Universitat Autònoma de Barcelona

Sheep experimental model for Rift Valley Fever Virus Infection for the study of immunopathogenesis, pathology and vaccinology

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Thesis

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Tesi doctoral presentada per Iván José Galindo Cardiel per accedir al grau de Doctor en Veterinària dins del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. Mariano Domingo Álvarez.

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El Dr. **MARIANO DOMINGO ÁLVAREZ**, Catedràtic d'Universitat del Departament de Sanitat i Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit al CReSA,

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Dr. Mariano Domingo Álvarez	Iván José Galindo Cardiel
Director	Doctorand

Para Raquel, Paula y Martina, mis razones para luchar día a día.

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Summary

Rift Valley Fever Virus (RVFV) is a mosquito-borne zoonotic phlebovirus that primarily affects ruminants by causing abortions and acute hepatitis with multifocal necrosis as major findings. Human RVF symptoms range from flu-like syndrome to retinitis and encephalitis. The increasing interest in RVFV and its potential impact on naive animal populations deserve revisiting experimental reproduction of RVFV infection, particularly in those animal breeds for which no data about their susceptibility to RVFV infection have ever been recorded. In this thesis, we show the susceptibility of 9–10 weeks old European sheep (Ripollesa breed) to RVFV infection, showing a mild, subacute form of disease. Four different viral isolates from field outbreaks efficiently replicated *in vivo* after subcutaneous experimental inoculation, and consistent viral loads in blood and virus shedding (variable in length depending on the RVFV isolate used) were detected, showing horizontal transmission to a noninfected, sentinel lamb. RVFV infection caused transient pyrexia in old lambs and no other clinical symptoms were observed, with the exception of corneal opacity ("blue eye") found in 3 out of 16 subcutaneously inoculated sheep. In order to better characterize this corneal opacity, in a preliminary approach, formalin-fixed and paraffin wax-embedded tissue from these ocular condition-affected animals was investigated by histopathology and quantitative real time reverse transcriptase polymerase chain reaction. Anterior uveitis with lymphoplasmacytic endotheliitis was diagnosed in these four RVFV-infected lambs. In order to evaluate the protection conferred by a single subcutaneous dose of a modified vaccinia virus Ankara (MVA) vectored vaccine encoding the Rift Valley Fever virus (RVFV) glycoproteins Gn and Gc in lambs, three groups of six to seven Ripollesa lambs of 5–7 weeks old were immunized as follows: one group received the vaccine (termed rMVA-GnGc), a second group received an MVA vector (vector control) and a third group received saline solution (non-vaccinated control). Fourteen days later, all animals were subcutaneously challenged with 10^5 $TCID_{50}$ of the virulent RVFV isolate 56/74 and vaccine efficacy assessed using standard endpoints. Two lambs (one from the vaccine group and one from the vector control group) succumbed to RVFV challenge, showing characteristic liver lesions. Lambs from both the vector control and non-vaccinated groups were febrile from days 2 to 5 post challenge (pc) while those in the rMVA-GnGc group showed a single peak of pyrexia at day 3 pc. RVFV RNA was detected in both nasal and oral swabs from days 3 to 7 pc in some lambs from the vector control and non-vaccinated groups, but no viral shedding could be detected in the surviving lambs vaccinated with rMVA-GnGc. To finish this manuscript, we characterize pathologically the new ocular detected condition in a secondary approach. Two groups of five lambs per group were selected (n=10) from the historical database of RVFV experimental infections performed in Center of Research in Animal Health (CReSA) (northeast Spain, NBS3 facility) in the basis of their clinical data, viremia and diagnosed

ocular and hepatic lesions (two previous experiments). The previously diagnosed anterior uveitis (8 out of 10) with lymphoplasmacytic endotheliitis (2 out of 10) was characterized. CD3, CD20 and lysozyme-positive mononuclear inflammatory infiltrates were observed in RVFV-positive paraffin-embedded eyes. CD20 labelling was only observed in infiltrates in anterior uvea. A novel T-cell dependent retinitis was also diagnosed in 5 out of 10 RVFV-infected lambs based on CD3-positive labelling. An immunochemistry protocol based on a murine monoclonal antibody was developed at CReSA BLS2 facility. In conclusion, adult sheep from this European breed are readily infected with RVFV without apparent clinical manifestations. A 5-10 weeks old European sheep (Ripollesa breed) challenge model has proven to be effective in vaccine testing because of its susceptibility to virus. Taken the vaccine data together, it is suggested that a single dose of the rMVA-GnGc vaccine may be sufficient to reduce RVFV shedding and duration of viremia but does not provide sterile immunity nor protection from disease. To our knowledge, this is the first pathological description of this ocular manifestation of RVFV infection in ruminants, although these lesions have been described in man. To the authors´ knowledge, this is the first description of retinal injury in RVFV-challenge sheep model.

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1. Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne zoonotic pathogen that primarily affects domestic ruminants but can also cause a devastating disease in humans. Outbreaks of RVFV have extended from sub-Saharan Africa to Middle East, raising several concerns about the potential spreading of the virus to Europe, Asia and Americas. Animal models are basic to develop new effective vaccines and accurate diagnostic tools. Rodent, small ruminant and non-human primate infection models are currently used in experimental studies of virus pathogenesis, vaccinology and transmission. Even after assuming the huge scientific advance done in this field, it's a reality that only two unsafe attenuated vaccines have been approved for widespread veterinary use. Recent advances in vaccine design has enabled the development of more potent prophylactic measures to combat viral infections. However, every step towards this way have to be joint to a deep knowledge about the virus and its associated pathology. In this thesis, we summarize several aspects of RVFV with particular emphasis on understanding the molecular components of the virus, the epidemiology, the comparative pathogenesis and the complete immune response elicited by RVFV that may lead to a better vaccine development, essential tools in the fight against this disease. We discuss an ovine model developed in Center of Research in Animal Health (CReSA) which let us know about an old-know but recently described ocular lesion in the natural host of RVFV, sheep, responsible of the maintenance of horizontal transmission to humans.

1.1. Rift Valley Fever Virus: etiology and characterization

1.1.1. The virus

Rift Valley fever (RVF) virus is a mosquito-borne, zoonotic, negative-sense, single-stranded, tripartite RNA virus of the family *Bunyaviridae* within the genus *Phlebovirus* (Nichol *et al.*, 2005; OIE, 2009; Terasaki and Makino, 2015). RVFV resistance to physical and chemical action is summarized in Table 1. Only one serotype is recognized but strains exist of variable virulence (OIE, 2009).

Table 1. Resistance to physical and chemical action of the Rift Valley Fever Virus. Adapted from OIE 2009.

Factor	Action
Temperature	Virus recoverable from serum after several months at 4°C or 120 minutes at 56°C.
pH	Resistant in alkaline environments but inactivated at pH <6.8
Chemicals/ disinfectants	Inactivated by lipid solvents (i.e. ether, chloroform, sodium deoxycholate), low concentrations of formalin and by strong solutions of sodium or calcium hypochlorite (residual chlorine should exceed 5.000 ppm).
Survival	Survives in freeze dried form and aerosols at 23°C and 50–85% humidity. Virus maintained in the eggs of certain arthropod vectors during inter-epidemic periods. Can survive contact with 0.5% phenol at 4°C for 6 months.

The RVFV genome is comprised of three RNA segments named the small (S-) (ambisense), medium (M-) and large (L-) segments (negative polarity) (Giorgi *et al.*, 1991; Ikegami and Makino, 2011) (Figure 1). RVFV has three segments with cRNA sequences on the ends of each segment which forms a circular, "panhandled" secondary structure, very similar to other bunyaviruses (Hewlett *et al.*, 1977; Pettersson and von Bonsdorff, 1975; Ronnholm and Pettersson, 1987). The L segment encodes the viral RNA-dependent RNA-polymerase (RdRp) used for replication and mRNA transcription. The M segment encodes two co-translationally processed glycoproteins of viral surface (Gn and Gc) and a nonstructural protein that can be expressed by itself (Nsm1) or in fusion with Gn (NSm2). While both N and L proteins are essential for viral transcription and replication, both Gn and Gc associate with host cell membranes to constitute the viral envelope (Fig. 1). The antisense-oriented S segment expresses the nucleoprotein (N protein), while its complementary-oriented S segment encodes the nonstructural protein NSs. (Gerrard and Nichol, 2007). The especial orientation of S segment is a characteristic feature of *Phleboviruses*. The surface of the assembled RVFV particles measure 90 to 110 nm in diameter and possess a lipid-bilayered, enveloped virion (Ellis *et al.*, 1979). The viral particle forms an ordered icosahedral shell of 122 capsomers which are composed of subunits of Gn and Gc heterodimers (Huiskonen *et al.*, 2009). Internal ribonucleoprotein complexes are layered proximal to the inner envelope of the virion. This fact suggests a possible interaction between the glycoproteins and the ribonucleoproteins (RNPs).

Such as represented in Figure 1, Gn and Gc associate with host cell membranes to constitute the viral envelope whereas both N and L proteins are essential for viral transcription and replication. The NSs protein is expressed very early upon infection and can act as a repressor of host cell transcription and is involved in the induction of cell-cycle arrest by activation of DNA damage signaling checkpoint protein kinase ATM (Baer *et al.*, 2012). RVFV NSs-mediated strategies for the inhibition of host's innate immune responses are summarized in the table 2 (Lorenzo *et al.*, 2015).

1.1.2. Structural and functional characterization

1.1.2.1. Viral RNA dependent RNA polymerase (RdRp)

Large (L) protein is the largest viral protein and is expressed on the L segment of the RVFV genome. L protein function has been largely characterized thanks to recombinant expression technology (Lopez *et al.*, 1995; Zamoto-Niikura *et al.*, 2009). L protein is capable of mRNA transcription of purified, transfected RVFV RNP complexes and may also play a role in the replication of the viral genome (Lopez *et al.*, 1995). The polymerase activity may be mediated through an SDD motif, suggesting that functionality is correlated with oligomerization of the protein. The SDD motif mediates the polymerization of recombinant L protein as well (Zamoto-Niikura *et al.*, 2009). Bunyaviruses use as primers for viral mRNA transcription a 5´-capped RNA (“cap-snatching” mechanism), generated by utilizing the viral endonuclease activity associated with L protein. Alignment of secondary crystal structures of different bunyaviral L proteins (plus RVFV) has shown that the endonuclease domain is conserved (Reguera *et al.*, 2010).

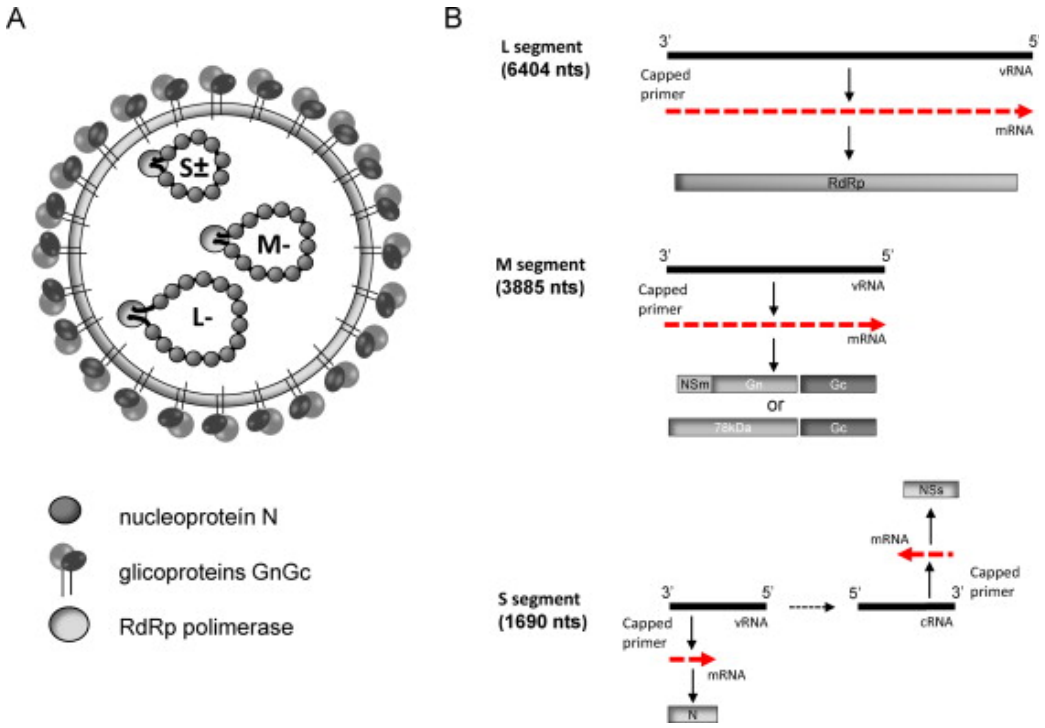


Figure 1. Organization of the RVFV viral particle and genome coding strategy. Printed from Lorenzo, López-Gil et al. 2015. (A) Schematic representation of the viral particle showing the viral glycoproteins Gn and Gc inserted into the virus envelope. Inside, the three genomic segments of single stranded RNA are shown forming complexes to both the viral RNA dependent RNA polymerase (RdRp) and the viral nucleoprotein N. The ± denotes that both genomic and antigenomic S segments can be encapsidated into the viral particle (Ikegami *et al.*, 2005b). Note the panhandle structures to depict the short stretches of complementary RNA at 5' and 3' ends of each RNA molecule. (B) Representation of each transcriptional unit of RVFV and translation strategies. The negative sense, vRNA, is transcribed after sequestration of short host's capped primers by the RdRp (which has endonucleolytic activity). The M segment is transcribed and can be translated into several mature polypeptides (the structural glycoproteins Gn and Gc and two non-structural proteins of 14 kDa (NSm) and 78 kDa) depending on the selective usage of five-in frame start codons. It has been suggested as a strategy for virus adaptation to both insect and mammalian hosts (in fact the 78 kDa protein has been found as a structural component of the viral particles when propagated in insect cell cultures). The S segment has a complex ambisense transcription strategy that includes the replication of vRNA to generate cRNA to act as a template for NSs transcription. The antigenomic S cRNA can be also packaged into viral particles (Ikegami *et al.*, 2005b) thus allowing earlier expression of NSs protein after infection.

1.1.2.2. Nucleoprotein (N)

RVFV nucleoproteins forms RNP complexes when associated with genomic viral RNA. Such RVFV RNPs are formed structurally by a nonhelical complex associate with glycoproteins to localize to the interior of the virion, unique among negative-sense RNA viruses (Raymond *et al.*, 2010). Native N seems not be directly involved in viral pathogenesis,

although it has been observed a partial degree of immune protection when recombinant N is analyzed (Lagerqvist *et al.*, 2009; Lorenzo *et al.*, 2010). However, recombinant N cannot elicit neutralizing antibodies. RVFV-immunized sheep and mice exhibit an increase the levels of proliferating memory cell pools and induction of IFN-gamma *in vitro* in the presence of recombinant N protein in recent studies. This fact suggests that this stronger level of immunity is mediated through the interactions of antigen-presenting cells (APCs) with T helper cells (Lagerqvist *et al.*, 2009; Lorenzo *et al.*, 2010; Lorenzo *et al.*, 2008).

1.1.2.3. Glycoproteins (Gn y Gc)

Both proteins in conjunction make up the heterodimeric unit of the viral surface. Gn is expressed near the N terminus of the M segment. Gc is located at the C-terminal end of the open reading frame (ORF) of the M segment. By using reverse genetics has been demonstrated that a single nucleotide substitution at nucleotide 847 of the M segment can determine the virulence phenotype of the RVFV ZH501 strain in mice (Morrill *et al.*, 2010). The coding region upstream from Gn (known as pre-Gn) plays a role in modulating virus-induced apoptosis as suggested by experiments using an attenuated strain of RVFV (MP-12) (Won *et al.*, 2007).

Table 2. RVFV NSs-mediated strategies for the inhibition of host's innate immune responses. Adapted from Lorenzo, López-Gil *et al*. 2015.

Action	Consequence	Reference(s)
Sequestration of p44 and degrading TFIIH p62 subunit and reducing expression of other protein subunits	Blocking type I IFN production. Disruption of host cell transcription	(Billecocq *et al.*, 2004; Bouloy *et al.*, 2001; Cyr *et al.*, 2015; Kalveram *et al.*, 2011; Le May *et al.*, 2004)
Enhanced replication in minigenome system	Regulation of viral RNA synthesis	(Ikegami *et al.*, 2005a)
Interaction with Sin3A-associated protein 30 (SAP30)	Repression of IFN-β transcription by maintaining the YY1 repressor complex	(Le May *et al.*, 2008)
Downregulation and degradation of PKR	Avoids phosphorylation of eIF2a for sustained translation	(Habjan *et al.*, 2009; Ikegami *et*

		al., 2009b; Kalveram *et al.*, 2013)
Chromatin deregulation	Binding to pericentromeric DNA repeats	(Mansuroglu *et al.*, 2010)
Interaction with several promoter regions	Modification of the expression of genes coding for coagulation factors	(Benferhat *et al.*, 2012)
Sequestration of nuclear PABP1	Avoids host mRNA polyadenylation favouring viral protein production	(Copeland *et al.*, 2013)
Recruiting of FBOX3	Proteasome degradation of the p62 subunit	(Kainulainen *et al.*, 2014)

Both recombinant Gc (Liu *et al.*, 2008) and Gn (de Boer *et al.*, 2010) can induce a complete immune protection from RVFV infection and form virus-like particles (VLPs) in mice. However, these VLPs were found to be highly pleiomorphic, suggesting that heterodimeric expression is required for homogenous expression of virion particle surfaces (Liu *et al.*, 2008). DNA vaccination studies using Gn have demonstrated that a neutralizing antibody response can be generated without coexpression of Gc in mice (Bhardwaj *et al.*, 2010).

Gn may ultimately be required for viral budding as well. This hypothesis is supported by the location of recombinant Gn protein into the Golgi complex via its Golgi localization signal, as opposed to retention of recombinant Gc in endoplasmic reticulum via its own expression (Gerrard and Nichol, 2002). Recombinant alphavirus replicon vector- derived glycoproteins can be exported to the cell surface in eukaryotic cell lines (Filone *et al.*, 2006). Interestingly, brief treatment with mildly acidic media has been shown to induce glycoprotein-mediated cell-to-cell fusion in a variety of cell types.

Viral entry mediated through glycoproteins may either (i) interact with a host receptor that is expressed in a wide variety of mammalian species, or (ii) not require a host-specific cellular receptor at all. However, different behaviors in viral entry has been shown by

analyzing another *Phlebovirus*. Uukuniemi virus-membrane fusion and its correspondent infection occur at low pH in only certain cell lines (Lozach *et al.*, 2010). Therefore, the exact mechanism of RVFV entry is not as yet clearly defined.

1.1.2.4. Nonstructural protein: M segment

The M segment encodes two co-translationally processed glycoproteins of viral surface (Gn and Gc) and a nonstructural protein that can be expressed by itself (Nsm1) or in fusion with Gn (NSm2) (Kakach *et al.*, 1988). Briefly, recombinant RVFV M segments expressed in a vaccinia virus platform encoded four proteins: Gn, Gc, and two proteins more initiated from ATG codons upstream from the Gn ORF with 14 kDa and 78 kDa respectively. In the case of the 78-kDa product, the translated product appeared to originate from the first coding ATG of the M segment and continue through the entire Gn-coding sequence. Current nomenclature designates the 78-kDa product as Nsm1, while the 14-kDa polypeptide has been designated NSm2 (Gerrard and Nichol, 2007).

Weingartl *et al.* suggest that the 78 kDa (Msm1) protein may be incorporated in the viral particles when the virus in propagated in mosquito c6/36 cells, highlighting the ability of the virus to infect mammalian cells upon mosquito bites (Weingartl *et al.*, 2014c). The 14 kDa (NSm) protein has demonstrated to play a role in the suppression of apoptosis in infected cells (Won *et al.*, 2007) and to associate with mitochondrial outer membranes (MOM) by meaning of the C-terminal region of the protein (Terasaki *et al.*, 2013). Deletion of NSm resulted in the reduced ability of RVFV to enter, replicate, and disseminate from the midgut epithelial cells in mosquitoes. Therefore, NSm appears to have a functional role in the vector competence of mosquitoes for RVFV at the level of the midgut barrier (Kading *et al.*, 2014). Nsm has demonstrated and interaction with several murine proteins, including the cleavage and polyadenylation specificity factor subunit 2 (Cpsf2), the peptidyl-prolyl cis–trans isomerase (cyclophilin)-like 2 protein (Ppil2) and the 25 kDa synaptosome-associated protein (SNAP-25) (Engdahl *et al.*, 2012). Moreover, it has been demonstrated that M segments lacking a portion of the 5’ UTR prevented efficient viral copackaging with both L and S segments, suggesting an active role for cis-acting RNA signals in this process of the pathogenesis (Terasaki *et al.*, 2011). However, NSm-deficient virulent RVFV strain (ZH-501) does not seem to affect virulence or lethality in experimental infections in rats (Gerrard *et al.*, 2007). Therefore, the exact role of NSm in RVFV pathogenesis is still inconclusive.

1.1.2.5. Nonstructural protein: S segment

The S segment of all pathogenic RVFV strains direct the synthesis of a NSs which has an undefined accessory function (Bouloy *et al.*, 2001). Recent evidences have shown that overexpression of the NS_S gene, but not the N, G_N or NS_M genes, correlated with mRNA nuclear accumulation (defect in nuclear mRNA export) in RVFV-infected cells (Copeland *et al.*, 2015). Transcription inhibition assays of RVFV-infected cells have demonstrated that nonstructural phosphoprotein (NSs) is expressed early during viral infection, although it should theoretically be expressed later than other viral components due to its antiviral-sense orientation (Figure 1) (Ikegami *et al.*, 2005b). The reason may be related to the small proportion of antiviral-sense S RNA packaged in the viral particles, for what is provided some initial inhibition of alpha/beta interferon (INF-α/β)-mediated responses. Moreover, NSs has been extensively characterized confirming an antagonistic activity against the IFN antiviral response (Bouloy *et al.*, 2001).

This inhibitory activity appears to be mediated through multiple mechanisms. One such mechanism involves the competitive inhibition of formation of the transcription factor TFIIH (Le May *et al.*, 2004). Le May *et al*. have demonstrated that NSs interacts with p44, a subunit of the transcription factor TFIIH, leading to a competitive inhibition of XPD, another component of TFIIH (Le May *et al.*, 2004). TFIIH transcriptional inhibition may play an important role in the suppression of the host antiviral response. Besides, a key component of RVFV virulence is its ability to form nuclear filaments through interactions between the viral nonstructural protein NSs and the host general transcription factor TFIIH. Cyr *et al*. have demonstrated that p62 (a subunit of TFIIH) binding to NSs through the ΩXaV motif is essential for degrading p62, forming nuclear filaments and enhancing RVFV virulence (Cyr *et al.*, 2015). Transcriptional suppression may also be done through its interaction with SAP30 (Le May *et al.*, 2008), thereby interfering with IFN-β expression. NSs can decrease cellular antiviral responses by disrupting the double-stranded RNA-dependent protein kinase R (PKR) activity (Habjan *et al.*, 2009; Ikegami *et al.*, 2009a). These mechanisms enable RVFV to infect the host cell while limiting IFN-mediated host antiviral responses.

1.1.2.6. Final comments about nonstructural proteins (NSm and NSs)

The induction of chromosome cohesion and segregation defects by the interaction of filamentous-formed NSs in the nucleus with some specific DNA regions of the host

genome could be related to RVFV pathology (Mansuroglu *et al.*, 2010). However, both NSm and NSs proteins are not essential for virus replication and propagation in cell cultures. Conversely, NSs-deficient mutants have demonstrated a more attenuated, avirulent phenotype than wild-type strains and could serve as potential attenuated vaccine candidates (Muller *et al.*, 1995).

1.2. Epidemiology

Rift Valley fever virus (RVFV) is an arthropod-borne pathogen that primarily affects ruminants in eastern and sub-Saharan Africa (Bird *et al.*, 2009; Faye *et al.*, 2007). Susceptibility of different breeds to RVFV varies considerably (OIE, 2009). The disease usually presents in an epizootic form over large areas of a country following heavy rains and sustained flooding, and is characterized by high rates of abortion and neonatal mortality, primarily in sheep, goats and cattle (OIE, 2009).

1.2.1. Host

The natural hosts of the RVFV are cited in the table 3 along with a selection of relevant references.

1.2.2. Transmission

RVFV is transmitted primarily through a variety of mosquito genera, in particular, *Aedes*, *Culex*, *Anopheles, Eretmapodites*, *Mansonia* among others (Boshra *et al.*, 2011a; Chevalier *et al.*, 2010; Ikegami and Makino, 2011; Jean Jose Nepomichene *et al.*, 2015; OIE, 2009). Recent studies about European and North-American mosquitoes' genera have shown a real capability of RVFV transmission, although the virus is currently limited to Africa and the Middle East (Chevalier *et al.*, 2010; Turell *et al.*, 2008). The virus regularly circulates in endemic areas between wild ruminants and hematophagous mosquitoes, although the clinical course of the disease is usually unapparent. Broad vector range of mosquitoes coupled with increased circulating virus is the main mechanism of expansion of the disease (OIE, 2009; WHO, 2007; WHO, 2008; WHO, 2010).

Table 3. Natural host of RVFV. Adapted from OIE 2009.

Species	Reference(s)
Domestic ruminants*	(Flick and Bouloy, 2005; Ikegami and Makino, 2011)
Wild ruminants**	(Botros *et al.*, 2006; OIE, 2009)
Rodents	(Ikegami and Makino, 2011; Ross *et al.*, 2012)
Domestic carnivores (dog, cat)	(Walker *et al.*, 1970a; Walker *et al.*, 1970b)
Non-human primates	(Ikegami and Makino, 2011; Ross *et al.*, 2012)
Human	(WHO, 2008)

* Cattle, sheep, goats, dromedaries.

** Buffaloes, antelopes, wildebeest, etc.

RVF outbreaks tend to be associated with periods of prolonged rainfall followed by a wet period (increased precipitations) which leads to an explosive hatching of RVFV-infected mosquito eggs. The virus can survive in dormant mosquito eggs for prolonged periods of time in arid conditions, mostly associated to certain *Aedes* species that act as main reservoirs for RVFV. Such periods are referred as inter-epizootic dry periods (Anyamba *et al.*, 2009; Chevalier *et al.*, 2010; Hendrickx and Lancelot, 2010). Floodwater *Aedes* mosquitoes are believed to transmit RVFV trans-ovarially to egg for long time, contributing to RVFV persistence despite the absence of mosquito egg hatching during the inter-epizootic dry periods (Davies *et al.*, 1985; Logan *et al.*, 1991). Precipitation cycles of 5–25 years produce RVF-immuno-naïve animal populations and when combined with introduction of virus, explosive outbreaks of the disease occur (OIE, 2009).

Several authors review some other transmission modes. We just cited them, because describing such specific mechanism amply exceeds the general review of this epidemiological introduction.

- Extrinsic incubation also occurs in vectors (OIE, 2009).
- Sylvatic cycle and inter-epidemic maintenance also occurs in some areas (Pepin *et al.*, 2010).
- Direct contamination: occurs in humans when handling infected animals and meat (Schwentker and Rivers, 1934; Woods *et al.*, 2002).

- Mechanical transmission by various vectors has been demonstrated in laboratory studies (Flick and Bouloy, 2005).
- Vertical transmission (Antonis *et al.*, 2013; Coetzer and Barnard, 1977).

1.2.3. Sources

Such as different authors have reviewed it, the source most common of infection for animal host are RVFV-infected wild fauna and vectors. However, for humans, nasal discharge, blood, vaginal secretions after abortion in animals, mosquitoes, infected meat, aerosols, and possibly consumption of raw milk, are the most common source of infection (Flick and Bouloy, 2005; OIE, 2009; Pepin *et al.*, 2010; WHO, 2008).

1.2.4. Occurrence

A new unrecognized sheep disease in the Kenyan Rift Valley was reported in 1912 by R. E. Montgomery (Montgomery, 1913), who described an outbreak of an ovine illness associated to liver necrosis. The disease had already been responsible for extensive losses in the sheep population for some years, particularly coincidental with “wet” years. In 1931, it was described an entity called “enzootic hepatitis of sheep” after an outbreak in a farm near the shores of Lake Naivasha in Kenya (Daubney *et al.*, 1931). This was the beginning of the way of this disease. Over the last three decades RVF virus has spread throughout Africa and since 2000 the geographic extent of the disease expanded to the Arabian Peninsula (Ahmad, 2000) and some Indian Ocean islands such as Madagascar, Comoros and Mayotte (Andriamandimby *et al.*, 2010; Roger *et al.*, 2011; Sissoko *et al.*, 2009) with the most recent epizootic occurring in Mauritania (Sow *et al.*, 2014) (Figure 2). Recent outbreaks in the Middle East have demonstrated the potential of the disease to spread beyond the African continent (Balkhy and Memish, 2003), highlighting the potential danger of the spreading of the disease. Europe is still RVFV-free.

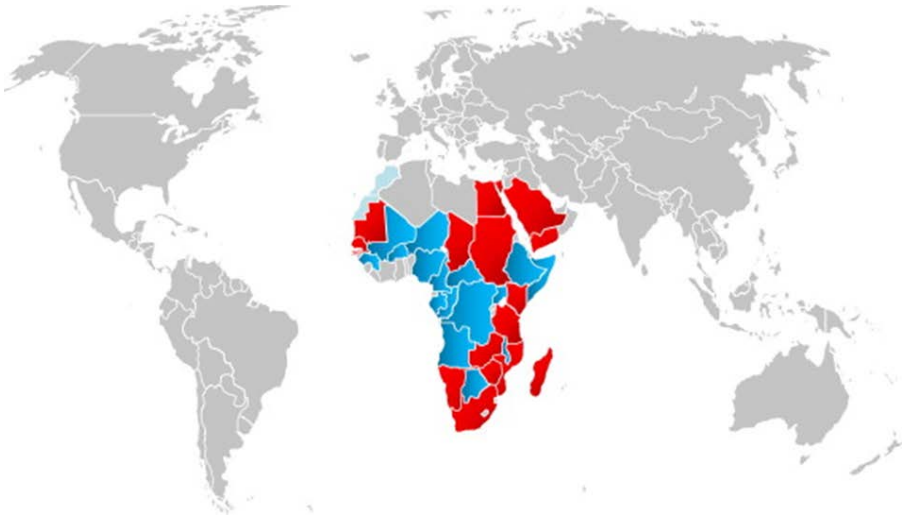


Figure 2. Current global distribution of RVF. Printed from Lorenzo, López-Gil et al. 2015. After the first description in Kenya the disease has since been reported in many African countries, the Arabian Peninsula, Madagascar and other Indian Ocean islands. Countries where important RVF outbreaks occurred are shown in red while those countries where both seropositive animals and occasional virus isolation has been reported are shown in blue. In light blue, countries where sero-positive animals (camels) have been recently detected (Di Nardo *et al.*, 2014; El-Harrak *et al.*, 2011) but not reported virus isolation.

1.2.5. Influence of climatic conditions

Factors such as rainfall, ocean temperature, and climate change play roles in determining the likelihood of an epidemic. RVFV's recent classification as a potential bioterrorist agent have renewed interest in the study of the virus as well as in RVF vaccinology (Rabinowitz *et al.*, 2006). Satellite imaging has been used to confirm historic importance of precipitation in RVF outbreaks and in forecasting high-risk areas for future outbreaks (OIE, 2009). Certain climate-based risk mapping models have found utility in prediction of future RVF outbreaks (Anyamba *et al.*, 2009; Anyamba *et al.*, 2010). Recently, an RVFV early warning system was developed using satellite measurements of sea surface temperatures, rainfall anomalies, and vegetation. Briefly, the systems states that a flooding period after an inter-epizootic dry period may cause an egg eclosion, so what infected *Aedine* mosquitoes can then bite wild ungulates or free-range livestock. Viremic animals can be bitten also by competent *Culicine* mosquitoes, therefore amplifying efficiently the virus allowing further spread to animals or humans. Satellite-based system could predict successfully human/ livestock outbreaks of RVF in the horn of Kenya, Sudan, and southern Africa from 2006 to 2008 (Anyamba *et al.*, 2009). Another risk assessment was made in Yemen and based on rainfall, mosquito vectors and seasonal importation of livestock (due to religious festivals) (Abdo-Salem *et al.*, 2011). The results showed a highest probability of having an outbreak during the festival season, when RVFV

transmission can occur via vector transmission (i.e., mosquitoes) and direct contact with infected livestock. In any matter, using an early warning system, in conjunction with mosquito vector control programs in "preoutbreak" periods, could be effective in preventing large-scale fatalities associated with RVF outbreaks. These epizootics come with devastating impacts for livestock production, causing particularly high rates of neonatal mortality and abortion in ruminants. Effective live attenuated RVF virus vaccines are available for livestock use, although safety issues preclude their distribution to non-endemic RVF areas (Ikegami and Makino, 2009; Indran and Ikegami, 2012). As preclude for the increased threat to public health, recent outbreaks in Kenya have resulted in increased human fatalities (Adam *et al.*, 2010; WHO, 2007), even reaching case fatality rates of >20% in different geographical settings (Al-Hazmi *et al.*, 2003; Hassan *et al.*, 2011). Recent evidences have shown that factors as older age, male gender, herder occupation, killing and butchering livestock, and poor visual acuity are associated with severe human RVF outbreaks (LaBeaud *et al.*, 2015).

Climatic change affecting northern Africa and southern Europe, joint to the increased trade with and traffic to the African continent provoke that it is only a matter of time before RVF outbreaks was set in Europe and Asia. Therefore, the implantation of a wider active RVFV surveillance program should be consider. Recently, a suspicious rickettsia-infected human case was finally ruled out of suffering RVFV (WHO, 2010), but the risk is still high. Therefore, better techniques to predict future outbreaks and safer vaccines have to be researched and refined in order to provide public health/veterinary officials advanced warning of pending RVFV transmissions.

1.3. Pathogenesis

RVF is an old-known but still uncontrolled disease. Several animal models have been used to understand the pathogenesis of RVF (Table 4) (Ikegami and Makino, 2011; Ross *et al.*, 2012; Terasaki and Makino, 2015).

1.3.1. Mouse

Several mouse models have been used to characterize the pathology associated with RVFV infection, because of mice are one of the most susceptible animal species to RVFV infection (Table 5) (Ross *et al.*, 2012). The models used for research of pathogenesis and

vaccinology have included BALB/c, IFNAR−/−, MBT/Pas, 129 and C57BL/6 strains. The most relevant characteristic about this model is that infected mice mimic the pathological findings associated to RVF in newborn lambs (Findlay and Daubney, 1931).

Table 4. Advantages and disadvantages of animal models for Rift Valley fever. Adapted from Ikegami and Makino 2011.

Model	Advantages	Disadvantages
Mouse	• Highly susceptible to RVFV. • Infected mice usually die in 2 weeks, and are suitable for RVFV challenge study. • Acute hepatitis at early stage, and lethal meningoencephalitis at late stage. • Cost-effective.	• No hemorrhagic fever. • No ocular disease.
Rat	• Varied susceptibility among inbred strains. • Suitable for studying host genes responsible for RVFV-resistant phenotype. • Similar pathological changes to those in mice. • A report suggests the presence of uveitis after aerosol challenge. • Cost-effective.	• The inbred strains of same name derived from different breeding colonies have different susceptibility to RVFV. • Age-dependent difference in susceptibility.
Hamster	• Highly susceptible to RVFV. • Similar pathological changes to those seen in mice. • Often used for experimental RVFV transmission by mosquitoes.	• No hemorrhagic fever. • No ocular disease. • Limited research resources.
Gerbil	• Encephalitis with minimum liver diseases.	• No significant disease except for encephalitis.

	• Useful for studying neuroinvasiveness.	• Age-dependent difference in susceptibility. • Limited research resources.
Rhesus monkey	• Lethal hemorrhagic fever. • Similar susceptibility to humans. • Important for testing the safety of vaccines or antivirals before clinical trial.	• No ocular disease reported. • Less than 20% develop hemorrhagic fever. • Requirement of ABSL4 or BSL3+ space to keep monkeys. • Expensive.
Sheep, ewe	• A report suggests the occurrence of hemorrhagic fever and edema of corneal and choroidal edema with inflammation. • High rate of abortion and fetal malformation. • Suitable for veterinary vaccine study.	• Susceptibility varies among different breeds. • Requirement of ABSL4 or BSL3+ for large animals. • Limited research resources. • Expensive.
Lamb	• Highly susceptible to RVFV. • Lethal acute hepatitis. • Important to evaluate the effect of colostrum from vaccinated ewes.	• Neurovirulence is not prominent. • No hemorrhagic fever. • No ocular diseases. • Requirement of ABSL4 or BSL3+ for large animals. • Limited research resources.

Table 5. The susceptibility of vertebrate hosts to Rift Valley fever virus. Adapted from Gerdes 2004.

