Development of novel diagnostic assays for the detection and surveillance of Rift Valley fever virus infections in ruminants and camels

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To my family
Parts of this work have already been published in the following journals:

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**Molecular and Serological studies on the Rift Valley fever outbreak in Mauritania in 2010.**

**Generation and application of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein NP and glycoproteins Gn and Gc.**
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Susanne Jäckel, Anne Balkema-Buschmann, Martin Eiden, Martin H. Groschup

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Susanne Jäckel, Martin Eiden; Anne Balkema-Buschmann, Malte Dauber, Frank T. Hufert, Katia Isselmou, Hermann Unger, Martin H. Groschup

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“Seroepidemiological and molecular studies of a Rift Valley fever outbreak in Northern Mauritania in 2010”
Martin Eiden, Susanne Jäckel, Anne Balkema-Buschmann, Ariel Vina-Rodriguez, Hermann Unger, Katia Isselmou, Martin H. Groschup

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“Molecular and seroepidemiological studies of a Rift Valley fever outbreak in Northern Mauritania in 2010”
Martin Eiden, Susanne Jäckel, Anne Balkema-Buschmann, Ariel Vina-Rodriguez, Hermann Unger, Katia Isselmou, Martin H. Groschup
Poster presentations:


“Novel serological and molecular diagnostic tools for RVFV detection and surveillance”
Martin Eiden, Susanne Jäckel, Anne Balkema-Buschmann, Janusz T. Paweska and Martin H. Groschup


“Neutralizing antibodies correlate closely with the presence of antibodies to Rift Valley Fever Virus glycoprotein Gn in small ruminants”
Susanne Jäckel, Martin Eiden, Anne Balkema-Buschmann, Mario Ziller, Janusz T. Paweska and Martin H. Groschup

“Generation and characterization of monoclonal antibodies against Rift Valley Fever Virus nucleocapsid protein and glycoproteins Gn and Gc”
Susanne Jäckel, Martin Eiden, Anne Balkema-Buschmann, Malte Dauber, Alejandro Brun and Martin H. Groschup

Endemic and Emerging Infectious Diseases of Priority in the Middle East and North Africa, Turkey/Istanbul, 18.06.-21.06.2012

“Novel serological tools for the diagnosis of Rift Valley Fever Virus”
Susanne Jäckel, Martin Eiden, Anne Balkema-Buschmann, Mario Ziller, Malte Dauber, Janusz T. Paweska and Martin H. Groschup
Arbozoonet Final meeting, Italy/Orvieto, 19.9-22.9.2012

“Novel serological tools for the diagnosis of Rift Valley fever”
Susanne Jäckel, Martin Eiden, Anne Balkema-Buschmann, Mario Ziller, Malte Dauber, Alejandro Brun, Petrus Jansen van Vuren, Janusz Paweska and M.H. Groschup


“Development and application of monoclonal antibodies against nucleocapsid protein and glycoproteins Gn and Gc of the Rift Valley Fever Virus”
Susanne Jäckel, Martin Eiden, Anne Balkema-Buschmann, Mario Ziller, Janusz T. Paweska and Martin H. Groschup

“Development and evaluation of an indirect ELISA for detecting antibodies against Gn protein of Rift Valley Fever Virus in small ruminants”
Susanne Jäckel, Martin Eiden, Anne Balkema-Buschmann, Mario Ziller, Janusz T. Paweska and Martin H. Groschup
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<td>BSL 3</td>
<td>Biosafety Level 3</td>
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<td>cELISA</td>
<td>competition ELISA</td>
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<td>CNERV</td>
<td>Centre National d’Elevage et de Recherche Vétérinaires</td>
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<tr>
<td>DIVA</td>
<td>Differentiating Infected from Vaccinated Animals</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>EU</td>
<td>European Union</td>
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<td>Gn</td>
<td>Glycoprotein n (-terminal)</td>
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<td>IgG</td>
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<td>Indirect immunfluorescence assay</td>
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<td>Large (-Segment)</td>
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<td>Loop-mediated isothermal AMPlification</td>
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<td>Abbreviation</td>
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<td>mab</td>
<td>monoclonal antibody</td>
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<td>N protein</td>
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<td>NP</td>
<td>Nucleocapsid protein</td>
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<td>Nss</td>
<td>non-structural protein (S-segment)</td>
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<td>Nsm</td>
<td>non-structural protein (M-segment)</td>
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<td>NTA</td>
<td>Nitrilotriacetic acid</td>
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<td>Polymerase chain reaction</td>
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<td>Percentage of positive control serum</td>
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<td>reverse transcription polymerase chain reaction</td>
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<td>Small (-Segment)</td>
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<td>VNT</td>
<td>virus neutralization test</td>
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Chapter 1  Introduction

