the foetal mesenchyme, exposing foetal endothelial cells and fibroblasts. The second route involves the haemophagous zone of the placenta where foetal trophoblasts are in direct contact with stagnant pools of maternal blood. The trophoblasts at the haemophagous zone are specialised to phagocytose erythrocytes to provide the foetus with iron[162]. Intriguingly, this mechanism might be hijacked by arboviruses to infect trophoblasts as was observed for RVFV (Figure 7.3; Chapter 2). Thus, infection of trophoblasts at the haemophagous zone could potentially occur through direct infection or via phagocytosis. The susceptibility of both maternal epithelial cells and trophoblasts at the foetal-maternal interface suggests that there is no physical barrier for the studied viruses to prevent vertical transmission. A physical barrier is here considered to be a cell layer that is not susceptible or permissive to virus infection. Remarkably, in Chapter 2 we observed that most maternal epithelial cells in the placenta are infected with RVFV and these cells seem to generate large amounts of viral progeny, while trophoblasts were only occasionally infected, at least at the early stages of infection.

Trophoblasts were found to be infected two days later, suggesting that an innate immune response initially counteracts virus replication in these cells. Moreover, in Chapter 3 we describe calcifications in the maternal epithelium of the ovine placenta in ewes that were challenged after receiving a single vaccination, suggestive of some challenge virus replication. However, no vertical transmission was detected, indicating that replication was limited to the maternal epithelium. Based on these observations, we can conclude that there is indeed a placental barrier. However this is not solely a physical barrier determined by cell susceptibility and permissivity. It is unlikely that cellular immunity plays an important role in creating the placental barrier, as foetal trophoblasts do not express any MHC molecules[157]. The innate immune system therefore is expected to play a large role at the placental barrier.

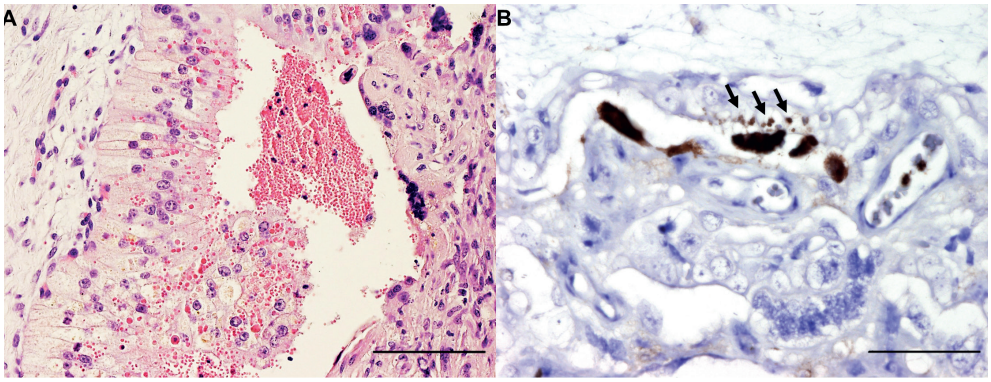

Figure 7.3. Phagocytosis of erythrocytes by trophoblasts in the haemophagous zone. (A) HE staining of the haemophagous zone of the ovine placenta at mid-gestation. Note the phagocytosis of erythrocytes by specialised trophoblasts[162]. (B) IHC detection of RVFV antigen 4 days post inoculation. Arrows indicate RVFV staining around phagocytosed erythrocytes.

The foetal and maternal immune systems function to protect the foetus from pathogens. The immune system of the placenta is complex, due to the delicate balance between protection of the foetus and prevention of immune-mediated rejection of the semi-allogenic foetus. Moreover, the maternal immune system adapts and changes throughout the different stages of pregnancy as each stage requires a unique immune status[292]. Interferons not only function in defence against viral pathogens, they also serve important functions during a healthy pregnancy. In the ovine placenta IFN-tau (IFN-τ), a type I interferon, is produced by foetal trophoblasts and is crucial for implantation and maintaining ovine pregnancy[293]. Additionally IFN-γ, a type II IFN, promotes the physiology of a healthy placenta by maintaining the decidua and modelling the maternal spiral arteries[294]. Type I and type III IFN signalling have an antiviral effect at the foetal-maternal interface. Type I IFNs bind the IFN alpha/beta receptor (IFNAR) which activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling cascade

that leads to the upregulation of hundreds of IFN-stimulated genes (ISGs), which act to restrict viral entry, replication and spread[294-296]. Type III IFNs use a different receptor, the IFN-lambda receptor (IFNLR), however the downstream signalling is similar to IFN I[214]. There are two main differences between IFN types I and III. The first is the receptor expression, IFNAR is expressed on all cell types whereas IFNLR is mostly restricted to epithelial cells[214,296,297]. Secondly, ISG expression induced by type I IFNs peaks early and declines rapidly, whereas more sustained expression occurs upon induction by type III IFNs[297-299]. The differences in receptor expression and ISG expression kinetics cause type I IFN to raise a highly potent and rapid immune reaction whereas type III IFN leads to a slower less potent reaction[297]. A recent study has demonstrated the importance of balance in antiviral immunity in the context of congenital infection. In this study ZIKV-infected IFNAR-negative mice displayed significantly less placental damage when compared to wt mice[300]. IFN-β, a type I IFN, expression led to abnormalities in placental morphology after ZIKV infection, resulting in more foetal death [300]. In the same study, human placental explants also showed remodelling of placental morphology due to IFN-β. Although IFN type I does have an antiviral effect in the placenta, IFN type III signalling likely plays a larger role[214,294]. While not much is known about IFN-λ expression in the ovine placenta, it has been demonstrated that human syncytial trophoblasts constitutively produce IFN-λ[301]. IFN-λ has been demonstrated to strongly restrict ZIKV infection in syncytial trophoblasts, indicating that ZIKV does not infect these cells but uses another pathway to gain access to the foetus[301]. Considering the relatedness of WSLV to ZIKV, this may explain why we did not observe WSLV infection in human term placental explants in Chapter 4. In our experiment with human placental explants we only studied the foetal villi of the human placenta, of which the outer layer consists of syncytiotrophoblasts. Possibly WSLV, like ZIKV is unable to infect these cells due to the constitutively expressed IFN-λ and utilises another pathway to cross the placenta.

The non-structural NSs protein of bunyaviruses is known to function as an antagonist of type I IFNs, however, for vertical transmission, type III IFNs are more relevant. The NSs protein of Heartland virus (HRTV), an emerging phlebovirus endemic to the US, is the only NSs protein which has been shown to antagonise both type I and type III IFN to date[302]. NSs of RVFV is able to suppress upregulation of IFN-β mRNA, and to inhibit general host transcription and degrade protein kinase K (PKR), an intracellular sensor of virus RNA[32]. The transcriptional shut-off will have consequences for type III IFN responses[303] as well as type I IFN responses. Activation of PKR leads to translational shut-off, therefore no new signalling proteins can be produced which impacts type III IFN signalling as well as type I IFN signalling[304], therefore it is likely that RVFV NSs is able to supress IFN-λ indirectly and at least to some degree. Additionally degradation of PKR also directly impacts IFN-λ induction. PKR activates the NF-κB pathway by degradation of the inhibitor of κB (iκB)[305] and NF-κB activation is required for the induction of IFN-λ1[306]. Thus PKR degradation will negatively impact IFN-λ induction as the NF-κB pathway will not be activated. However, vertical transmission still can occur without an NSs protein as was shown in a previous

study on the safety of a potential RVFV vaccine for pregnant ewes[184]. Inoculation of pregnant ewes with Clone 13, a naturally attenuated strain of RVFV with a large deletion in the NSs gene, resulted in congenital malformations in 40% of the lambs[184]. In Chapter 6 we provide the first evidence that SHUV NSs can antagonise innate immune responses. However follow-up research is needed to determine whether SHUV antagonises IFN-β, IFN-λ or both.

In conclusion, SHUV, RVFV and WSLV cross the ovine placental barrier by infection of maternal epithelial cells followed by infection of foetal trophoblasts. RVFV and WSLV are also capable of infecting foetal trophoblasts at the haemophagous zone. The placental barrier is in part formed by the innate immune system however how RVFV, WSLV and SHUV circumvent or antagonise innate immunity at the placental barrier is a topic for further research.

Question 3. How does gestational age affect the outcome of arbovirus infection?

In this thesis, vertical transmission of arboviruses in pregnant ewes was studied at one third gestation (Chapters 2, 3, 4 and 5) and at mid-gestation (Chapters 2 and 3). In addition, the susceptibility of human placental cells was determined using explants from term placentas (Chapters 2, 4 and 5). Gestational age is an important determinant of the outcome of infection by vertically transmitting arboviruses. Several cohort studies have demonstrated a strong relationship between maternal infection with ZIKV early in pregnancy with more severe brain abnormalities compared to maternal infection late in pregnancy[307,308]. Additionally, a recent cohort of children with a normal baseline neurologic examination whose mothers were infected with ZIKV during pregnancy were found to be more likely to experience developmental delays if infection had occurred during the third trimester compared to those exposed during the first or second trimester[309]. This indicates that although severe brain abnormalities such as microcephaly are rare after ZIKV infection during the third trimester of pregnancy, infection of the foetus during this period of gestation may still have severe consequences. It is feasible that developmental delays also occur in apparently healthy children born after maternal infection during the first and second trimester of pregnancy.