Highly susceptible	Susceptible	Moderately susceptible	Susceptible but unapparent infection	Resistant
Newborn lambs	Calves	Cattle	Camels	Birds
Newborn kids	Sheep	Goats	Equines	Reptiles
Puppies		Buffalo	Pigs	Amphibians
Kittens		Humans	Dogs	
Mice			Cats	
Hamsters			Guinea pigs	
			Rabbits	

The natural infection route has been analyzed by exposure to RVFV-infected mosquitoes. The expected pathogenesis result in circulation of virus or virus-infected cells from the inoculation site to regional lymph nodes by afferent lymphatic vessels. Viral replication could then occur in specific immune cell populations within the lymph nodes, resulting in a primary viremia, with subsequent spread via the bloodstream and widespread dissemination to different organs and tissues (liver, spleen, kidney) (Smith *et al.*, 2010). The incubation period of RVFV infection was established between 2-5 days. Wild-type (wt) RVFV ZH548 or ZH501 strains cause death most of the infected mice in 3 to 5 days (Bouloy *et al.*, 2001; Smith *et al.*, 2010; Vialat *et al.*, 2000), although other described wt isolates kill even faster (Findlay and Daubney, 1931; Mims, 1956). Ruffed fur with decreased activity in 2 to 3 days are seen in wt-infected mice, becoming more and more lethargic while lying with their back legs wide apart (Findlay and Daubney, 1931; Smith *et al.*, 2010). The clinical course is often very fast, killing the animal within one hour once the first signs are observed (Findlay and Daubney, 1931). Normal or decreased rectal temperature is often observed in wt RVFV-infected mice (Mims, 1956). Also, the clotting time of wt RVFV-infected blood is significantly extended by mixing with normal sera, suggesting that the shortage of coagulation factors is important for the extension of clotting time in infected mice (Mims, 1956). The liver is the major target organ of RVFV, while the enlargement of liver is not common (Findlay and Daubney, 1931; Smith *et al.*, 2010). Congestion and hemorrhage are common in the liver, spleen, lymph nodes, large intestine, kidneys and brain (Findlay and Daubney, 1931; Smith *et al.*, 2010), but are uncommon in the jejunum (Mims, 1956). Survivors occasionally have hind limb paralysis at days 8–9 post infection (p.i.) and die from encephalitis (Smith *et al.*, 2010).

Death appears to be a consequence of liver function failure due to fulminant hepatitis and hepatic necrosis. Such lesions are characterized by fulminant necrotizing hepatitis with coagulative necrotic foci leaving the portal space intact (McGavran and Easterday, 1963; Smith *et al.*, 2010; Tomori, 1979; Tomori and Kasali, 1979; Vialat *et al.*, 2000). Depletion of

glycogen in hepatocytes is also common at an early stage (Bouloy *et al.*, 2001; Findlay and Daubney, 1931; McGavran and Easterday, 1963). Survivors of the early hepatitis phase often are able to regenerate hepatocytes (Morrill *et al.*, 2010; Smith *et al.*, 2010). Mouse infected hepatocytes contain eosinophilic Feulgen-negative intranuclear inclusion bodies (INIB) (Chieco and Derenzini, 1999; Findlay and Daubney, 1931; Smith *et al.*, 2010). Filamentary-shaped INIB are formed by the NSs protein (Struthers and Swanepoel, 1982; Swanepoel and Blackburn, 1977), a viral nonstructural protein. The 10-to-17 amino acids at the carboxyl terminus of NSs are responsible for the formation of the filamentous structures via self-association (Yadani *et al.*, 1999). RVFV replication induces apoptosis in hepatocytes such as it has been shown by using either a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay or electron microscopy (Smith *et al.*, 2010). Apoptosis of lymphocytes were found in the thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues as well (Smith *et al.*, 2010). In the late stage of infection, however, lethal meningoencephalitis characterized by neuronal necrosis, microhemorrhages, and perivascular cuffs occurs in mice that survived the acute hepatitis (Smith *et al.*, 2010).

Viral antigen starts accumulating in hepatocytes at day 2, and their abundance increases extensively at day 3 p.i. (Smith *et al.*, 2010). Swollen endothelial cells can be observed in the liver (Findlay and Daubney, 1931); however, antigen detection in endothelial cells or Kupffer cells is not easy, so it has been hypothesized that these cells are not the primary targets of RVFV (McGavran and Easterday, 1963; Smith *et al.*, 2010). Also, RVFV antigen has been detected in odontogenic and gingival epithelium, lipocytes, pituicytes, olfactory neurons and multiple types of neurons in the brain, mononuclear phagocytes, cardiac myofibers, and in perineural, periosteal, adrenocortical, endosteal, perivascular, bone marrow stromal, fibroblastic reticular, and vascular smooth muscle cells, as well as in cells morphologically consistent with dendritic, pancreatic islet, and adrenal medullary cells; however, no viral antigen was reported in any ocular structure, including the retina (Smith *et al.*, 2010). In some mice that survived acute viral hepatitis, a sharp decrease in viral antigen occurred at 8 days p.i., and no virus could be detected in the sera, liver, lung, pancreas, large intestine and ovaries (Smith *et al.*, 2010).

Pathology appears to vary with route of exposure to RVFV through a not well understood mechanism. The succession of events following infection via the aerosol exposure might likely be an initial expansion in susceptible cells throughout the respiratory tract, thereby establishing a primary viremia that eventually reaches the liver, which becomes the principal site for virus replication and virus spread to other tissues including the brain (Dodd *et al.*, 2014; Reed *et al.*, 2013). Neuropathology development in aerosol-challenged mice is faster and more severe than through parenteral routes, because aerosol infection may allow direct invasion of the central nervous system via the olfactory nerves (Brown *et al.*, 1981; Dodd *et al.*, 2014; Reed *et al.*, 2013)

Liver is the main organ capable of sustaining a massive virus replication for both parenteral and aerosol routes of exposure, leading to the spread of the virus to secondary target organs. Hepatic virus replication or a strong unregulated immune response are the postulated causes of the liver failure, but the specific mechanism of necrosis remains inconclusive. In spite of the profuse immunostaining of viral antigen detected by immunohistochemistry (Reed *et al.*, 2012) or by chemiluminiscent viruses in IFNAR−/− mice (Gommet *et al.*, 2011), viral particles are scarcely to rarely seen in BALB/c mouse liver. In addition, macrophages, dendritic cells and granulocytes were main target cells for RVFV after intraperitoneal inoculation, such as it was demonstrated using GFP or luciferase expressing viruses. Intraperitoneal route affected mainly the thymus, spleen, liver and pancreas. Upon dermal and nasal inoculations, virus was observed in the lymph node draining the injected ear and in the lungs respectively (Gommet *et al.*, 2011).

The immunopathogenesis upon RVFV infection has been also studied in great detail in mice (Gray *et al.*, 2012). In ruminants, primates and C57BL/6 mice two different phases in the immune cell populations are observed after infection. First, there is an initial drop phase which starts from 48 h post-infection (hpi), with an evident decrease in lymphocytes, monocytes and platelet counts. This is followed by a recovery in the white blood cell (WBC) count coincidental with an increase of granulocyte colony stimulating factor (G-CSF) in serum (Easterday, 1965; Gray *et al.*, 2012). The level of non-pro-inflammatory chemokines was significantly elevated in liver, explaining the regular infiltrates of monocytes and granulocytes that are seen in RVFV-infected livers.(Gray *et al.*, 2012). In this line of argument, the level of IL-10 gene expression in the liver was found to be upregulated in infected mice (Jansen van Vuren *et al.*, 2011). A significant increase of IL-6, G-CSF and IL-12 (p40) as well as chemokines KC, MCP-1 and MIP-1α, starting at 48 hpi and decreasing to normal levels at 96 hpi, was observed in BALB/c OlaHsd mice. Hepatic upregulation of pro-apoptotic and pro-inflammatory genes and downregulation of anti-apoptotic ones was found significant as well (Jansen van Vuren *et al.*, 2011). Later after infection the expression of both IFN-β and IFN-γ genes was heavily upregulated, perhaps contributing to liver damage by means of the exacerbation of cytokine production.

1.3.2. Rat

Susceptibility of the Rat (*Rattus norvergicus*) to RVFV differs greatly among rat strains (Findlay and Howard, 1952). As an example, 10-to-15-week-old inbred rats from U.S. breeders exhibited three different clinic-pathological findings to subcutaneous (s.c.) RVFV inoculation (Peters and Anderson, 1981; Peters and Slone, 1982). Wistar-Furth (WF) and Brown Norway strains died within 4 days p.i. by liver necrosis so therefore were classified as highly susceptible to RVFV; however, Fisher 344, Buffalo, DA and Lewis strains were largely resistant to RVFV infection (Peters and Slone, 1982). Three adult inbreed rats from U.S. breeders were investigated as well (Anderson *et al.*, 1987). WF rats all died by day 2

p.i. with viral tissue titers reaching 9 $\log_{10}$ PFU/g. Lewis and MAXX rats were resistant to liver disease, but fatal necrotizing encephalitis developed in 16 and 44% of the rats, respectively. Observed ascending paralysis was caused by neural lesions characterized as mild-to-severe necrotizing encephalitis and encephalomyelitis with focal necrosis with neutrophilic infiltrate and perivascular cuffing primarily with lymphocytes. Five-to-6 $\log_{10}$ PFU/g of viruses could be detected in brain tissue of ACI and Maax strains but remain undetectable in the liver and blood in the presence of neutralizing antibody in the serum. Even using an immunosuppression model of Lewis rat, the reached virus titers are lower compared to WF, suggesting that this strain encodes a gene(s) important for the resistant phenotype (Anderson *et al.*, 1987). Bales *et al.* classified ACI and MAXX strains as moderate susceptible to RVFV infection. Briefly, in their study WF rats developed a rapidly progressing lethal hepatic disease after inhalational exposure; ACI rats were 100-fold less susceptible and developed fatal encephalitis after infection. Lewis rats developed fatal encephalitis after aerosol infection but do not succumb to parenteral inoculation. RVFV was found in the liver, lung, spleen, heart, kidney and brain of WF that succumbed after aerosol exposure. In contrast, RVFV was found only in the brains of ACI or Lewis rats that succumbed after aerosol exposure (Bales *et al.*, 2012). The intracranial injection of RVFV uniformly caused encephalitis in some of these rats, including the resistant Lewis strain (Anderson *et al.*, 1987). Surprisingly, inbred WF (WF/mol) and Lewis rats (Lewis/mol) from a European breeding colony are resistant and susceptible to RVFV, respectively. Furthermore, both WF and Lewis rats obtained from another European breeder were resistant to RVFV infection.

These changing susceptibilities to RVFV infection, from the opposite to those of the same strains from U.S. breeders to a complete resistance to infection, may point towards the possible genetic variability of inbred rats among different breeders (Ritter *et al.*, 2000). Cross-breeding experiments culminated in findings that indicated the resistance of WF/mol rat was segregated as a single Mendelian dominant locus (Ritter *et al.*, 2000). Findlay *et al*. also showed that the albino rat of the Glaxo strain had an age-dependent susceptibility to RVFV via the i.p. route infection (Findlay and Howard, 1952); rats younger than 15 days died in 2 to 4 days with extensive liver necrosis, whereas 26-day-old rats survived RVFV infection (Findlay and Howard, 1952).

1.3.3. Hamster

Such as we expose in table 5, the Syrian hamster (*Mesocricetus auratus*) is one of the most susceptible rodents to RVFV. Syrian hamsters have been commonly used for experimental transmission using infected mosquitoes (McIntosh *et al.*, 1973). Death occurs in 2 to 3 days p.i. after intraperitoneal inoculation with massive liver necrosis (Findlay and Daubney, 1931; Niklasson *et al.*, 1984). Animals that died early developed massive liver necrosis, whereas late death (by day 11 p.i.) was caused by encephalitis, mimicking the pathological course seen in mice (Findlay and Daubney, 1931).

Administration of low titers of neutralizing antibodies protects hamsters from fatal liver necrosis but not against late death (Niklasson *et al.*, 1984).

Scharton *et al*. describes the natural clinical course in golden Syrian hamsters challenged subcutaneously with the pathogenic ZH501 strain of RVFV. Peracute disease resulted in rapid lethality within 2 to 3 days of RVFV challenge. High titer viremia and substantial viral loads were observed in most tissues examined; however, histopathology and immunostaining for RVFV antigen were largely restricted to the liver. Acute hepatocellular necrosis associated with a strong presence of viral antigen in the hepatocytes indicates that fulminant hepatitis is the likely cause of mortality (Scharton *et al.*, 2015). Gowen *et al*. demonstrates that post-exposure vaccination with rMP12-C13 type is effective in limiting ZH501 replication and associated disease in standard pre-exposure vaccination and post-challenge treatment models of RVFV infection in hamsters (Gowen *et al.*, 2015).

1.3.4. Gerbil

Gerbils (*Meriones unguiculatus*) may be useful to studies related to neuroinvasiveness and encephalitis and the effect of age in the resistance/ susceptibility balance to RVF disease (Anderson *et al.*, 1988). Non-neuroadapted wt RVFV infection produces fatal encephalitis with minimal liver involvement in the gerbil, which represents a unique RVFV animal model. The gerbil has proven moderately susceptible to RVFV (table 5). The survival rate of 10-week-old gerbils after s.c. inoculation is reported to range from 50 to 100%, being clearly associated with the strain and inoculation dose (Anderson *et al.*, 1988). Death was reported to occur around 1 to 3 weeks in a dose-independent age-dependent manner. The used doses in the study were not related to mortality. The older the outbred or inbreed Tum (MON) gerbil was, the better survival rate (Anderson *et al.*, 1988). In fact, different neuroinvasiveness behavior of RVFV challenge indicate the presence of host factors influencing in an age-dependent manner (Anderson *et al.*, 1988).The infected gerbils exhibited hind-limb paralysis, generalized weakness and wasting (Anderson *et al.*, 1988).

Lesional pattern in Gerbils are related to minimal multifocal necrosis of hepatocytes at days 1 or 2 p.i. s.c. wt RVFV. The later neural findings are characterized as focal necrotizing encephalitis with neuronal necrosis, a neutrophilic infiltrate, and perivascular cuffing (Anderson *et al.*, 1988). Mild, necrotizing encephalitis without detectable infectious RVFV could be observed even in clinically normal RVF-infected gerbils (Anderson *et al.*, 1988).

1.3.5. Sheep

Fewer data related to pathogenesis of RVFV are available in breeds of sheep and these refer most to the pathology observed upon experimental infection for research in vaccinology field. The susceptibility for adult sheep appears to be more inconsistent than for young animals and between breeds, reflecting different host's genetic determinants (Gerdes, 2004). The disease in younger lambs follows a peracute course, with collapse and death occurring 12 h after the onset of pyrexia (40°C to 42°C). After the incubation period (12 to 36 hours p.i.), lambs become listless and exhibit abdominal pain (Gerdes, 2004). Daubney *et al*. originally reported an outbreak of RVF which was characterized as a high rate of abortion in pregnant ewes (95 to 100%) and high mortality of newborn lambs showing liver necrosis (Daubney *et al.*, 1931).

Studies of experimental infection of 1-4 day-old lambs via s.c. resulted in necrosis of isolated hepatocytes (12–18 h p.i.), focal coagulative necrosis of hepatocytes (24–33 h p.i.), and extensive hepatocyte necrosis (48–51 h p.i.) with a progressive increase in viral antigen, suggesting that hepatocytes are the primary target of RVFV (Van der Lugt *et al.*, 1996). No viral antigen could be detected in the endothelial or Kupffer cells in the liver (Van der Lugt *et al.*, 1996). The necrosis is predominantly centrilobular or midzonal, but no definite distribution pattern is standardized in liver necrosis (Coetzer, 1977; Van der Lugt *et al.*, 1996). Coetzer *et al*. observed that some infected lambs also exhibited necrosis in the villi at the distal jejunum and ileum and depletion of lymphocytes in the spleen, but no lesions were seen in the brain and eyes (Coetzer, 1977). Lambs older than one week were relatively resistant to RVFV infection, yet did exhibit fever (39 to 40 °C), conjunctivitis, viremia, diarrhea, nasal discharge, loss of appetite and, about 12 to 18 h prior to death, from decreased activity to prostration (Easterday *et al.*, 1962a; Easterday *et al.*, 1962b; Tomori, 1979).

Adult sheep or older lambs are less susceptible to RVFV and disease may vary from acute to unapparent, depending on the breed (Gerdes, 2004). Typically, there is an incubation period of 24 h to 72 h followed by an elevated temperature, lymphadenitis, and vomiting or diarrhea. The sheep is listless, becomes recumbent and may have hemorrhagic diarrhea. Icterus can develop and abortion may occur (Gerdes, 2004). Mortality varies from 10% to 70%, although in adults with a more sub-acute form of the disease it may only reach 20%, associated to liver multifocal necrosis (Easterday *et al.*, 1962b; Gerdes, 2004). Odendaal and Prezosky describes that the most characteristic post mortem lesions are multifocal or diffuse hepatic necrosis and wide-spread petechiae or ecchymosis on the serosal surfaces of the parenchymatous organs or the intestines in south African naturally RVFV-infected adult ruminants. Hemorrhages are also noted in the subcutaneous tissues, lymph nodes, and the mucosa of the abomasum. Free blood is sometimes found in the lumen of the small intestines (Odendaal *et al.*, 2014; Odendaal and Prozesky, 2010).

Odendaal *et al*. made an extensive histopathological characterization of naturally RVFV-infected liver (Odendaal *et al.*, 2014). The morphology of the lesions was very distinctive.

Briefly, histopathological hepatic lesions were similar in cattle and sheep but the extent of liver involvement varied with age. Randomly located, multifocal, focally extensive, or confluent bridging hepatocellular necrosis was diagnosed in adult animals. Neonates and fetuses were prompted to present a diffuse extensive cell death involving almost all hepatocytes. Multifocal hemorrhages and random to portal located, multifocal, mild to moderate infiltration of neutrophils and lymphocytes frequently accompanied the hepatic lesion. Scattered hyperplastic Kupffer cells were also present within the sinusoids. The involvement of hepatocytes within the limiting plate was also a common finding. Apoptosis was diagnosed in affected hepatocytes based on the presence of dissociation of cells, with shrinkage and rounding, hypereosinophilic cytoplasm, and karyorrhexis or karyolysis. Apoptotic bodies were identified as small cytoplasmic fragments. No affectation of the bile ducts was observed. Surviving hepatocytes usually had varying degrees of micro- or macrovesicular degeneration and anisokaryosis as it was previously described (Daubney *et al.*, 1931). A very distinctive feature in many cases (especially in neonates and fetuses) was randomly located foci of well circumscribed cytolysis. These foci varied in size and number and contained dense aggregates of hepatocellular debris, degenerate neutrophils, and scarce hyperplastic Kupffer cells. Rod to oval-shaped or round eosinophilic intranuclear inclusions bodies (INIB) were occasional observed in injured hepatocytes, which correlated with the observed INIB in rodents (Smith *et al.*, 2010). However, inclusion bodies were often difficult to detect and less common in the livers of adult animals compared to fetuses or neonates.

Nine- to ten-week-old young adult sheep (Ripollesa breed) that were subcutaneously inoculated with RVFV had corneal and choroidal edema with inflammatory infiltrate (anterior uveitis with lymphoplasmacytic endotheliitis), which could be associated with drainage failure or inadequate corneal dehydration after transient viremia (See Chapter 2, Figure 25) (Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). On the other hand, RVFV s.c. experimental infection of 7- to 11-month-old Yansaka sheep died during the viremic

febrile phase showing epistaxis (2 days p.i.~), severe bloody diarrhea, conjunctival

hemorrhage, widespread petechiae and ecchymoses in hairless areas, pulmonary edema/ hemorrhage, and thrombi formation in the blood vessels of the heart, kidneys and brain; all of the symptoms are associated to endothelial damage or disseminated intravascular coagulation (Olaleye *et al.*, 1996), such as it was observed in naturally RVFV infections (Odendaal and Prozesky, 2010). RVFV-infected West African Dwarf or the Ouda breed did not exhibit such rapid hemorrhagic symptoms and rather exhibited marked coagulative hepatic necrosis, and brain lesions, including mild gliosis, neural degeneration, neurophagia, and satellitosis (Olaleye *et al.*, 1996). Interestingly, Yansaka, West African Dwarf and Ouda also had increased prothrombin time, which may have indicated that hemorrhage was induced by a combination of vascular endothelial damage and an inability to clot blood in response to the damage (Olaleye *et al.*, 1996). The inconsistency of symptoms and mortality in adult sheep described in these publications suggest the

divergence of host genetic background, even within the same breed of sheep, affects susceptibility to RVFV infection.

Insufficient immunogenicity of inactivated vaccines or residual virulence of live-attenuated vaccines induced fetal malformations, abortions (reaching 50%) and variable ewe mortality (1 to 12,5%) after wt RVFV challenge in a pregnant sheep model (Yedloutschnig *et al.*, 1981). Dead or aborted lambs showed extensive liver necrosis typical of RVF (Yedloutschnig *et al.*, 1981). Immunization with a live-attenuated Smithburn vaccine strain could cause a teratogenic effect in the fetus (up to 33% of necropsies), including arthrogryposis, hydranencephaly, or mineralization of brain with or without *hydrop amnii* at 42 to 74 days of pregnancy in sheep (Coetzer and Barnard, 1977), such as it is observed in goats (Kamal, 2009). By immunizing with live-attenuated MP-12 vaccine strain either miscarried or produced lambs showing teratogenic effects at 28 to 56 days of gestation (11 out of 75 lambs from 50 vaccinated ewes), such as cerebellar hypoplasia, spinal hypoplasia, hydranencephaly, prognathia inferior, brachygnathia inferior, arthrogryposis, scoiliosis, lordosis, kyphosis, or dormed head (Hunter *et al.*, 2002). Remarkably, the abortion and teratogenous effects did not occur when pregnant ewes were vaccinated with MP-12 at the third trimester of pregnancy, i.e., at 90-110 days of gestation (Morrill *et al.*, 1991a; Morrill *et al.*, 1987). Immunization with a RVFV Clone 13 (C13) strain at 15 days of gestation, which has a 69% in-frame deletion of NSs (Muller *et al.*, 1995), did not cause abortion or fetal malformations and were protective against wt RVFV challenge (Dungu *et al.*, 2010). These data may imply the involvement of MP-12 NSs in the abortion of ewes and the teratogenic effects in lambs.

1.3.6. Nonhuman primates

Rhesus macaques (*Macaca mulatta*) are moderately susceptible to RVFV infection (table 5) (Findlay and Daubney, 1931). RVFV-infected macaques developed hyperthermia or fever (up to 39–40 °C), leukopenia and transient viremia at 1 to 4 days p.i. after i.p or intranasal inoculations, during a period of 24 to 120 h; however, some infected animals did not show any febrile reactions (Findlay and Daubney, 1931). However, no signs of disease or mortality were observed in subsequent experiments using different inoculation routes (Easterday, 1965). Rhesus macaques infected with a wt RVFV ZH501 strain developed a hemorrhagic fever-like illness after an intravenous inoculation (Morrill *et al.*, 1990; Peters *et al.*, 1988). Briefly, 13-20% of infected rhesus macaques became severely ill, moribund and were euthanized or die, 6,6 -41% recovered from illness, and 41-60% were clinically normal but showed a temporal viremia which reach the maximum viral titer around day 2. The affected monkeys exhibited lassitude, weakness, loss of appetite, petechiae, ecchymoses and bleeding from nares, gums or venipuncture sites. Hepatic necrosis, disseminated intravascular coagulation, and microangiopathic hemolytic anemia

were the pathological features in animals that developed the hemorrhagic syndrome. Dead monkeys showed clinically clear extensions of APTT, slight extension of PT, and a decrease in the number of platelets, possibly indicating a deficiency of coagulation factors and platelets. In a further study, evidence of hemostatic impairment including disseminated intravascular coagulation appeared to be more generalized (Cosgriff *et al.*, 1989). Lesions observed in these dead macaques were moderate focal or midzonal hepatic coagulative necrosis involving approximately 1/3 to 2/3 of hepatocytes, necrosis in the ventricular myocardium, fibrin thrombi in the glomeruli and small intertubular vessels of renal medulla in the kidneys, and mild depletion of lymphocytes from white pulp and the deposition of eosinophilic amorphous fibrin-like material in red pulp cords in the spleen (Peters *et al.*, 1988).

Morrill *et al*. suggested the importance of IFN-α in limiting viral replication. Serum interferon (IFN)-α was detected from 6–24 h p.i. in the surviving monkeys; and from 24–30 h p.i. in those dying. The delayed IFN response was preceded by viremia in most of lethally-infected monkeys (Morrill *et al.*, 1990). Besides, the administration of recombinant leukocyte A IFN (5 × 105 U, i.m) reduced the peak viremia titer by 100-times at 6 h after RVFV intravenous inoculation, and also cleared viruses by 48 h p.i. (Morrill *et al.*, 1989). No surviving monkeys developed signs of encephalitis or retinal complications in follow-up observations at two months to two years (Morrill *et al.*, 1990).

Green guenon (*Cercopithecus callitrichus*), sooty mangabey (*Cercocebus fuliginosus*) and Patas guenon (*Erythrocebus patas*), three species of African monkey, did not exhibit any febrile reaction even during the viremia period (Findlay, 1932). However, two brown capuchin monkeys (*Cebus fatuellus* and *Cebus chrysopus*) and two common marmosets (*Callithrix jacchus* and *Callithrix penicillata*), four species of South American monkeys, exhibited febrile reactions for 1 to 2 days upon RVFV infection. These findings may indicate that South American monkeys are more susceptible to RVFV infection than African monkeys (Findlay, 1932). RVFV-infected baboons (*Papio anubis*) had viremia for 3 to 4 days without developing significant clinical signs (Davies *et al.*, 1972).

Result heterogenicity of Rhesus monkey model in RVFV induced morbidity and mortality may be comparable as what it happens in sheep, prompting investigation into other alternatives. The common marmoset (*Callithrix jacchus*) is an alternative model for reproducing human RVF (Smith *et al.*, 2012). These animals appear to be more susceptible to RVFV infection than Rhesus monkeys in terms of morbidity, mortality and viremia. In a route-dependent way, diagnosis of acute hepatitis, delayed-onset encephalitis and hemorrhagic disease were made as in the most severe human RVF types. Interestingly, the highest rate of mortality was obtained when inoculation was made intranasally, perhaps reflecting the efficacy of aerosol transmission, which is probably one of the most important routes for human infection. The importance of aerosol transmission in RVFV lethality in African green monkeys (*Chlorocebus aethiopicus*) was confirmed in a recent

study in which most animals (83%) died at 10-11 days p.i. (Hartman *et al.*, 2014). Marmosets were equally sensitive to disease but showed reduced mortality rates (50%). That is the reason why both models could be useful to reproduce aerosolized RVFV infection, and to evaluate both therapeutic and prophylactic approaches as well.

1.3.7. Humans

Clinical signs of this disease in humans range from flu-like syndrome with fever, joint pains, photophobia and headaches to retinitis and encephalitis; complications may include hepatitis with generalized hemorrhages, thrombosis, retinitis and transient loss of vision, encephalitis, neurological symptoms and fatal hemorrhagic fever with thrombocytopenia in some individuals (Flick and Bouloy, 2005; Gerdes, 2004). Infected humans are usually involved in sheep herding and RVFV-infected tissue handling (Ikegami and Makino, 2011; LaBeaud *et al.*, 2015).

A wide description of human RVF is not a main objective in this work (point 2). A scheme of the pathological forms of Rift Valley fever in humans is shown in Figure 3.

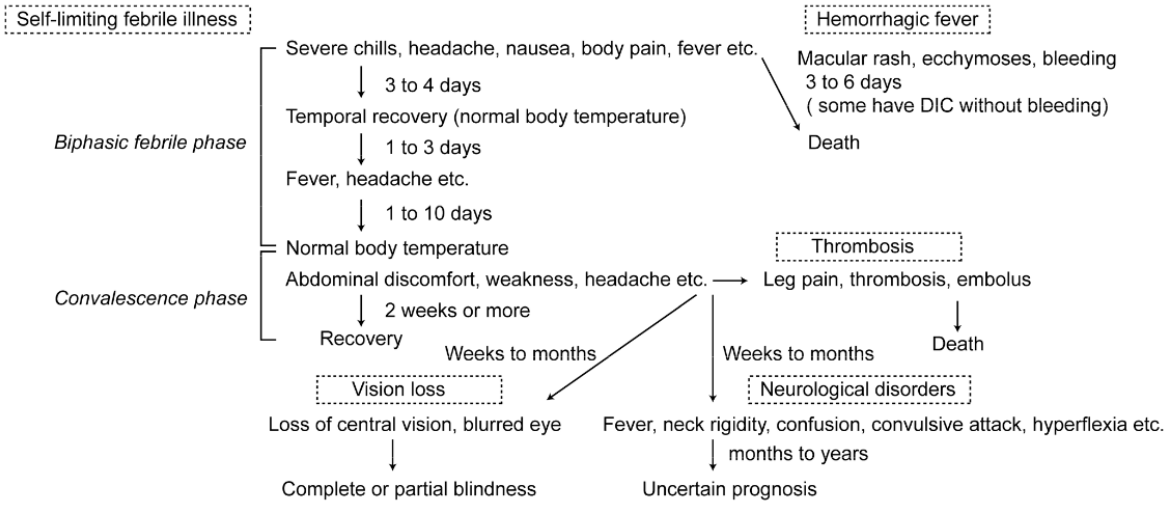


Figure 3. The pathological forms of Rift Valley fever in humans. Printed from Ikegami and Makino 2011.

1.3.7.1. Self-Limiting Febrile Illness

Typically, RVFV pathogeny is divided in two phases; biphasic febrile phase and convalescence phase (Figure 3). RVF incubation period is 4 to 6 days. The biphasic febrile phase extends for 3-4 days starting abruptly with severe chills, severe headache, malaise, dizziness, weakness, nausea and/or sensation of fullness over the liver region (Findlay and

Daubney, 1931; Francis and Magill, 1935; Kitchen, 1934). These symptoms are followed by hyperthermia or fever (38.8 °C to 39.5 °C); decreased blood pressure; pain in the back, shoulders, neck or legs; rigor; shivering; flushed face; red eye with sore; constipation; insomnia and/or photophobia. Occasionally, other symptoms are seen which include epistaxis, abdominal pain, lack of gustatory discrimination, vomiting and/or diarrhea (Daubney *et al.*, 1931; Findlay and Daubney, 1931; Joubert *et al.*, 1951; Kitchen, 1934; Mundel and Gear, 1951; Smithburn *et al.*, 1949). At 3rd day some patients may have a temporal recovery, often decreasing the body temperature to a normal level by the 4th day after the onset of symptoms. However, a second phase of temporal recurrence of high fever with a severe headache for a few days within 1 to 3 days after the recovery of body temperature (Findlay and Daubney, 1931; Mundel and Gear, 1951; Smithburn *et al.*, 1949). Moreover, high fever may extend for as much as 10 days (Findlay and Daubney, 1931). Occasionally, some patients may develop a massive coronary thrombosis after body temperature becomes normal (Mundel and Gear, 1951), persistent aching of legs for two weeks (Bird *et al.*, 2009; Kitchen, 1934), or persistent abdominal discomfort for weeks (Daubney *et al.*, 1931). The hepatomegaly and splenomegaly are not common.

In the convalescence phase, weakness, malaise, a tendency to sweat, frequent headaches, pain on motion of the eye, and a sense of imbalance are frequently shown by patients. Viremia has been demonstrated during the febrile period (3–4 days), whereas neutralizing antibody also starts appearing around the 4th day of the onset of symptoms (Easterday, 1965; Gear *et al.*, 1951; Kitchen, 1934; Sabin and Blumberg, 1947; Smithburn *et al.*, 1949).

1.3.7.2. Neurological Disorders

Neurological disease usually consist on a viral meningitis, encephalitis or meningoencephalitis, mainly characterized by temporal vision loss, sudden fever, rigor, retro-orbital headache, meningeal irritation, confusion, stupor and coma, convulsions, hypersalivation, teeth-griding, visual hallucinations, locked-in syndrome, and choreiform movement of upper limbs, hyperflexia and many white blood cells in cerebrospinal fluid (mostly lymphocytes) (CSF) (Alrajhi *et al.*, 2004; Maar *et al.*, 1979; van Velden *et al.*, 1977). Retinal hemorrhages associated to blindness has been described as well Some patients recover, some others no (Alrajhi *et al.*, 2004). An additional report described a patient who had persistent hemiparesis for four months after the onset of illness (Laughlin *et al.*, 1978). Histopathological lesions in brains were characterized by focal necrosis associated with an infiltration of round cells, mostly lymphocytes and macrophages, and perivascular cuffing (van Velden *et al.*, 1977).

1.3.7.3. Vision Loss

Ocular disorders range from maculopathy to retinopathy and are associated to the loss of central vision or blurred eye without a defined pathogenic timeline. Ocular affectation can be unilateral or bilateral (Deutman and Klomp, 1981; Salib and Sobhy, 1978; Siam *et al.*, 1980). Macular edema with exudates containing a white mass covering the macular area with or without retinal hemorrhage, vasculitis, infarction or vitreous haze are described in the affected eyes (Al-Hazmi *et al.*, 2005; Deutman and Klomp, 1981; Freed, 1951; Salib and Sobhy, 1978; Schrire, 1951; Siam and Meegan, 1980). Besides these findings, retinal detachment (Deutman and Klomp, 1981; Schrire, 1951), retinitis (Figure 4) (Khairallah *et al.*, 2010), uveitis (Al-Hazmi *et al.*, 2005; Siam and Meegan, 1980), or arterial occlusion (Al-Hazmi *et al.*, 2005; Ayoub *et al.*, 1978; Deutman and Klomp, 1981; Schrire, 1951; Siam *et al.*, 1978) has been reported in some cases. In many patients, chorioretinal scarring can remain in macular and paramacular areas in spite of the resorption of exudates, and a complete recovery of vision does not occur (Al-Hazmi *et al.*, 2005; Ayoub *et al.*, 1978; Deutman and Klomp, 1981; Freed, 1951; Salib and Sobhy, 1978; Siam *et al.*, 1978; Siam and Meegan, 1980). Some chronical RVFV-infected patients show partial improvement in vision after several months (Salib and Sobhy, 1978; Schrire, 1951; Siam *et al.*, 1978; Siam and Meegan, 1980).

1.3.7.4. Hemorrhagic Fever

The time to death in fatal RVF cases involving hemorrhagic course varies among cases (Abdel-Wahab *et al.*, 1978; Swanepoel *et al.*, 1979; Yassin, 1978). Typically, hemorrhagic manifestation starts suddenly. Patients experience fever, rigor, nausea, vomiting, headache, injected conjunctives, drowsiness, and/or body pains. Symptoms as macular rash over the entire trunk, ecchymoses on the arms, limbs, and/or eyelids, bleeding from the gums and/or gastrointestinal mucosal membrane, low blood pressure, hematemesis, melena, diarrhea, throat pain, pneumonitis, jaundice, and/or hepatosplenomegaly may also occur (Swanepoel *et al.*, 1979; Yassin, 1978). These patients typically show elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and reduction of platelet count and hemoglobin (Al-Hazmi *et al.*, 2003; Swanepoel *et al.*, 1979). Once patients become symptomatic, death occurs in 3 to 6 days in many cases; however, occasionally, death occurs in 12 to 17 days after the onset of symptoms. Histopathology shows diffuse necrosis of hepatocytes, mainly affecting the centrilobular area more than the portal area, which may indicate association with acute hepatic injury in this type of pathogenesis (Abdel-Wahab *et al.*, 1978; Swanepoel *et al.*, 1979). Some patients who do not exhibit jaundice or hemorrhage die from renal failure or disseminated intravascular coagulation (DIC), both accompanied by an elevation of ALT, AST, LDH or D-Dimer, or a decrease in platelet count (Al-Khuwaitir *et al.*, 2004). Typical hemorrhagic fever may also be associated with encephalitis and hepatic and gastro-intestinal necrosis (van Velden *et al.*, 1977), which demonstrates the neuroinvasiveness of RVFV in these patients.

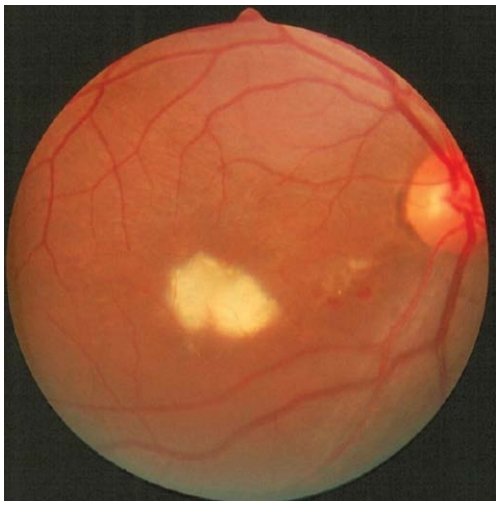


Figure 4. Fundus photograph of the right eye of a patient with Rift valley fever shows active retinitis in the macular region. Printed from Khairallah, Chee et al. 2010. The presence of retinal hemorrhages should be noted. Courtesy of Dr. E Abboud.

1.3.7.5. Thrombosis

A rare case of RVFV infection consisted on phlebitis, thromboembolism of different veins (vena cava, popliteal, pulmonary veins) with fatal multifocal pulmonary infarcts was described by Schwentker *et al*. (Schwentker and Rivers, 1934). Infection was confirmed but no RVFV lesions or antigens were detected in tissues. The neutralizing antibody showed up on the 6th day of illness, and its titer increased toward the 12th day.