Rift Valley fever virus is a vector-borne zoonotic virus affecting humans and a wide range of vertebrate hosts like cattle, camels, small ruminants and wildlife species. RVFV belongs to the family Bunyaviridae, genus Phlebovirus and is endemic in many countries on the African continent and the Arabian Peninsula.

Rift valley fever virus is an enveloped single-stranded negative-sense RNA virus with a three-segmented genome. The S-(small) segment encodes two proteins, the nucleocapsid protein (NP) and in complementary orientation a nonstructural protein (Nss). The M- (medium) segment codes for two structural proteins (glycoproteins Gn and Gc) and also for a nonstructural protein Nsm. The L- (large) segment includes the genetic information for the RNA-dependent RNA-polymerase (Bouloy et al., 2010; Ikegami et al., 2011, 2012).

RVFV is an arbovirus and is transmitted through bites of a wide range of mosquito genera including Aedes, Anopheles and Culex species; several of them were also found in Europe (Moutailler et al., 2008; Pépin et al., 2010). The virus epidemiology is characterized by a repeated emergence of enzootic circulation and epizootic outbreaks. The virus persists through inter-epidemic periods mainly by transovarial transmission and the survival in deposited mosquito eggs for many years during drought periods. This leads to an explosive increase in the mosquito population after long and heavy rainfalls and a subsequent massive spread of RVFV (Bird et al., 2009).

Transmission of the virus to humans occurs via direct contact to infected body fluids, tissues and/or excretions of livestock (Bird et al., 2009; Bouloy et al., 2010; Archer et al., 2013), mainly affecting exposed persons like farm workers, veterinarians or employees in a slaughterhouse (Archer et al., 2013). Based on the massive global trade of goods from Africa to other continents, the contagiousity of the virus and the presence of vector competent mosquito species, there is a high risk of virus introduction to non-endemic regions in other parts of the world, especially Europe and the United States (Chevalier et al., 2010; Rolin et al., 2013).
Human infections with RVFV are often asymptomatic. In most clinical cases, the infection takes a mild, febrile course of disease with influenza-like symptoms and is self-limiting. In 1-2 % of the cases, infections with RVFV lead to more severe symptoms like acute hepatitis, retinitis, hemorrhagic fever or encephalitis, in some cases with a lethal outcome. Up to 20 percent of the patients who develop a hemorrhagic syndrome with profound coagulopathy and multiple organ dysfunctions with renal failure decease eventually (Bird et al., 2009; Ikegami et al., 2011).