Gestational age will likely affect the pathogenesis of WSLV, another flavivirus, in a similar manner. In Chapter 4 we describe neuroinvasion of foetal brains by WSLV at one third of gestation. WSLV was detected throughout the foetal brains in neuronal progenitor cells and differentiated cells: neurons, oligodendrocytes, microglia and astrocytes. WSLV infection early in gestation could lead to more severe neurological complications in a manner similar to what is reported for ZIKV[307-309]. ZIKV causes congenital disease by infection of the neural progenitor cells associated with persistent inflammatory responses[310,311]. Infection of these neural progenitor cells earlier during gestation has greater consequences, as foetal development will be more severely affected. WSLV infection of differentiated cells

suggests that the foetal brain is also susceptible to infection at later stages of gestation. Infection at the final stage of gestation could lead to developmental problems during the first years of life rather than teratogenic malformations during gestation. In our study no lesions, necrosis or apoptosis were detected in foetal brains, which could suggest that WSLV does not cause pathology. However previous studies have reported that WSLV is associated with congenital malformations after experimental infection and infection in the field[141,146]. Therefore, it seems unlikely that these foetuses would have developed normally if they had been allowed to come to term.

Gestational age appears to play a much smaller role for wildtype RVFV infection. The damage caused by RVFV to the ovine placenta at one third- and mid-gestation, as described in Chapter 2, was highly similar. Infection of foetal maternal epithelial cells led to necrosis which resulted in severe haemorrhages in the placenta, and eventually in abortion. Infection at a later stage of gestation is expected to still cause abortion due to the extremely high susceptibility of maternal epithelial cells. As pregnancy progresses, the maternal epithelial cells are replaced by maternal syncytial cells, which originate from fusion of binucleate cells with the maternal epithelial cells. Both maternal epithelial cells and maternal syncytial cells are susceptible to RVFV infection (Chapter 2). Therefore, at the end of gestation, RVFV is likely still able to infect the ovine placenta and induce abortion of the foetus[291]. However infection during the first weeks likely results in resorption of the foetus rather than abortion[312].

Interestingly, RVFV vaccine strains that are able to cross the placenta are associated with congenital malformations rather than abortions. Makoschey *et al.* inoculated pregnant ewes at GD (gestation day) 50 or GD 120 with a high dose of Clone 13[184]. Two weeks after delivery, foetuses were necropsied and studied for congenital malformations, after which samples were collected for detection of RVFV RNA. Eight out of 20 lambs that were born from ewes inoculated at GD 50 had congenital malformations. In 6 lambs, viral RNA was detected in blood or organs and 1 lamb had pre-colostral antibodies. Out of the 28 lambs born from ewes inoculated at GD 120, no lambs suffered from congenital malformations, 5 lambs had pre-colostral antibodies and in 4 lambs viral RNA was detected in blood or organs. Unlike in humans where maternal IgG antibodies are actively transported across the placenta[313], in the ovine placenta there is no transfer of antibodies from mother to foetus[314]. The ovine foetus becomes immunocompetent between day 45 and 65 of gestation[315-317]. In accordance to this, the results reported by Makoschey *et al.* suggest that the foetal immune system is able to actively produce antibodies against Clone 13, which may prevent the formation of congenital malformations. This indicates that as pregnancy progresses and the foetal immune system develops, the foetal immune system may prevent viral infection or reduce the severity of infection.

To conclude, it seems likely that gestational age does not greatly affect the outcome of wildtype RVFV infection, resulting in destruction of the placenta and subsequent abortion

during most gestational stages. In contrast, gestational age probably has a large impact on the outcome of WSLV infection. Infection during early stages of gestation could cause more severe congenital disease, since the organs of the foetus are still developing. Infections at a later stage could still lead to developmental delays due to infection of differentiated brain cells, but the foetal immune system may reduce severity of the infection.

Question 4. What is the best model to study vertical transmission of arboviruses infecting humans?

The outbreak of ZIKV in 2015 has led to the development of *in vitro* and *in vivo* models to study the pathogenesis underlying vertical transmission in humans, as ZIKV infection was found to cause congenital disease in pregnant women[310]. *In vitro* models include primary cell cultures, placental explants and organoids. Research with primary cell cultures of placental cytotrophoblasts, endothelial cells, fibroblasts and Hofbauer cells (the resident macrophages of the placenta) demonstrated the susceptibility of these cells to ZIKV infection[318-320]. A placental organoid system mimicking the chorionic villi with differentiated cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts was also developed[321]. Placental explants exhibit natural physiology, cytoarchitecture and cellular complexity and have been used to research cell susceptibility of different arboviruses (this thesis;[79,322]) and innate immune responses after virus infection[241]. These tools are less expensive compared to animal experiments, can be used for high-throughput screenings and conditions can be controlled more rigorously. They are therefore excellent to study cell susceptibility, attachment factors, viral receptors, innate immune responses, essential host factors for virus replication, effectiveness of antivirals and their underlying mechanisms. Molecular tools such as reverse genetics systems can be employed to introduce mutations in viruses while systems such as CRISPR can be used to modify primary cells and cell lines. The development of these tools contributes significantly to the reduction and replacement of laboratory animals, however, *in vitro* models cannot fully recapitulate entire organs.

Therefore *in vivo* models are still an important tool to study vertical transmission of arboviruses. The anatomy of non-human primates (NPH) most closely resembles that of humans and NHPs have proven extremely valuable in ZIKV research[323-326]. However the high costs, specific animal facilities, limited availability and ethical issues associated with their use make the NHP model unattractive. The pig model has been used to study neurological disease of foetuses as pig brains resemble human brains[327,328]. Although the anatomy of the pig placenta, which is diffuse and endotheliochorial, differs significantly from the human placenta[154], the physiology, immunity and foetal development is similar[329], which makes the pig a suitable model for researching congenital malformations. However for studying virus interaction with the pig placenta the virus needs to be able to cross the placental barrier, which is not the case for ZIKV[327].

A large number of studies have been performed with small rodents such as mice (immunocompetent and immunocompromised), rats and guinea pigs[330,331]. Although small rodents have haemochorial placentas, like humans, the anatomy differs at the maternal-foetal interface. In the human placenta, maternal blood is separated from the foetal blood vessels by a layer of syncytiotrophoblasts and cytotrophoblasts. However, in the murine and rat placenta, maternal blood is in direct contact with a layer of mononucleated trophoblasts, which is surrounded by a double layer of syncytiotrophoblasts which are in close contact with foetal blood vessels[332]. Moreover, extravillous trophoblasts invade deep into the basal plate of the human placenta, a phenomenon that is absent in the murine and rat placenta[330].

Besides the difference in morphology, the placental endocrine functions of mice and rats also differ from humans during gestation. In humans, progesterone production shifts from the corpus luteum to the placenta at 6-8 weeks of pregnancy and progesterone levels stay high until term. In mice and rats, progesterone is produced by the corpus luteum throughout the gestation period and the levels drop to initiate parturition (Figure 7.4)[333]. A recent study on vertical transmission of RVFV in a pregnant rat model demonstrated that vertical transmission led either to resorption of the foetuses, congenital malformations or stillbirths[79]. The absence of abortion may be explained by the production of progesterone by the corpus luteum and not the placenta. RVFV infection of the rat placenta does not result in a drop in progesterone levels and therefore the pregnancy will not end in abortion, however resorption of the foetus and congenital malformations can still occur. The difference in endocrine functions of the rat and human placenta makes the rat model less suitable for research on the foetal outcomes after viral vertical transmission in humans. In the search for a suitable animal model not only the morphological features of the placenta should be considered, but the endocrine function, immune system and foetal development stages may be of equal importance.

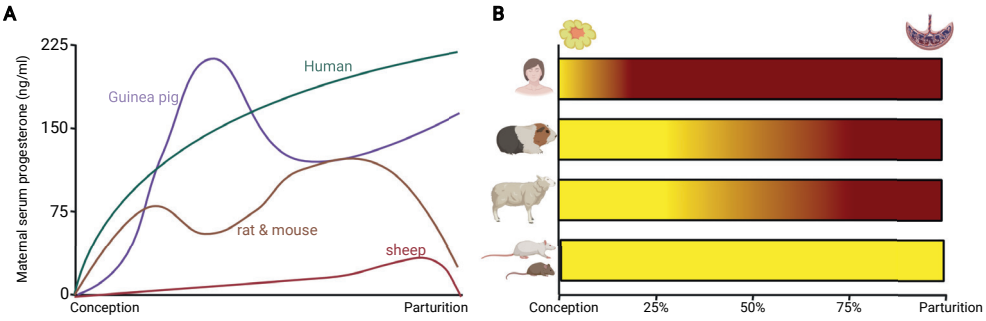

Figure 7.4. Progesterone circulation and production in humans, sheep, rats, mice and guinea pigs. (A) Circulating progesterone levels in humans, sheep, rats, mice, and guinea pigs throughout gestation. (B) Source of circulating progesterone (yellow: corpus luteum, red: placenta) during pregnancy. Adapted from Morrison et al, 2018.

As discussed in Chapter 2, the production of progesterone in pregnant ewes shifts at mid-gestation from the corpus luteum to the placenta. RVFV infection of the ovine placenta likely leads to abortion due to a drop in progesterone levels. However, as depicted in Figure 7.4, ovine progesterone levels during pregnancy are lower compared to humans, and contrary to humans, progesterone levels in ewes drop to initiate parturition at term[334]. Additionally, the synepitheliochorial placenta does not resemble the human placenta anatomically, although there are similarities between the haemophagous zone in the ovine placenta and the human placenta[154]. However due to the overall similarity in progesterone production and the similar anatomy at the haemophagous zone the ovine placenta could be interesting as an animal model for humans, despite its limitations.