1.3.7.6. Possible Vertical Infection

No increases in the total number of abortions were seen during an RVF outbreak in Egypt. The serological conversion rate of aborted women before and after outbreak was 31.1% and 27.5%, respectively (Abdel-Aziz *et al.*, 1980). An infected pregnant woman suffering a self-limiting febrile illness-like RVF deliver a positive-anti-RVFV IgM newborn baby who died on the 6th day after birth (Arishi *et al.*, 2006). A vertical transmission *in utero* might have occurred, although it is unknown whether the newborn baby died from RVF.

1.3.8. Other species

Other species affected by RVFV include goats, cattle, camels, dogs, cats, and ferrets, but does not cause any symptomatic diseases in rabbits, guinea pigs, birds, horses, pigs and other animals, as reviewed in detail previously (Daubney *et al.*, 1931; Easterday, 1965; Findlay and Daubney, 1931; Keefer *et al.*, 1972; Peters and Anderson, 1981; Scott, 1963;

Scott *et al.*, 1963; Shimshony and Barzilai, 1983; Walker *et al.*, 1970a; Walker *et al.*, 1970b; Weiss, 1957). To the best of our knowledge, the mechanism of species-specific susceptibility to RVFV infection is unknown.

Domestic ruminants including goats, cattle and camels are also susceptible to RVF (table 5) and some experimental and vaccine trials have been performed on this species, helping to better understand the pathogenesis of the virus (Barnard, 1979; Botros *et al.*, 2006; Coackley *et al.*, 1967a; Morrill *et al.*, 1997a; Morrill *et al.*, 1997b; Nfon *et al.*, 2012; Swai and Sindato, 2015; Weingartl *et al.*, 2014a; Yedloutschnig *et al.*, 1981; Yedloutschnig *et al.*, 1979). As it has been cited before, newborn lambs are most susceptible and mortality may vary from 70% to 100% such as happens in kids. Severe hepatic necrogranulomas, multifocal lymphocytic infiltration and hyperplastic overgrowth with vesicular elongated epithelium (even necrotic in some cases) were diagnosed in kids of RVF live attenuated vaccinated (Smithburn strain) goats (Kamal, 2009). Moreover, the author demonstrated that Smithburn vaccine is related to severe deleterious pathological changes in liver and cause abortion in pregnant does (Kamal, 2009). Camels may abort, in spite of not suffering illness, as do buffalo. Exotic ruminants may also play a role in the inter-epidemic circulation of virus (Beechler *et al.*, 2015; Swai and Sindato, 2015). Calves less than ten days old will also suffer a peracute form of RVF and die within 24 h. Jaundice is more commonly seen in older calves/ adult cattle, reaching a mortality rate in adults often less than 10% (Jouan *et al.*, 1989). The animal develops anorexia, diarrhea and dysgalactia and exhibits salivation and nasal discharge (Erasmus and Coetzer, 1981). Abortion may occur as a result of infection of the fetus and a wide propagation of the virus inside the liver of the foeti that leads to its death; a direct RVFV effect on the genital organs of the pregnant does that causes necrotic changes in the uteri; or as a result of a febrile reaction; the fetus is often autolyzed (Gerdes, 2004; Kamal, 2009).

Francis and Magill inoculated ferrets with RVFV-infected pharyngeal washes of accidental-infected laboratory assistants, observing a pulmonary involvement as main pathological feature (Francis and Magill, 1935). In spite of being one of the first non-rodent models ever used for assessing RVFV transmission, no further reports have been published.

1.4. Immunopathogenesis

As previously mentioned, RVFV NSs-mediated strategies for the inhibition of host's innate immune responses are summarized in Table 2 (Lorenzo *et al.*, 2015).

1.4.1. Rodents models

Transmission by mosquito bites can be considered the natural route of infection. Mosquito *Aedes* saliva co-injected intradermally with RVFV significantly increases the observed pathogenicity in C57BL/6NRJ mice (Le Coupanec *et al.*, 2013) as it has been demonstrated in other arboviruses (Edwards *et al.*, 1998; Reagan *et al.*, 2012; Styer *et al.*, 2011).

Rodents are quite susceptible to RVFV infection, leading to their use for evaluation of candidate vaccines and related immune mechanisms. RVFV is highly sensitive to the action of type-I interferon (IFN) as shown in mice, hamsters and non-human primates following administration of IFN inducers (such as polyriboinosinic-polyribocytidylic acid complexed with poly-L-lysine and carboxymethylcellulose -poly[ICLC]) or recombinant human IFN (Morrill *et al.*, 1989; Peters *et al.*, 1986). In addition, alpha/beta interferon receptor (IFNAR−/−) knockout mice are highly sensitive to RVFV, including the naturally avirulent Clone 13 RVFV strain that contains a 549 nucleotide deletion within the gene coding for the NSs protein (Bouloy *et al.*, 2001). NSs protein effectively blocks type I IFN production through a generalized repression of host cell transcriptional activity, which antagonizes the antiviral response (Table 2). NSs protein may also repress the transcriptional activity of hormone regulated genes, leading to an explanation about the high rates of abortion and teratogenicity observed in infected pregnant animals, or the fast and acute disease in newborns (Le May *et al.*, 2004).

A good marker for evaluation of natural or acquired RVFV immunity or vaccine potency is the antibody response elicited against the viral glycoproteins Gn and Gc. Some anti-Gn monoclonal antibodies have been used to map neutralizing epitopes within the Gn sequence (Besselaar and Blackburn, 1994). Such epitopes showed neutralizing activity *in vitro* against all RVFV isolates tested, which is correlated with the low sequence variation found between isolates (Battles and Dalrymple, 1988). However, antibodies raised against N are not able to neutralize RVFV *in vitro*, even knowing that the internal nucleoprotein N is a potent immunogen. Interestingly, N-based vaccines have conferred partial protection in some vaccination studies, pointing towards a lack of definition about the underlying mechanisms of immunity (Jansen van Vuren *et al.*, 2011; Lopez-Gil *et al.*, 2013; Spik *et al.*, 2006; Wallace *et al.*, 2006).

RVFV cellular immune response and its role in protection remains unclear. Recently, after immunization of BALB/c mice with recombinant MVA (Lopez-Gil *et al.*, 2013), adenovirus (Warimwe *et al.*, 2013) or DNA vaccines (Bhardwaj *et al.*, 2010), immunodominant CD8+ T-cell epitopes were identified in both the Gn and Gc glycoproteins. In contrast, glycoprotein-specific T-cell responses in the context of class-I molecules in vaccine target species (ruminants and humans) have yet to be identified; however, other potential T-cell epitopes have been identified using human modelling approaches. Polyfunctional CD8+ T-cell response in HLA-A2 transgenic mice was stimulated by N protein which carries HLA-A2 restricted epitopes (Xu *et al.*, 2013). There is an increased consensus in the important role

of antibodies in immunity to RVF and a potential involvement of a Th2-biased cellular response. RVFV clearance and protection from neurologic disease are dependent on CD4+ T cell and virus-specific antibody responses (Dodd *et al.*, 2013).

1.4.2. Ruminant models

The pathogenicity, transmission, strain virulence and immune responses upon experimental challenge with RVFV have been analyzed in great detail in ruminants, a main vaccine target species (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Capstick and Gosden, 1962; Coackley *et al.*, 1967b; Galindo-Cardiel *et al.*, 2012; Olaleye *et al.*, 1996; Swanepoel *et al.*, 1986; Tomori, 1979; Weingartl *et al.*, 2014a; Yedloutschnig *et al.*, 1981). The renewed interest in RVF vaccine development has leaded to standardize new ovine experimental models, which are very suitable for multiple reasons as reviewed (Table 4) (Kortekaas, 2014; Kortekaas *et al.*, 2011). Two ovine infection models have been used for vaccine efficacy studies: (i) a pregnant sheep model, which has inherent challenges related to pregnancy synchronization and the need for spacious high-containment experimental facilities (Antonis *et al.*, 2013; Baskerville *et al.*, 1992; Bird *et al.*, 2011; Dungu *et al.*, 2010; Harrington *et al.*, 1980; Hunter *et al.*, 2002; Morrill *et al.*, 1991a; Morrill *et al.*, 2013a; Yedloutschnig *et al.*, 1981), and (ii) a non-pregnant sheep model to monitor viremia levels, which is probably the easiest to implement but harder to interpret due to the heterogeneity of the results (Antonis *et al.*, 2013; Busquets *et al.*, 2010; Kortekaas *et al.*, 2012; Soi *et al.*, 2010; Weingartl *et al.*, 2014a; Yedloutschnig *et al.*, 1979). Inconsistency in detecting viremia or its duration in RVFV-inoculated sheep, changing RVFV titers, low mortality, and morbidity usually non-specific, are problematic facts of this latter model. In spite of the associated problems related to maintenance of newborn or very young lambs (less than one month old), seems reasonable to use these animals further for testing novel vaccine candidates, because of their higher susceptibility to disease than adult sheep (Coetzer and Ishak, 1982; Easterday *et al.*, 1962a; Van der Lugt *et al.*, 1996). In addition, the route of infection (usually subcutaneous, intramuscular or intravenous), breed of animals, the RVFV strain used and its passage history are responsible of the observed variation in ovine susceptibility to RVFV. A lack of an appropriate large animal infection model has delayed wider studies on the immunology, pathogenesis and vaccinology fields in target species.

Ovine RVFV infection consistently results in a severe leukopenia, being more severe in young lambs as compared to adult sheep (Easterday, 1965). In fact, at 3rd and 4th days p.i., the lowest counts of leukocytes were recorded in adult sheep (Easterday, 1965). Leukopenia was usually of short duration; even in animals with a fatal infection, the number of leukocytes began to return to pre-infection levels before death.

Such as we have previously described, mosquito bites can be considered the natural route of infection. Therefore, RVFV has been adapted to grow in both insect and mammalian cells for research. However, virus propagation has showed a marked heterogeneity in its results. For instance, the Egyptian ZH501 RVFV isolate propagated in insect (*Aedes albopictus*) C6/36 cells showed an increase of susceptibility of sheep after challenge than the same isolate propagated in monkey Vero E6 cells, suggesting a more efficient initiation of infection by using mosquito cells in subcutaneous challenges (Weingartl *et al.*, 2014a). However, no changes in susceptibility, viremia or IgM-related neutralizing antibodies (humoral response) were detected when using a South African RVFV strain (56/74) propagated in mammalian Vero E6 cells or in the insect C6/36 cells in a pilot study (Lorenzo *et al.*, 2015).

Sustained induction of IFN-γ following infection has reflected an activation of antigen-specific cellular responses against RVFV antigens using a panel of overlapping 15mer peptide pools spanning the whole mature GnGc sequence (Warimwe *et al.*, 2013). Stimulation of IFN-γ production in a challenge using insect cell-propagated RVFV challenge was higher than when using mammalian cell-propagated RVFV (Lorenzo *et al.*, 2015). Interestingly, one pool of peptides characterized by a putative single region/ epitope on the Gc polypeptide with characteristics of an immunodominant T-cell epitope was able to stimulate IFN-γ production in cells from all sheep, independently of the cells used for propagation (Lorenzo *et al.*, 2015). Further testing of these peptides are needed to better establish their capability of re-stimulation of IFN-γ production in T-cells from RVFV infected sheep of different breeds or genotypes, and to evaluate these responses in relation to protective immunity. However, these studies point to a possible role for IFN-γ in both the early innate antiviral response and in adaptive cellular immunity in sheep.

Nfon *et al*. revealed an earlier induction of systemic IFN-γ and IL-12, but not IFN-α, in a study about the innate immune response to infection with the Egyptian ZH501 RVFV infection in goats. Levels of TNF-α, IL-6 and IL1-β rose later in the course of infection in goats, correlating with the antagonistic function of the RVFV NSs protein. (Nfon *et al.*, 2012). In line with the observed variation depending of the virus propagation in sheep, baseline counts for CD172+ monocytes and dendritic cells (DCs) dropped only in goats challenged with the insect cell propagated virus but not in those receiving virus propagated in mammalian cells (Nfon *et al.*, 2012). A similar observation was found for both CD5+ and CD8+ T-cells as well as CD21+ B-cell counts. Direct infection of monocyte-derived cells has been demonstrated in *in vitro* infection experiments of monocyte-derived dendritic cells, leading to a plausible explanation for the reduction in the frequencies of monocyte-derived cells found in RVFV-infected goats (Nfon *et al.*, 2012) or monocyte-derived human CD14+ macrophages (McElroy and Nichol, 2012). Further, these data are supported by *in vivo* RVFV antigen-specific immunostaining of liver macrophages (Shieh *et al.*, 2010) and dendritic cells in mice (Smith *et al.*, 2010). Moreover, these results are in agreement with the proposed role for DC-SIGN (CD209), a surface highly-expressed protein of dendritic cells from mucosal, lymphoid and dermal tissues which is also present

in liver and alveolar macrophages, as an attachment factor for the entry of RVFV and other phleboviruses (Hofmann *et al.*, 2013; Lozach *et al.*, 2011). DC-SIGN is locating in cells involved on the activation of both innate and adaptive immune responses.

1.4.3. Human and Non-human primates models

In human sera, the proinflammatory chemokine CXCL-1 and the soluble CD40 ligand (sCD40L or CD154) were found elevated in non-fatal versus fatal cases, liking with disease severity the balance between proinflammatory and immunosuppressive cytokines (McElroy and Nichol, 2012). Conversely, both the IL-1 receptor antagonist (IL-1RA) and IL-10 levels were significantly higher in fatal cases than in non-fatal ones. However, larger studies on human sera are needed to determine the precise role of cytokine signalling upon RVFV infection.

Interferons have shown an antiviral activity. Macaques showed complete or marked protection to infective RVFV exposure after intravenous administration of recombinant or human IFN-α, either 24 h before infection or 6 h after infection, highlighting the importance of innate immunity in protection (Morrill *et al.*, 1989). In addition, a correlation between delayed interferon response and mortality was assessed by using Rhesus monkey model, pointing towards the need of an appropriate type I IFN response in limiting the severity of disease (Morrill *et al.*, 1990). Prophylaxis of monkeys with type II IFN (IFN-γ) 24 h before challenge has also obtained a protective effect, particularly on reduction of viremia titers (Morrill *et al.*, 1991b).

Rhesus monkey model has also been used for evaluation of safety and efficacy of candidate human vaccines. Several routes of inoculation including parenteral, aerosolized, oral and intranasal (Abdo-Salem *et al.*, 2011; Morrill and Peters, 2003; Morrill and Peters, 2011a; Morrill and Peters, 2011b) of the mutagenized attenuated MP12 vaccine (Caplen *et al.*, 1985) has been tested using rhesus monkeys for preclinical regulatory purposes. These studies have demonstrated a correlation between serum neutralization activity and reduced morbidity. A characteristic and unique biphasic neutralizing antibody response of naive (mock-vaccinated) monkeys was observed upon challenge. In fact, a delayed onset of neutralizing antibody responses to RVFV challenge could potentially explain the higher mortality rate in marmosets comparing to Rhesus monkeys (Smith *et al.*, 2012).

1.5. Vaccination and antiviral strategies

Epizootics causing particularly high rates of neonatal mortality and abortion in ruminants are increasing due to diverse factors, widely reviewed in the Point 1.2. Effective live attenuated RVF virus vaccines are available for livestock use, although safety issues preclude their distribution to non-endemic RVF areas (Ikegami and Makino, 2009; Indran and Ikegami, 2012). Besides, several outbreaks with increased human fatalities (Adam *et al.*, 2010; WHO, 2007), even reaching case fatality rates of >20% in different geographical settings (Al-Hazmi *et al.*, 2003; Hassan *et al.*, 2011), have prompted to the awareness of the community for the increased threat of RVFV to public health. Better techniques for new generation vaccines are needed more than ever. We describe a pinpointed selection of the trends in RVFV vaccinology.

1.5.1. Live attenuated vaccines

The only approved neurotropic partially attenuated vaccine, also called Smithburn strain, has been widely used in Africa from the late 1940s (Smithburn, 1949). This vaccine is a potent inducer for protection, but several contraindications consisting on abortions and RVF-related lesions in goats and cattle, has limited its use to areas threatened by an imminent outbreak (Bengis *et al.*, 2010; Botros *et al.*, 2006; Kamal, 2009).

New candidates appear to exhibit acceptable efficacy while offering less virulence. MP-12 is a human attenuated strain of RVFV derived from the virulent Egyptian strain ZH548 and have mutations in all three segments (Chevalier *et al.*, 2010). Besides, MP-12 displays a temperature-sensitive (ts) phenotype and does not replicate at 41°C. The ts mutation limits viral replication at a specific body temperature and may lead to an attenuation of the virus (Nishiyama and Ikegami, 2015). The vaccine is effective in sheep and cattle and its use is opened to endemic areas (Morrill *et al.*, 1991a; Morrill *et al.*, 1997a). In fact, recent evidences show that a single-shoot vaccination of MP-12 generates protective immunity to a virulent challenge with antibody duration of at least 2 years, with no evidence of a risk for vector transmission (Miller *et al.*, 2015). In spite of MP-12 can still be teratogenic in sheep thereby raising some of the same safety concerns, newborn lambs from immunized ewes acquire neutralizing antibodies via colostrum (Hunter *et al.*, 2002).

An individual clone of the human strain 74HB59, also called clone 13, was found to be highly immunogenic yet avirulent in mice and hamsters (Muller *et al.*, 1995). Clone 13 was found to have an in-frame deletion of most of the ORF of NSs. No noticeable pathogenic side effects, including abortions or male reproductive impairment, have been reported for this strain, while keeping its ability of conferring protection in pregnants ewes (Brown *et al.*, 2015; Dungu *et al.*, 2010; Lo *et al.*, 2015). RVFV clone 13 vaccine has shown to be safe to use and has high (>90%) immunogenicity in sheep and goats but moderate (> 65%) immunogenicity in cattle (Njenga *et al.*, 2015). The attenuated R566 strain contains the

three RVFV segments and is derived from clone 13 (S) and MP-12 (L, M) by reassortment. Complete protection was achieved in mice (Bouloy and Flick, 2009).

1.5.2. Inactivated vaccines

Formaline inactivated vaccines have also been developed for human use (Randall *et al.*, 1964; Randall *et al.*, 1962). Formalin-inactivated RVFV TSI-GSD-200 or formalin-killed Entebbe strain require several booster doses to maintain serum neutralization titers and to keep an effective level of immunity in humans and animals, respectively, what is a big disadvantage (Meadors *et al.*, 1986; Pittman *et al.*, 1999). Economic issues have done impractical its use in endemic areas. Currently, formalin-inactivated RVFV TSI-GSD-200 is being used for vaccination of military and laboratory personnel who are or may be in contact with the virus (Pittman *et al.*, 1999), a fast punctual need.

1.5.3. Virus-like particles

Enveloped virus-like particles (VLPs) create new opportunities to develop potent vaccines against pathogenic arboviruses, by creating complex assemblies of membranes and viral glycoproteins (Pijlman, 2015). Mammalian cell-derived VLPs carrying the neutralizing epitopes of RVFV glycoproteins can induce protective immunity in mice, without the risks of using a functional replicating virus (Naslund *et al.*, 2009; Pichlmair *et al.*, 2010). Baculovirus-infected insect cells RVFV VLPs needs the use of adjuvants to be efficient in mice and rats (Liu *et al.*, 2008; Mandell *et al.*, 2010). Economic limitations of the vaccine in the large-scale preparation of VLPs raise concerns about its use for widespread animal inoculation.

1.5.4. Recombinant viral vectors

In this approach, a gene encoding a major viral antigen is inserted into another non-virulent viral vector (recombinant virus), so that the cloned gene is expressed and the protein produced during viral infection. In theory, recombinant virus- infected animals are capable to react against the introduced antigen generating a humoral and cellular immune response. The vector virus can be unrelated to the original virus except for the introduced genes; therefore, this approach is somewhat analogous to an attenuated virus. Recombinant viruses can also induce secretory immunity if administered via an appropriate route. It is a safe and well-established approach in several potential vaccine candidates.

Complete protection associated to neutralizing RVFV antibodies has been achieved using a lumpy skin disease virus (LSDV), a nonreplicating, complex adenovirus, Venezuelan equine encephalitis virus, Newcastle disease virus (NCD), and capripoxvirus KS1 strain as vectors of Gn and Gc RVFV glycoproteins cDNA in mice, lambs and calves (Gorchakov *et al.*, 2007; Holman *et al.*, 2009; Kortekaas *et al.*, 2010a; Soi *et al.*, 2010; Wallace *et al.*, 2006). Furthermore, a single intramuscular inoculation NCD vector in lambs (encoding RVFV Gn/Gc) and calves (encoding RVFV-Gn) was sufficient to induce neutralizing antibodies (Kortekaas *et al.*, 2010a; Kortekaas *et al.*, 2010b). López-Gil *et al*. demonstrated protection in mice upon lethal RVFV challenge using a modified vaccinia Ankara (MVA) vector, also encoding Gn/Gc (E. López-Gil, unpublished observations).

Proteins NSm and NSs showed high efficacy in trials using veterinary species; therefore, they are now considered excellent vaccine candidates (Bird *et al.*, 2011; Dungu *et al.*, 2010; Weingartl *et al.*, 2014b). Reverse genetics have allowed doing NSm/NSs knockout mutants, which are quite stable either in being propagated in cell cultures or remaining immunogenic. In addition, splitting bunyavirus glycoprotein precursor genes have provided new opportunities to study bunyavirus genome packaging, offering new methods to develop next-generation live-attenuated bunyavirus vaccines (Brennan *et al.*, 2011; Wichgers Schreur *et al.*, 2014). Moreover, several RVFV variants, varying from viruses comprising two S-type segments to viruses consisting of four segments (RVFV-4s), of which three are M-type, could be rescued (splitting of the GnGc open reading frame) and were shown to induce a rapid protective immune response (Wichgers Schreur *et al.*, 2014). This genomic plasticity indicates that RVFV might behave as a viral vector to carry either mutant genes or even foreign antigens, as has been recently described for influenza Ha protein (Oreshkova *et al.*, 2014). Recent evidences have shown that a four-segmented RVFV variant lacking the NSs gene, created by splitting the M-genome segment, is able to induce sterile immunity in the natural target species after a single vaccination, preferably administrated via the intramuscular route (Wichgers Schreur *et al.*, 2015).

1.5.5. DNA vaccines

This approach of vaccination is supported by different advantages, such as (i) the fact that they can be administered to virtually anyone regardless of health status safely, and without any risk of reversion; (ii) multiple booster doses are no required for providing long lasting immunity; (iii) a DNA vaccine can be generated in large volumes at a much lower cost than some traditional vaccine types, making them a wonderful candidate to be given in emerging countries; (iv) the ability to store/ transport them under ambient conditions (i.e., no need for continuous refrigeration), which is very practical in endemic areas; and (v) their ability to express recombinant proteins that mimics viral ones, leading to a potent immune response against the antigen of interest.

The first approach for DNA RVFV vaccination have involved the recombinant DNA expression of one or both RVFV glycoproteins (Lagerqvist *et al.*, 2009; Lorenzo *et al.*, 2010; Spik *et al.*, 2006; Wallace *et al.*, 2006). Previous studies have demonstrated that neutralizing antibodies are induced by vaccinia virus-induced expression of the glycoprotein-encoding M segment in mice (Kakach *et al.*, 1988; Wasmoen *et al.*, 1988). Spik *et al*. described the first gene gun-delivered DNA vaccine encoding RVFV glycoproteins. Briefly, two DNA vaccines constructs were obtained, both of which express the viral M genome region encoding the envelope glycoproteins, Gn and Gc, but which differ in the amount of nonstructural M segment (NSm) coding information that is included; one beginning from the second AUG initiation site that encodes the nonstructural protein NSm (denoted NSm+); and, the second initiating translation from the fourth AUG site, thereby omitting NSm (denoted NSm−). Interestingly, NSm- was able to reach a complete protection inducing higher neutralizing antibodies titers than NSm+, which conferred a significant lower protection. RVFV DNA vaccine protected mice from challenge when delivered alone or in combination with other viral DNAs vaccines or adjuvants (Spik *et al.*, 2006). DNA vaccination studies using Gn have demonstrated that, a neutralizing antibody response associated to complete prevention against lethal RVFV challenge can be generated without coexpression of Gc in mice, using a Gn plasmid fused to trimers of C3d, a complement protein (self-adjuvant strategy) (Bhardwaj *et al.*, 2010). Such as it has been previously reviewed, mice and sheep vaccinated with a LSDV-vectored recombinant vaccine (rLSDV-RVFV) expressing the two RVFV glycoproteins (G1 and G2) developed neutralising antibodies and were fully protected when challenged, as were those vaccinated with a crude extract of truncated G2 glycoprotein (tG2) (Wallace *et al.*, 2006). By contrast, mice vaccinated with a DNA vaccine expressing G1 and G2 did not sero-convert with only 20% of them surviving challenge (Wallace *et al.*, 2006). Lagerqvist *et al*. described a 60% of protection after challenge of cDNA-vaccines encoding RVFV glycoproteins (Lagerqvist *et al.*, 2009). Lorenzo *et al*. described a dose-dependent protection in mice immunized with a construct encoding both mature glycoproteins, probably due to neutralizing antibodies induction (Lorenzo *et al.*, 2010).

The first approach for DNA RVFV vaccination have involved the recombinant DNA expression of nucleoprotein (Lagerqvist *et al.*, 2009; Lorenzo *et al.*, 2010). Lagerqvist *et al*. described a 50% of protection after challenge of cDNA-vaccines encoding RVFV nucleocapsid protein in mice (Lagerqvist *et al.*, 2009). Lorenzo *et al*. describes a partial protection in mice vaccinated with either N construct or a combination of both N and glycoproteins construct; interestingly, the combination of both vaccine constructs induced specific lymphoblast proliferation upon stimulation with a recombinant nucleoprotein (Lorenzo *et al.*, 2010). In a study performed by Lorenzo *et al*. with 6-month-old sheep, the sole immunization of DNA constructs encoding the glycoprotein precursor NSm/G2/G1 did not suffice to induce a detectable antibody response. In contrast, immunization of sheep with a plasmid vector encoding the viral nucleocapsid protein N elicited a potent and long lasting induction of antibodies but with low neutralizing titers. After DNA immunization, no antigen-specific proliferating cells were detected in ovine peripheral blood lymphocytes. Boosting with the attenuated vaccine strain MP12 was able to increase the

levels of proliferating memory cell pools and induction of IFN-gamma in response to purified virus or recombinant proteins, particularly in sheep vaccinated with a combination of both plasmid constructs. (Lorenzo *et al.*, 2008).

Ellenbecker *et al*. have recently used computational prediction and biochemical characterization of several novel RVFV RNA aptamers of nucleocapsid protein to demonstrate that the introduction of *in silico* generated RNA sequences into cells, prior to infection with RVFV, inhibited viral replication in cell culture. This proof of concept study demonstrates how the predictive power of bioinformatics and the empirical power of biochemistry can be jointly harnessed to discover, synthesize, and test new RNA sequences that bind tightly to RVFV N protein, leading to promising new vaccines products (Ellenbecker *et al.*, 2015).

2. Hypothesis and objectives

Such as we have described in the introduction, most of the promising novel candidate vaccines have not been extensively evaluated in the natural target species of RVFV. Robust challenge models are needed to properly evaluate the efficacy of the candidate vaccines, using the lowest number of animals possible. The refinement of these models should also consider the complete diagnosis, detection and proper evaluation of pathology related to candidate vaccines, mainly due to the safety concerns highlighted by currently licensed vaccines. Such optimized challenge models will facilitate future vaccination-challenge trials and significant inter-laboratory comparison of the results. Moreover, understanding the immune mechanisms involved in viral clearance and protection might be essential to design efficient vaccines in the future.

In order to better comply the general purposes planned in the project, the main objectives of this thesis are:

1) To establish a reproducible model of RVFV infection in 2-months old lambs.

2) To determine the pathogenicity of four different isolates RVFV available in CReSA.

3) To describe (3a) and characterized pathologically (3b) ocular injuries associated to RVFV infection.

4) To develop a new protocol for detection of RVFV antigens in ovine tissue using immunohistochemistry.

5) To check whether the inoculation of sheep with a recombinant vector strain of vaccinia virus Ankara (rMVA) that encodes the glycoproteins Gn and Gc RVFV is capable of conferring protection against a challenge with RVFV 56/74.

6) To study the immune response induced by T glycoproteins Gn and Gc.

Part of the results of these studies have been published in the papers, which are included as Annex 1, 2 and 3. The chapter 4 has been sent for publication at the time of presenting this manuscript. All the authors of these papers have given permission for including the material as part as this thesis.

3. Chapter 1: Experimental Infection of Young Adult European Breed Sheep with Rift Valley Fever Virus Field Isolates

3.1. Abstract

The increasing interest in Rift Valley fever virus (RVFV) and its potential impact on naive animal populations deserve revisiting experimental reproduction of RVFV infection, particularly in those animal breeds for which no data about their susceptibility to RVFV infection have ever been recorded. In this study we show the susceptibility of young European sheep (Ripollesa breed) to RVFV infection, showing a mild, subacute form of disease. Four different viral isolates efficiently replicated *in vivo* after subcutaneous experimental inoculation, and consistent viral loads in blood and virus shedding (variable in length depending on the RVFV isolate used) were detected, showing horizontal transmission to a noninfected, sentinel lamb. RVFV infection caused transient pyrexia in adult lambs and no other clinical symptoms were observed, with the exception of corneal opacity ("blue eye") found in 3 out of 16 subcutaneously inoculated sheep. In conclusion, adult sheep from this European breed are readily infected with RVFV without apparent clinical manifestations.

3.2. Introduction

Rift Valley fever virus (RVFV), a zoonotic mosquito-borne member of the *Phlebovirus* genus, belonging to the *Bunyaviridae* family, is responsible of significant economic losses in livestock production. The infection causes high mortality rates in lambs and adult animals by provoking bloody diarrhea, abortions in pregnant females, and stillbirths in sheep. Transmission to humans occurs by direct or indirect contact with infected animal body fluids and/or by mosquito bites (Al-Hazmi *et al.*, 2003; Sissoko *et al.*, 2009; WHO, 2007; WHO, 2008; Woods *et al.*, 2002). Human disease is characterized by febrile illness including myalgia, headache, and photophobia, which may rarely progress to retinitis, encephalitis, and/or fatal hemorrhagic fever (Flick and Bouloy, 2005; Gerdes, 2004).

Since the first significant epizootic episodes in South Africa in 1951, the disease expanded dramatically to North and East African countries (WHO, 2007) and out of Africa, in the Arabian Peninsula (Ahmad, 2000). Recently, large RVF outbreaks occurred in East African countries after heavy rainfall season and in Madagascar, Swaziland, and South Africa (Ahmad, 2000; Sissoko *et al.*, 2009; WHO, 2007). Because of climatic conditions, facilitating the expansion of mosquito niches (Anyamba *et al.*, 2009), global human and animal trade (Fevre *et al.*, 2006), and long-distance movement of RVF lineages (Bird *et al.*, 2007), RVF is considered an emerging threat for the nonendemic European countries.

Among animals, RVFV is spread primarily by the bite of infected mosquitoes, mainly *Aedes*, *Anopheles*, and *Culex* genus, in Africa (Pagès *et al.*, 2009) and also in the Mediterranean area, and thus mosquitoes have been able to act as potential vector of RVFV (Moutailler *et al.*, 2008).

The susceptibility of European ruminant species to RVFV infection is not well studied, and even less about their potential to act as natural amplifying hosts of this virus. Despite being an RNA virus, RVFV shows low (~5%) variability at the nucleotide level (Bird *et al.*, 2007). Differential pathogenicity between isolates has been reported (Anderson and Peters, 1988; Olaleye *et al.*, 1996; Swanepoel *et al.*, 1986). In this work, the pathogenicity of four different RVFV field isolates, from ruminant and mosquito origins, in a European sheep breed has been investigated. The potential of RVFV shedding as well as the risk of horizontal transmission to no-infected, in-contact lambs in the absence of competent mosquito vectors was also evaluated. The final aim of this work was to establish and characterize an infection sheep model for the assessment of future RVF vaccine prototypes, more particularly, a Modified-Vaccinia Ankara-based prototype designed by our research group.

3.3. Materials and Methods

3.3.1. Virus and preparation of viral inoculum

The four RVFV isolates used and their passage history during the study are listed in Table 6. Tissue culture infectious dose ($TCID_{50}$) titers of each virus stock were determined on BHK-21 cells by the method of Reed and Muench (Reed and Muench, 1938).

Table 6. Virus Isolates used in this study. Printed from Busquets, Xabier *et al*. 2010.

Name	Passage history	Isolation	Source	Reference
RVF 56/74 (WYJIUOWO)	#3 cer[a]	1974	Cow	(Barnard and Botha, 1977)
	#7 MDBK			
	#2 BHK-21			
	#MB[b]	1975	n.a.	n.a.
RVF 252/75	#2 VERO			
	#2 BHK-21			

RVF AN 1830	#MB	1956	Sheep	(Besselaar *et al.*, 1991)
	#1 VERO			
	#2 BHK-21			
	#1 VERO	1981	Mosquito	(Paweska *et al.*, 2003)
				(Wallace *et al.*, 2006)
RVF AR 20368	#6 MDBK			
	#2 BHK-21			

[a]Chicken embryo related cells.

[b]Mouse brain.

n.a., not available.

3.3.2. Animals

Animal procedures were approved by the Ethical and Animal Welfare Committee of Universitat Autònoma de Barcelona (UAB) in accordance with the EU directive 2010/63/EU for animal experiments. The experiments were conducted at Biosafety Level 3 (BSL-3) facilities of the Centre de Recerca en Sanitat Animal (CReSA-Barcelona). Nineteen lambs (Ripollesa breed) of both sexes, 9–10 weeks old at the time of experimental inoculation, were used. All animals were in-farm treated with insecticide to eliminate ectoparasites and then moved to the BSL3-CReSA's facility at 10 days before the infection. Lambs were fed following normal procedures used in conventional farms, with water supply ad libitum.

3.3.3. Experimental design, clinical and pathological sampling

All lambs were housed in the same box. Groups of four lambs were experimentally inoculated with each one of the RVFV isolates used. In all cases, 1×10^5 $TCID_{50}$ in 1 mL volume were inoculated subcutaneously, behind the elbow. A fifth group of three lambs remained noninoculated, albeit in contact with all the inoculated animals (as sentinels). Clinical signs, including rectal temperature and behavior, were daily recorded for 14 days. At 8 days' post-inoculation (dpi), two sheep from each group with negative quantitative retrotranscriptase polymerase chain reaction (qRT-PCR) blood results were euthanized and necropsied. The remaining sheep were sacrificed at 14 dpi.

Nasal and oral swabs were taken daily to track viral shedding collected with 1 mL of Trizol® (Invitrogen) for viral RNA extraction. Blood was also collected daily and processed as follows: 0.2 mL of total blood was lysed with 0.75 mL of Tri® reagent lysis buffer (Sigma) for viral RNA extraction. Serum samples from each animal taken at 0, 4, 8–9, and 14 dpi were analyzed for the presence of anti-RVFV antibodies.

From animals necropsied at 8 and 14 dpi, liver, kidney, lung, spleen, mesenteric lymph node, and inguinal lymph node samples were taken simultaneously paired for biological determinations and histological procedures (fixed in formalin 10%). One set of samples were immediately mixed with 0.5 mL RNAlater (Ambion), kept overnight at 2–8°C, and stored at −80°C until viral RNA extraction. Mediastinic lymph node, pancreas, ileum, adrenal gland, heart and cervical spinal cord were taken only for histology. Four-micrometer-thick sections of each paraffin-embedded block of studied animals were cut and processed for routine histological procedures (hematoxylin and eosin stain). All paraffin-embedded tissues were evaluated in a blinded fashion by the same pathologist.

3.3.4. RNA extraction and qRT-PCR

RNA from swabs and tissues was extracted using Trizol (Invitrogen) and from blood using Tri-reagent (Sigma), following the manufacturer's instructions. About 0.15 g of each tissue was homogenated in a volume of 0.75 mL of Trizol reagent. RNA was resuspended in 25 µL of nuclease-free water (Roche) and 3 µL was used for the amplification reaction in a total volume of 20 µL. RVFV RNA from samples was detected following a TaqMan one-step qRT-PCR specific for segment L of RVFV in a Fast7500 equipment (Applied Biosystems). The following primer pairs and TaqMan probe were designed previously (Mwaengo *et al*. unpublished) and synthesized by Tib Molbiol (Berlin, Germany); RVFV-fwd primer 5'-TTCTTTGCTTCTGATACCCTCTGT-3', RVFV-rev primer 5'-GTTCCACTTCCTTGCATCATCTG-3', and RVFV Taqman L probe 5'-(FAM)-TTGCACAAGTCCACACAGGCCCCT-(TAMRA)-3'. The amplification conditions were optimized to perform a one-step RT-PCR: 600 nM of RVFV-fwd, 900 nM of RVFV-rev, and 250 nM of RVFV-probe using OneStep RT-PCR Master Mix Reagents (Applied Biosystems) in a final volume of 20 µL. Then, the amplification cycles performed were reverse transcription at 50°C for 20 min, initial denaturing reaction at 95°C for 10 min, and 40 PCR cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s.