In livestock and wild animals, infections with RVFV cause significant morbidity and mortality. A characteristic sign for an infection with RVFV among ruminants are the so-called “abortion storms”. Independent from the stage of pregnancy massive abortions among pregnant sheep, goats or cattle over wide areas can be observed. Characteristic signs of the disease are inappetence, diarrhea and nasal discharge. Typical pathological findings are splenomegaly, gastrointestinal hemorrhage and massive lesions in the liver. Although RVFV is primarily a hepatotropic virus, viral antigen can be found in a wide range of organs and tissues like kidneys, adrenal glands, brain parenchyma, ovaries or the gastrointestinal tract. A reservoir for large amounts of virus antigen is the reticulo-endothelial system (Bird et al., 2009). Sheep lambs younger than one month are highly susceptible for an infection with RVFV with a mortality rate between 90 and 100 %. There is a peracute onset of the disease with a very short incubation period of 12 to 24 hours followed by a fast progress until death after 24 to 72 hours. The mortality rate among adult sheep is lower (up to 30 %) and the infection of older animals is characterized by fever, lethargy, nasal discharge and hematemesis. Newborn calves are also less susceptible to the virus than lambs shown by a lower case fatality rate between 10 and 70 %; for adult cattle this rate is even lower (5 to 10 %). Clinical signs of an infection with RVFV in cattle are fever, inappetence or hematochezia. Lactating cows show a temporary decreased milk production. A typical sign for a RVFV infection of pregnant cows are abortions, comparable to the findings in sheep. For cattle, it is often the only obvious indication for an infection with the virus (Bird et al., 2009). In goats, clinical signs are similar to those in sheep; however, morbidity and mortality rates are considerably lower (Bird et al., 2009). Although there is only little information available about
clinical signs in camels, RVFV infections are associated with widespread abortions (Meegan et al., 1979; Davies et al., 1985). In a recent outbreak in Mauritania two clinical forms were observed: a) acute cases were associated with different clinical signs like fever, severe ocular manifestations and icterus; camels died within a few days after the first appearance of hemorrhagic symptoms while b) in peracute cases, affected camels died within 24 hours after infection (El Mamy et al., 2011). Camels played an important role in RVFV dissemination and were involved during outbreaks in several African countries like Kenya, Nigeria, Sudan, Morocco, Egypt, Tanzania and Mauritania (Scott et al., 1963; Davies et al., 1985; Eisa, 1984; Ahmed Kamal, 2011; El-Harrak et al., 2011; Swai and Sindato, 2014; El Mamy et al., 2011, 2014). Wildlife animals also play a role in the transmission of RVFV especially for virus maintenance during enzootic periods (Bird et al., 2009). Natural infections were described in a wide range of wild mammals including bovidae, giraffidae, suidae and muridae. A particular role is assigned to African buffaloes and roof rats (Olive et al., 2012).

The first RVF outbreak was described in 1931 in Kenya in the Great Rift Valley (Daubney et al., 1931 referenced in Bird et al., 2009). More than 4500 lambs and ewes died during that outbreak. Until the late 1970’s, the virus was restricted to Sub-Saharan regions when in 1977 a huge RVF outbreak occurred in Egypt affecting humans and livestock. In 2000/2001, the disease was observed outside the African continent for the first time during an outbreak in Yemen and Saudi-Arabia and caused severe losses among humans and animals. The introduction of the virus was suspected to originate from the trade of subclinically infected small ruminants from Africa (Bird et al., 2009; Ahmed Kamal et al., 2011). Since 2006, several RVF outbreaks have occurred in different African countries: Kenya in 2006/7 (Munyua et al., 2010), Sudan in 2007 (Hassan et al., 2011), Tanzania in 2007 (Jost et al., 2010) and Madagascar in 2008/9 (Chevalier et al., 2011). Recently, outbreaks were reported in South Africa, Namibia, Saudi Arabia, Botswana and Mauritania in 2010 (Faye et al., 2014; Métras et al., 2013; Monaco et al., 2013) with more than 14 500 clinical cases and over 9000 deaths among livestock and wild animals (Promedmail 2010; OIE 2010/2011).

RVF epizootics are preceded generally by heavy rainfalls. Especially during the outbreak in 2010, there was a strong correlation between heavy precipitation, a massive increase in the mosquito population in the northern regions and the occurrence of RVFV (El Mamy et al., 2011, 2014). During this outbreak, 13 humans out of 63 affected died after the infection with the virus. Additionally, sheep, goats and camels were involved leading to 173 laboratory confirmed cases and 21 fatalities (El Mamy et al., 2011).

In September 2013, a new RVF outbreak was reported in Senegal. Affected species were Dorcas gazelles, cattle and goats (OIE 2013; Promedmail 2013). Just a short time later, in October 2013, an additional RVF outbreak in southern Mauritania occurred affecting camels and small ruminants and leading to 291 clinical cases as well as three deaths among them so far (OIE 2013).