Interestingly, the guinea pig placenta resembles the human placenta more closely in anatomy, physiology and endocrine function, when compared with the placenta of mice, rats and sheep[333]. Similar as in humans, syncytio- and cytotrophoblasts separate maternal blood from foetal blood and extravillous trophoblasts invade deep into the basal plate. Additionally, progesterone production shifts at mid-gestation from the corpus luteum to the placenta, and progesterone levels stay high during pregnancy[334].

It is important to note that for a suitable animal model, the animal needs to be susceptible to the virus. Guinea pigs seem an excellent model to study vertical transmission of arboviruses infecting humans, however RVFV infection does not lead to any symptomatic disease in guinea pigs[70,171]. However it remains possible that pregnant guinea pigs are susceptible to RVFV infection, due to the high susceptibility of placental cells and may therefore be a good model for the human.

Question 5. How can vaccination prevent vertical transmission?

Vaccines are the most successful and cost-effective tool for prevention of veterinary and human diseases[335]. As use of the WSLV vaccine remains limited[141] and no SHUV vaccine is available yet, this question will focus on vaccination strategies for the prevention of RVF. In Chapter 3 the efficacy of the live-attenuated RVFV-4s vaccine was tested in pregnant ewes. Live-attenuated RVF viruses are generally highly efficacious, however those currently on the market are unsafe for use in pregnant ewes as they are able to cause congenital malformations[141,179,182,184]. The safety of RVFV-4s was studied previously and no vertical transmission occurred which was demonstrated by the absence of viral RNA in blood and organs of the foetuses[228]. Both single and double vaccination with RVFV-4s in pregnant ewes elicited a strong neutralising antibody response within one week after inoculation (Chapter 3). However, challenge with wildtype RVFV led to a higher increase in antibody responses in ewes that received a single vaccination compared to ewes that were vaccinated twice, which indicates that some challenge virus replication may have occurred. The detection of viral RNA in a single placentome, calcifications in several placentomes and placentome eversions in placentas from ewes that were vaccinated once corroborate this finding. Importantly, no vertical transmission was observed.

However as observed in Chapter 2, the ovine placenta is extremely susceptible to RVFV infection, therefore even a strong neutralising antibody response could be insufficient to completely prevent virus replication in maternal epithelial cells, especially when the ewes are challenged intravenously with a high dose of RVFV. Interestingly, no signs of infection were observed in the placenta of ewes that received double vaccination. As both groups displayed similar neutralising antibody levels at the moment of challenge infection, another unknown mechanism prevented vertical transmission in these animals. While T-cell responses were suggested to contribute to sterile immunity that prevents vertical transmission[336], more research is warranted to firmly establish the role of T-cell responses.

Currently no veterinary RVF vaccine has been approved for use outside endemic areas[337]. Emergency vaccination can be used to control outbreaks, however routine (yearly) vaccination would be most effective to prevent RVFV outbreaks in livestock and human populations[337-339]. Vaccination of livestock on a routine basis would not only prevent vertical transmission and thus the resulting abortion storms in sheep herds, but would likely also decrease death of newborn animals and decrease the number of human cases[337]. Routine infection of livestock would increase the overall level of herd-immunity in the main amplifier of RVFV during an outbreak. Over time routine vaccination therefore would contribute to a potential decrease in virus activity in these areas[337]. When considering a routine vaccination program, the timing of vaccination should be considered. As discussed in this thesis, pregnant ewes are extremely vulnerable and it is therefore preferable to vaccinate ewes before gestation. Newborn animals have an immature immune system and therefore it is also not ideal to vaccinate at a young age. Moreover maternally-derived antibodies that are provided via colostrum may interfere with the generation of vaccine-induced neutralising antibody responses[340]. However, young animals are extremely vulnerable to RVFV infection. It is therefore of the utmost importance to study whether maternal derived antibodies of vaccinated ewes protect their offspring, and to determine the duration of this protection. Taking all these considerations to mind, it may be best to vaccinate animals as they reach sexual maturity, which is at 6 months for ewes. As an ewe may become pregnant before vaccination, vaccine safety in the pregnant animal is of importance. To decrease virus activity during outbreaks, vaccination of rams should also be considered to reduce the number of susceptible species that can amplify RVFV.

Final thoughts and suggestions for further research

In this thesis, we studied the vertical transmission of arboviruses in mammals, with the aim to understand the pathologic mechanisms underlying abortion, stillbirth or congenital malformations. Our efforts have identified the primary target cells and tissues of RVFV, SHUV and WSLV infection. However, more research is warranted to understand the underlying pathological mechanisms in greater detail. Interesting lines of research for future studies can be organised into three main topics: diagnostics, vaccine studies and fundamental research. Prioritizing of RVFV by the Coalition for Epidemic Preparedness Innovations

(CEPI) and the World Health Organization (WHO) has led to awareness of RVFV epidemics and their consequences[173,174]. This highlighted the importance of diagnostic assays such as enzyme-linked immunosorbent assays (ELISAs), virus neutralization tests (VNTs), and quantitative polymerase chain reactions (qPCRs) that can be used for RVFV surveillance in endemic areas[237,341,342]. However similar diagnostic tools for neglected arboviruses such as WSLV and SHUV remain extremely limited. The research tools developed in this thesis could therefore be developed into diagnostic tests for these neglected viruses. The qPCRs developed for the detection of SHUV and WSLV RNA in several tissues could be validated for diagnostic purposes. The WSLV NS1 protein produced for immunisation of rabbits could be uses to set-up ELISAs. NS1 has been used for several other flaviviruses such as ZIKV, yellow fever virus (YFV), WNV and DENV to develop ELISA assays for diagnostic purposes[343]. Additionally the sheep sera collected during our animal trials, which may be positive for WSLV or SHUV IgM antibodies, could serve as a positive control during the development of diagnostic assays, as well as our rabbit polyclonal antisera against WSLV-NS1 and SHUV-Gc_{head}. An interesting application for SHUV-Gc_{head} protein would be via its use in a vaccine. Gc_{head} of the closely related SBV has already been shown to be highly immunogenic, and application as a subunit or DNA vaccine conferred protection in 66% of the animals[190]. Moreover another vaccine study used a Modified Vaccinia virus Ankara (MVA) vector that produces SBV-Gc_{head}. Immunisation with this MVA-based vaccine led to an efficient SBV-specific antibody response, which protected the animals from challenge with SBV[191].

Chapter 3 describes the efficacy of the RVFV-4s vaccine in pregnant ewes. Although we demonstrate that a single vaccination is enough to prevent vertical transmission, many questions remain to be answered. In this study ewes were challenged 2-3 weeks after vaccination. In the field farmers would vaccinate possibly once per year, so it is important to know whether and how long the immunity lasts. Moreover it is important to know whether the offspring are protected from RVFV as well. As newborn lambs are the most susceptible to RVFV infection, it would be highly advantageous when vaccination of the mother confers protection to the foetus via maternally-derived antibodies.

Our results demonstrate the high susceptibility of ovine maternal epithelial cells and trophoblasts for RVFV, WSLV and SHUV. However the receptors and/or attachment factors on these cells needed for viral entry remain unknown. Our ovine placental cells 16 (OPC-16) cell line that is described in Chapter 6 could help elucidate essential host proteins for viral entry, replication, assembly and release. A genome-wide clustered regularly interspaced short palindromic repeats (CRISPR) screen to identify host factors could be performed with OPC-16 cells[344,345]. As previously demonstrated, CRISPR screens can be used to uncover several important host pathways for ZIKV, DENV, WNV and other flaviviruses[346-348]. Similarly to ovine placental cell lines, CRIPSR screens with human trophoblast cell lines could identify important entry factors, host proteins or pathways that influence infection of human placentas. The identification of essential host factors may greatly increase our

understanding of the molecular processes involved in arbovirus infection and its potential for vertical transmission.

For successful infection of the ovine or human placenta viruses need to evade the host innate immune system. As discussed in Question 2 of this chapter, both type I and type III IFNs play a role in antiviral immunity of the placenta. For bunyaviruses NSs is an important innate immunity antagonist[32] and as such may play an important role in transplacental transmission. However as Clone 13 is able to pass the placental barrier and is still able to cause congenital malformations, NSs is unlikely to play a large role in transplacental transmission. Ovine and human placental cell lines could be used to study the interaction of NSs with the innate immune system. In our pregnant ewe studies samples were taken from the placenta and stored in RNAlater, which stabilises and protect RNA. Transcriptomic analysis of these samples can identify the up- and downregulated genes, indicating which intracellular signalling pathways are important for infection of the ovine placenta. Similarly, transcriptomic and/or qPCR analyses on human placental explants infected with a wildtype virus or NSs deletion mutants could provide further insight into the role of NSs in the suppression of IFN type I or III induced genes in the human placenta.

A transcriptomic approach could also be employed to better understand WSLV infection of the foetal brain. Although we observed in Chapter 4 that neuronal progenitor cells as well as differentiated neurons, oligodendrocytes, astrocytes and microglia were infected with WSLV, no apoptosis or necrosis was detected. Foetal brain samples from our investigation were also stored in RNAlater. These samples are available to investigate the mechanisms that lead to congenital malformations in foetuses, for example via transcriptomic analyses. An approach that has previously revealed that immune and cell death pathways were mostly responsibly for neuronal disease caused by WNV and chikungunya virus (CHIKV) in mice[349]. Additionally more research is warranted to study the effect of WSLV infection in foetal brains on foetal development.