RVFV-RNA quantitation was achieved by using an *in vitro* translated RVFV L-specific RNA of 135 nucleotides as a standard. Briefly, a 135-bp L gene (positions 2872 to 3006) was PCR amplified with the aforementioned forward and reverse primers and directly subcloned within the T7 polymerase expression pGEMT vector (Mwaengo *et al*. unpublished). The complete insert including the upstream T7 promoter was amplified with a vector-specific primer (M13-40: 5'-GTTTTCCCAGTCACGAC-3') and the RVFV-rev primer using OneStep RT-PCR reagents (Qiagen). The PCR product was *in vitro* transcribed

to RNA with T7 polymerase (RiboMax; Promega). Finally, *in vitro* translated RVFV L-specific RNA was purified after several DNAse treatments and quantified by spectrophotometrical measurement (Qubit; Invitrogen). The number of RNA copies was calculated as previously described (Fronhoffs *et al.*, 2002). Serial 10-fold dilutions of this *in vitro* translated RVFV L-specific RNA were performed and the standard curves were generated. The limit of detection of this one-step qRT-PCR assay was as low as 3.89 copies/reaction of the in vitro transcribed RNA, equivalent to 1.44 $\log^{10}$ RVFV RNA copies per swab or 1.62 $\log^{10}$ RVFV-RNA copies per mL of blood or per gram of tissue.

3.3.5. Sequencing and phylogenetic analysis

RVFV M segment fragments corresponding to the most variable sequence (Bird *et al.*, 2007) were amplified by conventional RT-PCR upon RNA isolation from sheep blood or infected cells using specific primers. Sequence alignments and neighbor-joining distance trees were generated using the ClustalX 2.0 package software.

3.3.6. Serological tests

Sera from animals inoculated were evaluated for the presence of neutralizing antibodies. Heat-inactivated serum samples were serially diluted (twofold or threefold, starting at dilution of 1:4) in Dulbecco's modified Eagle's medium containing 2% fetal bovine serum, mixed with an equal volume (50 µL) of medium containing 4×10^3 pfu of a viral stock (MP12 strain). This mixture was added to Vero cell monolayers seeded in 96-well plates. After 3 days at 37°C, cells were fixed and stained with 0.75% crystal violet and 10% formaldehyde. The neutralization titer of each sample was determined as the reciprocal of the highest dilution of serum at which 75% neutralization was observed.

Detection of anti-RVFV IgM and IgG antibodies was performed as described previously (Lorenzo *et al.*, 2008). Briefly, ELISA plates were coated with rabbit polyclonal serum anti-RVFV diluted 1/2000 in carbonate/ bicarbonate buffer (pH 9.6). After blocking reactive sites with 5% skim milk in phosphate-buffered saline, 5×10^3 pfu of MP12 virus strain was added to a 50 µL volume. After three washing steps with 0.1% Tween-20 in phosphate-buffered saline, diluted heat-inactivated sheep sera were added to each well and incubated. After further washing steps, bound sheep antibodies were detected with either anti-sheep total IgG or IgM (Zymed-Invitrogen) HRP conjugates. Plates were incubated for 1 h at 37°C, washed three times, and 50 µL of soluble tetra methyl benzidine substrate (Sigma) was added to each well. The plates were then incubated in the dark at room temperature for a further 5 min. The reaction was stopped by the addition of 50 µL of 3 N H_2SO_4 and the optical density was determined at 450 nm.

Interferon (IFN)-γ assays were performed at 14 dpi using the whole blood bovine IFN test (Bovigam TB; Prionics). Whole sheep blood was first incubated overnight in the presence and absence of 10^5 $TCID_{50}$ of virus and the levels of IFN-γ secreted in plasma were then measured by Enzyme-linked Immuno Assay (EIA). The results were expressed for each sample in A450 units after subtraction of the negative control values.

3.4. Results

3.4.1. Infection-associated clinical signs

Lambs #21, #22, #25, #27, #29, #31, #33, #34 and #40 were euthanized at 8 dpi in the basis of its negativity to viremia.

The group 1 of animals (#21, #22, #23 and #24) reached fever to 42ºC in 2 dpi (Figure 5). No fever was recorded from 6 dpi in any case of the group 1. The only incidental clinical observation was pulmonary rumors in lamb #24, without mucus. This lamb showed an unilateral conjunctivitis in the left eye (1 out of 14 dpi).

Figure 5. Individual daily body (rectal) temperature of sheep during the whole experiment for experimental group 1: RVFV 56/74 (WYJIUOWO).

The group 2 of animals (#25, #26, #27 and #28) reached fever of 42ºC from 2 to 6 dpi (Figure 6). No fever was recorded from 6 dpi of the group 2, excepting lamb #26 at 9 dpi. Lamb #26 showed bilateral mucus (3 out of 14 dpi), marked apathy (2 out of 14 dpi) and bilateral conjunctivitis (6 out of 14 dpi).

Figure 6. Individual daily body (rectal) temperature of sheep during the whole experiment for experimental group 2: RVFV 252/75.

The group 3 of animals (#29, #30, #31 and #32) reached fever up to 42ºC in 2 dpi, from 2 to 5 dpi (Figure 7). Punctual fever pikes were recorded in all lambs of the group 3 in

several points of the experiment. Lamb #29 showed mild bilateral conjunctivitis (1 out of 14 dpi). Lamb #32 showed mild unilateral (right) conjunctivitis (3 out of 14 dpi).

Figure 7. Individual daily body (rectal) temperature of sheep during the whole experiment for experimental group 3: RVFV AN 1830.

The group 4 of animals (#33, #34, #35 and #36) reached fever up to 41,5ºC in 2 and 3 dpi, from 2 to 5 dpi (Figure 8). No fever was recorded from 6 dpi in any case of the group 4. No clinical signs were recorded in all experiment in group 4.

Figure 8. Individual daily body (rectal) temperature of sheep during the whole experiment for experimental group 4: RVFV AR 20368.

The group 5 of animals (#38, #39 and #40) was controls (unchallenged lambs) acting as sentinels. However, as they act as sentinels, lamb #38 reached fever up to 41,5ºC in 4 dpi, from 4 to 7 dpi (Figure 9). Lamb #39 only showed fever at 1 dpi, what it was considered incidental. No fever was recorded in lamb #40. No clinical signs were recorded in all experiment in group 5.

Figure 9. Individual daily body (rectal) temperature of sheep during the whole experiment for experimental group 5: Control (sentinel). Note this group is only composed by three lambs.

Comparing mean records of temperature between experimental groups, it was observed that all inoculated lambs showed fever from 2 to 5 dpi (Figure 10). Very few viral isolate-independent clinical signs associated with RVFV infection were observed during the infection. No icterus, hemorrhages or loss of appetite was recorded. No mortality was recorded either. The most evident clinical sign was a sharp increase in body temperature (pyrexia) in nearly all inoculated sheep, reaching 41.5°C–42°C at 2–3 dpi, depending on the individuals, with the exception of sheep #31 (inoculated with sheep isolate AN 1830), which did not present pyrexia throughout the study.

Figure 10. Mean daily body (rectal) temperature of groups during the whole experiment for experiment. Bars express standard error.

3.4.2. RVFV quantitation in blood, swabs, and tissues

Viral loads in blood, oral and nasal hyssops were quantified by qRT-PCR, quantitative retrotranscriptase polymerase chain reaction. All lambs were checked to confirm RVFV negativity in the beginning of the experiment (D0).

Lambs from the group 1: RVFV 56/74 (WYJIUOWO) reached viral loads in blood from 2 to 5 dpi about 2 to 6,5 $\log_{10}$ RNA copies/mL (Figure 11). The higher and long-lasting viremia was observed in the lamb #21, which reached the highest load in 3 dpi. No viremia was observed from 6 dpi in group 1. These findings correlate with infection-associated clinical signs observed in the group 1. Oral and nasal viral excretion were recorded from 3 to 6 dpi in the group 1, excepting for the lamb #24 which had not shedding in all the experiment.

Lambs from the Group 2: RVFV 252/75 reached viral loads in blood from 2 to 14 dpi about 2,5 to 7,5 $\log_{10}$ RNA copies/mL (Figure 12). The most of animals of the group 2 showed viremia from 2 to 4 dpi. The higher and long-lasting viremia was observed in the lamb #26 in which, remarkably, it was shown a persistent viremia until the end of the experiment. The higher viral load was observed in this lamb between 2 and 3 dpi. These findings correlate with infection-associated clinical signs observed in the group 2, in which lamb #26 was, by far, the more affected animal with bilateral conjunctivitis and nasal discharge.

Lambs from the Group 3: RVFV AN 1830 reached viral loads in blood from 2 to 14 dpi about 2 to 7 $\log_{10}$ RNA copies/mL (Figure 13). The most of animals of the group 3 showed viremia from 2 to 4 dpi. The higher viral load was observed in lamb #32 at 3 dpi. The long-lasting viremia was observed in the lamb #30 in which, remarkably, it was shown a persistent viremia until the end of the experiment. These findings correlate with infection-associated clinical signs observed in the group 3, in which lambs #30 and #32 showed pikes of fever from 2 dpi to the end of the experiment.

Lambs from the Group 4: RVFV AR 20368 reached viral loads in blood from 2 to 5 dpi about 1,6 to 4,9 $\log_{10}$ RNA copies/mL (Figure 14), which was the lowest range of all the RVFV-inoculated groups. The most of animals of the group 4 showed viremia from 2 to 4 dpi. Lamb #36 showed a delayed weak viremia at 4 dpi. No infection-associated clinical signs were observed in this group either.

Lambs from the Group 5: Control (sentinel) was not suspected to be viremic. However, lamb #38 reached a viral load in blood from 4 to 5 dpi about 5 $\log_{10}$ RNA copies/mL (Figure 15), which was interpreted as a delayed viremia correlated to a contact-associated RVFV infection. The rest of animals of the group 5 did not show viremia in any case. No infection-associated clinical signs were observed in the group 5 either.

Taking together all these findings, we observed that most animals from all groups showed a short increase in viral loads (2–5 days), correlating with pyrexia and peaking at 3 dpi. Remarkably, two lambs (#26, 252/75 group, and #30, AN1830 group) showed a long-lasting detection of viral RNA until the end of the experiment (14 dpi).

Despite all RVFV isolates were injected at the same concentration, they differentially replicated *in vivo*. All sheep inoculated with AN1830 isolates (group 3) displayed high viral load, ranging 6–8 $\log_{10}$ RNA copies/mL, with the exception of sheep #31, which remained negative for viral RNA detection (and no pyrexic). Also, similarly high viral loads were found in one out of four sheep in the groups inoculated with either the 56/74 cow isolate (groups 1) or the 252/75 isolate (group 2) ($\geq 10^6$ viral RNA copies/mL). In contrast, the AR20368 mosquito isolate (group 4) showed the lowest viral load in blood (only 3–4 $\log s_{10}$ RNA copies/mL).

Interestingly, all of the sheep with higher viral loads in blood showed also a longer window for RNA detection. Sheep #21 inoculated with isolate 56/74 (group 1) was positive for RVFV-RNA detection between 2 and 6 dpi, whereas in sheep #26 RNA was detected for 12 days. Similarly, in the group inoculated with the AN1830 isolate (group 4), viral RNA was detected for 4, 12, and 6 days in sheep #29, #30, and #32, respectively. In the remaining sheep, the viral RNA was detected for 2–3 days only.

Regarding viral shedding, nasal swabs became positive for RVFV RNA by qRT-PCR, 1 day after the peak in the viral load detected in blood (at 3 dpi) for most animals, lasting from 1 to 6 days (3 days in average). Exceptions to this trend were all sheep infected with the AN 1830 isolate (group 3), shedding virus as early as 2 dpi, and sheep inoculated with the AR 20368 strain (group 4), where few nasal swabs scored positive for RVFV-RNA (Figures 13 and 14). Oral swabs became RVFV-RNA positive between 1 and 4 days after the peak of RNA detection in blood samples, lasting for only 1–3 days for most of the animals. Again, only few cases of low RVFV excretion were reported in the AR 20368 group (group 4). Although nasal swabs showed slightly higher viral loads than oral swabs, both viral loads were consistently lower than those found in blood. There was a clear correlation between viral loads in blood and shedding levels. However, viral excretion occurred even when the viral load in blood was low, as illustrated by the AR 20368 group (group 4).

Interestingly, three of the lambs showing high viral load in blood at 3 dpi (#26, 252/75 group 2; #30 and #32, AN 1830 group 3), ranging between 6.43 and 8.35 $\log_{10}$ viral RNA copies/mL, as well as longer viral load in blood (up to 14 dpi), scored positive for RVFV-RNA in their kidneys, as detected by qRT-PCR, with viral RNA copies ranging between 1.53 and 2.53 $\log_{10}$/g at 14 dpi.

No positivity was found by the qRT-PCR assay in tissue samples from liver, lung, spleen and lymph nodes taken at 8 or 14 dpi.

3.4.3. Horizontal transmission of RVFV to noninfected, in-contact sentinels

Pyrexia was also recorded in one out of three sentinels, peaking at 4 dpi, delayed 2 days with respect to the inoculated sheep (Figure 9). This sheep showed highest viral loads (around 5 $\log_{10}$ RVFV RNA copies/mL) at 4–5 dpi (Figure 15). Sequencing of the variable RVFV M segment region obtained from sheep #38 showed the highest percent of identity with the corresponding sequence of AR20368 mosquito isolate (group 4). Therefore, the horizontal infection of sheep #38 was most probably originated by the mosquito isolate.

(a)

(b)

(c)

Figure 11. Individual daily viral load levels in blood samples (a), oral (b) and nasal (c) viral excretion measured by qRT-PCR during the whole experiment for Group 1: RVFV 56/74 (WYJIUOWO). The limit of detection (LoD) for the qRT-PCR used is shown by the horizontal red line.

(a)

(b)

(c)

Figure 12. Individual daily viral load levels in blood samples (a), oral (b) and nasal (c) viral excretion measured by qRT-PCR during the whole experiment for Group 2: RVFV 252/75. The limit of detection (LoD) for the qRT-PCR used is shown by the horizontal red line.

(a)

(b)

(c)

Figure 13. Individual daily viral load levels in blood samples (a), oral (b) and nasal (c) viral excretion measured by qRT-PCR during the whole experiment for Group 3: RVFV AN 1830. The limit of detection (LoD) for the qRT-PCR used is shown by the horizontal red line.

(a)

(b)

(c)

Figure 14. Individual daily viral load levels in blood samples (a), oral (b) and nasal (c) viral excretion measured by qRT-PCR during the whole experiment for Group 4: RVFV AR 20368. The limit of detection (LoD) for the qRT-PCR used is shown by the horizontal red line.

(a)

(b)

(c)

Figure 15. Individual daily viral load levels in blood samples (a), oral (b) and nasal (c) viral excretion measured by qRT-PCR during the whole experiment for Group 5: Control (sentinel). The limit of detection (LoD) for the qRT-PCR used is shown by the horizontal red line.

3.4.4. RVFV-specific immune responses upon *in vivo* infection

The dynamics of IgG and IgM and the development of neutralizing antibodies was similar in all infected groups, independently of the strain used and the levels of viral loads in blood (Figures 16, 17, 18, 19 and 20). Anti-RVFV IgG dynamics started with a slight raising of the titers at 4 dpi, followed by a rapid peak at 8 dpi and a second peak or slight increase at 12 dpi. Anti-RVFV IgM dynamics started with a similar increase to IgG of the titers at 4 dpi, followed by a most pronounced peak at 8 dpi and a quick decrease of the titers at 12 dpi. These Ig dynamics are consistent between the strains used. Neutralizing antibody responses were clearly detected at 8 dpi in all groups, coincidental with a peak in anti-RVFV IgM titers, with maximum titers (up to 1/5000) at the end of experiment (14 dpi), with a concomitant increase in anti-RVFV IgG titers (Figures 16, 17, 18, 19 and 20). Neutralizing antibody titers (1/2500) were also found in the infected sentinel at 14 dpi, as well as at low levels at 8 dpi, accordingly with a delay in detection of RVFV-RNA in blood and virus excretion.

To assess whether other immune mechanisms, such as cell-mediated immune responses, were induced upon RVFV infection, we tested the levels of specific IFN-γ induction in blood at 14 dpi (Figure 21). Although the overall IFN-γ levels were low, IFN-γ induction was much higher in those sheep infected with cow (Group 1: 56/74) and mosquito (Group 4: AR20368) isolates, both showing the lowest and shortest viral loads in blood (Figures 11 and 14, respectively). Contrarily, lower IFN-γ levels were found in those lambs with long, persistent viremia (Group 2: 252/75 and Group 3: AN1830) (Figures 12 and 13, respectively).

(a)

(b)

(c)

Figure 16. RVFV-specific immune responses upon *in vivo* infection for Group 1: RVFV 56/74 (WYJIUOWO). (a) Humoral IgG responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (b) Humoral IgM responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (c) Neutralizing antibody response, of individual sheep at the indicated days post infection.

(a)

(b)

(c)

Figure 17. RVFV-specific immune responses upon *in vivo* infection for Group 2: RVFV 252/75. (a) Humoral IgG responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (b) Humoral IgM responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (c) Neutralizing antibody response, of individual sheep at the indicated days post infection.

(a)

(b)

(c)

Figure 18. RVFV-specific immune responses upon *in vivo* infection for Group 3: RVFV AN 1830. (a) Humoral IgG responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (b) Humoral IgM responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (c) Neutralizing antibody response, of individual sheep at the indicated days post infection.

(a)

(b)

(c)

Figure 19. RVFV-specific immune responses upon *in vivo* infection for Group 4: RVFV AR 20368. (a) Humoral IgG responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (b) Humoral IgM responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (c) Neutralizing antibody response, of individual sheep at the indicated days post infection.

(a)

(b)

(c)

Figure 20. RVFV-specific immune responses upon *in vivo* infection for Group 5: Control (sentinel). (a) Humoral IgG responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (b) Humoral IgM responses found in serum from inoculated sheep at different times post inoculation measured by antigen capture ELISA. (c) Neutralizing antibody response, of individual sheep at the indicated days post infection.

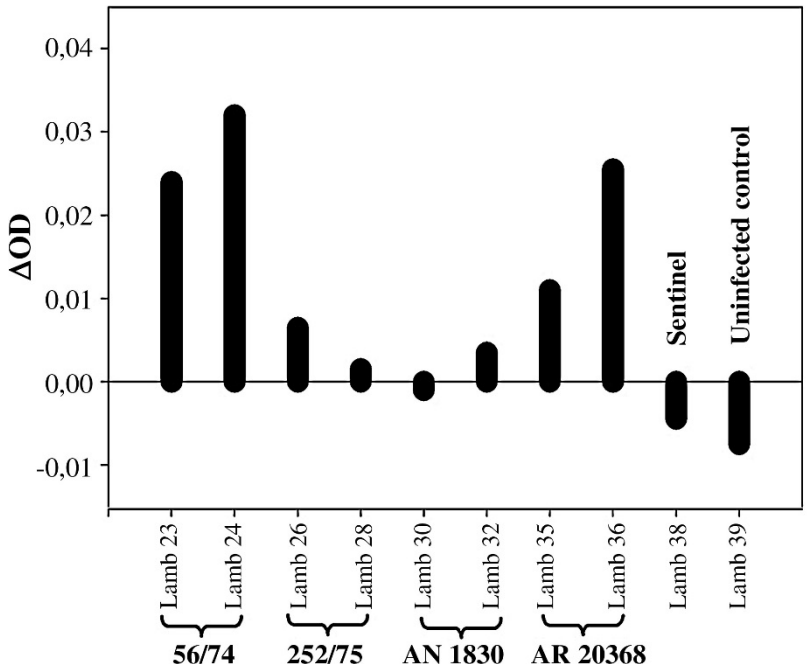


Figure 21. Levels of interferon-γ released in virus-stimulated sheep blood cultures taken at 14 dpi. Printed from Busquets, Xabier *et al*. 2010.

3.4.5. Pathological observations

A well-defined, infiltrative, multifocal, white-spotted, less than 2-mm-diameter hepatic nodules were observed in lambs #26, #28 (14 dpi), and #33 (8 dpi) as major gross finding (Figure 22). Other hepatic related finding was related to multifocal reddish nodules with vascular injection in diaphragmatic areas of both lobules in lamb #28. Both findings suggested mild multifocal necrotizing hepatitis, acute (#28) or subacute (#26, #28 and #33). Multifocal lymphoplasmocytic cholangiohepatitis (mild: lamb #34; moderate: lambs #22 and #25; severe: lambs #29 and #33), diffuse lymphoplasmocytic cholangitis (mild: lambs #23, #24, #27, #31, #32, and #35; moderate: lamb #28; severe: lamb #26, associated to fibrosis), and multifocal chronic biliary hyperplasia (related to all cited hepatic lesions) in different intensities were observed histopathologically. Mild multifocal lymphoplasmocytic cholangitis associated to scattered individual hepatocyte necrosis surrounded by minimal neutrophilic infiltrations were observed in #21, #22, #25, #28, #34, #39 and #40. The rest of the lambs showed no hepatic lesions.

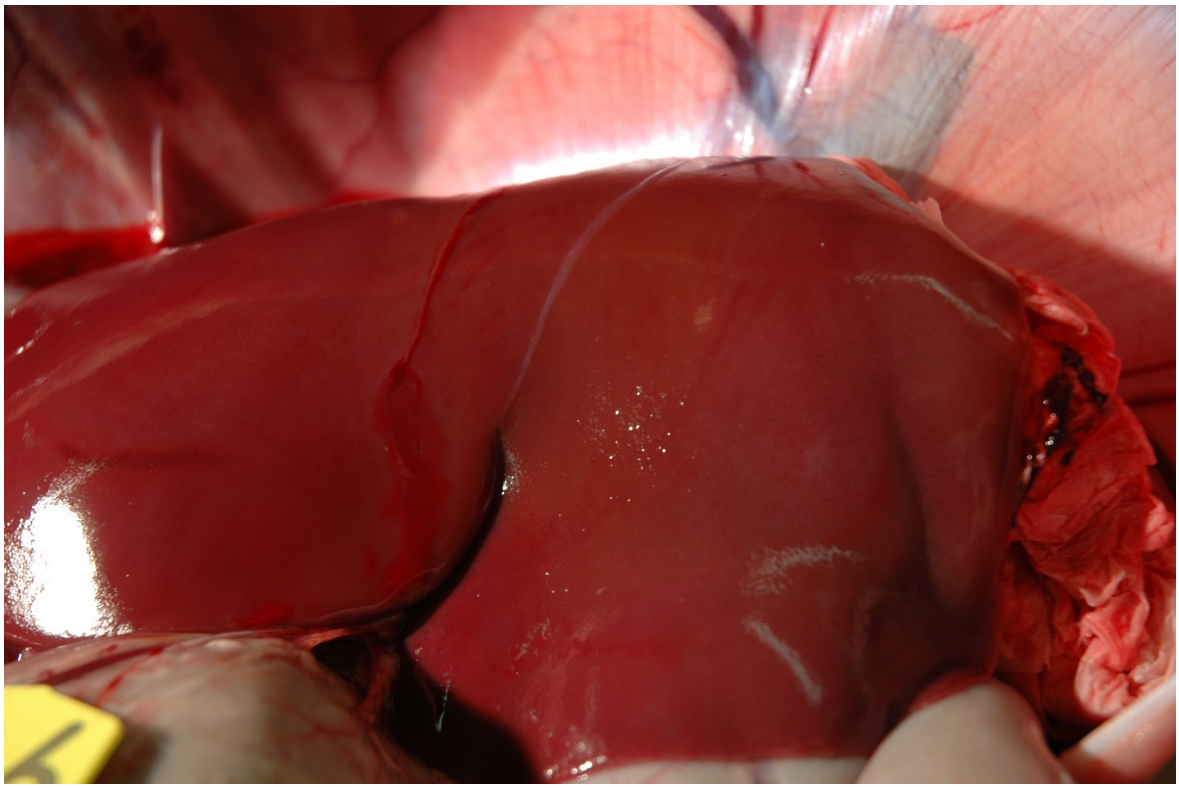


Figure 22. Hepatic lesions observed in lamb #26. Arrow heads shows the gross location of the whitish nodules in the hepatic lobules, clearly observed after transversal cutting (no shown).

As it was described in clinical results, moderate to severe keratoconjunctivitis with marked vascular injection was observed unilateral (#24, #32) or bilateral (#26, #29). Corneal opacity ("blue eye") was found at 8–9 dpi in lambs #26 (252/75 group) and #29 and #32 (AN1830 group), being bilateral for sheep #26 and #32 (Figure 23). Moderate chronic squamous corneal metaplasia, thickness of fibrous stroma, and mild hypertrophy of the posterior corneal endothelium were observed in the three affected corneas. Besides these findings, corneal edema associated with a mild diffuse transmural inflammatory infiltrate was observed in three lambs. Mild perivascular choroidal edema was found at posterior ocular chamber, closely related to retina, in lambs #26 and #32. A complete pathological description of these ocular findings is made in Chapter 2: Lymphoplasmacytic endotheliitis and anterior uveitis.

(a) (b)

Figure 23. Gross ocular findings observed in the experiment. (a) marked keratoconjunctivitis of right eye of lamb #24. (b) Corneal opacity ("blue eye") in left eye of the lamb #26

Pulmonary findings were mild bilateral lacking of lung collapse (#23, #24, #26) and dorso-caudal reddish consolidated areas in apical lobules with variable peri-bronchial fibrosis (#24, #30) consistent with mild chronic bronchopneumonia (Figure 24). Mild interstitial mononuclear inflammatory infiltrates with mild pneumocytic hypertrophy and mild to moderate chronic multifocal bronchoalveolar lymphoid tissue hyperplasia were observed in some lambs (#21, #22, #24, #25, #27, #30, #32, #33, #34, #35, #38, #39 and #40: 13 out of 19). However, the gross finding in lamb #26 was not correlated with its histology, what it was assumed it could be a wrong gross data report. Severe focally diffuse chronic pleuritic fibrosis with marked BALT hyperplasia was observed too in lamb #30. A moderate bronchointerstitial inflammatory pattern was observed in lambs #30 and #33. The chronicity involved in the pathogenesis of these pulmonary findings let us to hypothesize that they were not related with challenge.

Figure 24. Pulmonary findings in lamb #30. These chronic lesions were not related with challenge.

The only renal gross finding was a bilateral reddish pelvis with white cords in renal calix in lamb #26. Histologically, mild multifocal peri glomerular to interstitial lymphoplasmocytic and/or mononuclear infiltrates with glomerular retraction and mild proteinosis was observed in lambs #25 and #27, consistent with mild chronic glomerulonephritis. Moderate renal congestion was histologically confirmed in lambs #35 and #39. Lamb #36 showed a moderate multifocal chronic tubular mineralization.

No gross lesions were recorded in adrenal glands. The only remarkable finding was an acute focally extensive cortico-medullar hemorrhage in the adrenals of the #25 and #32 lambs.

Moderate subacute multifocal lymph node hypertrophy with marked follicles and variable degree of redness was observed in all lambs. Histologically, mild to moderate subacute follicular lymphoid hyperplasia with mild trabecular edema, cortical histiocytic infiltration intermixed with monocytes and an increase of mitosis in germinal centers was consistently observed in all necropsied lambs. Marked dilatation of lymphoid medullar sinus was seen in lamb #22. In the rest of lambs, the more the hyperplasia, the wider sinus dilation. Moderate to marked acute splenic congestion was seen in 17 out of 19 animals, closely related to euthanasia method (barbital overdose). Tangible body macrophages were seen in several lymph nodes of lamb #33 and #39. Moderate focally diffuse chronic splenic fibrosis was detected in lamb #26.

No gross lesions were recorded related to cardiac system. Intraneuronal (Purkinje cells) miocardial sarcocystosis with mild focal perivascular lymphoplasmacytic pericarditis was diagnosed in lamb #25. A mild multifocal lymphoplasmacytic monocytic myositis with segmental fiber degeneration and minimal to mild vacuolization was observed in lamb #22. The rest of animals remained within the normal limits.

No gross findings were related to intestine or pancreas. Ileum histology showed mild lymphangiectasia with a mild to moderate increase of eosinophils and macrophages in lamina propia. Intraepithelial lymphocytes showed a slight increase from normal tissues in all lambs diagnosed, clearly correlated with the detected general lymphoid hyperplasia. Mild diffuse endocrine pancreas hyperplasia (Langerhans islets) with a minimal to mild fibrosis was observed in #26, #27, #29 and #32. Moderate focally extensive interlobar hemorrhage in pancreas was observed in lamb #40.

No gross findings were observed in cervical spinal cord, although the limited open field did not let us to confirm any diagnosis. Histologically, the only relevant finding was an acute multifocal hemorrhages in dorsal and ventral spinal horns in the lamb #24. Lambs #28 and #32 showed a minimal focal neuronal megacariosis with minimal perilesional astrogliosis of gray matter. Mild reactive gliosis was observed in lambs #29 and #40.

3.5. Discussion

The risk of the introduction of Rift Valley fever in Europe was assessed in 2005 by a panel of experts (EFSA, 2005). In this work, we contribute with some data on the pathogenicity of four African isolates of RVFV in a European breed of young sheep. The data obtained allow withdrawing some conclusions with respect to the consequences of a RVFV infection of ruminants in Europe. The lack of a severe, fatal disease might be explained by

different results, such as the age of the animals used (9 weeks old), as previously reported (Kamal, 2009). Alternatively, the passage history of these strains in cell culture might also influence the moderately virulent phenotype observed. In agreement with this last hypothesis, higher viral loads in blood were reached after inoculation with RVFV isolates 252/75 and AN1830, which were less passaged in cell culture than 56/74 and AR20368 (Table 16). The fact that these virus strains were isolated from different animal species might also influence their pathogenic effects. Curiously enough, the two viruses showing lower replication levels were isolated from mosquito and cow.

In all infected animals, viral RNA was detected in blood and quantified by qRT-PCR with inter- and intra-group variations. However, all of them peaked at the same day (3 dpi), starting at 2 dpi, coincidental with the peak of fever. Our results are in good agreement with those from previous studies using different viruses and/or sheep breed (Olaleye *et al.*, 1996). In all animals infected with the AN1830 sheep isolate, viral loads in blood were detected for longer periods (at least 4 days), being able to persist in one lamb in blood and also in kidney throughout the experimental procedure. These same animals shed virus for longer periods and from earlier time points: as soon as at 2 dpi. Interestingly, the only virus isolated from the mosquito reservoir (AR20368) yielded the lowest virus RNA levels in blood and swabs among tested viruses, all isolated from ruminant, which it may reflect an incomplete adaptation to the mammalian host.

A correlation was observed between RVFV-RNA load in blood and kidney, which is considered a secondary target organ (Kamal, 2009), with the threshold being around 6.43 $\log_{10}$ RVFV RNA copies/mL at 3 dpi in blood. Therefore, RVF 252/75 and RVF AN 1830 isolates would persist in kidney for longer time than the other isolates once reached certain viremia titers. Interestingly, viral RNA copies were detected in kidney samples from both sheep displaying bilateral blue eye. Viral infections such as RVFV has been related to major ocular changes in humans and animals (Al-Hazmi *et al.*, 2005; Gerdes, 2004; Khairallah *et al.*, 2010). Our findings suggest a chronic corneal sequel, which could be associated with drainage failure or inadequate corneal dehydration after a transient viremia (lost of corneal deturgescence). Severe deleterious hepatic pathological changes in kids (necrogranulomas) and hyperplastic overgrowth with vesicular elongated epithelium (even necrotic in some cases) and lymphocytic infiltration were diagnosed in RVF live attenuated vaccinated (Smithburn strain) goats (Kamal, 2009). Our results do not show any relation between the presence of hepatic lesion and virus load or excretion in contrast to that reported by Kamal (2009). This fact may point us toward two possibilities with the RFV-infected lambs: (1) a recovery phase of the RVF infection or (2) a chronic hepatic sequel after hepatic replication. Further studies will be carried out to characterize the observed ophthalmic and hepatic lesions. Taken together the pathological diagnoses, it was concluded that hepatic and ocular findings were the only findings clearly RVFV challenge-related. The rest of findings were considered as incidental following criteria of evidence-based clinical approach (Geyman, 2000).

Although the route of transmission is not known, the virus isolate responsible for the infection observed in one of the three sentinel animals was, surprisingly, the AR20368 mosquito isolate, as it was stated by genome sequencing. As sheep inoculated with this isolate did not display the highest nasal and oral shedding titers, other factors might be involved in the observed transmission. Also, as all inoculated animals were housed in the same box, potential cross-contamination events cannot be ruled out. In any case, the high and long viral load in blood observed might be advantageous for virus dissemination by competent vectors, and therefore, competence studies with the four virus isolates could be addressed in the future. Further, these long-term viremic infected lambs may be potentially dangerous (Easterday *et al.*, 1962a; Erasmus and Coetzer, 1981; Woods *et al.*, 2002), for example, in the case of being slaughtered, for its risk of viral transmission to human by direct contact with infected fluids. Our results confirm that horizontal RVFV transmission is not an unusual event, as it has been previously described with in-contact American sheep becoming infected with the RVFV Zagazig 501 isolate (Harrington *et al.*, 1980). Therefore, if infection pressure inside herd is high enough, it could be stated that some strains of RVFV could be horizontally transmitted, independently of vector presence.

Induction of a neutralizing antibody response is a good correlate for controlling infection and protection after challenge with RVFV (Harrington *et al.*, 1980; Hubbard *et al.*, 1991; Morrill *et al.*, 1991a). For most animals, virus was cleared from the body fluids before neutralizing antibodies were detected, and thus it is reasonable to speculate that other early immune mechanisms play a role in the elimination of the virus, most probably related with nonspecific innate immune responses. Although a clear *in vitro* neutralizing activity was observed in all sera, this immune response was undistinguishable from the virus isolate used. In contrast, a clear correlation was observed between specific IFN-γ detection at 14 dpi and viral clearance, with persistently infected sheep showing almost no specific IFN-γ responses. Curiously enough, persistently infected cells also showed the lowest IFN-γ response to the unspecific phytohemagglutinin (PHA) stimulation, suggesting a transient immune suppression. Understanding the immune mechanisms involved in viral clearance and protection might be essential to design efficient vaccines in the future.

In conclusion, adult lambs from the European Ripollesa breed are susceptible to experimental infection with African RVFV isolates of different origins. Transient pyrexia, rising viral loads in blood, and viral shedding were recorded for all viruses, with no severe clinical signs of disease. Further, both specific humoral and cellular responses were detected after viral infection, thus opening the possibility of using this animal model to test potential vaccines against RVFV.

4. Chapter 2: Lymphoplasmacytic Endotheliitis and Anterior Uveitis in Sheep Infected Experimentally with Rift Valley Fever Virus

4.1. Summary

Lymphoplasmacytic endotheliitis and anterior uveitis was diagnosed in four lambs infected experimentally with field isolates of Rift Valley fever virus (RVFV). Formalin-fixed and paraffin wax-embedded tissue from these animals was investigated by histopathology and quantitative real time reverse transcriptase polymerase chain reaction. To our knowledge, this is the first pathological description of this ocular manifestation of RVFV infection in ruminants, although these lesions have been described in man.

4.2. Introduction

Rift Valley fever virus (RVFV) is a negative-sense, segmented, single-stranded RNA *Phlebovirus* (family *Bunyaviridae*) transmitted by mosquito vectors. This zoonotic infection can lead to high mortality in ruminants by causing bloody diarrhea, liver failure, abortion and stillbirth. Sheep are highly susceptible to RVFV infection depending on the virus strain and the breed and age of the animals (Busquets *et al.*, 2010; Coetzer and Ishak, 1982; Olaleye *et al.*, 1996). Human disease is characterized by febrile illness, including myalgia, headache and photophobia, which may progress rarely to retinitis, encephalitis and/or fatal hemorrhagic fever (Gerdes, 2004).

The present report describes lymphoplasmacytic anterior uveitis, resembling the ocular alterations in human patients, in four 9–10-week-old Ripollesa breed lambs infected experimentally with different strains of RVFV.

4.3. Material and Methods

4.3.1. Study design and pathological evaluation

Four experimental groups of four lambs per group (n = 16) were inoculated subcutaneously behind the elbow with 1×10^5 $TCID_{50}$ (in a 1 ml volume) with one of four different strains of RVFV. Three additional lambs were not inoculated and served as controls (Busquets *et al.*, 2010). Lambs were observed daily for clinical signs and there was daily recording of rectal temperature and blood sampling for detection of RVFV RNA.

Two non-viraemic lambs in each group were killed 8 days postinoculation (dpi) and the remaining animals were killed at 14 dpi. All lambs underwent necropsy examination. Tissue samples were taken routinely from several organs, but the eyes were only collected from four lambs that showed clinical evidence of ocular disease (see below) during the experiment (Busquets *et al.*, 2010). The entire globes were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (4 µm) of each eye were prepared through the sagittal central axis, perpendicular to the long posterior ciliary artery (Peiffer *et al.*, 1999), and were stained with haematoxylin and eosin (HE), Gram stain and periodic acid–Schiff (PAS). Only the ocular lesions are described in this report.