Since RVFV continuously expands its geographical range and causes increasing numbers of epidemics and outbreaks, the demand for safe and accurate diagnostic tests is rising. The infection with RVFV can be diagnosed with different methods such as molecular detection of nucleic acids or viral antigen, virus isolation and by serological assays. Viral nucleic acids can be detected by quantitative real-time RT-PCR assays which target the M-segment (Drosten et al., 2002) or L-segment (Bird et al., 2007), as well as with real-time reverse transcription-loop-mediated isothermal amplification (LAMP) assays (Peyrefitte et al., 2008; Le Roux et al., 2009). Viral antigen can be detected in affected tissues by immunohistochemistry (Odendaal et al., 2014).

Furthermore, there are several classical methods for serological testing of field samples for RVFV specific antibodies including indirect immunofluorescence, haemagglutination inhibition, complement fixation and virus neutralization tests (Fafetine et al., 2007; Paweska et al., 2007). In addition, enzyme-linked immunoassays (ELISAs) are used which employ inactivated viruses from infected
mouse brains or lysates of RVFV infected cells (Paweska et al., 2003, 2005a,b; LaBeaud et al., 2007, 2008). There are further ELISAs published which are based on recombinant nucleocapsid protein (NP) as antigen (Jansen van Vuren et al., 2007, 2009; Paweska et al., 2008; Williams et al., 2011). Moreover, competition or antigen capture ELISAs are used which utilize monoclonal antibodies against RVFV nucleocapsid protein (Zaki et al., 2006; Martin-Folgar et al., 2010; Fukushi et al., 2012; Kim et al., 2012).

Finally, a novel multiplex bead-based suspension LUMINEX assay for antibody detection was established (van der Wal et al., 2012).

The aim of the here presented work was to develop and establish novel diagnostic tools for the detection of Rift Valley fever virus infections with emphasis on a safe, reliable, fast and robust analysis of field samples. As the above mentioned classical methods as well as several of the published ELISAs need live or inactivated viruses they all have one great disadvantage in common as they can only be done under BSL3 conditions. ELISA assays based on recombinant antigens do not need special safety conditions, so they can be used not only in well-equipped laboratories, but also in endemic African countries having more basic technical facilities and lacking skills among the laboratory staff for working under BSL 3 conditions. Furthermore, all known serological assays with one exception use nucleocapsid protein as antigen, so they can only detect antibodies directed against this structural protein (Paweska et al., 2007; Fafetine et al., 2007; Kim et al., 2012). For a profound analysis of immunological reactions after an infection with RVFV it is essential to detect antibodies against different viral proteins, especially against all structural proteins including not only NP, but also the two glycoproteins Gn and Gc. Comparing the results would provide new insights into the reaction of the immune system of RVFV infected livestock and camels. Furthermore, the use of ELISAs detecting antibodies that are directed against different antigens supports the development and use of vaccine candidates working according to the DIVA concept in order to differentiate naturally infected from immunized animals (Kortekaas, 2014). Additionally, it is also essential to have serological assays based on different test principles, for example indirect immunofluorescence, ELISA or Western Blot, so that antibodies directed
against varying conformations of the used antigen will be detected. As not only serological testing of field samples is important for the investigation of a RVFV outbreak, but also the detection of viral antigens and genomes, it is crucial to provide a range of assays for this purpose. An important aspect hereby is the development of different sensitive real-time PCR assays with a high specificity and varying target regions on the RVFV genome additionally to the already published protocols by Drosten (2002) and Bird (2007) et al. and with regard to up-to-date data from GenBank. In acute outbreak situations it is also important to analyze organ samples by immunohistochemistry, which can also be helpful in animal infection studies, to understand the pathogenesis in humans and animals.

For the above mentioned purposes, an indirect ELISA based on bacterially expressed glycoprotein Gn was designed and validated with field sera from small ruminants from several African countries. The novel assay was tested in a seroprevalence study with field samples from a RVF outbreak in Mauritania and compared with commercially available test systems. In addition, the samples were analyzed by conventional and novel molecular PCR assays to detect viral RNA and were subsequently subjected to further molecular characterization. Finally, a set of monoclonal antibodies against the nucleocapsid protein and both glycoproteins Gn and Gc was generated and comprehensively examined. These novel mabs represent useful tools for pathogenicity studies in affected animals and the establishment of novel serological assays in RVFV diagnostics including the development of competitive ELISAs and immunohistochemistry protocols to enable studies on the tissue tropism and route of RVFV spread in infected animals.