The results in this thesis contribute to a better understanding of vertical transmission of arboviruses. Understanding the underlying mechanisms of vertical transmission will contribute to the development of safe vaccines for pregnant animals and humans. Additionally, gaining more knowledge on neglected arboviruses will advance our capability to correctly prioritise viruses that may become a risk in the future and will therefore lead to better preparedness.

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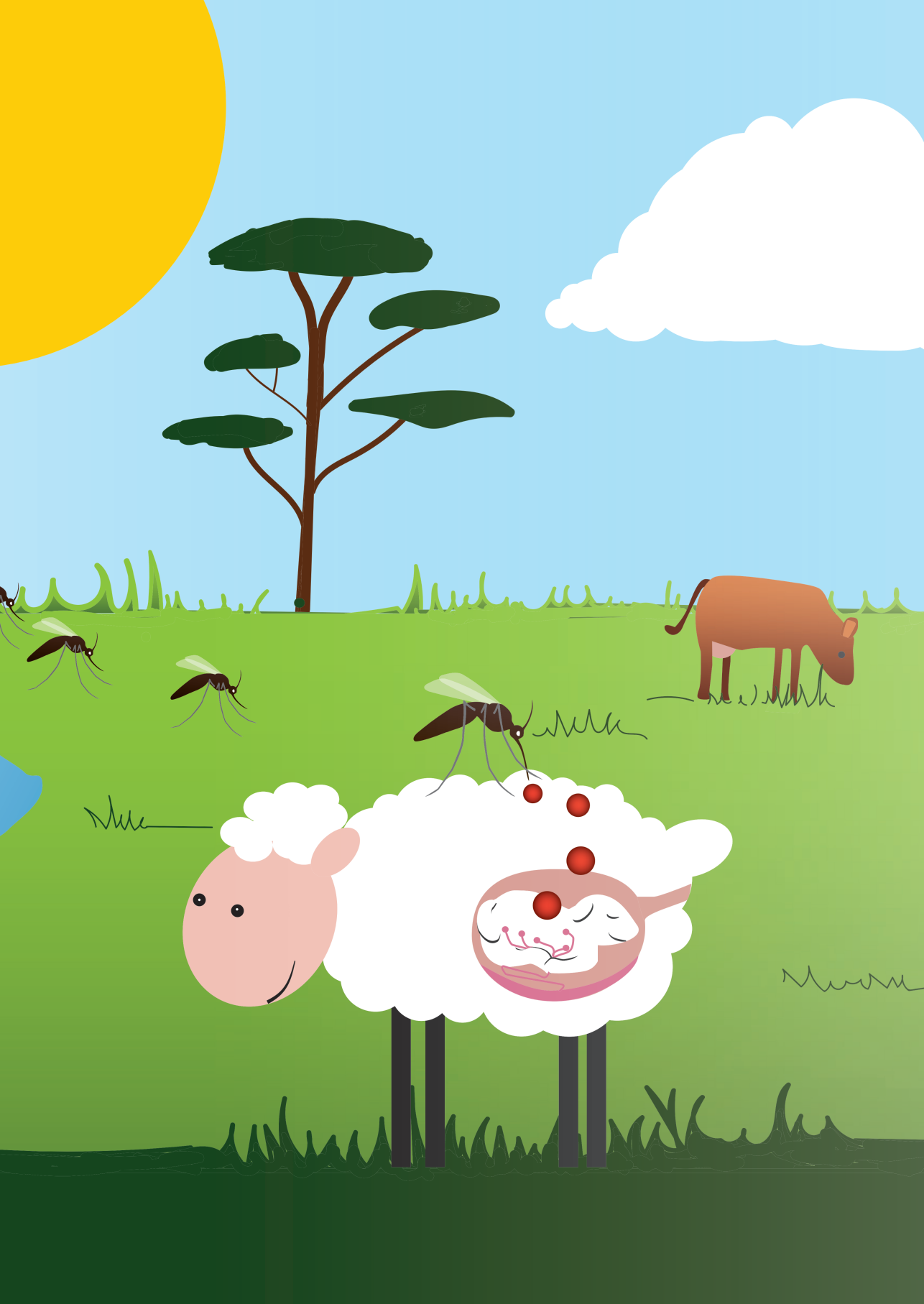

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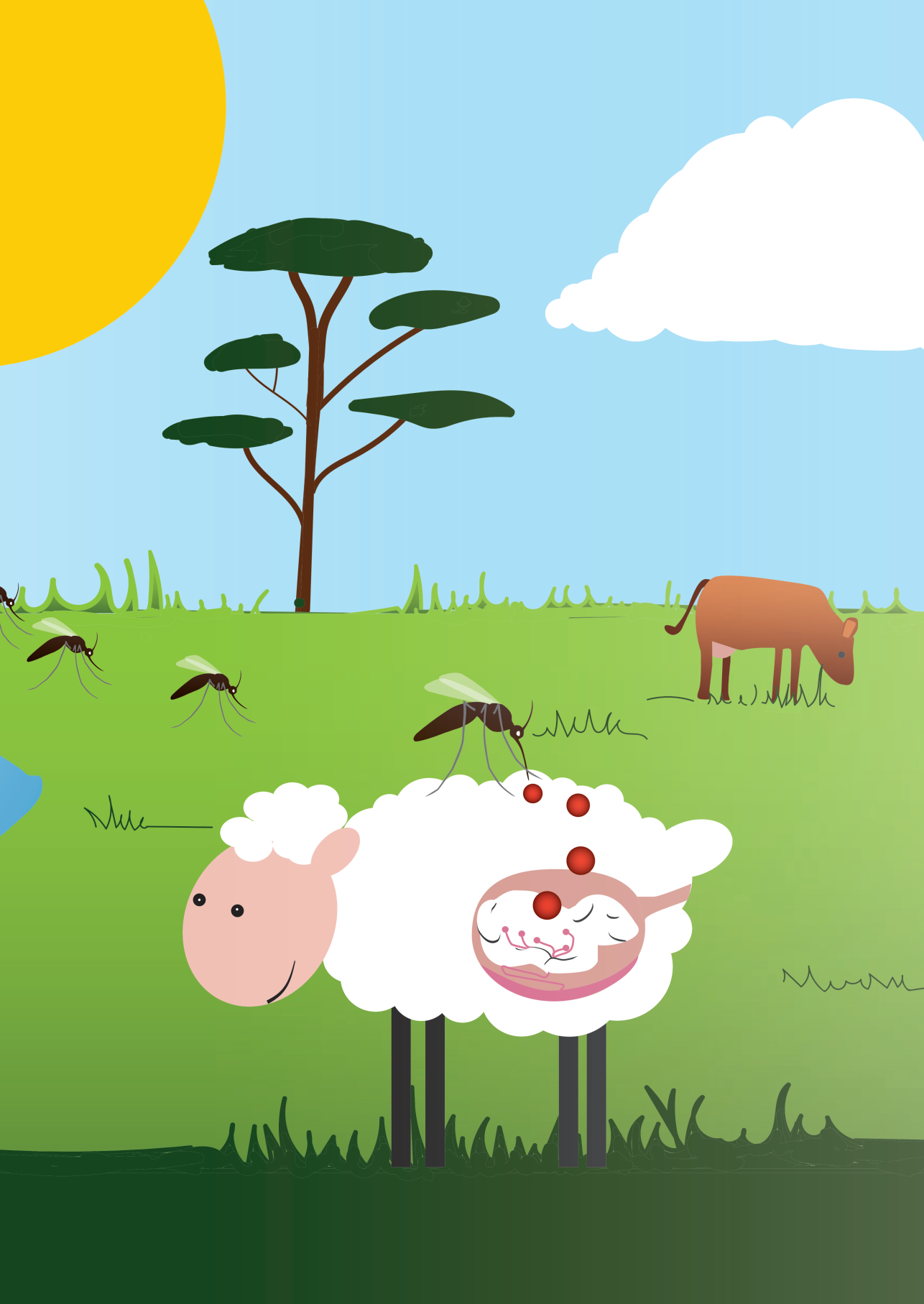


Summary

Summary

In 2015 an outbreak of the then relatively unknown Zika virus demonstrated how poorly the world is prepared for epidemic viral diseases. Although the virus was already discovered in 1947, few human cases were reported until the outbreak of 2015 in Brazil. The estimated number of current human cases is between 500,000 and 1.5 million, and thousands of babies have been born with birth defects such as microcephaly caused by this virus. ZIKV is an arbovirus and is spread from host to host by mosquitoes. Additionally, ZIKV is a zoonotic virus, which means that the virus is transmitted from animals to humans and is able to cause disease in humans. A knowledge gap was highlighted by this outbreak, as very little was understood about the mechanisms that underly vertical transmission (transmission from mother to foetus), and little was known about the pathology that leads to congenital malformations. It is therefore important that we bridge this knowledge gap by studying neglected viruses that, like ZIKV, have the potential to transmit vertically. Doing so, we will be better prepared to identify and prioritise potential arboviral threats to humans, and also to animals. Characterisation of the mechanisms underlying the ability of these viruses to transmit vertically could additionally facilitate the development of efficient countermeasures such as antivirals and vaccines. In this Thesis, I therefore focussed on the mechanisms underlying vertical transmission of neglected arboviruses of different virus families that are zoonotic or have zoonotic potential and that have the ability to transmit vertically.