4.3.2. RNA extraction and qRT-PCR

To assess the possible role of RVFV in the causation of the ocular lesions, a quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using primers specific for the M segment of RVFV. RNA was extracted from the formalin-fixed and paraffin wax-embedded ocular tissues. Four sections (20 µm) were processed as described by Sorg and Metzler (1995) with some modifications (Sorg and Metzler, 1995). Briefly, sections were dewaxed by washing for 15 min in xylene, twice more for 10 min in xylene and twice more for 10 min in absolute ethanol at room temperature. After air drying, all sections were incubated with 1 ml of digestion buffer (1 mg of proteinase K per ml, 20 mM Tris–HCl [pH 8.0], 10 mM EDTA [pH 8.0], 1% sodium dodecyl sulphate) at 45°C with shaking (300 rpm) overnight. Each tube was divided in two equal parts; the tissue from each tube was homogenized in 1 ml Trizol reagent (Invitrogen, Paisley, UK) and RNA extraction was carried out following the manufacturer's instructions. RNA was resuspended in 40 µl nuclease-free water (Ambion, Austin, Texas, USA) and 3 µl was used as template for RT-PCR reactions in a total volume of 20 ml, as described by Busquets *et al*. (2010).

4.4. Results

4.4.1. Ocular pathology findings

Diffuse complete corneal opacity ('blue eye') was observed bilaterally in lambs #26 and #32, and unilaterally in lamb #29 at 8–9 dpi (Figure 25), and this lasted from 1 to 5 days in the different animals. The eyes from lamb #24 were also collected because small corneal erosions were seen at necropsy examination.

Major histological findings in the eyes are summarized in Figure 26 (lamb #26). Lesions ranged from mild (lambs #24 and #32) to marked (lambs #26 and #29). Corneal lesions consisted mainly of mid-zonal to diffuse oedema with a few scattered neutrophils, and peripheral mild superficial and mid-stromal neovascularization. A moderate mixed inflammatory infiltrate composed of neutrophils and mononuclear cells (i.e. lymphocytes, plasma cells and some macrophages) was seen in the limbus and in the peripheral cornea. The corneal endothelium was sometimes swollen or hypertrophic, had focal areas of attenuation with occasional karyorrhectic nuclei and loss of continuity. Multifocal infiltration of mononuclear cells beneath this endothelium was interpreted as endotheliitis. In the anterior uvea (i.e. iris and ciliary bodies) there was perivascular to diffuse inflammatory infiltration of plasma cells, lymphocytes and fewer macrophages. The density of these cells increased towards the filtration angle where they were organized into a sheet. The inflammation did not extend to the choroid in any eye. An incipient thin pre-iridal fibrovascular membrane was observed in one case (lamb #26). A loose, slightly basophilic fibrillar material with enmeshed cells, mainly macrophages and a few neutrophils, some of them karyorrhectic or degenerate, was seen in the anterior chamber in the more severely affected lambs. Clumps of these cells were also attached to the anterior surface of the iris or to the corneal endothelial lining (keratic precipitates). Retinal findings consisted of occasional minimal perivascular mononuclear cells with rare neutrophils, but a definitive diagnosis of retinitis was not made. No bacteria, protozoa or fungi were detected in Gram- and PAS-stained sections.

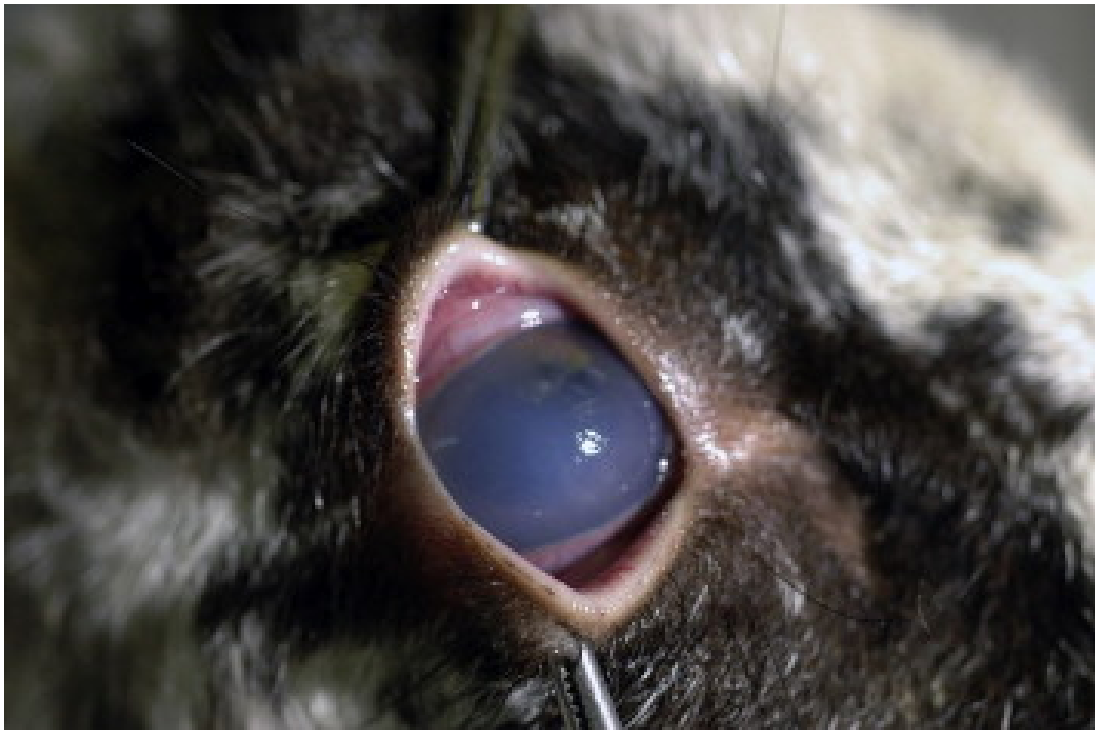


Figure 25. Diffuse corneal opacity (‘blue eye’) in the eye of RVFV-infected lamb #26. Printed from Galindo-Cardiel, Busquets *et al*. 2012.

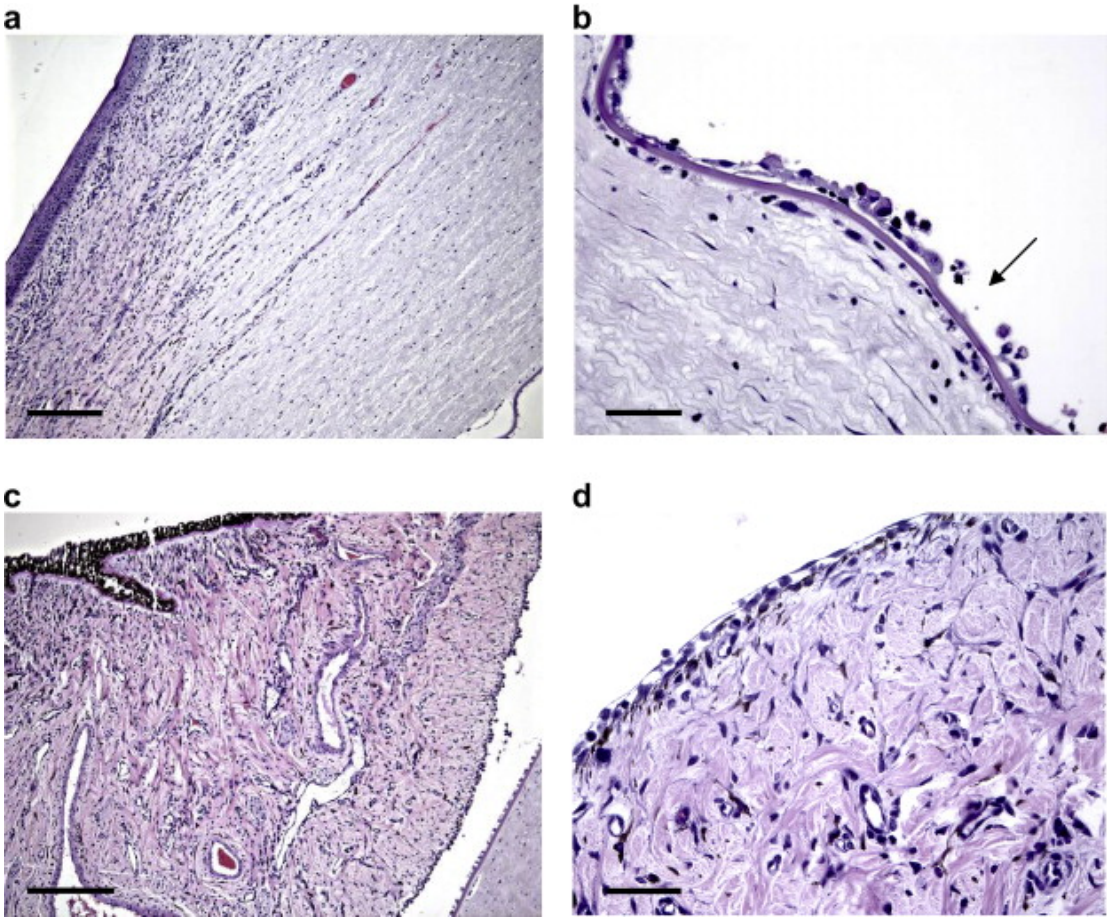


Figure 26. Microscopical findings in the eye of RVFV-infected lamb 26. Printed from Galindo-Cardiel, Busquets et al. 2012. (a) Anterior cornea. Chronic (subacute) lymphoplasmacytic keratitis with superficial and mid-stromal corneal neovascularization. HE. Bar, 250 µm. (b) Posterior cornea. Corneal oedema, endothelial damage with attenuated and hypertrophic cells, focal loss of continuity (arrow), keratic precipitates and lymphocytic infiltration. HE. Bar, 50 µm. (c) Uvea and iris. Perivascular to interstitial lymphoplasmacytic infiltration. HE. Bar, 250 µm. (d) Anterior surface of the iris. Pre-iridal fibrovascular membrane with lymphoplasmacytic uveitis. HE. Bar, 50 µm.

4.4.2. RVFV quantification in paraffin-embedded tissues

Viral RNA was detected only in the eyes of lambs #26 and #29, coinciding with those animals showing more severe ocular lesions; the viral load was 3.01 and 4.33 log RVFV RNA copies/100 µg of ocular tissue, respectively. Of these two animals, only lamb 26 was still viraemic at 14 dpi (the end of the experiment). Viral RNA was also detected in kidney samples from lambs #26 and #32, but not from lamb #29. Although a strong correlation between RVFV RNA loads has been observed between blood and kidney tissue (Busquets *et al.*, 2010), our findings show that RNA from RVFV may still persist in the kidney or the eye in detectable amounts after the viraemic phase of the disease.

4.5. Discussion

The present study has characterized ocular lesions following experimental infection of lambs with three different South African isolates of RVFV. Detection of viral RNA in two of the lambs may suggest an active systemic or residual ocular infection. RVFV can cause major ocular disease in man, leading to blindness (i.e. anterior uveitis, retinitis, chorioretinitis, macular and paramacular scarring, vascular occlusion, and optic atrophy) (Al-Hazmi *et al.*, 2005; Al-Hazmi *et al.*, 2003; Gerdes, 2004; Khairallah *et al.*, 2010; Yoser *et al.*, 1993). However, no histological descriptions are available from human cases so, an unequivocal correlation between the findings of this study and the human condition should be made with caution. Interestingly, similar kinetics are observed between the human and ovine infections, with the onset of human ocular clinical signs occurring 5–14 days after local outbreaks of RVFV in ruminants (Khairallah *et al.*, 2010). Infectious viral anterior uveitis with or without chorioretinal injury in ruminants has been described in malignant catarrhal fever, bluetongue and rinderpest infections (Holzhauer and Vos, 2009; Rossiter, 2001; Vikoren *et al.*, 2006; Whiteley *et al.*, 1985), but no descriptions have been made of these lesions in ruminant RVFV infections.

The major gross finding in this study was corneal edema. This change occurs under three circumstances, which are not mutually exclusive: corneal ulceration, corneal endothelial dysfunction and vascular leakage from corneal neovascularisation (Peiffer *et al.*, 1999). Histopathological observations suggested that the corneal edema was related to endothelial damage (lymphocytic endotheliitis) and that the mid-stromal corneal neovascularization was secondary to the anterior uveitis. An immune-mediated mechanism has been proposed as the underlying cause of viral endotheliitis and anterior uveitis in ruminants (Holzhauer and Vos, 2009; Whiteley *et al.*, 1985), and a similar pathogenesis may be assumed in these cases.

No retinal-associated clinical or pathological blindness was observed in this study. The detection of virus RNA in kidney, blood and eye suggested two clinicopathological possibilities: (1) a recovery phase of RVFV infection with different ocular viral clearance or, (2) a pathological sequel (Al-Hazmi *et al.*, 2005; Al-Hazmi *et al.*, 2003; Gay, 2006; Khairallah *et al.*, 2010). Surprisingly, lamb 24 showed mild anterior uveitis without corneal edema. This fact may suggest that subclinical uveitis may be more prevalent in sheep infected with RVFV. The ocular changes were not followed up for a longer time because of the experimental design, so further studies should be carried out to confirm the present observations.

The findings of this study suggest that sheep may be used as a model for studying RVF-associated ocular pathology in man, although further phenotyping and genotyping studies are needed. RVFV infection should be included in the list of viral entities that can cause uveitis in ruminants.

5. Chapter 3: Efficacy assessment of an MVA vectored Rift Valley Fever vaccine in lambs

5.1. Summary

The present study has evaluated the protection conferred by a single subcutaneous dose of a modified vaccinia virus Ankara (MVA) vectored vaccine encoding the Rift Valley Fever virus (RVFV) glycoproteins Gn and Gc in lambs. Three groups of six to seven lambs were immunized as follows: one group received the vaccine (termed rMVA-GnGc), a second group received an MVA vector (vector control) and a third group received saline solution (non-vaccinated control). Fourteen days later, all animals were subcutaneously challenged with 10^5 $TCID_{50}$ of the virulent RVFV isolate 56/74 and vaccine efficacy assessed using standard endpoints. Two lambs (one from the vaccine group and one from the vector control group) succumbed to RVFV challenge, showing characteristic liver lesions. Lambs from both the vector control and non-vaccinated groups were febrile from days 2 to 5 post challenge (pc) while those in the rMVA-GnGc group showed a single peak of pyrexia at day 3 pc. RVFV RNA was detected in both nasal and oral swabs from days 3 to 7 pc in some lambs from the vector control and non-vaccinated groups, but no viral shedding could be detected in the surviving lambs vaccinated with rMVA-GnGc. Together, the data suggest that a single dose of the rMVA-GnGc vaccine may be sufficient to reduce RVFV shedding and duration of viremia but does not provide sterile immunity nor protection from disease. Further optimization of this vaccine approach in lambs is warranted.

5.2. Introduction

Rift Valley Fever virus (RVFV) is a mosquito-borne bunyavirus that causes a zoonotic disease. The virus is composed of a tripartite RNA genome, comprising large (L), medium (M) and small (S) genome segments. The viral nucleoprotein N and the virulence-associated non-structural protein NSs are encoded in the S segment in ambisense orientation while the L segment encodes the viral polymerase, and the M segment encodes at least two non-structural proteins (termed NSm and 78 kDa) and the structural glycoproteins Gn and Gc (Bouloy and Weber, 2010; Gerrard and Nichol, 2007). RVFV is widely distributed in the African continent, the Arabian Peninsula (Balkhy and Memish, 2003) and Indian Ocean islands (Gerdes, 2004; Pepin *et al.*, 2010; Sissoko *et al.*, 2009), where it has caused major disease outbreaks in both human and livestock. Newborn lambs and gestating ewes are extremely vulnerable to RVFV infection, which results in very high rates of mortality and abortion, respectively. RVF is now considered an emerging threat for European countries due to trade and globalization and the ability of the virus to replicate in and be transmitted by numerous mosquito species (Pepin *et al.*, 2010). As well as improving virus detection it is desirable to develop better vaccines against RVFV in

susceptible animal species. Currently licensed vaccines do not meet the safety standards required for use in Europe.

Such as it has been described in point 1.5. of this manuscript, two classical RVF vaccines have been used to control recent outbreaks in South Africa; a formalin inactivated vaccine (Harrington *et al.*, 1980) and a live attenuated virus strain (Smithburn, 1949). Both these vaccines retain some flaws such as low immunogenicity and potentially adverse side effects, respectively. A live-attenuated vaccine termed 'Clone 13' has been licensed for use in several countries in Africa, being safer for pregnant ewes (Dungu *et al.*, 2010) although it has been shown that Clone 13 replicates and is excreted in saliva in competent mosquito species (Amraoui *et al.*, 2012; Muller *et al.*, 1995). In addition, there is a need for human vaccines that could be deployed among populations at risk including farmers, veterinary and/or medical personnel at risk after a RVF outbreak.

Current knowledge in the mechanisms involving the induction of immunity against RVFV in mammals may help in the design of novel vaccines with the potential for use in humans. Some of the most promising approaches tested to date for the generation of RVF vaccines are based on the use of attenuated recombinant viruses rescued by means of reverse genetics (Bird and Nichol, 2012; Morrill *et al.*, 2013a; Morrill *et al.*, 2013b; Oreshkova *et al.*, 2013). Other approaches have used live viral vectors such as adenoviruses (Holman *et al.*, 2009; Warimwe *et al.*, 2013), alphaviruses (Gorchakov *et al.*, 2007; Heise *et al.*, 2009), paramyxoviruses (Kortekaas *et al.*, 2010a) and members of the Poxviridae family (Papin *et al.*, 2011; Soi *et al.*, 2010; Wallace *et al.*, 2006) to deliver immune relevant RVFV antigens. Recently, we observed that a single immunization with a recombinant modified vaccinia virus Ankara encoding the RVFV glycoproteins Gn and Gc (rMVA-GnGc) can protect mice against lethal RVFV challenge (Lopez-Gil *et al.*, 2013). In the present work we evaluate the immunogenicity and protective efficacy of the rMVA-GnGc vaccine in sheep, a major target species for the virus.

5.3. Materials and methods

5.3.1. Virus and vaccines

The South African virulent RVFV 56/74 isolate used for sheep challenge studies was kindly provided by the Agricultural Research Council-Onderstepoort Veterinary Institute (South Africa). The virus was chosen in the basis of experimental results described in Chapter 1 (point 3) and its availability in the laboratory. This virus was originally isolated from cattle (Barnard and Botha, 1977) and subsequently propagated 3 times in chicken embryo-related cells and seven times in MDBK cells. Two additional passages in BHK-21 cells were

performed to generate a viral stock. $TCID_{50}$ titers of the virus stock were determined on Vero cells by the method of Reed and Muench (Reed and Muench, 1938).

The MVA vaccine was generated by homologous recombination in MVA infected chicken embryo fibroblast (CEF) cells of a recombinant plasmid encoding GFP as marker and RVFV glycoprotein GnGc sequences in a bi-cistronic expression cassette as described (Lopez-Gil *et al.*, 2013). The virus was grown in permissive DF-1 cells (ATCC# CRL-12203), the supernatants and lysed cells harvested and virus pelleted by ultracentrifugation through 36% sucrose cushion. Titration of vaccine virus was performed in DF-1 cell monolayers. Both vaccine virus and challenge virus stocks were stored at −80 °C until use.

5.3.2. Experimental design, clinical records and sampling procedure

Animal procedures were approved by the Ethical and Animal Welfare Committee of Universitat Autònoma de Barcelona (UAB) in accordance with the EU directive 2010/63/EU for animal experiments. The experiments were conducted at Biosafety Level 3 (BSL-3) facilities of the Centre de Recerca en Sanitat Animal (CReSA-Barcelona). Nineteen lambs (Ripollesa breed) of both sexes, aged 5–7 weeks at the time of vaccination were used. All animals were in-farm treated with insecticide to eliminate ectoparasites and then moved to the BSL-3 facility 5 days prior to the vaccination. Lambs were fed as per procedures used in conventional farms, with water supply provided *ad libitum*.

All lambs were housed in the same animal box. The lambs were distributed into three groups. The first two groups, composed of 6 lambs each, were immunized with 10^8 plaque-forming units (pfu) of the rMVA-GnGc vaccine (vaccine group) or with an rMVA only encoding GFP (vector control group), respectively. The third group (infection control; n = 9) received saline solution. In all groups, lambs were inoculated subcutaneously using 25G needle injection, behind the elbow. One lamb from the vaccine group (#124) accidentally received an incomplete dose of the rMVA-GnGc vaccine.

Two weeks after immunization the lambs were challenged with 10^5 $TCID_{50}$ of RVFV 56/74 subcutaneously. Clinical signs, including rectal temperature and behavior, were recorded daily until the end of the experiment. Animals were considered febrile if rectal temperature was above 40.2 °C. This threshold was derived based on the mean plus three standard deviations of the rectal temperatures recorded seven days before the challenge.

Two lambs belonging to RVFV-infection control group (lambs #138 and #141) were euthanized at 4 dpi for pathogenic evaluation of the RVFV 56/74 challenge. All lambs were

necropsied and liver and ocular tissues fixed in 10% formalin for histopathological studies. Two lambs died during the experiment (#125 and #130). A wider tissue collection for histology was sampled, consisting in liver, kidney, lung, ileum, pancreas, heart, spleen, cervical spinal cord, mediastinic and mesenteric lymph nodes, and both eyes. Hepatic and ocular lesional scoring was assessed as a matter of comparison between pathogenic behavior between groups after challenge, taking into account we had RVFV-challenge related mortality too.

The liver was evaluated by two pathologist grading hepatic necrosis, inflammatory infiltrates and hepatocytic individual eosinophilia (a preliminary degree of hepatocyte death). Necrosis was scored from absence (0), presence of clusters of neutrophils (5-6) surrounding individual necrotic/ degenerated hepatocytes (1), evidence of mild to moderate multifocal to random hepatic necrosis (2) to diagnosis of severe to very severe confluent extensive hepatic necrosis (3). Inflammatory infiltrates were scored from absence (0), to presence of peribiliar segmental inflammatory infiltrate in portal areas (no cuffing) (1), or peribiliar perivascular portal mononuclear cuffing (2). Hepatocytic individual eosinophilia was scored from no tintorial changes in hepatocytes (0) to an increase of cytoplasmic eosinophilia and nuclear pycnosis in hepatocytes (multifocal, individual) (1).

The eyes were evaluated by two pathologist grading lesions in endothelium and uvea, searching specifically for a confirmatory diagnosis of the presence of anterior uveitis with lymphoplasmacytic endotheliitis (Galindo-Cardiel *et al.*, 2012). Lesions in endothelium were scored from not endothelial lesion neither neovascularization or inflammation (0), presence of scarce segmental endothelium hypertrophy with minimal periendothelial edema (1), mild diffuse corneal endothelium hypertrophy with occasional mid-stromal neovascularization and corneal edema (2), to grade 2 lesions plus occasional multifocal corneal and/or subendothelial leucocytic infiltration (probably mixed) (3). Uveal lesions were scored from absence (0), to minimal (1) to mild (2) perivascular or multifocal mixed inflammatory infiltration with minimal/mild perivascular to diffuse uveal edema, or mild diffuse mixed inflammatory infiltration with mild diffuse uveal edema (3).

Blood, as well as nasal and oral swabs were collected every two days' post-challenge for viral RNA extraction and/or virus isolation. After collection, the blood samples in EDTA were directly frozen to −80 °C and nasal and oral swabs were mixed in DMEM prior −80 °C storage. Serum samples were obtained from each animal on the day of challenge and at days 5, 9 and 16 post-challenge for analysis of anti-RVFV antibodies.

5.3.3. Immunological assays

Specific anti-nucleoprotein N antibodies were detected using a competitive ELISA (ID-VET) as per manufacturer's instructions. Neutralizing antibody titers were determined as described (Lopez-Gil *et al.*, 2013). Briefly, heat inactivated serum samples were serially diluted (twofold), starting at a dilution of 1:20 in DMEM medium containing 2% FBS, mixed with an equal volume (50 µl) of medium containing 10^3 pfu of a stock of the MP-12 RVFV strain. After one-hour incubation at 37 °C, this mixture was added to Vero cell monolayers seeded in 96-well plates. After 3 days at 37 °C, cells were fixed and stained with 2% crystal violet and 10% formaldehyde. The neutralization titer of each sample was defined as the reciprocal of the highest serum dilution resulting in 50% neutralization.

5.3.4. Virus detection and isolation

RNA from nasal and oral swabs was extracted using Trizol® (Invitrogen) and from blood using Tri-reagent® (Sigma), according to manufacturer's instructions. RVFV RNA from samples was detected using a TaqMan one-step RT-qPCR specific for RVFV L segment as described previously in chapter 1 (Busquets *et al.*, 2010). Positive samples on RT-qPCR were processed for virus isolation as follows. Blood samples were first lysed by dilution in sterile distilled water and 10× phosphate buffered saline (PBS) added to restore physiological conditions (final blood dilution 1:20). Hundred µl of log2 dilutions (starting at 1:40) were added to 96-well cultured Vero cells in quintuplicate. After incubation for 90 min supernatants were aspirated and substituted with fresh media (DMEM supplemented with 2% FBS). After 6 days the cells were fixed and stained with 10% formaldehyde and 2% crystal violet. $TCID_{50}$ end point titers were calculated by the Reed and Muench method (Reed and Muench, 1938). Twofold dilutions of infected swabs, either nasal or oral, were prepared in cell culture medium with antibiotics and incubated onto Vero cells in quadruplicate and processed as described above.

5.3.5. Statistical analyses

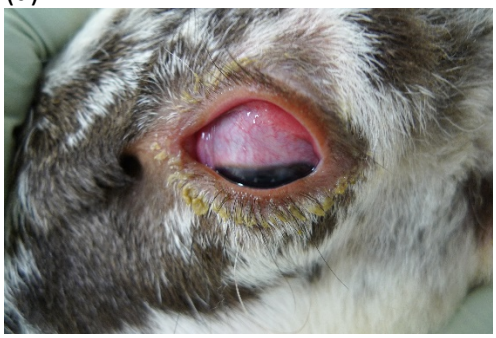
Statistical comparisons were made using the Kruskal–Wallis test and adjustment for multiple testing done by the Dunn's post hoc test. All calculations were performed using the GraphPad 5.0 software (Prism).

5.4. Results

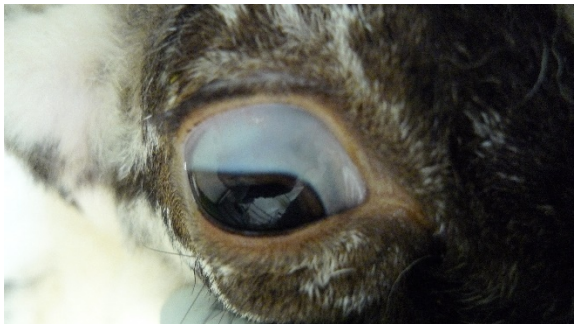
5.4.1. Clinical findings in lambs

After immunization no fever, abnormal behavior or apparent clinical display was observed in any of the lambs. However, morbidity was observed over the duration of the experiment in all groups upon challenge with the RVFV 56/74 virus. To quantify the extent of morbidity among the groups, a clinical score was applied based on severity of illness. Briefly, severity was categorized according to the relative intensity of mucosal irritation associated or not to vascular hyperemia (1: mild, 2: moderate, 3: severe, 4: very severe). Gross and clinical evaluation was marked in the basis of the findings scored in Figure 27.

(a)



(b)



(c)

Figure 27. Clinical scoring of the MVA vaccine experiment. (a) Mucosal irritation with marked diffuse hyperemia (left lamb, graded as severe) comparing with normal pale mucosa (right lamb, graded as normal). Conjunctival irritation with marked diffuse hyperemia and variable corneal lesions; (b) Lamb graded as severe, note the purulent secretions over the eyelids, (c) Lamb graded as normal.

The earliest clinical display observed after challenge was mucosal hyperemia, noticed in all lambs as early as day 2 post-challenge (pc), being more evident in the MVA control and saline groups as compared to the vaccine (rMVA-GnGc) group. By day 4 pc this manifestation reached similar intensity in lambs from the vaccine group (Figure 28).

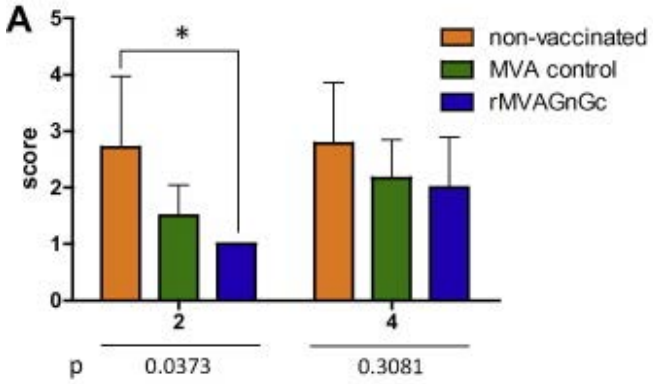


Figure 28. Clinical evaluation in challenged sheep. (A) Early clinical signs (mucosal hyperemia) recorded at days 2 and 4 post challenge (dpc). Severity was categorized according to the relative intensity of irritation (1: mild, 2: moderate, 3: strong, 4: very strong). The graph bars depict median with interquartile range from each group of sheep. The p values are indicated for each day post challenge. Asterisk denotes statistical significance ($p < 0.05$).

Later, more severe signs of disease were observed in all groups. The MVA control group displayed such signs from days 4 to 11 pc, but severity of illness among the rMVA-GnGc vaccinated lambs had decreased by day 5 pc. Lambs receiving saline also displayed clinical signs for a longer duration than the rMVA-GnGc group, but their overall clinical severity was lower than that observed in the MVA control group (Figure 29). Mild to severe bilateral conjunctivitis with mild to severe hyperemia, variable degree of corneal erosions and purulent exudates was a major clinical feature found in 13 out of 19 lambs across all groups.

(a)
(b)
(c)

Figure 29. Clinical evaluation in challenged sheep. Late clinical signs scored for each individual sheep from the rMVA-GnGc group (a), rMVA-GFP control group (b) and the non-vaccinated group (c), according to the following categorization: mild to strong conjunctivitis (0.5–1.5), if bilateral (+0.5); inappetence or apathetic behavior (+1); respiratory distress (+1).

Rectal temperatures were taken daily both after immunization and challenge. As expected, pyrexia was absent in all lambs from all groups before the challenge (data no shown). After virus inoculation, all lambs showed an increase in temperature albeit differences were observed between the groups (Figure 30). Comparing the data together, all groups showed a sharp increase of fever between 2 and 3 dpi. However, rMVA-GgGn vaccine group decrease its mean temperature by 4 dpi, showing a slightly lower biphasic temperature dynamics comparing to the other groups.

Figure 30. Pyrexia in challenged sheep. Mean temperatures for each group are displayed in the figure. Red horizontal line indicates threshold of fever.

Among rMVA-GnGc vaccinated lambs fever peaked at day 3 post challenge (14 days' post-vaccination (Figure 31a) with the exception of the lamb that received an incomplete dose of the vaccine (lamb #124, see 5.3. Material and Methods), which peaked at day 2 instead, suggesting a vaccine dose effect. In this group only one lamb (#121), showed a secondary onset of fever on day 5 post challenge. This lamb was the only sheep from this group showing a strong bilateral conjunctivitis at day 7 post challenge.

(a)

(b)

(c)

Figure 31. Individual daily rectal temperatures (a), rMVA-GnGc vaccine group (b) rMVA-GFP vector group and (c) RVFV-infection control group. Note the lambs #125 and #130 were found dead after a high fever peak. Red horizontal line indicates threshold of fever.

The duration of fever lasted longer in both control groups (from day 2 to day 5), with an earlier peak of fever at day 2 post challenge (Figure 31b and 31c). Curiously, in these control groups the onset of fever was asynchronous, with some lambs peaking at 3, 4 and 5 days' post challenge. Lamb #132 from the MVA control group had a secondary peak of fever on day 9 (Figure 31b) and showed pale mucosa until the end of the study.

Three lambs showed even more severe signs such as lack of appetite, lethargy and respiratory distress. Lambs #125 (rMVA-GnGc vaccine group) and #130 (rMV-GFP vector group) succumbed to the RVFV challenged and their results are showed in the next point. The third lamb which showed marked clinical signs (#132), also from the rMVA-GFP vector group, survived although showing extremely pale mucosa. At day 11 post challenge all remaining sheep in the rMVA-GnGc vaccinated group displayed no clinical signs, whereas in both control groups some lambs still displayed signs of disease (Figure 29).

5.4.2. Pathology

Hepatic and ocular lesional scoring of all groups are shown in Figure 32. No statistics have been made between the two timelines (5 dpi and 14 dpi) because of the death of two lambs was unexpected, which let us to realize some pathogenic trend analysis by scoring the lesions, but without any statistical consistence for significant conclusions (lack of enough number of evaluated tissues in the two periods). The group 5 dpi is composed

only by lambs #125, #130 (both naturally dead), #138 and #141 (both euthanized). In spite of this fact, there is a suggestive trend in which the higher hepatic lesion, the lower ocular pathology.

Figure 32. Hepatic and ocular lesional scoring. Bars are depicted from the mean of the pathologic scores graded by two pathologists.

As we already stated, two of the most clinical severely affected died, one from the rMVA-GnGc vaccine group (lamb #125, Figure 33) and one from the rMVA-GFP vector group (lamb #130, Figure 34), at days 4 and 5 post challenge, respectively. At necropsy, both dead animals revealed as fatal lesion a moderate to severe hepatomegaly with multiple friable whitish foci that were diagnosed as severe multifocal hepatic necrosis, a characteristic injury of RVF disease (2/2). Accumulation of serous (#125) or bloody (#130) transudate at the pleural and peritoneal cavities was also found. Severe segmental hemorrhagic enteritis with severe focally extended necrosis of mucosa was observed in lamb #125. Also, splenomegaly and swelling of peripheral and visceral lymph nodes with multifocal petechiae to widely spread hemorrhages and edema was found in both naturally dead lambs. Marked bilateral redness of lungs were also noted (2/2) with consolidated dorso-caudal areas in lamb #130.

(a) (b)
(c) (d)

Figure 33. Necropsy findings of lamb #125. (a) Severe subcutaneous edema with diffuse mild ictericia (arrow). Note the some multifocal petechiae are noted in the subcutaneous musculature (arrowheads). (b) Severe multifocal to focally extensive hepatic necrosis with severe multifocal acute petechiae to ecchymosis. (c) Mild segmental to diffuse pulmonary congestion with moderate amount of serous pericardial transudate. (d) Severe segmental hemorrhagic enteritis. The inlet shows an opened intestine portion plenty of fresh unclotted blood.

Comparative histopathology between both naturally dead lambs is shown in Figures 35 and 36. The main histopathological finding in both lambs were centrolobulillar to diffuse hepatic necrosis (2/2) with multifocal hemorrhages (2/2), mild multifocal mononuclear and neutrophilic infiltration associated to necrotic foci (2/2), and mild biliary hyperplasia (1/2, #130) intermixed with intralesional bacterial colonies (1/2, #130). Other relevant lesions observed in these lambs were multifocal severe lymphocytolysis in spleen and lymph nodes with marked lymphoid depletion, cortical lymphangiectasia and variable degree of extramedullar hematopoiesis (2/2). Other findings consisted on bacterial thromboembolism with vascular fibrinoid necrosis in myocardium, lung, adrenal, spleen, and kidney (1/2, #130), mild interstitial pneumonia (1/2, #130), and pulmonary congestion with bronchoalveolar associated tissue depletion (1/2, #125). No anterior uveitis was diagnosed in these lambs. However, bacterial thromboembolism in choroid, and iris with bilateral uveal hyperemia, and mild bilateral leukocytosis were observed in lamb #130. Diffuse acute renal tubular necrosis was observed in lamb #125. In this animal, a diffuse segmental enteric necrosis with multifocal hemorrhages and mild to moderate

neutrophilic infiltration in lamina propia was confirmed. Mild unilateral uveal hyperemia was observed in lamb #125.

(a)

(b) (c)

Figure 34. Necropsy findings of lamb #130. (a) Severe multifocal to focally extensive hepatic necrosis with severe multifocal acute petechiae to ecchymosis. Subcapsular multifocal to focally extensive renal hemorrhages which went deeper to renal cortex (cutting no shown) (arrow). Close to kidney, it can be appreciated multifocal hemorrhages in perirenal fat tissue. (b) Diffuse severe pulmonary congestion with moderate bloody serous transudate. Note the caudal pulmonary lobule is partly consolidated (arrow). (c) marked lymphadenopathy with lymphadenomegaly and diffuse hemorrhagic necrosis of lymph nodes in all cavities, note some mesenteric lymph nodes (arrow).