The assays will be applied in cooperation with African partners in order to implement affordable and efficient diagnostic alternatives for surveillance and rapid discovery of novel Rift Valley fever outbreaks.
Chapter 2 A novel indirect ELISA based on glycoprotein Gn for the detection of IgG antibodies against Rift Valley fever virus in small ruminants

S. Jäckel, M. Eiden, A. Balkema-Buschmann, M. Ziller, P. Jansen van Vuren, J.T. Paweska, M.H. Groschup

Abstract

Rift Valley fever virus (RVFV) is an emerging zoonotic pathogen that causes high morbidity and mortality in humans and livestock. In this paper, we describe the cloning, expression and purification of RVFV glycoprotein Gn and its application as a diagnostic antigen in an indirect ELISA for the specific detection of RVF IgG antibodies in sheep and goats. The performance of this Gn based ELISA is validated using a panel of almost 2000 field samples from sheep and goats from Mozambique, Senegal, Uganda and Yemen. All serum samples were also tested by virus neutralization test (VNT), the gold standard method for RVFV serological testing. Compared to the VNT results the Gn based ELISA proved to have an excellent sensitivity (94.56%) and specificity (95.57%). Apart from establishing this new diagnostic assay, these results also demonstrate a close correlation between the presence of RVFV Gn and neutralizing antibodies.

Research in Veterinary Science 2013 Oct; 95 (2):725-730
DOI: 10.1016/j.rvsc.2013.04.015
Chapter 3 Molecular and Serological Studies on the Rift Valley Fever Outbreak in Mauritania in 2010

S. Jäckel, M. Eiden, B.O. EL Mamy, K. Isselmou, A. Vina-Rodriguez, B. Doumbia, M.H. Groschup

Abstract

Rift Valley fever virus (RVFV) is a vector-borne RNA virus affecting humans, livestock and wildlife. In October/November 2010, after a period of unusually heavy rainfall, a Rift Valley fever outbreak occurred in northern Mauritania causing clinical cases in cattle, sheep, goats and camels, 21 of which were of lethal outcome. The aim of this study was to obtain further information on the continuation of RVF virus activity and spread in animal species in Mauritania after this outbreak. We therefore tested sera from small ruminants, cattle and camels for the presence of viral RNA and antibodies against RVFV. These sera were collected in different parts of the country from December 2010 to February 2011 and tested with three different ELISAs and an indirect immunofluorescence assay. The results show a high seroprevalence of RVFV IgM and IgG antibodies of about 57% in all animals investigated. Moreover, in four camel sera, viral RNA was detected emphasizing the important role camels played during the latest RVF outbreak in Mauritania. The study demonstrates the continuous spread of RVFV in Mauritania after initial emergence and highlights the potential role of small ruminants and camels in virus dissemination.

Transboundary and Emerging Diseases 2013 Nov; 60 (Issue Supplement s2): 31-39
DOI:10.1111/tbed.12142
Chapter 4  Generation and application of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein and glycoproteins Gn and Gc

Susanne Jäckel, Martin Eiden, Malte Dauber, Anne Balkema-Buschmann, Alejandro Brun, Martin H. Groschup

Abstract
Rift Valley fever virus (RVFV) is a vector-borne virus that causes high neonatal mortality in livestock and deadly haemorrhagic fever in humans. In this paper, we describe the generation of monoclonal antibodies (mabs) against all three structural proteins of RVFV (glycoproteins Gn and Gc and nucleocapsid protein NP). After immunization of BALB/c mice with individual recombinant proteins, a total of 45 clones secreting ELISA-reactive monoclonal antibodies against NP, Gn and Gc epitopes were obtained. Twelve clones were directed to NP, 28 to Gn, and 5 to Gc. Western blot analysis revealed that most of the mabs were reactive to linearized epitopes on recombinant as well as native virus proteins. Six mabs against NP, 21 against Gn and all mabs against Gc also detected conformational epitopes, as shown by indirect immunofluorescence on RVFV-infected cells. All of the mabs were evaluated for their use in a competition enzymelinked immunosorbent assay (ELISA) for the detection of a RVFV infection. Several mabs were identified that competed with polyclonal rabbit serum, and one of them – mab Gn123, raised against Gn protein – was selected for a proof-of-principle study with field sera from a recent Rift Valley fever outbreak. The novel Gn-based competition ELISA demonstrated high performance, offering a promising alternative and addition to serological assays based on nucleocapsid protein.