Rift Valley fever virus (RVFV) is a phlebovirus of the order *Bunyavirales* that is pathogenic to ruminants and humans. It was first discovered in 1931 in Kenya, and since then outbreaks have been reported throughout the African continent. In the year 2000, RVFV was detected for the first time outside of Africa, on the Arabian Peninsula. RVFV is able to infect wild and domesticated ruminants, camelids, and humans. Sheep, however are the most susceptible species. Newborn lambs seldomly survive RVFV infection, and in adults the mortality rate is below 30%. Characteristic of RVFV outbreaks are abortion storms in sheep herds, in which all pregnant ewes may abort simultaneously. The detrimental outcome of RVFV infection of pregnant ewes is well described, however the pathology that leads to abortion has not yet been elucidated. In the work described in Chapter 2 of this Thesis, pregnant ewes at one third- or mid-gestation were inoculated with RVFV and necropsied at different timepoints post inoculation. Tissues were analysed by PCR, virus isolation, and immunohistochemistry (IHC) to identify primary target cells and tissues. RVFV was shown to replicate efficiently in maternal epithelial cells before the virus infects foetal trophoblasts. Moreover, the virus was shown to bypass the maternal epithelial cell layer by directly targeting foetal trophoblasts in the haemophagous zone, a region of the ovine placenta where maternal blood is in direct contact with foetal cells. Abortion was associated with widespread necrosis of placental tissues accompanied by severe haemorrhages. Increasing evidence suggests that RVFV is abortogenic in humans as well, warranting more research on the interaction of RVFV with the human placenta.

Therefore, human placental explants were infected with RVFV and analysed by PCR and IHC. Experiments with human placental explants revealed that RVFV replicates efficiently in both cyto- and syncytiotrophoblasts. The susceptibility of syncytiotrophoblasts to RVFV infection is interesting, as these cells are resistant to other vertically transmitted arboviruses such as ZIKV. These studies demonstrate that RVFV targets the foetal-maternal interface in both ovine and human placentas.

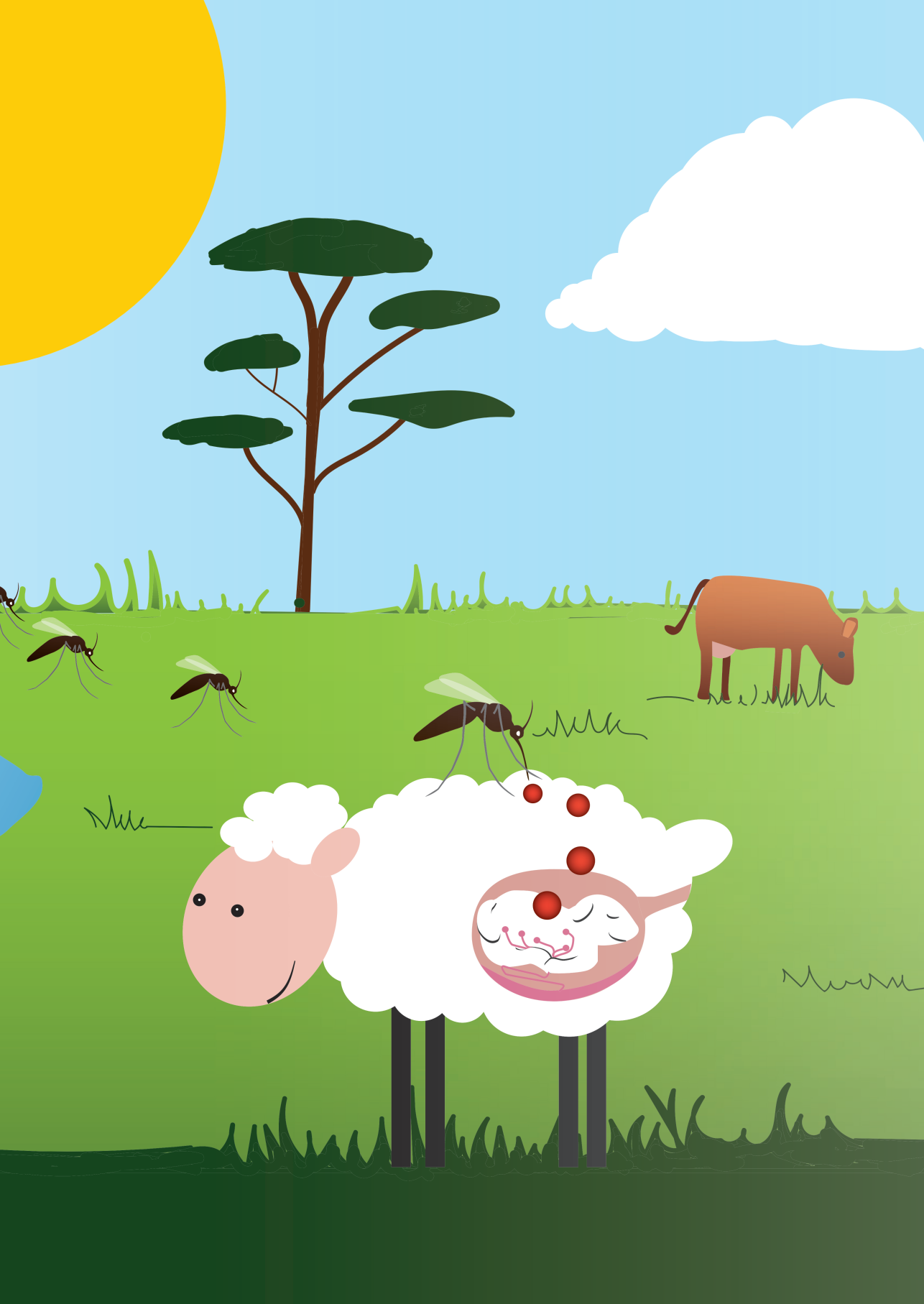
The most effective means to prevent RVFV-mediated abortions is through vaccination. Live-attenuated vaccines can provide long-lasting protection by a single vaccination and are therefore preferred to inactivated vaccines. Currently available live-attenuated vaccines, however, may transmit to the ovine foetus, resulting in stillbirths, congenital malformations or abortion. We have previously reported the development of a novel live-attenuated RVFV vaccine that was shown to be completely safe for pregnant ewes, even after application of an overdose. This novel vaccine virus, named vRVFV-4s, was developed by splitting the M genome segment and deleting the major virulence determinant NSs. The vaccine was shown to provide complete protection after a single vaccination of lambs, goats and cattle. Chapter 3 of this Thesis reports the efficacy of the vRVFV-4s vaccine in pregnant ewes. Both a single vaccination and a double vaccination were evaluated in two independent experiments, anticipating the extremely high susceptibility of pregnant ewes for the virus. A single vaccination with vRVFV-4s was demonstrated to be sufficient to protect both pregnant ewes and to prevent vertical transmission to the ovine foetus.

An infection with the Wesselsbron virus (WSLV), a flavivirus endemic to the African continent, causes similar clinical signs as RVFV infection and is therefore included in the differential diagnosis of RVF. WSLV is primarily spread by mosquito vectors belonging to the *Aedes* genus and mostly affects sheep and goats. Other animals, such as cattle, camelids and horses are also susceptible. WSLV is a zoonotic virus, as human cases were reported in several countries. In humans, WSLV infection causes fever, headaches, myalgia and arthralgia. Most reported cases, however, were the result of a laboratory-acquired infection. Nevertheless, prevalence in humans is likely underestimated as WSLV undergoes little to no surveillance in hospitals. Similar to RVFV, susceptibility of animals decreases with age. In young sheep, WSLV is lethal in up to 30% of the cases, whereas adult sheep recover after a mild febrile illness. WSLV infection of pregnant animals results in congenital malformations or abortion. The pathogenesis leading to congenital malformations, abortion and stillbirths remains unknown, despite the availability of reports on the gross pathology of WSLV infection of pregnant ewes. In Chapter 4 of this Thesis, we report a study in which pregnant ewes were infected with WSLV and 8 days later subjected to detailed pathology by PCR and IHC. The virus was mainly detected in foetal trophoblasts of the placenta. Interestingly, high viral RNA levels were detected in foetal brains and IHC revealed WSLV in neural progenitor cells, differentiated neurons, oligodendrocytes, microglia and astrocytes. Our study demonstrates that WSLV efficiently crosses the maternal–foetal interface and is highly neuroinvasive in the ovine foetus.

Shuni virus (SHUV) is a neglected orthobunyavirus that was discovered in the 1960s in Nigeria and has since then been detected in South Africa, Zimbabwe and Israel. The virus was isolated from several species of field-collected biting midges and mosquitoes. Additionally it was shown to disseminate in laboratory-reared biting midges, suggesting that members of the families *Culicidae* and *Ceratopogonidae* may function as vectors. In horses, ruminants and several wildlife species SHUV infection leads to severe neurological disease. Importantly, SHUV infection of ruminants is associated with congenital malformations and occasionally stillbirth or abortion. The detection of antibodies in human sera suggests that the virus may have zoonotic potential. To understand how SHUV crosses the ruminant placenta, pregnant ewes were experimentally infected and subjected to detailed clinical- and histopathological examination. We found that SHUV, like WSLV and RVFV, targets maternal epithelial cells and foetal trophoblasts. In Chapter 5, we show that SHUV infects syncytiotrophoblasts of human term placental explants, warranting further research into the potential risk of SHUV infection during human pregnancies.