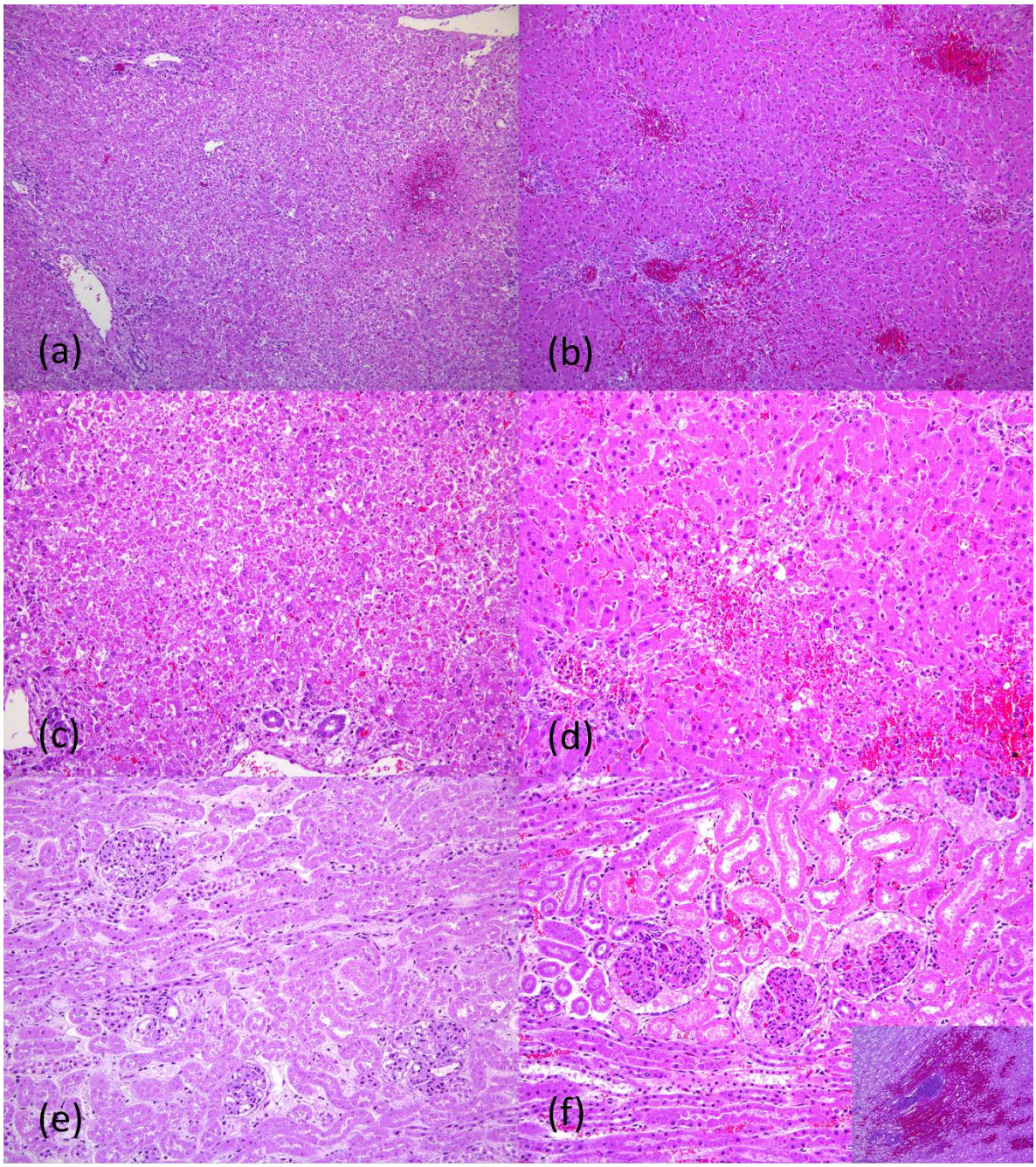


Figure 35. Comparative hepatic and renal histopathological findings of the naturally dead lambs. (a) Liver, lamb #125. Severe acute diffuse hepatic necrosis with multifocal hemorrhages. HE. 5x. (b) Liver, lamb #130. Severe acute centrolobulillar to bridging hepatic necrosis with multifocal hemorrhages. HE. 5x. (c) Liver, lamb #125. Extensive and massive hepatocytolysis in absence of inflammation is observed. HE. 20x. (d) Liver, lamb #130. Different stages in viral hepatocytolysis pathogenesis are observed. Note individual hepatocyte pycnosis, a preliminary lytic stage (arrow). HE. 20x. (e) Kidney, lamb #125. Severe diffuse acute renal tubular necrosis. HE. 20x. (f) Kidney, lamb #130. Severe diffuse acute renal tubular necrosis with moderate glomerular retraction and proteinosis in Bowman space. Inlet shows a bacterial thromboembolism with extensive hemorrhage, consistent with an infarct. HE. 20x (inlet 5x).

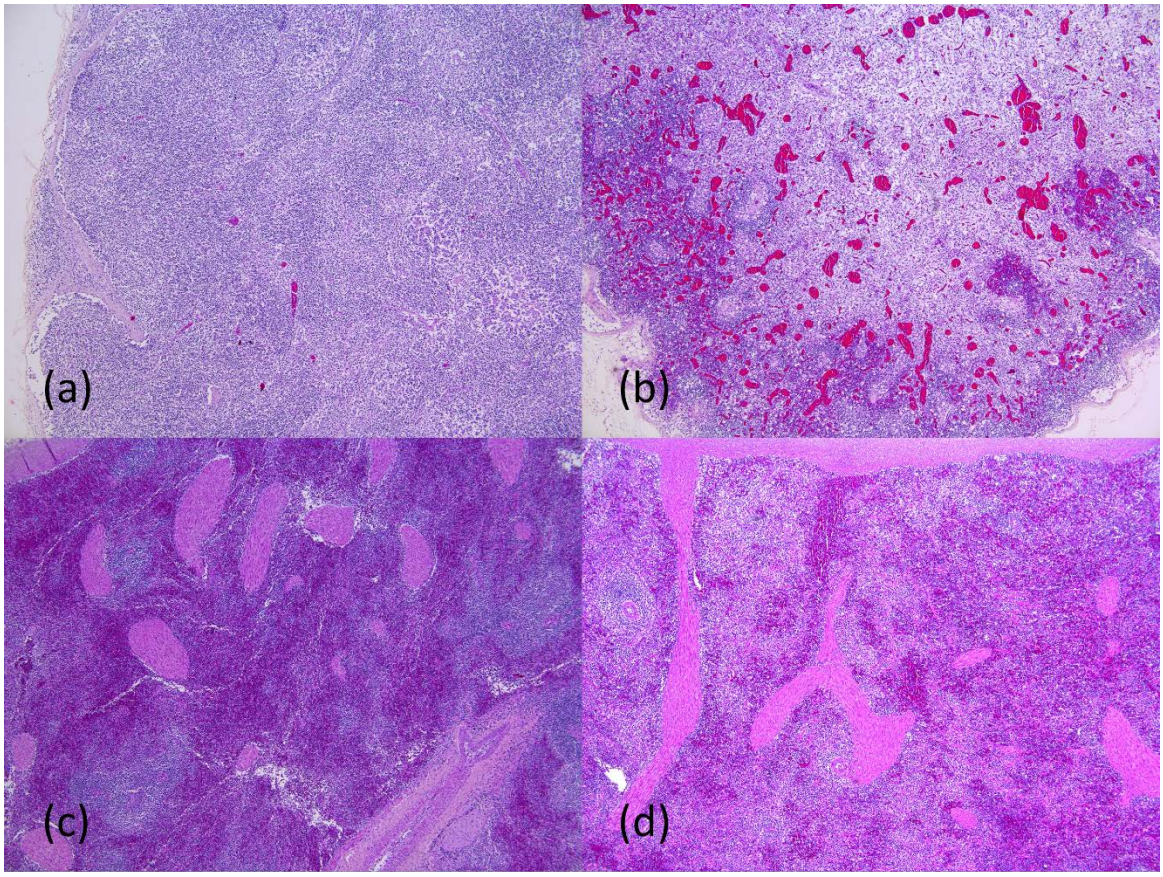


Figure 36. Comparative lymph node and splenic histopathology of the naturally dead lambs. (a) Mesenteric lymph node, lamb #125. Severe acute lymphoid depletion with marked lymphoblastic infiltration of germinal centers (arrow). HE. 5x. (b) Mesenteric lymph node, lamb #130. Severe acute lymphoid depletion with multifocal lymphocytolysis, severe congestion and multifocal erythrocyte extravasation. HE. 5x. (c) Spleen, lamb #125. Moderate to severe lymphoid depletion (WP/RP ratio 1:5). HE. 5x. (d) Spleen, lamb #130. Severe lymphoid depletion (WP/RP ratio 1:8). HE. 5x.

Two lambs were euthanized for pathogenic evaluation of RVFV challenge (lambs #138, #141) (Figure 37). Lamb #138 showed as major finding a moderate hepatomegaly with multifocal whitish spots distributed for all hepatic parenchyma. Lamb #141 showed the same multifocal whitish spotted pattern but without the hepatomegaly. The rest of organs and cavities of both lambs were considered within normal limits. Lamb #138 was

euthanized in the basis of the clinics, which showed a mild bilateral conjunctivitis at 2-3 dpi but was not observed during the necropsy. Lamb #141 never showed any appreciable clinical sign. Histopathology findings were restricted mostly to liver, lymphoid tissue (Figure 38) and eyes (Figure 39). Severe multifocal centrolobulillar necrotizing hepatitis with variable degree of neutrophilic and mononuclear infiltration, individual hepatocytolysis and hepatocyte vacuolation with pycnosis was diagnosed in both lambs. Lymphoid tissue showed a slight to mild lymphoid depletion diagnosed in the basis of the lacking of follicular centers. However, germinal centers were very active, with lymphoblastic activation and sinusal mononuclear and neutrophilic infiltration. Ocular globes showed mild bilateral anterior iridal fibroblast hypertrophy (1/2, lamb #138) and perivascular or multifocal lymphoplasmacytic infiltration associated to episcleral conjunctiva (lymphoplasmacytic conjunctivitis) with segmental endothelial hypertrophy (1/2, lamb #141).

(a) (b)

Figure 37. Necropsy findings in liver of euthanized lambs. (a) Multifocal withish nodules consistent with moderate to severe acute multifocal hepatic necrosis was diagnosed in lamb #138. (b) The same finding was observed in liver from lamb #141.

Similar hepatic (2/5) and ocular (4/5) lesions were also observed in rMVA-GFP vector group. Multifocal to randomly distributed clusters of pyknotic to hypereosinophilic hepatocytes surrounded by some neutrophils (interpreted as single hepatocyte necrosis) with mild lymphocytic periportal infiltrates, and mild centrolobulillar microvacuolar hepatocytic degeneration were observed in the vaccinated group at the end of experiment (3 out of 5). Minimal lymphoplasmacytic endotheliitis with anterior iridal fibroblast hypertrophy, and minimal to mild anterior uveitis were observed bilaterally in all vaccinated lambs with different pathological severity.

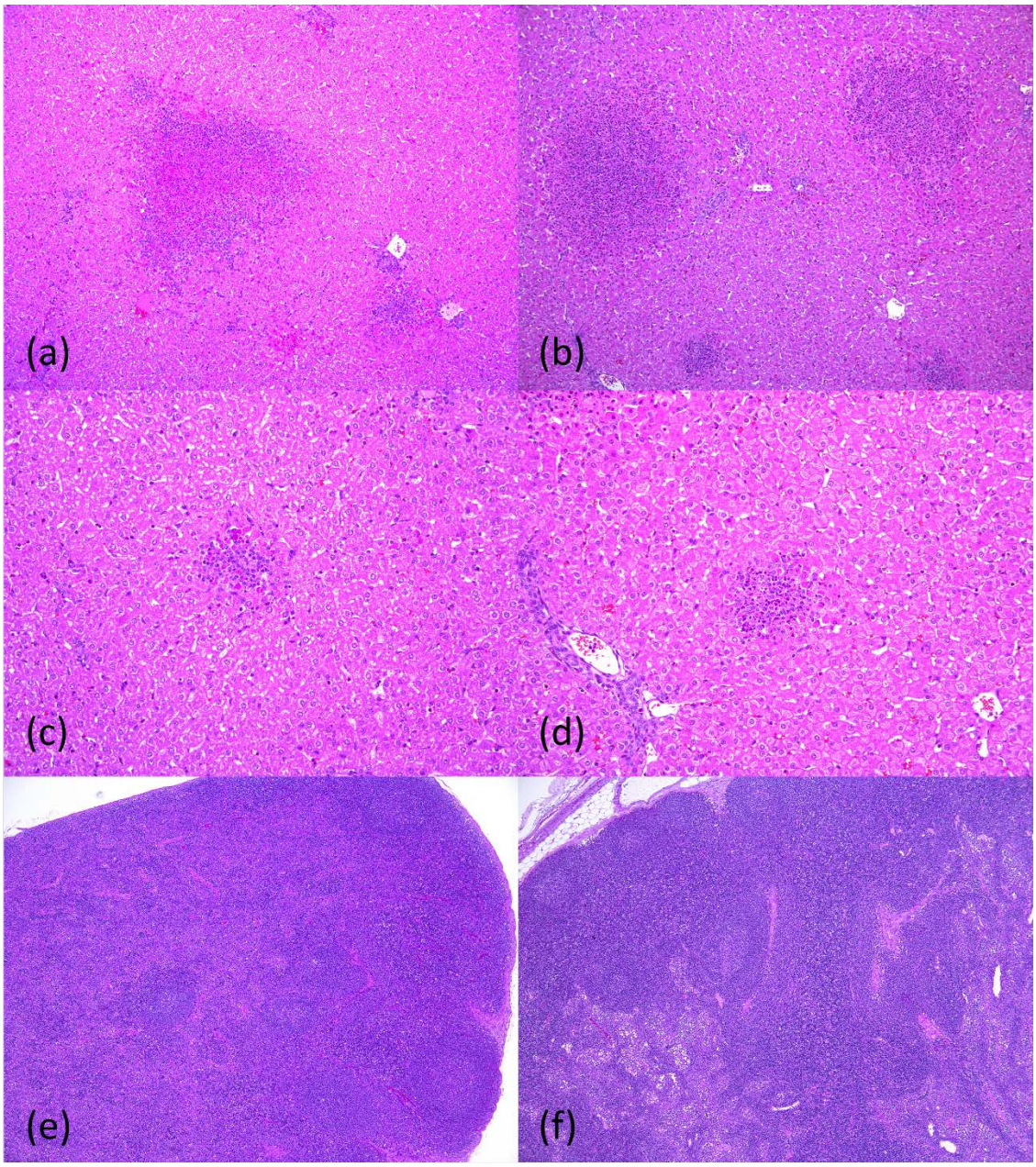


Figure 38. Comparative hepatic and lymph node findings in euthanized lambs. Severe multifocal centrolobulillar necrotizing hepatitis with variable degree of mixed inflammatory infiltrate (a, lamb #138) (b, lamb #141). HE. 5x. Characteristic foci of necrotic hepatocytes intermixed with variable number of neutrophils and mononuclear cells are consistently seen in centrolobulillar spaces (c, lamb #138) (d, lamb #141). HE. 20x. (e) Lamb #138 and (f) lamb #141 mesenteric lymph nodes. Mild follicular lymphoid depletion with lymphoblastic activation (no shown at this magnification) but with a regular lymphoid structure, close to normal. HE. 5x.

Similar but milder hepatic lesions were observed in RVFV-infected non-vaccinated group at 14 dpi. Mild to moderate multifocal necrotizing hepatitis with mixed inflammatory infiltration was observed in 3 out of 7 lambs. Similar ocular lesions were observed bilateral or unilateral in all lambs from infection group. Mostly of them showed from scarce segmental endothelium hypertrophy with minimal periendothelial edema (4 out of 7) to mild diffuse corneal endothelium hypertrophy with occasional mid-stromal neovascularization and corneal edema (2 out of 7).

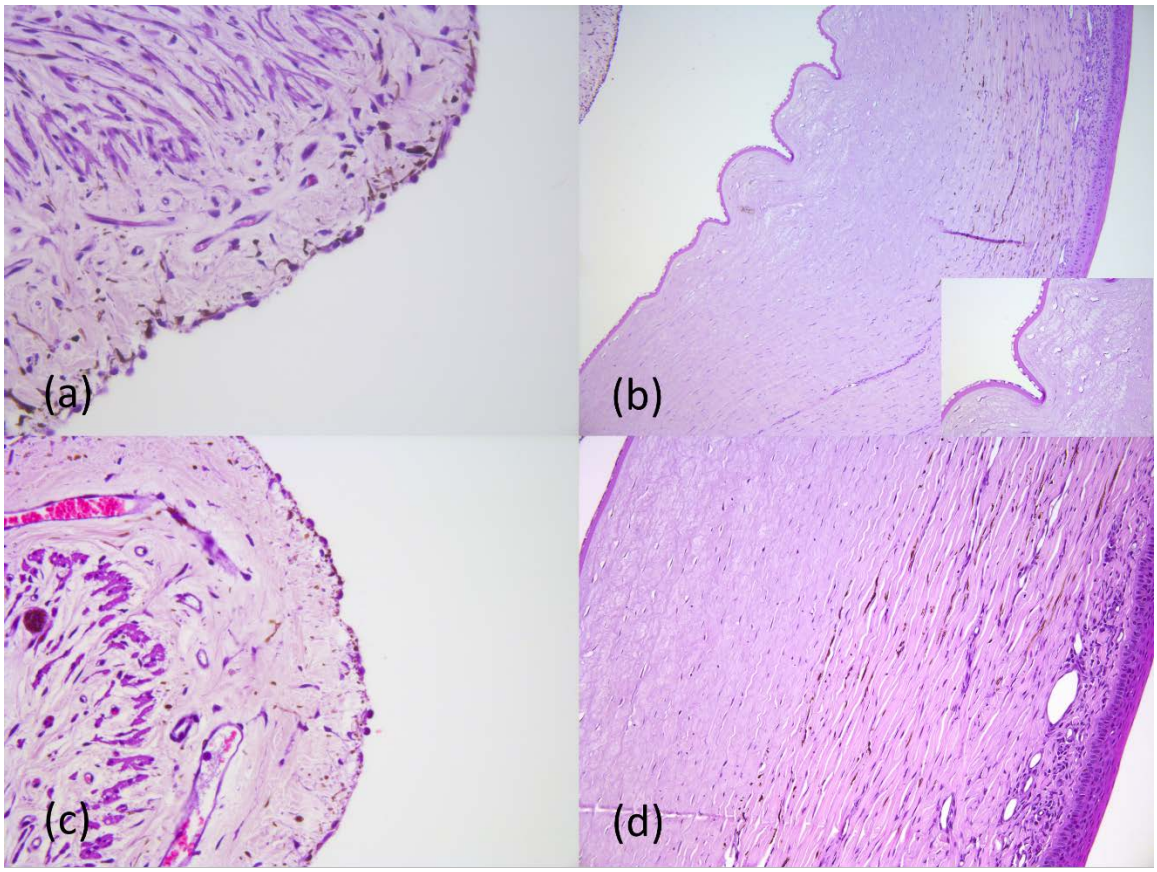


Figure 39. Endothelial and uveal findings in RVFV-challenged lambs. Anterior uvea, iridal membrane. Preiridal fibroblast hypertrophy (note the rounded nuclei) with moderate neovascularization, mild (a, lamb #138) and minimal (c, lamb #141). HE. 40x. Cornea, episclera and anterior chamber. Multifocal perivascular lymphoplasmacytic conjunctivitis with neovascularization (arrows) and corneal edema (arrowheads), both mild (b, lamb #138) (d, lamb #141). HE. 10x. (b inlet, lamb #138) moderate segmental endothelial hypertrophy, closely related to corneal edematized areas. HE. 40x.

5.4.3. Virus detection and isolation

To determine more precisely the level of efficacy achieved by the vaccine, blood and swabs samples taken from lambs at different days' post challenge were analyzed by RT-qPCR and virus titration in cell culture (Table 7). Viral RNA in blood was not detected at day 1 post challenge. However, it was detected at day 3 post challenge in most of the lambs from all groups. At day 5 post challenge two sheep from both the RVFV-infection control group (non-vaccinated lambs) and the rMVA-GnGc vaccine group were positive for viral RNA detection in blood (Figure 40a, 40b, and 40c). However, no virus was isolated at this time point in both groups (Figure 40d, 40e and 40f). Interestingly, four lambs from the rMVA-GFP vector group were positive at day 5 post challenge and live virus was isolated from one of them. This lamb was found dead at day 5 post challenge (#130).

Overall, nasal and oral viral shedding post-challenge was more pronounced in both control groups than in the rMVA-GnGc group. At least one lamb in each of the control groups was positive for RVFV RNA in nasal swabs at day 7 post challenge, whereas in the rMVA-GnGc group only two lambs, the one that received an incomplete dose (#124) and the one that died shortly after infection (#125), were positive at day 3 post challenge by RT-qPCR. Virus was isolated from controls at day 5 post challenge and only in one lamb at day 3 in the rMVA-GnGc group. In contrast to what was observed for the viremia, more virus was detected in nasal swabs in the group receiving saline. With respect to oral swabs viral RNA was still detected at day 5 post challenge in some lambs from both control groups but not in the rMVA-GnGc group. In addition, virus could be isolated at day 5 in one lamb, and even at day 7 pc, viral RNA was detected in one of the animals from the MVA control group.

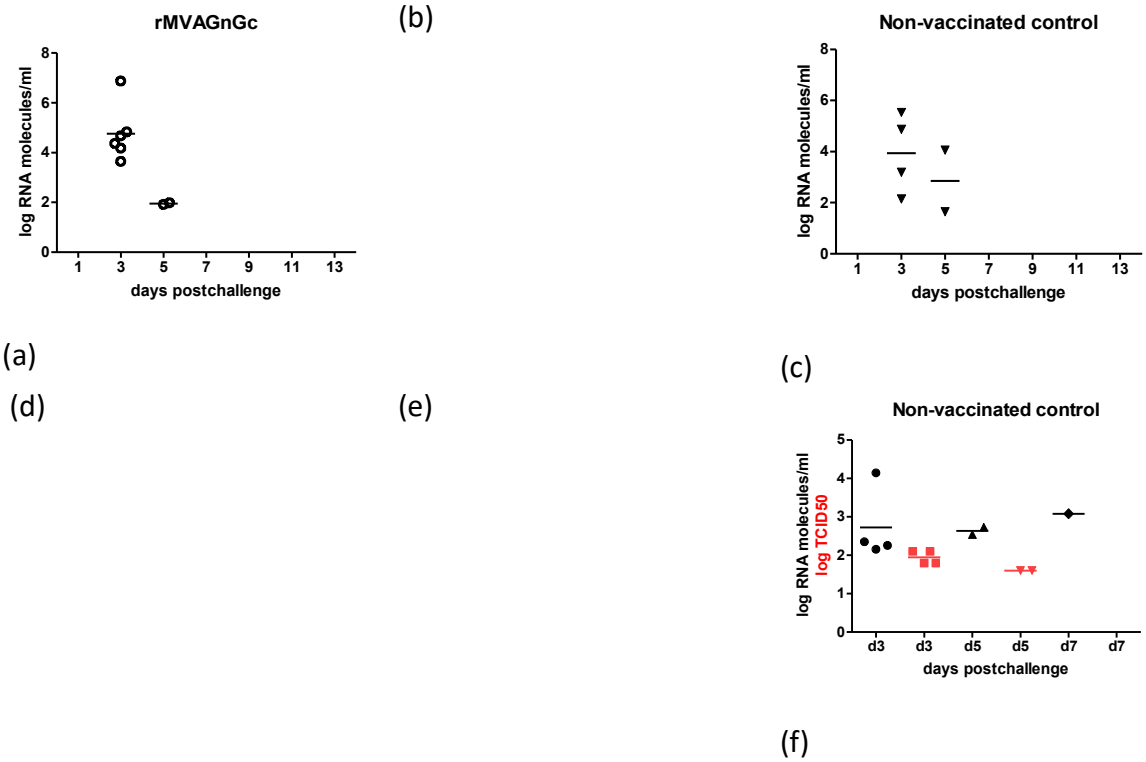


Figure 40. Graphic charts for virus detection in blood by RT-PCR (log RNA molecules/mL) of rMVA-GnGc (a), rMVA-GFP vector (b) and RVFV-infected non-vaccinated (c) groups. Virus isolation by culture (log RNA molecules/mL) and nasal swab virus detection (expressed in log $TCID_{50}$, in red letters) by RT-PCR are shown for rMVA-GnGc (d), rMVA-GFP vector (e) and RVFV-infected non-vaccinated (f) groups.

5.4.4. Antibody detection

The level of antibodies raised against the RVFV viral nucleoprotein N was quantified by a commercial competition ELISA (Figure 41). As early as 5 days' post challenge (19 days' post immunization) all lambs from the rMVA-GnGc group had seroconverted. In contrast, only four lambs from both control groups had comparable anti-N antibody titers in serum.

Complete seroconversion in lambs from control groups occurred later, between 6 and 9 days' post challenge (20 and 23 days' post immunization respectively).

Table 7. Quantification of viral RNA and virus isolation in blood, nasal and oral swabs of lambs.

Sample	Blood												Nasal swab											
dpc[d]	1		3		5		7		9		13		3		5		7		3		5		7	
Lamb	RNA[e]	VI[f]	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI	RNA	VI
121[a]	neg		4.7	3.7	neg		neg		neg		neg		neg		neg		neg		neg		neg		neg	
122[a]	neg		4.8	3.5	neg		neg		neg		neg		neg		neg		neg		neg		neg		neg	
123[a]	neg		4.4	neg	2.0	neg	neg		neg		neg		neg		neg		neg		neg		neg		neg	
124[a]	neg		3.7	1.8	1.9	neg	neg		neg		neg		2.2	–	neg		neg		neg		neg		neg	
125[a]	neg		6.9	⩾4.7									3.4	2.3					2.1	neg				
126[a]	neg		4.2	3	neg		neg		neg		neg		neg		neg		neg		neg		neg		neg	
127[b]	neg		2.7	1.8	neg		neg		neg		neg		neg		–		neg		neg		neg		neg	
128[b]	neg		5.8	4.2	1.9	neg	neg		neg		neg		3.7	neg	3.6	1.6	2.2		neg		1.8	neg	neg	
129[b]	neg		7.8	⩾4.7	4.2	neg	2.7	neg	2.1	neg	neg		3.0	neg	3.0	1.6	4.1		2.0	neg	2.2	neg	neg	
130[b]	neg		7.3	4.4	3.7	1.8							4.2	neg	3.2	neg			1.8	neg	2.4	1.8		
131[b]	neg		2.9	1.8	neg		neg		neg		2.1	neg	neg		neg		neg		neg		neg		neg	
132[b]	neg		6.8	4.9	2.5	neg	2.7	neg	neg		1.6	neg	3.9	2.3	3.0	1.9	neg		neg		neg		2.6	neg
133[c]	neg		neg		neg		neg		neg		neg		2.2	2.1	neg		neg		neg		neg		neg	
134[c]	neg		neg	neg	neg		neg		neg		neg		neg		neg		neg		neg		neg		neg	
135[c]	neg		4.9	3.0	neg		neg		neg		neg		2.2	1.8	2.5	1.6	neg		neg		neg		neg	
136[c]	neg		neg		neg		neg		neg		neg		2.3	2.1	neg		neg		1.7	–	3.5	neg	neg	
137[c]	neg		5.5	4.5	neg		neg		neg		neg		4.1	1.8	2.7	1.6	3.1		2.5	–	2.0	neg	neg	
139[c]	neg		3.2	neg	1.6	neg	neg		neg		neg		neg	neg	neg		neg		neg		neg		neg	
140[c]	neg		2.1	1.8	4.1	neg	neg		neg		neg		neg	neg	neg		neg		neg		neg		neg	

[a] rMVA-GnGc group.

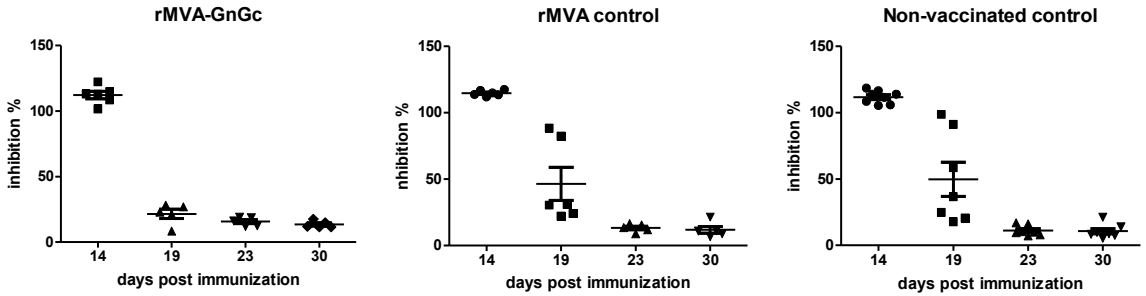
[b] rMVA-GFP vector group.

[c] RVFV infection control group (non-vaccinated group).

[d] Days post challenge.

[e] Detection of viral RNA (values in log_{10} viral GEC/ml).

[f] Virus isolation (Infectious dose in log_{10} $TCID_{50}$/mL); neg: negative result.



(a) (b) (c)

Figure 41. Detection of anti-nucleoprotein antibodies after viral challenge. The figure shows individual seep serum tested in a competition ELISA. Serum samples were assayed for the presence of anti N antibodies at 0, 5, 9 and 16 days after virus challenge (corresponding to 14, 19, 23 and 30 days' post immunization).

A similar acceleration of the antibody responses was observed for RVFV neutralizing antibodies (Figure 42). While no pre-challenge (day 14 post immunization) RVFV neutralizing antibodies were detected in any of the control groups, one lamb from the rMVA-GnGc group showed low but detectable titers of neutralizing antibodies. Moreover, by day 5 and 9 post challenge (19 and 23 days' post immunization, respectively) the titers of RVFV neutralizing antibodies differed significantly ($p = 0.0160$ and $p = 0.0021$, respectively), with the vaccine group showing the highest titers and faster induction of antibodies (Figure 43).

(c)

(a) (b)

Figure 42. Neutralizing antibody kinetics. 50% end point titers were measured at 0, 5, 9 and 16 days after virus challenge (corresponding to 14, 19, 23 and 30 days' post immunization). Red horizontal line indicates the sensitivity threshold of the VNT assay.

Figure 43. Neutralizing antibody kinetics. Scattered plots with mean values are depicted. Bars indicate mean ± SD. Significant ($p < 0.05$) p values are indicated for determined days' post challenge. Asterisks denote statistical significance ($*p < 0.05$; $**p < 0.01$).

5.5. Discussion

The aim of this work was to evaluate the performance of a modified vaccinia virus Ankara expressing the RVFV glycoproteins (rMVA-GnGc) as a potential single shot vaccine candidate against RVF in a sheep model of infection. Recent studies have evaluated the protective efficacy of other poxviral vectors such as capripoxvirus and lumpy skin disease virus (LSDV) in sheep (Soi *et al.*, 2010; Wallace *et al.*, 2006). Two doses of these vaccines, expressing the viral glycoproteins, provided protection against fever and viremia after virulent RVFV challenge. In mouse models these vectors provided incomplete or no protection when administered in a single dose format. This fact was also noted for a recombinant vaccinia virus (VACv) expressing the RVFV glycoproteins (Papin *et al.*, 2011).

López-Gil *et al* (2013) have shown that a single inoculation of the rMVA-GnGc vaccine was sufficient to protect mice from a lethal challenge (Lopez-Gil *et al.*, 2013). Moreover, no apparent or very mild clinical display was observed and, most importantly, a remarkable absence of detectable viremia confirmed the achieved protection. Therefore, the promising data obtained in the mouse model prompted us to test the level of protection of a single administration of rMVA-GnGc in sheep, one of the natural targets of RVFV. From the point of view of field veterinary vaccines, single dose vaccination is a preferred choice over

multiple administrations, taking in account economical and practical reasons, particularly in developing countries. Recommendations for new RVFV vaccine developments include single dose delivery as an advantage over repeated administrations, thus minimizing the risks of transmission by needle injections in endemic RVFV areas (Kortekaas *et al.*, 2011).

We used an ovine model of infection that had been previously characterized (Busquets *et al.*, 2010). In this study the subcutaneous challenge of lambs with 10^5 $TCID_{50}$ of the 56/74 RVFV strain caused two fatalities and clear clinical signs were observed in all lambs. Intriguingly, the only two lambs that died after the challenge belonged to the groups inoculated with MVA virus (sheep from rMVA-GnGc and rMVA-GFP groups) but none of the lambs challenged in the saline group died. Perhaps the mechanisms of anti-vector immunity rendered both lambs more susceptible to disease or, alternatively, these two lambs were affected by other non-adverted pathologies that could have enhanced the virulence of the challenge. Despite the death of one of the six sheep in the rMVA-GnGc group, some partially protective effects could be attributable to the immunization with rMVA-GnGc.

The most obvious consequence of vaccination was a 24 h delay in the onset of fever and early clinical signs such as mucosal irritation. This observation was serendipitously supported by the earlier onset of fever of a sheep (#124) which received an incomplete dose of this vaccine. In addition, virus detection experiments also confirmed the partially protective effects of this vaccine. Even though at 3 days' post-challenge fever peaked in the rMVA-GnGc group (mean pyrexia peaked at day 2 post challenge in both control groups) both the RNAemia and viremia levels found at this time point among the groups did not reach sufficient statistical significance (P values of 0.1399 and 0.1948, respectively). It is noteworthy that the levels of viremia and viral shedding were measured in samples collected at day three post challenge, one day after the peak of fever observed for both controls but simultaneous to the peak of fever in lambs from the rMVA-GnGc group. Since it has been shown that the peak of fever usually occurs simultaneously with the peak of viremia (Busquets *et al.*, 2010), perhaps differences in virus titers at day 2 pc would have been more significant than at day 3 pc.

Interestingly, more differences were found between the groups with respect to viral shedding. In the saline group infectious virus was isolated from nasal swabs at day 3 post challenge in 4 of 7 lambs and virus isolation was still possible at later times after the challenge (up to seven days' post challenge) both in nasal and oral swabs. In contrast, virus shedding was detected only at day 3 post challenge in the rMVA-GnGc group among lambs that died after challenge.

Though neutralizing antibodies were barely detectable in only one out of five lambs just before the challenge (2 weeks after vaccination), a clear priming effect could be observed in the rMVA-GnGc group since neutralizing antibodies after challenge developed faster and to higher titers than in both control groups. Therefore, it is plausible that during the first days after challenge a certain level of neutralizing antibodies was reached, able to reduce further virus replication and spreading. Additionally, it was observed that vaccinated lambs showed accelerated anti-N antibody responses even though they were vaccinated with an MVA vector expressing only RVFV glycoproteins. This fact could suggest a synergistic effect of antibodies whereby a stronger neutralizing antibody response directed against the RVFV glycoproteins accelerates the processing of nucleoprotein antigen from neutralized virus particles or virus infected cells thus facilitating the development of anti-N antibodies. Alternatively, an enhanced replication of the virus in the vaccinated animals would make more N antigen available for immune recognition. Though very unlikely that this fact could be effectively translated for field diagnosis, the different kinetics of the antibody response against a non-vaccine antigen (in this case the immunogenic nucleoprotein N) would help to distinguish vaccinated from non-vaccinated animals after experimental virus exposure.

Our results suggest that a single subcutaneous immunization of lambs with rMVA-GnGc does not confer full protection from disease; however, it appears able to delay the onset and severity of clinical signs as well as the duration of illness. Moreover, viremia and virus shedding appears to be delayed and reduced in extent. Therefore, further optimization of rMVA-GnGc vaccination regimen in lambs is needed, for instance by varying the route of administration, using adjuvants or by increasing the amount of recombinant virus delivered. Since MVA is not capable of replication in sheep the amount of input virus may be a key determinant for the magnitude of the immune response mounted by the host. MVA vaccines delivered in previous trials against other pathogens in veterinary species such as calves, and/or ponies were administered with at least one or two booster doses (Antonis *et al.*, 2007; Breathnach *et al.*, 2006; Chiam *et al.*, 2009). Experiments are underway to test the protection elicited with a booster dose of rMVA-GnGc in sheep as well as for potentiating the immune response elicited by a single administration of this experimental vaccine. In conclusion, the rMVA-GnGc vaccination regimen presented in this study achieved only limited protection against clinical disease in lambs; however, it was able to reduce the extent of viremia and virus shedding warranting further optimization trials.

6. Chapter 4: Pathological characterization of anterior uveitis in Rift Valley Virus infected lambs.

6.1. Summary

Rift Valley Fever Virus (RVFV) is a mosquito-borne zoonotic phlebovirus that primarily affects ruminants by causing abortions and acute hepatitis with multifocal necrosis as major findings. Human RVF symptoms range from flu-like syndrome to retinitis and encephalitis. A 9-10 weeks old European sheep (Ripollesa breed) challenge model has proven to be effective in vaccine testing because of its susceptibility to virus. Two groups of five lambs per group were selected (n=10) from historical database of RVFV experimental infections performed in IRTA-CReSA (northeast Spain, NBS3 facility) in the basis of their clinical data, viremia and diagnosed ocular and hepatic lesions. An anterior uveitis (8 out of 10) with lymphoplasmacytic endotheliitis (2 out of 10) was characterized. CD3, CD20 and lysozyme-positive mononuclear infiltrations were observed in RVFV-positive paraffin-embedded eyes. CD20 labelling was only observed in anterior uvea. A novel T-cell dependent retinitis was also diagnosed in 5 out of 10 RVFV-infected lambs based on CD3-positive labelling. To the authors´ knowledge, this is the first description of retinal injury in RVFV-challenge sheep model.