Archives of Virology 2014 Mar; 159 (3): 535-46
DOI: 10.1007/s00705-013-1867-4
Chapter 5  Discussion

Rift Valley fever virus is an arthropod borne virus affecting humans as well as livestock and wildlife animals and causes high losses among ruminants and wildlife species with high morbidity and mortality rates. The infection especially affects young animals and can cause massive abortions in pregnant sheep and goats (Bird et al., 2009; Pépin et al., 2010). As animals, humans and different environmental factors are all involved in the infection cycle of RVFV, there is an urgent need to implement control strategies against RVFV spread and amplification at an early stage to prevent the development of large outbreaks and the distribution of the virus from one country to another. This is important particularly for countries in which the virus is endemic with a substantial risk of intermittent epizootics and sporadic epidemics. Moreover, the virus is capable of infecting more than 30 mosquito species including various Aedes and Culex species; some of them are also found in Europe (Chevalier et al., 2010). Therefore, there is a high risk of a virus introduction into Europe followed by a rapid dissemination in the naive populations in the EU member states (Moutailler et al., 2008; Bird et al., 2009). In any cases, a continuous molecular and serological surveillance is a key component of the prevention and control of RVF outbreaks. Therefore, the development of rapid, sensitive and reliable serological and molecular diagnostic methods is of utmost importance and is closely linked to establishing new molecular tools for the identification and antigenic characterization of the virus.

To realize these aims, a comprehensive approach was carried out by cloning and expressing the three structural proteins (nucleocapsid protein NP and both glycoproteins Gn and Gc) of RVF virus. Genome sequences of RVFV strain MP-12 were cloned into bacterial expression vectors and purification protocols were established for each of the proteins. All proteins contained an N-terminal histidine-tag to allow their purification on Ni-NTA-chelating columns. Despite of the high protein yields which were achieved after purification and refolding, precipitation during the purification process did not occur since both recombinant glycoproteins lacked the
hydrophobic transmembrane domain. NP was also soluble in an N-terminally truncated form. These recombinant proteins were used as immunogens and as antigens in newly developed diagnostic assays. Although they lacked posttranslational modifications, they were highly immunogenic demonstrated by excellent antibody titers that were elicited in vaccinated rabbits and Balb/c mice. Monoclonal antibodies (mabs) were eventually obtained from these mice by clonal selection of antibody secreting hybridoma cells. Cell supernatants from hybridoma cell clones were screened for mabs reacting to NP, Gn and Gc antigens correspondingly by newly established indirect ELISAs. Positive clones were subsequently characterized by western blotting using bacterially expressed protein as well as lysate of RVFV strain MP-12. Subsequently, indirect immunofluorescence assays adapted from a commercial kit for human samples (Euroimmun) were used for their further characterization. In total, 28 mabs directed to Gn protein, five to Gc and twelve to nucleocapsid protein were identified by indirect ELISA. In Western blot, these mabs did not only react with recombinant bacterially expressed antigen, but also with linearized epitopes of the virus by using infected cell culture as antigen. Furthermore, mabs also reacted with conformational epitopes as shown by indirect immunofluorescence assay. Due to their excellent immunogenicity, the bacterially expressed proteins were also suitable antigens for other relevant assays like ELISA and Western Blot.