SHUV, like all bunyaviruses, has a three-segmented genome. RNA viruses with segmented genomes are known for their ability to occasionally reassort their genome segments with related viruses. Reassortment events can create new viruses with a potentially different tropism and/or virulence. In Chapter 6, we report the development of a reverse genetics system to investigate the ability of SHUV to reassort with Schmallenberg virus (SBV), a related orthobunyavirus that is endemic to Europe. Rescue of a reassortant containing the L and S segments of SBV and the M segment of SHUV was demonstrated, but failure to rescue alternative reassortants suggests the risk of reassortment in nature is low. We additionally used our reverse genetics system to explore the role of the SHUV NSs protein, the major virulence factor of closely related orthobunyaviruses. Experiments with recombinant SHUV and a corresponding NSs mutant suggested that the NSs protein of SHUV functions as an antagonist of host innate immune responses. This novel reverse genetics system can now be used to study SHUV virulence factors and virus-host interactions, and to elucidate the molecular mechanisms that drive reassortment events.

In conclusion, the research reported in this Thesis contributes to our knowledge on the transmission routes of arboviruses across the placental barrier by the identification of potential cell and tissue targets in ovine and human placentas. Additionally, important tools were developed to study these viruses that can be further employed in the future by us and others to study virus-host interactions.



Samenvatting

Samenvatting

In 2015 kreeg een relatief onbekend virus, het Zika virus, grote naamsbekendheid door een uitbraak in Brazilië. Het virus werd gelinkt aan kinderen die geboren werden met één of meerdere afwijkingen. Een veel voorkomende afwijking is een kleiner hoofd, ook wel microcefalie genoemd. Daarnaast werd er bij sommige mensen die geïnfecteerd waren met Zika een andere afwijking gedetecteerd, het Guillan-Barré syndroom, een afwijking aan het zenuwstelsel. Ondanks dat het Zika virus al in 1947 ontdekt is, waren er tot 2015 maar weinig humane gevallen bekend. Op dit moment wordt het aantal humane gevallen echter geschat tussen de 500,000 en 1,5 miljoen. Daarbovenop zijn duizenden baby's met een geboorteafwijking als gevolg van Zika geboren. Het Zika virus is een arbovirus, wat betekent dat het via geleedpotigen (arthropods in het Engels) wordt overgedragen, in dit geval door muggen. Daarnaast is het ook een zoönotisch virus, wat wil zeggen dat het overgedragen kan worden van dier op mens. Het is daarbij ook in staat om ziekte te veroorzaken in zowel mens als dier. Deze uitbraak wees ons op een kennistekort op het gebied van virussen die verticale transmissie (transmissie van moeder op foetus) veroorzaken en de onderliggende mechanismes die verticale transmissie mogelijk maken. Het is heel belangrijk dat dit kennistekort verminderd wordt, en dat kunnen we doen door virussen die "verwaarloosd" zijn in het onderzoek, maar die net als het Zika virus de mogelijkheid hebben om verticaal te kunnen worden overdragen te bestuderen. Meer onderzoek naar deze virussen zorgt ervoor dat we potentiële bedreigingen beter kunnen identificeren en prioriteren waardoor we in de toekomst beter voorbereid zijn op soortgelijke uitbraken. Het karakteriseren van de mechanismes die verticale transmissie mogelijk maken kan daarnaast het ontwikkelen van effectieve bestrijdingsmethodes zoals antivirale middelen en vaccins faciliteren. In dit proefschrift heb ik daarom onderzoek gedaan naar de onderliggende mechanismes die leiden tot verticale transmissie van verwaarloosde arbovirussen van verschillende families die zoönotisch of potentieel zoönotisch zijn. De vraag hoe de virussen de placenta oversteken staat hierbij centraal.

Eén van deze virussen is het Rift Valley fever virus (RVFV), een phlebovirus behorende tot de orde *Bunyavirales*. Het is ontdekt in 1931 in Kenia en sindsdien worden uitbraken gerapporteerd in het hele Afrikaanse continent. In het jaar 2000 werd RVFV voor het eerst buiten Afrika gedetecteerd, namelijk in het Arabisch schiereiland. Dit virus kan ziekte veroorzaken in verschillende wilde en tamme herkauwers, kamelen en in de mens. Schapen zijn het meest gevoelig voor RVFV infectie. Pasgeboren lammetjes overleven een infectie zelden en in volwassen dieren is de mortaliteit rond de 30%. Het meest karakteristieke aan een RVFV uitbraak in kuddes schapen is echter de zogenaamde abortus storm. Hierbij treedt er abortus op bij een groot deel van, zo niet alle, drachtige dieren op bijna hetzelfde moment. Deze dramatische uitkomst van een RVFV uitbraak wordt al beschreven sinds de jaren 30, maar de onderliggende mechanismes die tot abortus leiden zijn nog niet beschreven. Daarom wordt in hoofdstuk 2 van dit proefschrift de pathologie onderliggend aan abortus bestudeerd in drachtige ooien. Drachtige ooien op een derde of de helft van

de dracht werden geïnfecteerd met RVFV en vervolgens werden de ooien op verschillende tijden opgeofferd. Verschillende organen werden geanalyseerd met PCR, virus isolatie en immuunhistochemie (IHC) om de primaire doelwitten van het virus te identificeren. Hierbij werd gevonden dat RVFV op 2 verschillende manieren de placenta kan oversteken. Bij de eerste manier infecteert RVFV verschillende cellen van de schapen placenta aan zowel de maternale, als foetale kant van de placenta. Vooral de maternale epitheel cellen bleken erg gevoelig voor RVFV infectie wat heeft geleid tot veel cel schade in deze cellen. Daarnaast kan het virus via gespecialiseerde zones in de placenta, zogenaamde haemofage zones, het maternaal epitheel omzeilen om direct de foetale kant van de placenta te infecteren. Abortus wordt waarschijnlijk veroorzaakt door de ernstige schade aan de placenta in de vorm van necrose en zware bloedingen. Daarnaast is er steeds meer bewijs dat RVFV ook een miskraam kan veroorzaken in zwangere vrouwen. Om hier meer kennis over te krijgen hebben wij humane placentale explantaten geïnfecteerd met RVFV en geanalyseerd met PCR en IHC. Hieruit blijkt dat RVFV heel efficiënt kan repliceren in cytotrofoblasten en syncytiotrofoblasten, twee celtypes uit de foetale kant van de humane placenta. Dit is een hele interessante bevinding aangezien syncytiotrofoblasten vaak resistent zijn tegen virus infecties, bijvoorbeeld tegen Zika virus infectie. Deze studies laten zien dat de cellen die de scheiding vormen tussen de maternale en foetale kant van de placenta geïnfecteerd kunnen worden door RVFV in zowel de schapen als mensen.

De meest effectieve manier om te voorkomen dat schapen geïnfecteerd worden met RVFV is door ze te vaccineren. Levend-verzwakte vaccins zorgen voor een langdurige bescherming tegen het virus na 1 vaccinatie, en hebben daardoor de voorkeur boven geïnactiveerde vaccins. Helaas zijn de levend-verzwakte vaccins die op dit moment op de markt zijn niet veilig voor drachtige ooien, aangezien deze vaccins kunnen leiden tot geboorteafwijkingen, abortus of doodgeboren lammeren. In eerdere publicaties is beschreven hoe een nieuw vaccin is ontwikkeld in ons lab, genaamd vRVFV-4s. Dit vaccin is ontwikkeld door het middelste genoom segment van RVFV te splitsen in 2 delen, waardoor in totaal 4 genoom segmenten ontstaan. Daarnaast is het eiwit wat voor virulentie zorgt verwijderd. Er is ontdekt dat het toedienen van een hoge dosis van dit vaccin veilig is voor drachtige ooien en ook dat het vaccin goed beschermt na een enkele vaccinatie in lammeren, kalveren en jonge geiten. In hoofdstuk 3 van dit proefschrift wordt gekeken of dit vaccin ook drachtige ooien beschermt door zowel eenmalige als tweemalige vaccinaties te evalueren. Twee keer vaccineren wordt hier ook meegenomen omdat drachtige ooien zeer gevoelig zijn voor infectie met RVFV. Uit de resultaten is gebleken dat ook in drachtige ooien één keer vaccineren voldoende is om transmissie van de moeder naar de foetus te voorkomen.