6.2. Introduction

Rift Valley fever (RVF) virus is a mosquito-borne, zoonotic, negative-sense, single-stranded, tripartite RNA virus of the family *Bunyaviridae* within the genus *Phlebovirus* (Nichol *et al.*, 2005; OIE, 2009). The virus primarily affects ruminants but can also be lethal in humans. In adult sheep or older lambs, the disease varies from subclinical or unapparent infection to high fever, lymphadenitis, diarrhea, abortion, different degrees of multifocal hemorrhages, and acute hepatitis associated to jaundice and multifocal to focally extent necrosis, which is the final death cause (Anderson and Peters, 1988; Boshra *et al.*, 2011a; Easterday *et al.*, 1962a; Gerdes, 2004). Human symptoms of this disease range from flu-like syndrome with fever, joint pains, photophobia and headaches to retinitis and encephalitis; complications may include hepatitis with generalized hemorrhages, thrombosis, retinitis and transient loss of vision, neurological symptoms and fatal hemorrhagic fever with thrombocytopenia in some individuals (Flick and Bouloy, 2005; Gerdes, 2004). Infected humans are usually involved in sheep herding and RVFV-infected tissue handling (Ikegami and Makino, 2011).

The pathogenicity, transmission, strain virulence and immune responses upon experimental challenge with RVFV have been analyzed in great detail in ruminants, a main vaccine target species (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Capstick and Gosden, 1962; Coackley *et al.*, 1967b; Galindo-Cardiel *et al.*, 2012; Olaleye *et al.*, 1996; Swanepoel *et al.*, 1986; Tomori, 1979; Weingartl *et al.*, 2014a; Yedloutschnig *et al.*, 1981). Moreover, RVFV's recent classification as a potential bioterrorist agent have renewed interest in the study of the virus as well as in RVF vaccinology in sheep (Rabinowitz *et al.*, 2006). This renewed interest has leaded to standardize new ovine experimental models (Kortekaas, 2014; Kortekaas *et al.*, 2011). Recently, an anterior uveitis with lymphoplasmacytic endotheliitis was described as an occasional finding in a new-standardized ovine model, suggesting that Ripollesa sheep may be used to study RVF-associated ocular pathology in man (Galindo-Cardiel *et al.*, 2012). Therefore, it is needed a deeper and better characterization of the RVFV pathogenesis in experimentally infected Ripollesa sheep. This chapter describes further phenotyping of the recently described ocular entity and summarize important facts in the pathological dynamics of the proposed RVFV-challenge ovine model.

6.3. Material and Methods

6.3.1. Animal selection and experimental design

Two groups of five lambs per group were selected (n=10) from historical database of RVFV experimental infections performed in IRTA-CReSA (northeast Spain, NBS3 facility). All lambs were challenged subcutaneously behind the elbow with 1×10^5 $TCID_{50}$ (in a 1 ml volume) with one of four different African strains of RVFV (Busquets *et al.*, 2010). Briefly, the South African virulent RVFV 56/74 isolate used for challenge of lambs #125, #130, #135, #138, #140 and #141 was kindly provided by the Agricultural Research Council-Onderstepoort Veterinary Institute (South Africa) (Barnard and Botha, 1977; Busquets *et al.*, 2014) (Barnard and Botha 1977, Busquets, Lorenzo et al. 2014). As previously described, lamb #24 was inoculated with RVF 56/74 (WYJIUOWO) isolate, lamb #26 with RVF 252/75 isolate, and lamb #29 and #32 with RVF 1830 isolate (Busquets *et al.*, 2010). Each animal was chosen for the study by the concurrence of at least 4 out of 5 of these criteria: 1) unilateral or bilateral gross ocular lesions were observed in any time during the challenge experiment; 2) systemic clinical signs of the disease were recorded (any combination of these; fever, mild to moderate conjunctivitis, innapetence, apathy, mild to severe diffuse mucosal redness, and respiratory distress); 3) RVFV viremia was detected during at least one day and; 4) hepatic and/or ocular lesions were recorded in the pathological report; and 5) the animal died unexpectedly or was euthanized before the ending of the experiments. The lambs were grouped by the timing of the decease, before of 8 days' post-infection –dpi- (Group ≤ 8 dpi) or just in the end of the experiments (Group = 14 dpi).

Non-lesionated hepatic and ocular tissues from three RVFV-negative lambs were used as negative controls for haematoxylin-eosin (HE) evaluation, Gram and periodic acid–Schiff (PAS) staining, immunohistochemistry (IHC) and retrotranscriptase real time quantitative polymerase chain reaction (rRT-qPCR). Formalin-fixed paraffin-embedded lymph node tissues from these animals were used as positive controls for immunohistochemistry of CD3, CD20 and lysozyme. Three RVFV-positive frozen liver tissues from historical database were selected as positive controls for rRT-qPCR (data no shown).

6.3.2. Real time PCR

RNA from paraffin-embedded tissues was extracted using Trizol® (Invitrogen) and from blood using Tri-reagent® (Sigma), according to manufacturer's instructions. RVFV RNA from samples was detected using a TaqMan one-step rRT-qPCR specific for RVFV L segment as described previously (Busquets *et al.*, 2010).

6.3.3. Histopathology

All lambs underwent necropsy examination. Hepatic samples and ocular globes were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (4 µm) of liver and eye were stained with HE, Gram stain and PAS for pathological examination. Ocular slides were prepared through the sagittal central axis, perpendicular to the long posterior ciliary artery (Peiffer *et al.*, 1999). Liver and eye sections were evaluated in a blinded fashion by two pathologists. Scoring used for pathological characterization and evaluation is described in Table 8.

6.3.4. Immunohistochemical characterization of inflammatory infiltrates in affected eyes

Immunohistochemistry (IHC) for CD3 (pan-T cell marker), CD20 (pan-B cell marker) and lysozyme (histiocytic marker) was performed in eye, in order to better characterize the mononuclear inflammatory infiltrate observed in ocular lesions. All are methods previously described with brief modifications (Mason *et al.*, 1989; Matsuda *et al.*, 2010; Morsky, 1988). The technical conditions and the antibodies used are summarized in Table 9. The quantity of CD3, CD20 and lysozyme antigens were scored from 0 (-) to 3 (+++) following different criteria (Table 8).

Table 8. Scoring of histopathological lesions, RVFV, CD3, CD20 and lysozyme immunohistochemistry.

	Scoring			
	0 (-)	1 (+)	2 (++)	3 (+++)
Liver	Absent	Scattered multifocal necrotic hepatocytes with minimal perilesional neutrophilic infiltration.	Moderate multifocal hepatic necrosis with minimal to mild perilesional mononuclear/ neutrophilic inflammatory infiltration.	Severe multifocal to bridging hepatic necrosis with minimal to mild perilesional inflammatory infiltration.
Eye*	Absent	Mild lymphoplasmacytic anterior uveitis.	Moderate lymphoplasmacytic anterior uveitis.	Severe lymphoplasmacytic anterior uveitis with mild to moderate endotheliitis.
RVFV	No labelling	Multifocal-distributed, mild, globular to diffuse, cytoplasmic and interstitial positive labelling in hepatocytes and necrotic tissue.	Multifocal to focal extensive-distributed, moderate, globular to diffuse, cytoplasmic and interstitial positive labelling in hepatocytes and necrotic tissue. Some mononuclear inflammatory cells are positive.	Focal extensive to bridging-distributed, moderate to severe, globular to diffuse, cytoplasmic and interstitial positive labelling in hepatocytes and necrotic tissue. Many mononuclear inflammatory cells are positive.
CD3	No labelling	Less than 1 cell of mononuclear cells per field (5 fields, x40) shows a cytoplasmic to membranous strong positive labelling.	From 2 to 6 mononuclear cells per field (5 fields, x40) shows a cytoplasmic to membranous strong positive labelling.	More than 6 mononuclear cells per field (5 fields, x40) shows a cytoplasmic to membranous strong positive labelling.

CD20	No labelling	Less than 1 cell of mononuclear cells per field (5 fields, x40) shows a membranous strong positive labelling.	Less than 5 cell of mononuclear cells per field (5 fields, x40) shows a membranous strong positive labelling.	No observed.
Lysozyme	No labelling	Less than 1 cell of mononuclear cells per field (5 fields, x40) shows a cytoplasmic strong positive labelling.	Less than 5 cell of mononuclear cells per field (5 fields, x40) shows a cytoplasmic strong positive labelling.	More than 6 mononuclear cells per field (5 fields, x40) shows a cytoplasmic strong positive labelling.

*Adapted from and based on the pathological descriptions of Galindo-Cardiel *et al*. 2011.

Table 9. Antigen retrieval, antibodies and detection method of CD3, CD20 and lysozyme used in eyes of RVFV-positive lambs.

	CD3	CD20	Lysozyme
Antigen retrieval	EDTA*, pH 9, 20´, 96ºC.	Sodium Citrate 10mM, ($C_6 H_5 Na_3 O_7$-2 H_2O), pH 6, 20´, 98ºC.	Protease type XIV, 0,1%, TBS**, 8´, 37ºC, (Sigma Aldrich, Madrid, Spain).
Primary antibody	Polyclonal rabbit anti-human CD3 (Ref. A0452, Dako, Glostrup, Denmark), 1:100, BSA***.	Polyclonal rabbit anti-human CD20 (Ref. PA5-32313, Thermo Scientific, Rockford, USA), 1:300, BSA.	Polyclonal rabbit anti-human Lysozyme (Ref. A0099, Dako, Glostrup, Denmark), 1:1000, BSA.
Secondary antibody	EnVision+System-HRP Labelled Polymer Anti- Rabbit (ref. K4002, Dako, Glostrup, Denmark).		
Detection method	Avidin–biotin complex (ABC) method.		

* EDTA: Ethylenediaminetetraacetic acid.

**TBS: Tris-buffered saline.

***BSA: Bovine seroalbumin.

6.3.5. Immunohistochemistry of RVFV

With the aim to develop a new protocol for detection of RVFV antigens in ovine tissue using IHC, four in-house developed antibodies and three commercial antibodies were used in a pilot optimization with RVFV-positive liver samples from lambs #125 and #130. The technical conditions and the antibodies used are summarized in Table 10. After the pilot optimization of RVFV IHC, an avidin–biotin–peroxidase method with primary monoclonal mouse anti-IgG2a RVFV

10H3-4E4-3D5 (Biotem, Apprieu, France) was used to detect RVFV in all the selected samples. Hepatic and ocular sections were placed on silane (3-

[triethoxysilyl]-propylamine)-coated slides, dewaxed and rehydrated by passage through alcohol solutions. Endogenous peroxidase was inactivated by incubation in H_2O_2 3% in methanol for 30 min at room temperature. Antigen retrieval was performed by incubation of the slides with Target Retrieval Solution 10X (Dako, Glostrup, Denmark) Tris-buffered saline (TBS) for 20 min at 96°C. After washing with PBS, blocking of non-specific binding was carried out for 1 h with bovine albumin 2% in TBS. The monoclonal RVFV antibody was used diluted at 1 in 1000 in TBS-albumin and incubated overnight at 4°C. After three

washes in TBS, the EnVision+System-HRP Labelled Polymer Anti-Mouse secondary antibody (Dako, Glostrup, Denmark) were each applied to the slides for 60

min. After washing three times with TBS, sections were finally incubated in 3',3-diaminobenzidine (DAB) and H_2O_2 for 10 min before counterstaining with Mayer's haematoxylin. Negative controls were established by omission of primary antibody. The quantity of RVFV antigens was scored from 0 (-) to 3 (+++) following the criteria defined in Table 8.

6.3.6. Statistical analysis

SAS 9.1.3 software (SAS institute Inc., Cary, North Carolina) was used for statistical analysis. The Wilcoxon-Mann–Whitney test was used to compare viral loads in liver and eye by rRT-qPCR; histopathological lesions; and CD3, CD20, lysozyme and RVFV expression by IHC between groups. Statistical significance was taken at $P < 0.05$.

6.4. Results

6.4.1. Clinical pathology

Two out of ten lambs were dead because of a severe acute hepatic necrosis associated to RVFV infection (#125, #130). A wide description of the necropsy findings is described in Chapter 3. However, the hepatic viral load in lethal cases was lower than observed in euthanized lambs, even in those killed at the same time (4 dpi). The statistical comparisons between both groups (≤8 dpi and =14 dpi) are shown in Table 11. No significant association of viremia and ocular viral loads were found between groups.

Table 10. Antibodies, antigen retrieval, dilutions and detection method for diagnosis of RVFV used in eyes of RVFV-positive lambs.

Antibodies	Antigen retrieval	Dilutions
In-house		
mAb antiN RVFV A9f12 HRPO	No treatment	1:500
	Protease K	1:1000
		1:2000
Rabbit policlonal serum antiRVFV Capture ELISA 1/2000 19/04	Sodium Citrate	1:100
	Protease K	1:250
		1:500
Pool M4 80,40,20 + MP12 Challenge 6/03/09	No treatment	1:100
	Sodium Citrate	1:250
		1:500
Anti- NPRVFV F1D11	No treatment	1:400

Commercial[¥]		
	No treatment	1:50
15G6-4B8 (IgG2b)	Proteinase K	1:100
	Target Retrieval Solution 10x (Dako)	1:250
		1:500
10H3-4E4-3D5 (IgG2a)		1:1000
		1:2000
8E10-4A4 (IgG1)		

All the in-house developed antibodies were supplied from the *Centro de Investigación en Sanidad Animal* (CISA-INIA) (28130 Valdeolmos, Madrid, Spain). All the commercial antibodies were supplied from Biotem Antibodies (38140 Apprieu, France). [¥] The same protocols were performed in the three antibodies. All antibodies were incubated using EnVision+System-HRP Labelled Polymer Anti- Rabbit/ Anti-Mouse (Dako, Glostrup, Denmark); the detection system was based on avidin–biotin complex (ABC) method.

Table 11. Wilcoxon-Mann-Whitney test results for group trend comparisons. Only positive relationships have been cited in the table. The rest of analyzed variables were equally distributed between groups.

Statistics	rRT-qPCR Liver	HP Liver	IHC Liver	CD20 uvea vs. retina*
Mann-Whitney (U)	2,500	1,500	2,500	
Wilcoxon (W)	17,500	16,500	17,500	
Z	-2,353	-2,394	-2,362	-1,890[b]
Sig. asint. (bilateral)	,019	,017	,018	,059
Sig. exact [2*(sig. unilateral)]	,032[a]	,016[a]	,032[a]	

* Wilcoxon signed-rank test. Only CD20 labelling was close to significance in a rank-biserial correlation. The rest of variables were considered not significant.

[a] No corrected for the tie.

[b] Based on positive ranks.

6.4.2. Pathology

Histopathological and immunohistochemical data are shown in Figure 27. Histopathological hepatic lesions were similar in all evaluated sheep but the extent of liver involvement varied accordingly to scoring (Table 8). Randomly located, multifocal, focally extensive, or confluent bridging hepatocellular necrosis was diagnosed as major hepatic lesion (Table 12). These foci varied in size and number and contained dense aggregates of hepatocellular debris, degenerate neutrophils, and scarce hyperplastic Kupffer cells. Apoptosis was diagnosed in affected hepatocytes based on the presence of dissociation of cells, with shrinkage and rounding, hypereosinophilic cytoplasm, and karyorrhexis or karyolysis. Apoptotic bodies were identified as small cytoplasmic fragments. Interestingly, a very distinctive feature of some cases showed randomly located foci of well-circumscribed cytolysis or individual hepatocyte degeneration with varying degree of perilesional mixed (chronic active) inflammatory infiltration. In the periphery of hepatic necrosis, a mild micro- or macrovesicular degeneration with anisokaryosis was usually observed in surviving hepatocytes. Random to portal located, multifocal, minimal to mild infiltration of neutrophils, lymphocytes and plasma cells were frequently accompanied to hepatic lesion, depending of the severity and chronicity. Scattered hyperplastic

Kupffer cells were also present within the sinusoids of non-necrotic hepatic tissue. Neither hemorrhages nor an especial involvement of hepatocytes within the limiting plate was found in the affected livers. Minimal to mild hyperplasia of cholangiocytes with mild multifocal proliferation of ducts was observed in several cases of the group =14 dpi, probably related to a recovery phase of infection. No inclusion bodies were detected in the selected cases. Histopathological ocular lesions were widely described in previous reports (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). Briefly, mild to severe lymphoplasmacytic anterior uveitis with variable mild lymphoplasmacytic endotheliitis were diagnosed in 8 out 10 cases. In lamb #141 a mild lymphoplasmacytic conjunctivitis with mild corneal erosion was diagnosed associated to a minimal anterior uveitis (Table 12). Gram and PAS stains were negative in all ocular and hepatic tissues evaluated.

A cytoplasmic to membranous strong labelling was observed in T cells stained with CD3. Surprisingly, positive cytoplasmic strong labelling of individual mononuclear cells within retinal structures (perivascular, interstitial) was clearly observed in 5 out of 10 cases (Table 12), although the group trend comparisons of the finding was not significant (Table 11). No foci of mononuclear inflammatory infiltrates in retina were observed in HE sections. However, mild vascular leakage with hypertrophic endothelium and an increase of intravascular and perivascular located mononuclear cells was observed in the CD3-positive retinas. A membranous strong labelling was observed in B cells stained with CD20. In contrast with CD3 labelling, CD20-positive mononuclear cells were only located in anterior uvea or ocular anterior chamber (Table 12) and there was a clear trend to significance (uvea positive, retina negative) between groups (Table 11). A diffuse strong cytoplasmic labelling was observed in histiocytes stained with lysozyme only in lambs #26 and #29. Either CD20-positive or lysozyme-positive mononuclear cells were related to the diagnosed hypopyon and mixed mononuclear infiltration of uvea, only seen in the more severely affected lambs (Table 12) such as it was previously described (Galindo-Cardiel *et al.*, 2012). In spite of these findings, no correlation of CD20, CD3 or lysozyme labelling between groups were observed in ocular tissues (Table 11).

All pilot testing done with the polyclonal in-house developed antibodies were negative to RVFV antigen in hepatic tissues (data no shown). The pilot study carried out with mononuclear commercial antibodies determined the better protocol to visualize conclusive RVFV-positive labelling in affected cells (Figure 44). Briefly, multifocal, focal extensive or bridging-distributed, moderate to severe, globular to diffuse, cytoplasmic and interstitial RVFV-positive labelling in hepatocytes and necrotic tissue was consistently observed in 4 out of 5 lambs from group <8 dpi. Many inflammatory cells were positive too (Table 11). No RVFV-positive labelling was observed in livers from group =14 dpi. RVFV-positive labelling was observed in the two most affected eyes (lambs #26 and #29), mainly related to mononuclear inflammatory cells.

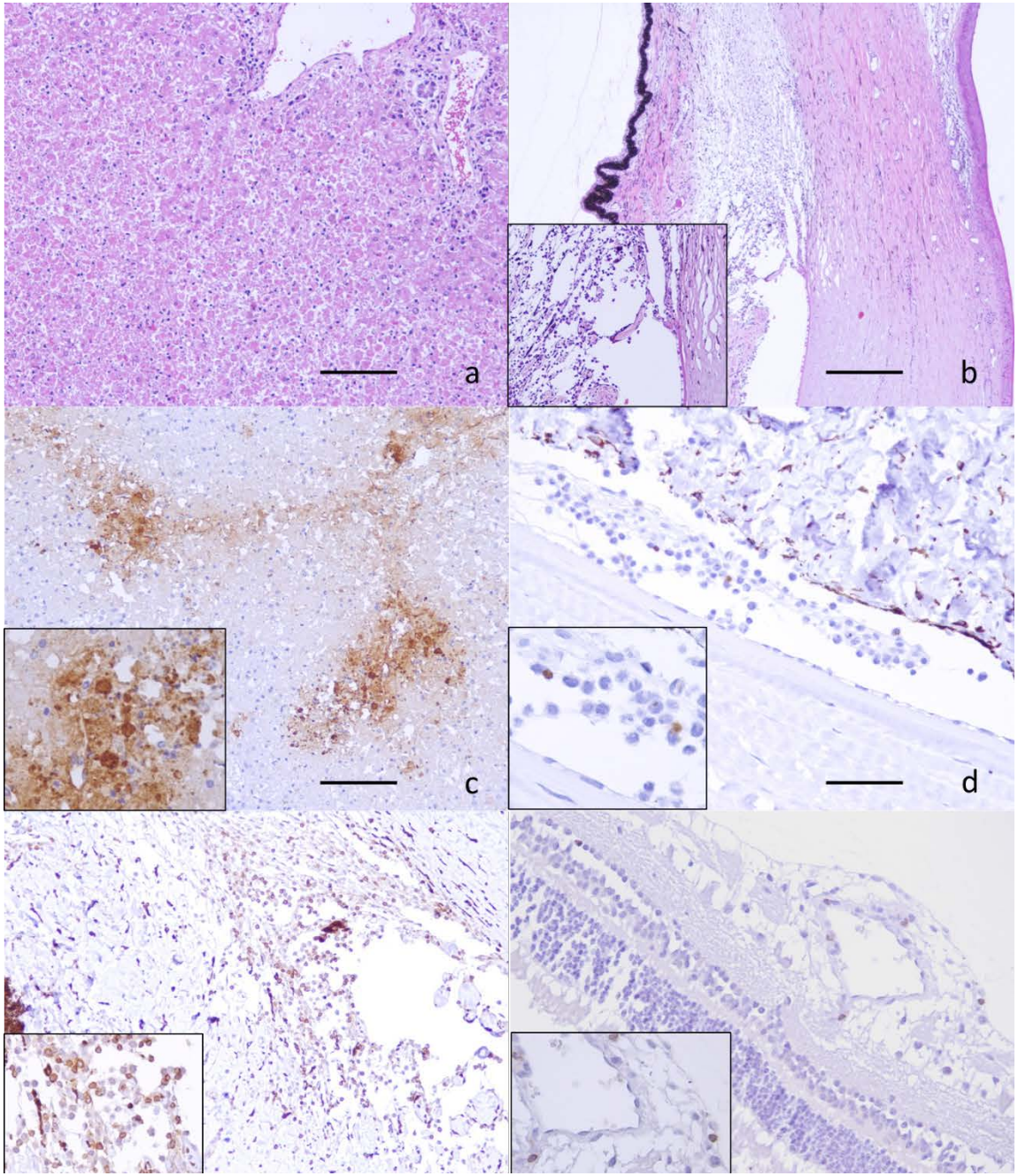

a
b
c
d

Figure 44. Histopathological and immunohistochemical data of RVFV-infected Ripollesa lambs. (a) Lamb #125. Liver. Severe bridging hepatic necrosis. Note the absence of inflammatory infiltrates. HE. Bar 250 µm. (b) Lamb #26. Anterior uvea, cornea. Chronic (subacute) lymphoplasmacytic anterior uveitis with lymphoplasmacytic endotheliitis and superficial and mid-stromal corneal neovascularization. HE. Bar, 350 µm. Inlet Bar, 250 µm. (c) Lamb #125. Liver. RVFV positive multifocal to bridging strong labelling of necrotic tissue and hepatocytes. IHC. Bar, 250 µm. Inlet Bar, 125 µm. (d) Lamb #26. Anterior uvea. RVFV-positive cytoplasmic strong labelling of individual mononuclear cells. IHC. Bar, 250 µm. Inlet Bar, 125 µm. (e) Lamb #26. Anterior uvea. CD3 positive membranous to cytoplasmic strong labelling in mononuclear infiltrates. IHC. Bar, 250 µm. Inlet Bar, 125 µm. (f) Lamb #26. Retina. CD3 positive membranous to cytoplasmic strong labelling in two individual mononuclear cells (perivascular, interstitial). IHC. Bar, 250 µm. Inlet Bar, 125 µm. (g) Lamb #26. Anterior uvea. CD20 positive membranous strong labelling in mononuclear infiltrates. IHC. Bar, 250 µm. Inlet Bar, 125 µm. (h) Lamb #26. Anterior uvea. Lysozyme positive cytoplasmic strong labelling in monocytes. IHC. Bar, 250 µm. Inlet Bar, 125 µm.

Table 12. Clinical, histopathological, immunohistochemical findings (RVFV, CD3, CD20, and lysozyme) and viral concentration (rRT-PCR) results in liver and eyes from the studied RVFV-positive lambs are shown.

ID	Clinics (dpi)	Viremia	rRT-qPCR¥		RVF-IHC							
			Liver	Eye	Liver	Eye	Liver	Eye	CD3	CD20	Lis	CD3	CD20	Lis
Group ≤ 8 dpi														
#125	Dead, 4 dpi	NE	6,44	3, 84	+++	-	+++	-	-	-	-	-	-	-
#130	Dead, 5 dpi	NE	5,18	3, 15	+++	-	++	-	-	-	-	-	-	-
#138	Euthanasia, 4 dpi	(0/5)	34,72	-	++	+	+	-	-	-	-	-	-	-
#141	Euthanasia, 4 dpi	(1/2)	32,89	2, 8	++	+	++	-	+	-	-	-	-	-
#29	Euthanasia, 8 dpi	(4/10)	NE	4, 31	+	+++	-	+	+++	+	+++	+	-	+
Group = 14 dpi														
#24	Euthanasia, 14 dpi	(2/15)		-	+	+	-	-	-	-	-	+	-	-
#26	Euthanasia, 14 dpi	(13/15)	NE	3,	+	+++	-	+	+++	++	++	+	-	-

				26											
#32	Euthanasia, 14 dpi	(7/15)	NE	3, 82	+	+	-	-	-	-	-	+	-	-	
#135	Euthanasia, 14 dpi	(1/5)	-	-	-	+	-	-	+	+	-	-	-	-	
#140a	Euthanasia, 14 dpi	(2/5)	-	4, 42	-	-	-	-	-	-	-	-	-	-	
#140b				-	-	++		-	++	+	-	+	-	-	

Viremia data expressed in RVFV-positive days/ all days of the experiment before death. ¥ Reversal retrotranscriptase polymerase chain reaction (rRT-PCR) results are given in log nº RNA copies/ 100 µgr. HP: histopathology; Hepatic lesions: (+) scattered multifocal necrotic hepatocytes with minimal perilesional neutrophilic infiltration; moderate (++) to severe (+++) multifocal hepatic necrosis with minimal perilesional inflammatory reaction; Ocular lesions: (+/-) vascular leakage in choroid and uvea with minimal to mild lymphoplasmacytic conjunctivitis; mild (+), moderate (++) or severe (+++) lymphoplasmacytic anterior uveitis as described in Galindo-Cardiel *et al* 2010. RVF-IHC: Rift Valley Fever virus immunohistochemistry. U-IHC: uveal immunohistochemistry. R-IHC: retinal immunohistochemistry. Lis: Lysozyme. ** No evaluated (NE). Both eyes of lamb #140 were included because of the selected criteria used for this characterization. Data from negative and positive controls are not included.

6.5. Discussion

Fewer data related to pathogenesis of RVFV are available in breeds of sheep and these refer most to the pathology observed upon experimental infection for research in vaccinology field. Differential pathogenicity between isolates has been reported (Anderson and Peters, 1988; Olaleye *et al.*, 1996; Swanepoel *et al.*, 1986). However, RVFV shows low (~5%) variability at the nucleotide level, despite being an RNA virus (Bird *et al.*, 2007). Moreover, the susceptibility for adult sheep appears to be more inconsistent than for young animals and between breeds, reflecting of different host's genetic determinants even within the same breed of sheep (Busquets *et al.*, 2010; Gerdes, 2004). Two out of ten RVF-affected experimentally challenged lambs (#125, #130) died associated to acute hepatic widespread necrosis. Moreover, the rest of clinically affected viremic older lambs of the reviewed experiments suffered fever and/ or ocular involvement (conjunctivitis, corneal edema, aka blue eye) as the only clinical observed symptoms, which is in accordance with the sub-acute form of the disease that may reach 20% of mortality in adults (Easterday *et al.*, 1962b; Gerdes, 2004). In contrast, no relevant or considered incidental gross lesions were observed in selected animals (data no shown) such as it happens with natural RVFV strains, in which widespread acute hemorrhages associated to endothelial damage or disseminated intravascular coagulation are consistently seen among others (Odendaal *et al.*, 2014; Odendaal and Prozesky, 2010; Olaleye *et al.*, 1996). Major histopathological lesions were mainly focused in liver, and they were similar in Ripollesa lambs to other breeds, although the extent of liver involvement varied somehow. Randomly located, multifocal, focally extensive, or confluent bridging hepatocellular necrosis was diagnosed with scattered distributed minimal to mild mixed to mononuclear inflammatory foci, hyperplastic Kupffer cells and apoptotic hepatocytes. No hemorrhages or biliary affectation was diagnosed in our cases, such it was previously described (Daubney *et al.*, 1931). Intranuclear inclusions bodies (INIB) were neither detected in the selected older lambs, which is more in accordance with adult sheep findings (Baskerville *et al.*, 1992; Lorenzo *et al.*, 2015). In contrast, naturally affected or experimentally infected ovine and murine fetuses and neonates (younger lambs) show multifocal rod to oval-shaped or round eosinophilic INIB (Daubney *et al.*, 1931; Smith *et al.*, 2010). A positive correlation between hepatic viral load, liver lesion and its RVF-positive labelling was assessed for group ≤8 dpi, pointing towards a progressive hepatic clearance of RVFV in time. This fact may explain the absence of INIB in the affected animals, confirming the recovery phase observed in long lasting experimentally RVFV-infected Ripollesa older lambs (group =14 dpi) (Busquets *et al.*, 2014; Busquets *et al.*,

2010; Galindo-Cardiel *et al.*, 2012). This timeline correlates also with convalescence phase (10-30 dpi) of human disease, which highlights another relevant coincidence of this model (Ikegami and Makino, 2011).

This retrospective study has characterized the diagnosed ocular lesions of experimentally infected older lambs with RVFV isolates. The onset of human ocular clinical signs occurring 5–14 days after local outbreaks of RVFV in ruminants are in accordance with the observed RVFV-infected sheep kinetics (Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012; Khairallah *et al.*, 2010). Anterior uveitis associated to or not with lymphoplasmacytic endothelitis, corneal edema and corneal erosions was diagnosed in 8 out of 40 RVFV-challenged older lambs, leading to a 20% of apparent prevalence of the lesion (data no shown) (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). However, no retinal-associated clinical or pathological partial blindness were observed in the reviewed studies. This fact may be related to the lacking of standards on ocular clinical exploration of sheep in experimental conditions, which should include functional test. The clinical diagnosis is totally based on the simple observation of the animal behavior and corneal checking during the exploration. In spite of this, the apparent prevalence for ocular involvement in RVFV infection in humans, which varies from 10 to 25% of clinical cases, is in accordance with apparent ocular pathology prevalence in Ripollesa breed (Ikegami and Makino, 2011). A previous report had already suggested a more prevalent uveitis, associated or not to corneal edema, in sheep infected with RVFV (Galindo-Cardiel *et al.*, 2012).

CD3, CD20 and lysozyme positive labelling is correlated with the presence of mononuclear infiltrations, in the already described anterior uveitis and lymphoplasmacytic endotheliitis. Surprisingly, corneal edema was only involved in 2 out of 11 studied eyes, in accordance with previous report which suggest a secondary involvement of cornea due to vascular damage (endothelium and neo-mid-stromal vascularization) (Galindo-Cardiel *et al.*, 2012). Anterior uveal mononuclear B cell-positive infiltration was observed in the selected cases, suggesting that a strong antibody production is also involved in RVFV ocular pathogenesis. B cells and CD4(+) T cells, but not CD8(+) T cells, are required for robust IgG and neutralizing antibody responses in neurologically affected RVFV-challenged mice, even in the presence of a functional innate response, and such requirement correlated with viral clearance from peripheral tissues, including eye (Dodd *et al.*, 2013; Dodd *et al.*, 2014). However, other report describes a robust CD8+ T cell response associated to a strong neutralizing antibody also in RVFV-challenged vaccinated mice (Warimwe *et al.*, 2013). Regrettably, no further T cell phenotyping was done in the selected cases, but the consistent distribution of T cell infiltration in the eyes of both

groups suggest a central role of CD3-positive cells in the ocular pathogenesis in ruminants. Moreover, a novel presumptive diagnosis of retinitis was attempted based on the CD3-positive labelling of mononuclear cells associated to the vascular alterations (5/10), which is the first description of such lesion in RVFV-infected ruminants, to the best of the author ´s knowledge. Two basic pathogenic mechanisms of acute anterior uveitis based on cytokine production of resident uveal macrophages associated to neutrophilic infiltration (relatively short-lived), or a delayed-type hypersensitivity immune response associated to soluble antigens (delayed onset but extended duration) have been described in experimental models of rat and mice (Smith *et al.*, 1998). The observed lysozyme-positive monocytes closely related to hypopyon in the most severe cases of anterior uveitis, and the lacking of neutrophils in the affected eyes, may suggest that the first mechanism is not clearly involved in the Ripollesa ocular lesion. An immune-mediated mechanism has been also proposed as the underlying cause of viral lymphocytic endotheliitis and anterior uveitis in ruminants (Holzhauer and Vos, 2009; Whiteley *et al.*, 1985), and therefore it was suggested in previous cases in Ripollesa breed (Galindo-Cardiel *et al.*, 2012). Moreover, lacking of apoptosis evidence or grossly immune-complex depositions in ocular tissue does not allow confirm a clear delayed immune-mediated hypersensitivity (type III or IV), based on the selected cases. However, hepatic viral clearance (significant, Table 21) among with the persistency in ocular lesions between groups (no significant, Table 21), no RVFV-positive labelling in ocular tissue (just in mononuclear infiltrates), and consistent RNA viral detection in affected eyes (7 out of 11) after viremia phase, may suggest an ongoing active viral infection, in progress for clearing. Such pathologic situation may lead to more or less pronounced sequels in the survivors (Al-Hazmi, Ayoola et al. 2003, Al-Hazmi, Al-Rajhi et al. 2005, Gay 2006, Khairallah, Chee et al. 2010). It is worthy to mention that different ocular viral clearance with an increase of eye lesion severity was intuitively observed, but not significantly confirmed, in the recovery phase of RVFV infection observed at 14 dpi (Table 21). Such as it was described in previous reports, this apparently lack of clearance of RVFV in eyes may correspond with two clinical phases: (i) a particular ocular progression to permanent sequel; (ii) the progression of the ocular lesion is slower than hepatic one and the time of experiment was not enough to confirm the pathogenesis (Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). Whether Ripollesa susceptibility to RVFV ocular disease correlate to their own host genetic determinants, remains unknown because is out of the scope of this paper. However, recent evidence demonstrated that three different polymorphisms of Toll like receptor 8 (TLR8) in humans are significantly associated to eye disease (Hise *et al.*, 2015), so further genotyping studies are needed to confirm the special ocular susceptibility to RVFV of Ripollesa breed.

The Ripollesa sheep breed, in the middle way from lambing to puberty, has demonstrated a moderate sub-acute clinical susceptibility with major liver involvement to RVFV infection

under experimental conditions. Moreover, a T cell-dependent retinitis has been diagnosed, suggesting that Ripollesa sheep may be used as a model for studying RVF-associated ocular pathology in man. However, there are still unconfirmed data in ocular pathogenesis of RVFV in sheep, so further studies should be carried out to better characterize the timeline of the viral uveitis in clinically affected RVFV-positive Ripollesa sheep. RVFV infection should be included in the list of viral entities that can cause uveitis and retinitis in ruminants.

7. General discussion

Rift Valley fever virus (RVFV) is an arthropod-borne pathogen that primarily affects ruminants in eastern and sub-Saharan Africa (Bird *et al.*, 2009; Faye *et al.*, 2007). Susceptibility of different breeds to RVF varies considerably (OIE, 2009; Terasaki and Makino, 2015). The disease usually presents in an epizootic form over large areas of a country following heavy rains and sustained flooding, and is characterized by high rates of abortion and neonatal mortality, primarily in sheep, goats and cattle (OIE, 2009). Factors such as rainfall, ocean temperature, and climate change play roles in determining the likelihood of an epidemic. The risk of the introduction of Rift Valley fever in Europe was assessed recently by scientific community (EFSA, 2005; Mansfield *et al.*, 2015). RVFV's recent classification as a potential bioterrorist agent have renewed interest in the study of the virus as well as in RVF vaccinology (Rabinowitz *et al.*, 2006). Therefore, robust challenge models are needed to properly evaluate the efficacy of the most of the promising novel candidate vaccines, using the lowest number of animals possible. The refinement of these models should also consider the complete diagnosis, detection and proper evaluation of pathology related to candidate vaccines, mainly due to the safety concerns highlighted by currently licensed vaccines. As a surprisingly fact, many candidate vaccines have not been extensively evaluated in the natural target species of RVFV, sheep, which may be caused because the lack of standardization of reproducible ovine models. In this work, we contribute with some data to establish a reproducible model of RVFV infection in 2-months old lambs of a European breed, Ripollesa sheep; we have better defined the pathogenicity of four African isolates of RVFV; we have tested the efficacy of a potential MVA-vectored vaccine candidate; we have described and characterized pathologically ocular injuries associated to RVFV infection; and, we have developed a new protocol for detection of RVFV antigens in ovine tissue using immunohistochemistry. The data obtained allow withdrawing some conclusions with respect to the consequences of a RVFV infection of ruminants in Europe.