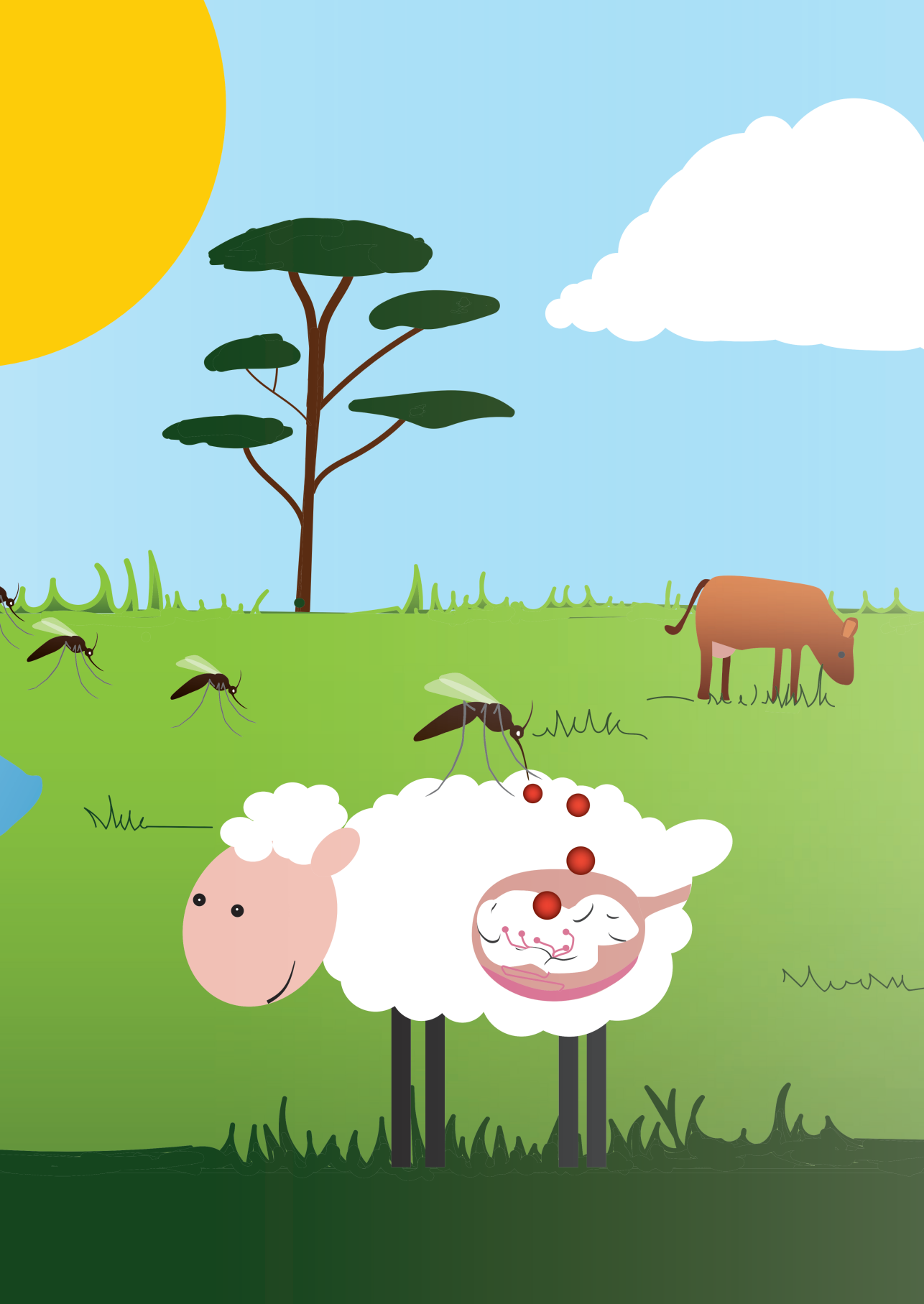
Een ander arbovirus dat voorkomt op het Afrikaanse continent is het Wesselsbron virus (WSLV). Het is een flavivirus dat vergelijkbare klinische symptomen veroorzaakt als RVFV infectie. WSLV wordt voornamelijk verspreid door muggen behorende tot het genus *Aedes* en het veroorzaakt voornamelijk ziekte in schapen en geiten. Andere dieren,

zoals runderen, kamelen en paarden, zijn ook vatbaar voor infectie. Daarnaast is WSLV een zoönotisch virus, er zijn namelijk verschillende humane gevallen geregistreerd in verschillende landen. In de mens leidt WSLV infectie tot koorts, hoofdpijn, spierpijn en pijn in de gewrichten. De meeste humane infecties zijn echter ontstaan door incidenten op een lab. Desalniettemin wordt de prevalentie van WSLV in de mens waarschijnlijk onderschat aangezien er weinig tot geen diagnostiek in ziekenhuizen aanwezig is om WSLV te detecteren. Net als voor RVFV neemt de gevoeligheid voor het virus af naarmate dieren ouder worden. In jonge schapen kan de mortaliteit oplopen tot 30%, terwijl volwassen schapen vaak alleen last hebben van milde koorts. WSLV infectie in drachtige dieren veroorzaakt geboorteafwijkingen, miskramen of doodgeboren lammeren. De pathogenese die leidt tot deze uitkomsten is nog onbekend, ondanks de grote hoeveelheid aan pathologische informatie die beschikbaar is over de uitkomst van WSLV infectie in drachtige schapen. In hoofdstuk 4 van dit proefschrift rapporteren wij over een studie waarin drachtige ooien geïnfecteerd werden met het WSLV virus om na 8 dagen een gedetailleerde pathologische studie uit te voeren door middel van analyses via PCR en IHC. We hebben hierbij gevonden dat WSLV vooral foetale trofoblasten infecteert in de placenta. Daarnaast werd er veel virus gedetecteerd in de foetale hersenen en IHC analyse liet zien dat WSLV zich door de hele hersenen van de foetus verspreidt. Hierbij werden verschillende hersencellen geïnfecteerd, waaronder neurale voorloper cellen, gedifferentieerde neuronen, oligodendrocyten, microglia en astrocyten. Deze studie laat dus zien dat WSLV heel efficiënt de placenta kan oversteken en zeer invasief is voor het foetale brein.

Naast WSLV en RVFV is ook het Shuni virus (SHUV) bestudeerd in dit proefschrift. Dit is een verwaarloosd orthobunyavirus dat al in de jaren 60 ontdekt werd in Nigeria. Sindsdien is het gedetecteerd in Zuid-Afrika, Zimbabwe en Israël, maar waarschijnlijk zijn er meer landen waar dit virus voorkomt. Het virus is geïsoleerd uit diverse knutten en muggen uit het veld en het is aangetoond dat 2 kolonies lab gegroeide knutten ook SHUV kunnen verspreiden. In paarden, herkauwers, en verschillende wilde dieren (waaronder giraffen, neushoorns en krokodillen) leidt infectie met SHUV tot ernstige neurologische symptomen. Daarnaast leidt infectie in drachtige herkauwers tot geboorteafwijkingen en soms ook tot miskramen of doodgeboorte. Ook SHUV is mogelijk een zoönotisch virus, aangezien antilichamen zijn gevonden in dierenartsen in Zuid-Afrika. Om meer te weten over de interactie van SHUV met zowel de schapen placenta als de humane placenta is de studie gedaan die beschreven is in hoofdstuk 5. Drachtige ooien werden geïnfecteerd en na 7 dagen werd er uitgebreid klinisch en histopathologisch onderzoek gedaan. Hier werd gevonden dat SHUV ook maternale epitheelcellen en foetale trofoblasten kan infecteren in de schapen placenta. Daarnaast laten we zien dat SHUV net als RVFV syncytiotrofoblasten kan infecteren in humane placentale explantaten. Deze bevindingen geven aan dat er meer informatie nodig is om het potentiele risico van SHUV in de mens, en met name in zwangere vrouwen, te bepalen.

Zoals alle bunyavirussen heeft ook SHUV een genoom dat uit 3 segmenten bestaat, een groot (L), middel (M) en klein (S) segment. Het is bekend van RNA virussen met gesegmenteerde genomen dat zij hun genoom segmenten kunnen uitwisselen met gerelateerde virussen. Het uitwisselen van genoom segmenten kan er voor zorgen dat nieuwe virussen ontstaan die mogelijk andere soorten kunnen infecteren en ook gevaarlijker kunnen zijn doordat ze een ernstiger ziektebeeld kunnen veroorzaken. In hoofdstuk 6 bekijken we de mogelijkheid van SHUV om een nieuw virus te vormen met het gerelateerde virus Schmallenberg. Schmallenberg virus (SBV) is een veterinair virus dat in Europa voorkomt en ook in drachtige herkauwers ernstige geboorteafwijkingen veroorzaakt. Om dit aan te tonen maken wij gebruik van een zogenoemd Reverse Genetics systeem. Dit hebben wij ontwikkeld voor zowel SBV als SHUV en hiermee kunnen deze virussen geproduceerd worden in het lab. Simplistisch gezegd doen we dit door de verschillende genoomsegmenten aan cellen toe te voegen. Wij hebben laten zien dat een virus kan ontstaan met het L en S segment van SHUV en het M segment van SBV. Andere combinaties bleken niet mogelijk, wat aangeeft dat de kans dat dit in de natuur spontaan zou gebeuren erg klein is. Daarnaast hebben wij het Reverse Genetics systeem gebruikt om de rol van één eiwit van SHUV te bestuderen. Het NSs eiwit is namelijk een belangrijk eiwit wat tegen het aangeboren immuun systeem van cellen werkt. Dit is al bewezen voor NSs eiwitten van andere gerelateerde virussen en dit hebben wij ook kunnen laten zien voor SHUV. Dit systeem kan in de toekomst gebruikt worden om verdere studies te doen aan SHUV en de interactie met host cellen en om de moleculaire mechanismes die voor het uitwisselen van genoom segmenten zorgen te onderzoeken.

Tot slot, het onderzoek in dit proefschrift draagt bij aan de kennis over transmissie routes van arbovirussen over de placenta door de identificatie van cellen en weefsels die gericht worden geïnfecteerd in zowel schapen als humane placenta's. Daarnaast zijn belangrijke methodes en technieken ontwikkeld om deze verwaarloosde virussen beter te bestuderen. Deze kunnen in de toekomst bijdragen aan zowel de diagnostiek van deze virussen in ziekenhuizen als in vervolgonderzoek.



Appendices

Dankwoord

Eind mei 2016 had ik nog nooit van het Centraal Veterinair Instituut (CVI, nu WBVR) gehoord en ongeveer een maand later in juli begon ik daar met veel plezier aan het PhD traject wat geresulteerd heeft in dit mooie boekje. Ondanks dat het in het begin een beetje wennen was, een instituut is toch echt wel anders dan een universiteit, kijk ik met veel plezier terug op de afgelopen 4,5 jaar! Er is een heel leger aan mensen nodig om een proefschrift succesvol af te ronden en gelukkig had ik een hele hoop fijne mensen in de buurt die daaraan hebben bijgedragen! Iedereen heel erg bedankt daarvoor! Natuurlijk wil ik ook een aantal mensen in het bijzonder bedanken, het dankwoord is niet voor niets het populairste en meest gelezen stuk uit een proefschrift.

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ik het zwaar had met het PhD leven zorgde je voor een wandeling buiten voor advies en een luisterend oor, zelfs in de regen. **Rianka**, los van alle hulp die je me hebt gegeven op het lab was je ook een uitstekend sport maatje! Je hebt ervoor gezorgd dat ik weer regelmatig ging sporten. Naast het bootcampen waren we heel goed in lekkere recepten koken en hebben we samen de break-out run overleefd waar we heel erg trots op mogen zijn! **Mirriam**, wat was het gezellig dat je mijn kamergenootje werd na de verhuizing! Helaas was je het laatste jaar wat vaker "binnen", maar dan moesten we maar extra lang bijkletsen als je er wel was. En als we elkaar misliepen hing er soms een vrolijke post-it aan mijn scherm om me bijvoorbeeld te feliciteren met de Venloop. **Jet**, jij bent een echte vakrot. Dankzij jouw ervaring kon ik heel veel dingen veel efficiënter uitvoeren. Daarnaast waren jouw zelfgemaakte Brabantse worstenbroodjes mijn favoriete traktatie van het jaar. **Erick**, it was so nice to have you back for your PhD at the institute! It was always great to have lunch breaks together and talk about travelling or surviving PhDs. I'm sure you will do extremely well and will write an amazing thesis, with beautiful pictures of course! Good luck! **José**, wat leuk dat jij onze groep kwam versterken! Dankje voor je wandeltips en gezelligheid. **Nadia,** jij bent een geweldig voorbeeld van een powervrouw. Je doet goed onderzoek, weet wat je wilt en bent niet bang om dat te laten merken en dat is inspirerend. **Jan**, ook al ben je niet vast onderdeel van de arbogroep, wij hebben wel een periode samengewerkt aan mijn project wat heel erg prettig was! En blijf vooral foto's posten van lekker eten.

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Ik begon mijn PhD op vleugel 16 op de edelhertweg, maar na een jaar gingen we naar het mooie nieuwe gebouw 2.15. Naast dat de labs daar allemaal nieuw en schoon en opgeruimd waren, kwamen er ook nog eens heel veel mensen bij elkaar die elkaar normaal niet zagen en dat was heel erg leuk. Zo kreeg ik een nieuw kamergenootje en wat was dat goed gematcht! **Renate**, ik ben echt heel erg blij dat wij bij elkaar op de kamer ingedeeld werden. Net als Mirriam hielden we niet van een opgeruimd bureau, en wel van leuke versiering op de kamer, met als hoogtepunt natuurlijk de Jamie poster! Niet alleen konden we eindeloos kletsen, we konden elkaar ook heel erg goed helpen bij ons werk. Sorry dat je hulplijn nu weg is! **Alle bewoners van 2.15,** en in het bijzonder **Judith, Astrid, Bregtje, Frank, Stephanié** en **Annemieke,** bedankt voor de gezelligheid tijdens de koffiepauzes, lunchpauzes, alle taart en de tosti's. **Conny**, heel erg bedankt voor het organiseren van de leuke PV activiteiten! **Eline**, jij bent een maestro in het plannen van leuke escaperooms door heel Nederland! Gelukkig hebben we nog heel veel plannen die we kunnen gaan uitvoeren als de wereld weer normaal is. En je bent ook een geweldige hulp geweest bij

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PE&RC was een hele fijne graduate school om bij te zitten. Ik vond het erg leuk om deel uit te maken van de PhD council (PPC) en evenementen te organiseren. **Lennart, Claudius** en **Amber** bedankt voor het organiseren van o.a. de PhD weekenden. Die hebben mij erg geholpen! **Jeroen** en **Naomi,** not only were we together part of the PPC, we also did all the weekends together which I really enjoyed!

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Toen ik aan mijn bachelor stage begon bij Virologie in Utrecht kwam ik in de leukste studentenkamer terecht. Ex-viro's **Marlies, Jimmy, Anne, Melle, Armando** en **Wouter**, ook al voelt het heel raar om weer terug op de afdeling te zijn zonder jullie, ik vind het echt superleuk dat we nog steeds samen leuke dingen doen en hoop dat we dat nog een hele tijd blijven doen!

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About the author

Judith Oymans was born on December 17th 1991 in Venlo, the Netherlands, where she attended primary school (St. Willibrordus school) and graduated bilingual VWO (English) at College Den Hulster. In 2010 Judith went to Utrecht University for the bachelor's programme Biomedical Sciences. Here she developed an interest for viruses, as her first passioned choice for the curriculum was the Virology class by Dr. Raoul de Groot, which she took with great interest. To pursue this interest further she finished her bachelor's in 2014 with a thesis at the lab of Prof. Dr. Dr. Frank van Kuppeveld under supervision of Dr. Mark Bakkers and Dr. Raoul de Groot, where she studied the dynamic interaction of the hemagglutinin esterase (HE) protein of lineage A betacoronaviruses with sialic acids. This thesis was then extended with an 11-month internship during her master's programme Molecular and Cellular Life Sciences. After that she wrote a literature thesis at Radboud Institute for Molecular Life Sciences in Nijmegen under supervision of Dr. Ronald van Rij titled "The Piwi-associated RNA pathway and antiviral response in insects". For her final internship Judith went to Oxford University where she performed a 7-month internship at the lab of Prof. Dr. Ervin Fodor under supervision of Dr. Aartjan te Velthuis. There, she studied the function of the priming loop of the influenza RNA dependent RNA polymerase in replication initiation, elongation and realignment. After graduating with a Master of Science degree in 2016, Judith started a PhD at Wageningen University and Research that was mostly executed at Wageningen Bioveterinary Research in Lelystad, the Netherlands, under supervision of Prof. Dr. Jeroen Kortekaas, Dr. Paul Wichgers Schreur and Dr. Gorben Pijlman. This thesis is the result of this PhD, focusing on vertical transmission of arboviruses in mammals. Her research led to various publications and was presented at several scientific meetings. During the major part of her PhD, Judith joined the PE&RC PhD council at Wageningen University and Research, where she participated in the social events committee and the PE&RC day committee. In this role, she organised the PE&RC day, the annual symposium for PhD candidates in 2018. Furthermore, in April 2018 she organised and hosted the annual Dutch Arbovirus Research Network (DARN) meeting in Lelystad together with Jeroen Kortekaas. In October 2020 she returned to the lab of Prof. Dr. Frank van Kuppeveld at Utrecht University where she participates in the Corona Accelerated R&D in Europe (CARE) project for her post-doctoral research.

List of publications

Oymans J, van Keulen L, Vermeulen GM, Wichgers Schreur PJ, Kortekaas J. Shuni Virus Replicates at the Maternal-Fetal Interface of the Ovine and Human Placenta. *Pathogens*. 2020 Dec 29;10(1):E17. doi: 10.3390/pathogens10010017. PMID: 33383649.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Vertical transmission of neglected arboviruses

Writing of project proposal (3ECTS)

- The role of mononuclear phagocytes in the vertical transmission of arboviruses

Post-graduate courses (3.9 ECTS)

- Basics in statistics; PE&RC, Wageningen, the Netherlands (2016)
- Immunology summer school; FEBS, Hvar, Croatia (2017)

Laboratory training and working visits (1.8 ECTS)

- Development of tools to study arbovirus infection in the central nervous system; University of Veterinary Medicine, Hanover, Germany (2017)
- Diagnostic tools to identify neglected arboviruses; University of Pretoria, South Africa (2019)

Invited review of (unpublished) journal manuscript (1 ECTS)

- Eurosurveillance: RVFV outbreak in Mayotte (2019)

Competence strengthening / skills courses (2.6 ECTS)

- Research integrity; WGS (2016)
- Brain training; WGS (2017)
- WUR PhD carousel; WGS (2017)
- Workshop on scientific communication; Epizone (2017)
- Reviewing a scientific paper; WGS (2018)
- Presenting with impact; WGS (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.7 ECTS)

- PE&RC Weekend for first years (2016)
- PE&RC Day (2016, 2018)
- PE&RC Weekend for mid years (2018)
- PE&RC Weekend for last years (2020)

Discussion groups / local seminars / other scientific meetings (5.4 ECTS)

- Virology meetings; WBVR (2016-2020)
- Dutch annual symposium; Virology (2017-2019)
- PhD Discussion group; WBVR (2018-2020)

International symposia, workshops and conferences (4.7 ECTS)

- Negative strand virus; oral presentation; Verona, Italy (2018)
- Dutch arbovirology network meeting; oral presentation in 2018; the Netherlands (2017-2020)

Lecturing / supervision of practicals / tutorials (3 ECTS)

- Molecular virology (2016-2019)
- Cell biology and health (2017-2019)

Supervision of MSc students (3 ECTS)

- SHUV and SBV NSs protein
- SHUV and SBV NSs protein
- Interaction between arboviruses and human placentas

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