In the beginning of this work, we showed the susceptibility of 9–10 weeks old European sheep (Ripollesa breed) to RVFV infection, showing a mild, subacute form of disease. Four different viral isolates efficiently replicated *in vivo* after subcutaneous experimental inoculation, and consistent viral loads in blood and virus shedding (variable in length depending on the RVFV isolate used) were detected, showing horizontal transmission to a noninfected, sentinel lamb (Busquets *et al.*, 2010).

Once all the pathological data were evaluated, we detected and described a new ocular condition in these old lambs, consisting in an anterior uveitis with lymphoplasmacytic endotheliitis in the blue-eye-diagnosed animals (Galindo-Cardiel *et al.*, 2012).

With the aim of contributing to develop a new safer recombinant vaccine, the research group evaluated the protection conferred by a single subcutaneous dose of a modified vaccinia virus Ankara (MVA) vectored vaccine encoding the Rift Valley Fever virus (RVFV) glycoproteins Gn and Gc in the proposed model. Briefly, three groups of six to seven lambs were immunized as follows: vaccine group (termed rMVA-GnGc), MVA vector control group (vector control) and control group (non-vaccinated saline-treated animals). Fourteen days later, all animals were subcutaneously challenged with 10^5 $TCID_{50}$ of the virulent RVFV isolate 56/74 and vaccine efficacy assessed using standard endpoints. One lamb from the vaccine group and another lamb from the MVA vector control group succumbed to RVFV challenge, showing characteristic liver lesions. Lambs from both the vector control and non-vaccinated groups were febrile from days 2 to 5 post challenge (pc), while those in the rMVA-GnGc group showed a single peak of pyrexia at day 3 pc. RVFV RNA was detected in both nasal and oral swabs from days 3 to 7 pc in some lambs from the vector control and non-vaccinated groups. No viral shedding could be detected in the surviving lambs vaccinated with rMVA-GnGc. Together, the data suggest that a single dose of the rMVA-GnGc vaccine may be sufficient to reduce RVFV shedding and duration of viremia but does not provide sterile immunity nor protection from disease (Busquets *et al.*, 2014).

The need of a better pathological characterization of the new ocular condition in the proposed model made us to define a specific retrospective research about RVFV-associated anterior uveitis prevalence in the available database. Briefly, two groups of five lambs per group were selected (n=10) from such historical database of the RVFV experimental infections performed in IRTA-CReSA (northeast Spain, NBS3 facility) in the basis of their clinical data, viremia and diagnosed ocular and hepatic lesions. An anterior uveitis (8 out of 10) with lymphoplasmacytic endotheliitis (2 out of 10) was characterized. CD3, CD20 and lysozyme-positive mononuclear infiltrations were observed in RVFV-positive paraffin-embedded eyes. CD20 labelling was only observed in anterior uvea. A novel T-cell dependent retinitis was also diagnosed in 5 out of 10 RVFV-infected lambs based on CD3-positive labelling (Galindo-Cardiel, *et al*., under revision). This work let us also define an optimized immunochemical diagnostic tool. Understanding the pathogenesis and immune response following RVFV infection is key to find novel vaccines or antiviral treatments.

The pathogenicity, transmission, strain virulence and immune responses upon experimental challenge with RVFV have been analyzed in great detail in ruminants, a main vaccine target species (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Capstick and Gosden, 1962; Coackley *et al.*, 1967b; Galindo-Cardiel *et al.*, 2012; Olaleye *et al.*, 1996; Swanepoel *et al.*, 1986; Tomori, 1979; Weingartl *et al.*, 2014a; Yedloutschnig *et al.*, 1981). Differential pathogenicity between isolates has been reported (Anderson and Peters, 1988; Olaleye *et al.*, 1996; Swanepoel *et al.*, 1986). However, RVFV shows low (~5%) variability at the nucleotide level, despite being an RNA virus (Bird *et al.*, 2007). The influence of genetics in susceptibility is out of doubt; a single nucleotide substitution at nucleotide 847 of the M segment can determine the virulence phenotype of the RVFV ZH501 strain in mice (Morrill *et al.*, 2010). Moreover, the susceptibility for adult sheep appears to be more inconsistent than for young animals and between breeds, reflecting of different host's genetic determinants even within the same breed of sheep (Busquets *et al.*, 2010; Gerdes, 2004). This phenomenon is also observed in rats, which show marked age and breed-dependent susceptibility to RVFV infection (Findlay and Howard, 1952; Ritter *et al.*, 2000). No genetic studies to determine genetic host factors in RVFV pathogenesis have been done in sheep, to the best of authors´ knowledge. The lack of a severe, fatal disease might be explained by different results, such as the age of the animals used (9 weeks old), as previously reported (Kamal, 2009). Alternatively, the historical passage in cell culture, and even the type of culture used, of these strains might also influence the observed moderately virulent phenotype. The lower number passage, the higher viral loads detected. In our first study, higher viral loads in blood were reached after inoculation with RVFV isolates 252/75 and AN1830, which were less passaged in cell culture than 56/74 and AR20368 (Table 6) (Busquets *et al.*, 2010). The specie from the strain was isolated might also affect their pathogenic effects. Curiously enough, the two viruses showing lower replication levels were isolated from mosquito and cow. Viremia results showed an inter- and intra-group variations in RVFV-infected Ripollesa sheep, such as we described previously (Busquets *et al.*, 2014; Busquets *et al.*, 2010). However, all tested animals peaked at the same day (3 dpi), starting at 2 dpi, coincidental with the peak of fever. Our results are in good agreement with those from previous studies using different viruses and/or sheep breed (Olaleye *et al.*, 1996). For instance, viremia was detected for longer periods (at least 4 days) from earlier time points (as soon as at 2 dpi) in most of the animals infected with the AN1830 sheep isolate, being able to persist in one lamb in blood and also in kidney throughout the experimental procedure (Busquets *et al.*, 2010). Interestingly, the mosquito-isolated tested virus (AR20368) yielded lowest viremia and virus shedding than

ruminant-isolated tested viruses, which it might reflect an incomplete adaptation to the mammalian host, or a viral propagation heterogeneity associated to the adaptation to grow in both insect and mammalian cells for research. For instance, the Egyptian ZH501 RVFV isolate propagated in insect (*Aedes albopictus*) C6/36 cells showed an increase of susceptibility of sheep after challenge than the same isolate propagated in monkey Vero E6 cells, suggesting a more efficient initiation of infection by using mosquito cells in subcutaneous challenges (Weingartl *et al.*, 2014a). However, no changes in susceptibility, viremia or IgM-related neutralizing antibodies (humoral response) were detected when using a South African RVFV strain (56/74) propagated in mammalian Vero E6 cells or in the insect C6/36 cells in a pilot study (Lorenzo *et al.*, 2015). In any case, the lower replication and shedding but still active viremia observed in our studies might explain the regular field observations that detect viral circulation in endemic areas between wild ruminants and hematophagous mosquitoes, associated to a usually unapparent clinical course of the disease (Ikegami and Makino, 2011; Le Coupanec *et al.*, 2013; Logan *et al.*, 1991). Broad vector range of mosquitoes coupled with increased circulating virus leads to the main mechanism of expansion of the disease (WHO, 2007; WHO, 2008; WHO, 2010). Recent studies about European and North-American mosquitoes genera have shown a real capability of RVFV transmission as preclude for the increased threat to public health, after the unexpected virulence for humans of the RVF Egyptian 1977 outbreak (Al-Hazmi *et al.*, 2003; Hassan *et al.*, 2011). The virus is currently limited to Africa and the Middle East (Chevalier *et al.*, 2010; Turell *et al.*, 2008), and no RVF incursions have yet been described out of the affected areas.

RVFV is transmitted primarily through a variety of mosquito genera, in particular, *Aedes, Culex, Anopheles, Eretmapodites, Mansonia* among others (Boshra *et al.*, 2011b; Chevalier *et al.*, 2010; Ikegami and Makino, 2011; OIE, 2009). The AR20368 mosquito isolate showed a real potential to spread to a sentinel animal, independently of vector presence, associated to a high infection pressure of the studied herd (Busquets *et al.*, 2014; Busquets *et al.*, 2010). RVFV survives at aerosols at 23°C and 50–85% humidity, very similar to climatic conditions of experimental box (Table 1) (OIE, 2009). However, the sheep inoculated with this isolate did not display the highest nasal and oral shedding titers, therefore other factors might be involved in the observed transmission. Also, as all inoculated animals were housed in the same box, potential cross-contamination events cannot be ruled out. In fact, direct contact with sheep body fluids has been significantly associated with infection in humans (Woods *et al.*, 2002), so it may play a role between animals too. In any case, the observed high and long-lasting viremia in the proposed model might be advantageous for virus dissemination by competent vectors, and therefore, competence studies with the four virus isolates could be addressed in the future. Further, these long-term viremic infected lambs may be potentially dangerous

(Easterday *et al.*, 1962a; Erasmus and Coetzer, 1981; Woods *et al.*, 2002), for example, in the case of being slaughtered, for its risk of viral transmission to human by direct contact with infected fluids. In-contact American sheep became infected with the RVFV Zagazig 501 isolate in the absence of vectors, confirming the horizontal RVFV transmission of some strains (Harrington *et al.*, 1980).

Sustained induction of IFN-γ following infection has reflected an activation of antigen-specific cellular responses against RVFV antigens using a panel of overlapping 15mer peptide pools spanning the whole mature GnGc sequence (Warimwe et al., 2013). Several studies in RVFV-infected sheep have shown a stronger level of immunity mediated through the interactions of antigen-presenting cells (APCs) with T helper cells. Such level of immunity may be caused by an increase of the levels of proliferating memory cell pools and induction of IFN-gamma *in vitro* in the presence of recombinant N protein (Lagerqvist *et al.*, 2009; Lorenzo *et al.*, 2010; Lorenzo *et al.*, 2008). However, recombinant N cannot elicit neutralizing antibodies. Neutralizing antibody induction is correlated with the control of the infection and protection after challenge with RVFV (Harrington *et al.*, 1980; Hubbard *et al.*, 1991; Morrill *et al.*, 1991a). Most animals involved in our studies cleared the virus from the body fluids before neutralizing antibodies were detected. Therefore, other early immune mechanisms as nonspecific innate immune responses might play a role in the elimination of the virus.

For instance, serum levels of inflammatory cytokines in RVF human patients have been shown to be indicative of severe disease. Chemokines and pro- and anti-inflammatory cytokines were detected at significantly increased (IL-8, CXCL9, MCP-1, IP-10, IL-10) or decreased (RANTES) levels when comparing fatal cases to infected survivors and uninfected controls, or when comparing combined infected patients to uninfected controls. These results suggest that regulation of the host innate inflammatory responses plays an important role in the outcome of RVFV infection in humans (Jansen van Vuren *et al.*, 2015). The regular infiltrates of monocytes and granulocytes that are seen in RVFV-infected livers of mice may be explained by the significant increase of the level of non-pro-inflammatory chemokines (Gray *et al.*, 2012). In this line of argument, the level of IL-10 gene expression in the liver was found to be upregulated in infected mice (Jansen van Vuren *et al.*, 2011). A significant increase of IL-6, G-CSF and IL-12 (p40) as well as chemokines KC, MCP-1 and MIP-1α, starting at 48 hpi and decreasing to normal levels at 96 hpi, was observed in BALB/c OlaHsd mice. Hepatic upregulation of pro-apoptotic and pro-inflammatory genes and downregulation of anti-apoptotic ones was found significant as well (Jansen van Vuren *et al.*, 2011). Later after infection the expression of both IFN-β and IFN-γ genes was heavily upregulated, perhaps contributing to liver damage by means

of the exacerbation of cytokine production in mice. Levels of TNF-α, IL-6 and IL1-β rose later in the course of infection in goats, correlating with the antagonistic function of the RVFV NSs protein. (Nfon *et al.*, 2012). Stimulation of IFN-γ production in a challenge using insect cell-propagated RVFV challenge was higher than when using mammalian cell-propagated RVFV (Lorenzo et al., 2015). In our studies, although a clear *in vitro* neutralizing activity was observed in all tested sera, this immune response was undistinguishable from the virus isolate used. In contrast, a clear correlation was observed between specific IFN-γ detection at 14 dpi and viral clearance, with infected sheep at the end of experiment showing almost no specific IFN-γ responses. Curiously enough, persistently infected cells (at the end of experiment) also showed the lowest IFN-γ response to the unspecific phytohemagglutinin (PHA) stimulation, suggesting a transient immune suppression. Interestingly, one pool of peptides characterized by a putative single region/epitope on the Gc polypeptide with characteristics of an immunodominant T-cell epitope was able to stimulate IFN-γ production in cells from all sheep, independently of the cells used for propagation (Lorenzo *et al.*, 2015). Regrettably, no cytokine or innate markers were tested in our ovine model, leading towards a deeper research in this topic. Understanding the immune mechanisms involved in viral clearance and protection might be essential to design efficient vaccines in the future.

In our preliminary results, two out of ten RVF-affected experimentally challenged lambs (#125, #130) died associated to acute hepatic widespread necrosis (Busquets *et al.*, 2010). Hepatic virus replication or a strong unregulated immune response are the postulated causes of the liver failure, but the specific mechanism of necrosis remains inconclusive (Gommet *et al.*, 2011; Reed *et al.*, 2012). Moreover, the rest of clinically affected viremic older lambs of the reviewed experiments suffered fever and/ or ocular involvement (conjunctivitis, corneal edema, aka blue eye) as the only clinical observed symptoms, which is in accordance with the sub-acute form of the disease that may reach 20% of mortality in adults (Easterday *et al.*, 1962b; Gerdes, 2004). The apparent natural mortality associated to insufficient immunogenicity of experimental vaccines tested at CReSA or natural virulence of South African RVFV strains used for challenging in our studies is established in 5% (2 out of 40 animals), in accordance with another ovine experimental models based on pregnant ewes (Busquets *et al.*, 2014; Yedloutschnig *et al.*, 1981). Lymphadenopathy associated with congestion and necrosis, with multifocally extensive subcutaneous edema and petechiae were observed only in naturally death lambs, according with natural RVFV-infected sheep. Euthanatized lambs show characteristic hepatic lesions (chapter 3). No relevant or considered incidental gross lesions were observed in the rest of studied animals based on clinical-evidence medicine (Geyman, 2000). However, a strong strain-dependent correlation was observed between RVFV-RNA load in blood at 3 dpi and kidney, which is considered a secondary target organ (Kamal,

2009). Interestingly, viral RNA copies were detected in kidney samples from sheep displaying bilateral blue eye. These viremia and gross lesional findings in the proposed model are in contrast with natural RVFV infections, in which widespread acute hemorrhages associated to endothelial damage or disseminated intravascular coagulation are consistently seen in viremic animals, among others findings (Odendaal *et al.*, 2014; Odendaal and Prozesky, 2010; Olaleye *et al.*, 1996).

Major histopathological lesions were mainly focused in liver, kidney and lymph node, and they were similar in Ripollesa lambs to other breeds, although the extent of liver involvement varied somehow. Randomly located, multifocal, focally extensive, or confluent bridging hepatocellular necrosis was diagnosed with scattered distributed minimal to mild mixed to mononuclear inflammatory foci, hyperplastic Kupffer cells and apoptotic hepatocytes. No hemorrhages or biliary affectation was diagnosed in our cases, such it was previously described (Daubney *et al.*, 1931). Intranuclear inclusions bodies (INIB) were neither detected in the selected older lambs, which is more in accordance with adult sheep findings (Baskerville *et al.*, 1992; Lorenzo *et al.*, 2015). In contrast, naturally affected or experimentally infected ovine and murine fetuses and neonates (younger lambs) show multifocal rod to oval-shaped or round eosinophilic INIB (Daubney *et al.*, 1931; Smith *et al.*, 2010). Severe deleterious hepatic pathological changes in kids (necrogranulomas) and hyperplastic overgrowth with vesicular elongated epithelium (even necrotic in some cases) and lymphocytic infiltration were diagnosed in RVF live attenuated vaccinated (Smithburn strain) goats (Kamal, 2009). Hepatic necrosis as major lesion was in accordance with most of the used experimental models, mice, non-human primates and, with less extent, hamsters (Niklasson *et al.*, 1984; Peters *et al.*, 1988; Ross *et al.*, 2012). Other experimental models as rats, gerbils or dogs show other pathological patters (neurological, mixed) after RVFV infection, with variable susceptibility (Anderson and Peters, 1988; Anderson *et al.*, 1988; Peters and Anderson, 1981). Other species affected by RVFV include goats, cattle, camels, dogs, cats, and ferrets, but does not cause any symptomatic diseases in rabbits, guinea pigs, birds, horses, pigs and other animals, as reviewed in detail previously (Daubney *et al.*, 1931; Easterday, 1965; Findlay and Daubney, 1931; Keefer *et al.*, 1972; Peters and Anderson, 1981; Scott, 1963; Scott *et al.*, 1963; Shimshony and Barzilai, 1983; Walker *et al.*, 1970a; Walker *et al.*, 1970b; Weiss, 1957).

Severe diffuse acute renal tubular necrosis (naturally death lambs), mild to severe acute lymphoid depletion with marked lymphoblastic infiltration of germinal centers, multifocal lymphocytolysis, and multifocal lymph node congestion were seen as secondary major histopathological findings. Ovine RVFV infection consistently results in a severe leukopenia, being more severe in young lambs as compared to adult sheep (Easterday,

1965). In fact, at 3rd and 4th days p.i., the lowest counts of leukocytes were recorded in adult sheep (Easterday, 1965). Leukopenia was usually of short duration; even in animals with a fatal infection, the number of leukocytes began to return to pre-infection levels before death. In line with the observed variation depending of the virus propagation in sheep, baseline counts for CD172+ monocytes and dendritic cells (DCs) dropped only in goats challenged with the insect cell propagated virus but not in those receiving virus propagated in mammalian cells (Nfon *et al.*, 2012). A similar observation was found for both CD5+ and CD8+ T-cells as well as CD21+ B-cell counts. Direct infection of monocyte-derived cells has been demonstrated in *in vitro* infection experiments of monocyte-derived dendritic cells, leading to a plausible explanation for the reduction in the frequencies of monocyte-derived cells found in RVFV-infected goats (Nfon *et al.*, 2012) or monocyte-derived human CD14+ macrophages (McElroy and Nichol, 2012). Our results are in agreement with lymphoid depletion observed but no characterization was made in our cases. Further studies should be carried out for determining the dynamics of B and T cells in Ripollesa sheep after RVFV infection.

RVFV-positive hepatic immunostaining was correlated with major lesions, such as it has been observed in previous studies (Van der Lugt *et al.*, 1996). No viral antigen could be detected in the endothelial or Kupffer cells in the liver, such as it has been described in lambs (Van der Lugt *et al.*, 1996). The necrosis was predominantly centrilobular or midzonal too, but no definite distribution pattern was standardized in liver necrosis in our cases, correlating with published results (Coetzer, 1977; Van der Lugt *et al.*, 1996). In-house made mouse-derived antibodies failed to stain virus in our RVFV-positive ovine samples. However, specific murine-derived antibodies consistently stained RVFV in our cases. According to scoring made for evaluating the RVFV immunostaining, some to many mononuclear inflammatory cells were positive (++ or +++) (Table 8). These data are supported by *in vivo* RVFV antigen-specific immunostaining of liver macrophages (Shieh *et al.*, 2010) and dendritic cells in mice (Smith *et al.*, 2010).

The results of the experimental infection of Ripollesa lambs did not show any relation between the presence of hepatic lesion and virus load or excretion in contrast to that reported by Kamal (2009). However, the retrospective study of ocular condition revealed a positive correlation between hepatic viral load, liver lesion and its RVF-positive labelling for group ≤8 dpi, pointing towards a progressive hepatic clearance of RVFV in time, confirming the recovery phase observed in long lasting experimentally RVFV-infected Ripollesa older lambs (group =14 dpi) (Galindo-Cardiel, *et al*., under revision). These facts are in accordance with the RVFV pathogenesis observed in mice, in which an increase of viral antigens detected at 3 dpi is followed by a sharp decrease occurred at 8 days p.i.

Besides, no virus could be detected in the sera, liver, lung, pancreas, large intestine and ovaries (Smith *et al.*, 2010). This recovery may also explain the absence of INIB in the affected animals (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). This timeline correlates also with convalescence phase (10-30 dpi) of human disease, which highlights another relevant coincidence of this model (Ikegami and Makino, 2011; Terasaki and Makino, 2015).

Part of the aim of this thesis was to evaluate the performance of a modified vaccinia virus Ankara expressing the RVFV glycoproteins (rMVA-GnGc) as a potential single shot vaccine candidate against RVF in a sheep model of infection. López-Gil *et al* (2013) have shown that a single inoculation of the rMVA-GnGc vaccine was sufficient to protect mice from a lethal challenge (Lopez-Gil *et al.*, 2013), although other recombinant vaccinia virus (VACv) expressing the RVFV glycoproteins in a mouse model did fail to protect (Papin *et al.*, 2011). In this study the subcutaneous challenge of lambs with 10^5 $TCID_{50}$ of the 56/74 RVFV strain caused two fatalities and clear clinical signs were observed in all lambs. Intriguingly, the only two lambs that died after the challenge belonged to the groups inoculated with MVA virus (sheep from rMVA-GnGc and rMVA-GFP groups) but none of the lambs challenged in the saline group died. Our results suggest that a single subcutaneous immunization of lambs with rMVA-GnGc does not confer full protection from disease. However, complete protection has been achieved using a lumpy skin disease virus (LSDV), a nonreplicating, complex adenovirus, Venezuelan equine encephalitis virus, Newcastle disease virus (NCD), and capripoxvirus KS1 strain as vectors of Gn and Gc RVFV glycoproteins cDNA in mice, lambs and calves (Gorchakov *et al.*, 2007; Holman *et al.*, 2009; Kortekaas *et al.*, 2010a; Soi *et al.*, 2010; Wallace *et al.*, 2006). Furthermore, a single intramuscular inoculation NCD vector in lambs (encoding RVFV Gn/Gc) and calves (encoding RVFV-Gn) was sufficient to induce neutralizing antibodies (Kortekaas *et al.*, 2010a; Kortekaas *et al.*, 2010b). The final cause of the failure of protection remains inconclusive in our studies.

Such as it has been extensively discussed in chapter 3, some partially protective effects could be attributable to the immunization with our prototype. rMVA-GnGc vaccination appears able to delay the onset and severity of clinical signs as well as the duration of illness. Moreover, viremia and virus shedding appears to be delayed and reduced in extent. Therefore, further optimization of rMVA-GnGc vaccination regimen in lambs is needed, for instance by varying the route of administration, using adjuvants or by increasing the amount of recombinant virus delivered. Reviewing results of other vaccine vectors, it may be reasonable to use the intramuscular route as a successful administration route in future research (Kortekaas *et al.*, 2010a; Kortekaas *et al.*, 2010b;

Kortekaas *et al.*, 2011; Wichgers Schreur *et al.*, 2015). The amount of input virus may be determinant for host immune response, because of MVA is not capable of replication in sheep. MVA vaccines delivered in previous trials against other pathogens in veterinary species such as calves, and/or ponies were administered with at least one or two booster doses (Antonis *et al.*, 2007; Breathnach *et al.*, 2006; Chiam *et al.*, 2009). Experiments are underway to test the protection elicited with a booster dose of rMVA-GnGc in sheep as well as for potentiating the immune response elicited by a single administration of this experimental vaccine. From the point of view of field veterinary vaccines, single dose vaccination is a preferred choice over multiple administrations, taking in account economical and practical reasons, particularly in developing countries. Recommendations for new RVFV vaccine developments include single dose delivery as an advantage over repeated administrations, thus minimizing the risks of transmission by needle injections in endemic RVFV areas (Kortekaas *et al.*, 2011).

The pathological characterization and retrospective studies have characterized the new diagnosed ocular condition of experimentally infected older lambs with South African RVFV isolates. The onset of human ocular clinical signs occurring 5–14 days after local outbreaks of RVFV in ruminants are in accordance with the observed RVFV-infected sheep kinetics (Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012; Khairallah *et al.*, 2010). RVFV can cause major ocular disease in man, leading to blindness (i.e. anterior uveitis, retinitis, chorioretinitis, macular and paramacular scarring, vascular occlusion, and optic atrophy), and also in animals (Al-Hazmi *et al.*, 2005; Al-Hazmi *et al.*, 2003; Gerdes, 2004; Khairallah *et al.*, 2010; Yoser *et al.*, 1993). However, no histological descriptions are available from human cases so, an unequivocal correlation between the findings of these studies and the human condition should be made with caution. Infectious viral anterior uveitis with or without chorioretinal injury in ruminants has been described in malignant catarrhal fever, bluetongue and rinderpest infections (Holzhauer and Vos, 2009; Rossiter, 2001; Vikoren *et al.*, 2006; Whiteley *et al.*, 1985), but no descriptions have been made of these lesions in ruminant RVFV infections. Therefore, there was a clear need for a better pathological characterization.

The major ocular gross finding in these studies was corneal edema. Our findings suggest a chronic corneal squeal, which could be associated with drainage failure or inadequate corneal dehydration after a transient viremia (loss of corneal deturgescence). This change occurs under three circumstances, which are not mutually exclusive: corneal ulceration, corneal endothelial dysfunction and vascular leakage from corneal neovascularisation (Peiffer *et al.*, 1999). Histopathological findings suggested that the corneal edema was related to endothelial damage (lymphocytic endotheliitis) and that the mid-stromal

corneal neovascularization was secondary to the anterior uveitis. Anterior uveitis associated to or not with lymphoplasmacytic endothelitis, corneal edema and corneal erosions was diagnosed in 8 out of 40 RVFV-challenged older lambs, leading to a 20% of apparent prevalence of the lesion (Table 12) (Busquets *et al.*, 2014; Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). However, no retinal-associated clinical or pathological partial blindness were observed in our studies. This fact may be related to the lacking of standards on ocular clinical exploration of sheep in experimental conditions, which should include functional test. The clinical diagnosis is based on the simple observation of the animal behavior and corneal visual checking during the exploration. Formal vision testing has proven to be a helpful, low-technology tool for RVF screening during human outbreaks in high-risk rural settings (LaBeaud *et al.*, 2015). Maybe it should be worthy to include some kind of visual test during the clinical evaluation of RVFV challenge trials to better detect ocular conditions. In spite of the differences in diagnosis, the apparent prevalence for ocular involvement in RVFV infection in humans, which varies from 10 to 25% of clinical cases, is in accordance with apparent ocular pathology prevalence in Ripollesa breed (Ikegami and Makino, 2011; LaBeaud *et al.*, 2015). Our group had already suggested a more prevalent uveitis, associated or not to corneal edema, in sheep infected with RVFV in a previous report (Galindo-Cardiel *et al.*, 2012).

As we have shown in the already described anterior uveitis and lymphoplasmacytic endotheliitis, CD3, CD20 and lysozyme positive labelling is correlated with the presence of mononuclear infiltrations (point 6.5. Chapter 4). Surprisingly, corneal edema was only involved in 2 out of 10 studied lambs, in accordance with our previous observations which suggest a secondary involvement of cornea due to vascular damage (endothelium and neo-mid-stromal vascularization) (Galindo-Cardiel *et al.*, 2012). Anterior uveal mononuclear B cell-positive infiltration was observed in the selected cases, suggesting that a strong antibody production is also involved in RVFV ocular pathogenesis. B cells and CD4(+) T cells, but not CD8(+) T cells, are required for robust IgG and neutralizing antibody responses in neurologically affected RVFV-challenged mice, even in the presence of a functional innate response, and such requirement correlated with viral clearance from peripheral tissues, including eye (Dodd *et al.*, 2013; Dodd *et al.*, 2014). However, other report describes a robust CD8+ T cell response associated to a strong neutralizing antibody also in RVFV-challenged vaccinated mice (Warimwe *et al.*, 2013). To reinforce this hypothesis, the nucleocapsid protein of RVFV has been described as a potent human CD8+ T cell antigen that elicits memory responses (Xu *et al.*, 2013). Baseline counts for CD172+ monocytes and dendritic cells (DCs) dropped only in goats challenged with the insect cell propagated virus but not in those receiving virus propagated in mammalian cells (Nfon *et al.*, 2012). A similar observation was found for both CD5+ and CD8+ T-cells as well as CD21+ B-cell counts. Regrettably, no further T cell phenotyping was done in the

selected cases, but the consistent distribution of T cell infiltration in the eyes of both groups in the retrospective study suggest a central role of CD3-positive cells in the ocular pathogenesis in ruminants.

Moreover, a novel presumptive diagnosis of retinitis was attempted based on the CD3-positive labelling of mononuclear cells associated to the vascular alterations (5/10), which is the first description of such lesion in RVFV-infected ruminants, to the best of the author´s knowledge. Human ocular disorders range from maculopathy to retinopathy and are associated to the loss of central vision or blurred eye without a defined pathogenic timeline. In humans, as happens in Ripollesa sheep, ocular affectation can be unilateral or bilateral (Busquets *et al.*, 2010; Deutman and Klomp, 1981; Galindo-Cardiel *et al.*, 2012; Salib and Sobhy, 1978; Siam *et al.*, 1980). Macular edema with exudates containing a white mass covering the macular area with or without retinal hemorrhage, vasculitis, infarction or vitreous haze are described in the affected eyes by using retinal photography or ophthalmoscopy (Al-Hazmi *et al.*, 2005; Deutman and Klomp, 1981; Freed, 1951; Salib and Sobhy, 1978; Schrire, 1951; Siam and Meegan, 1980). Such findings were not found in our lambs, but hypopyon, a matter of exudative lesion, was diagnosed in 2 out of 10 RVFV-infected lambs of the retrospective study. In accordance with human clinical observations, CD3-dependent retinitis (Khairallah *et al.*, 2010) and uveitis (Al-Hazmi *et al.*, 2005; Siam and Meegan, 1980) have been diagnosed in RVFV-infected Ripollesa sheep. Besides these findings, retinal detachment (Deutman and Klomp, 1981; Schrire, 1951) or arterial occlusion (Al-Hazmi *et al.*, 2005; Ayoub *et al.*, 1978; Deutman and Klomp, 1981; Schrire, 1951; Siam *et al.*, 1978) has been reported in some human cases. Histological artifacts did not let to clearly state if retinal detachment was a real lesion or not. In the pathologists´ opinion, retinal detachment is associated to other clinical findings in ruminants, which were not observed in our cases (Peiffer *et al.*, 1999), supporting the histologic artifact presence in paraffin-embedded eyes. In many human patients, chorioretinal scarring can remain in macular and paramacular areas in spite of the resorption of exudates, and a complete recovery of vision does not occur (Al-Hazmi *et al.*, 2005; Ayoub *et al.*, 1978; Deutman and Klomp, 1981; Freed, 1951; Salib and Sobhy, 1978; Siam *et al.*, 1978; Siam and Meegan, 1980). Even more, some chronical RVFV-infected human patients show partial improvement in vision after several months (Salib and Sobhy, 1978; Schrire, 1951; Siam *et al.*, 1978; Siam and Meegan, 1980). Regrettably, nor evolution of retinitis neither clinical vision evolution was not established in our studies because of such condition was an incidental finding. A specific experimental design to characterize the ocular condition should be carried out to assess the clinical and pathological timeline.

Two basic pathogenic mechanisms of acute anterior uveitis based on cytokine production of resident uveal macrophages associated to neutrophilic infiltration (relatively short-lived), or a delayed-type hypersensitivity immune response associated to soluble antigens (delayed onset but extended duration) have been described in experimental models of rat and mice (Smith *et al.*, 1998). Direct infection of monocyte-derived cells as has been demonstrated in *in vitro* infection experiments of monocyte-derived dendritic cells, leading to a plausible explanation for the reduction in the frequencies of monocyte-derived cells found in RVFV-infected goats (Nfon *et al.*, 2012) or monocyte-derived human CD14+ macrophages (McElroy and Nichol, 2012). Further, these data are supported by *in vivo* RVFV antigen-specific immunostaining of liver macrophages (Shieh *et al.*, 2010) and dendritic cells in mice (Smith *et al.*, 2010). The observed lysozyme-positive monocytes closely related to hypopyon (exudative lesion) in the most severe cases of anterior uveitis, and the lacking of neutrophils in the affected eyes, may suggest that the first mechanism is not clearly involved in the Ripollesa ocular lesion. An immune-mediated mechanism has been also proposed as the underlying cause of viral lymphocytic endotheliitis and anterior uveitis in ruminants (Holzhauer and Vos, 2009; Whiteley *et al.*, 1985), and therefore it was suggested in previous cases in Ripollesa breed (Galindo-Cardiel *et al.*, 2012). Moreover, lacking of apoptosis evidence or grossly immune-complex depositions in ocular tissue does not allow confirm a clear delayed immune-mediated hypersensitivity (type III or IV), based on the selected cases. However, hepatic viral clearance (significant, Table 11) among with the persistency in ocular lesions between groups (no significant, Table 11), no RVFV-positive labelling in ocular tissue (just in mononuclear infiltrates), and consistent RNA viral detection in affected eyes (7 out of 11) and in kidneys (2 out of 11) after viremia phase, may suggest an ongoing active viral infection, in progress for clearing. Such pathologic situation may lead to more or less pronounced sequels in the survivors (Al-Hazmi *et al.*, 2005; Al-Hazmi *et al.*, 2003; Gay, 2006; Khairallah *et al.*, 2010). It is worthy to mention that different ocular viral clearance with an increase of eye lesion severity was intuitively observed, but not significantly confirmed, in the recovery phase of RVFV infection observed at 14 dpi (Table 12). Such as it was described in previous reports, this apparently lack of clearance of RVFV in eyes may correspond with two clinical phases: (i) a particular ocular progression to permanent sequel; (ii) the progression of the ocular lesion is slower than hepatic one and the time of experiment was not enough to confirm the pathogenesis (Busquets *et al.*, 2010; Galindo-Cardiel *et al.*, 2012). Whether Ripollesa susceptibility to RVFV ocular disease correlate to their own host genetic determinants, remains unknown because is out of the scope of this paper. However, recent evidence demonstrated that three different polymorphisms of Toll like receptor 8 (TLR8) in humans are significantly associated to eye disease (Hise *et al.*, 2015), so further genotyping studies are needed to confirm the special ocular susceptibility to RVFV of Ripollesa breed.

The Ripollesa sheep breed, in the middle way from lambing to puberty, has demonstrated a moderate sub-acute clinical susceptibility with major liver involvement to experimental infection with African RVFV isolates of different origins. Transient pyrexia, rising viral loads in blood, and viral shedding were recorded for all viruses, with no severe clinical signs of disease. Further, both specific humoral and cellular responses were detected after viral infection, thus opening the possibility of using this animal model to test potential vaccines against RVFV. Such optimized challenge models will facilitate future vaccination-challenge trials and significant inter-laboratory comparison of the results. Moreover, understanding the immune mechanisms involved in viral clearance and protection might be essential to design efficient vaccines in the future. Besides, a T cell-dependent retinitis has been diagnosed, suggesting that Ripollesa sheep may be used as a model for studying RVF-associated ocular pathology in man. However, there are still unconfirmed data in ocular pathogenesis of RVFV in sheep, so further studies should be carried out to better characterize the timeline of the viral uveitis in clinically affected RVFV-positive Ripollesa sheep. RVFV infection should be included in the list of viral entities that can cause uveitis and retinitis in ruminants.

8. Conclusions

1. Nine to ten-week old European sheep (Ripollesa breed) are moderately susceptible to experimental infection with African RVFV isolates of different origins, as it is shown by transient pyrexia, minimal to mild clinical signs, detection of viral loads in blood, and viral shedding after challenge trials.

2. Both specific humoral (Anti-RVFV IgG and IgM) and cellular (IFN-γ) responses were detected after viral infection, showing the potential of the proposed model to test new RVFV vaccines.

3. A single subcutaneous immunization of lambs with rMVA-GnGc does not confer full protection from disease; however, it appears to delay the onset and severity of clinical signs as well as the duration of illness. Moreover, viremia and virus shedding appear to be delayed and reduced in extent.

4. Anterior uveitis associated to or not with lymphoplasmacytic endothelitis, corneal oedema and corneal erosions was diagnosed, leading to a 20% of apparent prevalence of the lesion in the proposed model; this is the first pathological description of this ocular manifestation of RVFV infection in ruminants.

5. CD20 labelling in affected eyes was only observed in anterior uvea, suggesting that a strong antibody production is also involved in RVFV ocular pathogenesis.

6. A novel T-cell dependent retinitis was also diagnosed based on CD3-positive labelling, leading towards a new experimental model for human condition.

7. Lysozyme-positive mononuclear infiltrations associated to CD20-positive cells and without neutrophilic infiltration were observed in RVFV-positive paraffin-embedded eyes, suggesting that an immune-mediated mechanism, more than a cytokine-based mechanism, may be related to the diagnosed uveitis in RVFV-infected sheep.

8. A new immunochemical diagnostic tool based on a murine monoclonal antibody was successfully developed for ovine paraffin-embedded tissues.

9.

9. References

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