A variety of classical serological methods like virus neutralization test, indirect immunofluorescence, complement fixation or haemagglutination inhibition tests are commonly used for the detection of RVFV specific antibodies in serum samples from humans and animals besides ELISAs. The production of all these conventional assays requires the handling of live viruses under biosafety level 3 conditions which can only be realized in highly specialized laboratories (Fafetine et al., 2007; Jansen van Vuren et al., 2007). The VNT represents the gold standard method for detecting specific antibodies against RVFV (Swanepoel et al., 1986; Pawseska et al., 2008). However, it is a very laborious and time consuming method which has to be carried out under BSL 3 conditions. Therefore, this test is hardly suitable for larger
seroepidemiological studies and for rapid investigations in outbreak situations (Fafetine et al., 2007).

RVFV glycoproteins Gn and Gc are also strong immunogens in affected animals (Pépin et al., 2010; van der Wal et al., 2012; Jäckel et al., 2013a,b) and are considered to be the major target for neutralizing antibodies. In ruminants, neutralizing antibodies were already found four to eight days post infection. Recently, a study was published where sheep were immunized with a glycoprotein GnGc subunit vaccine and neutralizing antibodies were detected in all animals as they showed a strong antibody response in the plaque reduction neutralization test (PRNT$_{80}$) (Faburay et al., 2014). Therefore, recombinant Gn protein was chosen as antigen in the here described indirect ELISA to capture specific serum antibodies. Antibody binding was detected using HRPO conjugated Protein G followed by a color-producing substrate reaction. The novel Gn based indirect ELISA was validated by testing almost 2000 field samples from sheep and goats from Senegal, Uganda, Yemen and Mozambique (Jäckel et al., 2013a) for which the VNT titers were already known. 331 of all tested sera were positive in the Gn based ELISA as well as in the VNT. A good correlation was shown for the VNT titers and the corresponding ELISA PP values for the reactive sera. Overall, the Gn ELISA showed a high performance with sensitivity and specificity rates of 94,56% and 95,57%, respectively. These results also highlight the importance of neutralizing antibodies for the adaptive immune response to RVFV which was also shown by Faburay et al., 2014, who immunized sheep with a GnGc subunit vaccine and induced a strong neutralizing antibody response in all animals.

This is the first report of an indirect Gn based ELISA. All ELISAs reported before were based on NP antigen and recently published NP based indirect ELISAs had a similar performance as an evaluation with human sera yielded a sensitivity of 99,72 and a diagnostic specificity of 99,62% (Paweska et al., 2007). The testing of 1023 sera from African buffalos gave a sensitivity of 98,7% and a specificity of 99,36 % (Paweska et al., 2008). Fafetine et al. (2007) described the validation of a NP based indirect ELISA with 100 cattle sera and 613 serum samples from small ruminants: this ELISA had a sensitivity of 99,4% and 100% and a specificity of 98,3% and 100%,
respectively. Furthermore, in another publication 1262 field samples from small ruminants (sheep and goats) were tested with an indirect IgG NP ELISA which displayed a specificity of 99.81% and 100% and a sensitivity of 88.04 and 98.46% for sheep and goats, respectively (Fafetine et al., 2012). For all these studies the VNT was used as gold standard method except for the study from Fafetine et al. (2012) where the results of the indirect ELISA were compared to those of a sandwich ELISA. The sensitivity and specificity of the above mentioned assays is partly higher than for the Gn ELISA. The reason for a reduced specificity in comparison to the NP based ELISAs are a number of “false positive” samples which reacted in the Gn ELISA, but were negative in VNT. An explanation for these results could be the presence of non-neutralizing antibodies against Gn protein which was already shown for envelope glycoproteins of other viruses (Burton et al., 2002). A small number of sera did not react with Gn antigen in the indirect ELISA, but were positive in the virus neutralization test. Apparently, a set of neutralizing antibodies within the serum does not bind to the antigen possibly due to the lack of neutralizing epitopes which were masked by conformational variations of the recombinant antigen in contrast to the fully glycosylated native protein. For this reason the sensitivity is minimal lower when compared to other indirect assays. Furthermore, neutralizing activity during infection can be triggered also by other proteins of the virus like glycoprotein Gc (Boshra et al., 2011; Pépin et al., 2010; Ikegami and Makino, 2009) or the nonstructural proteins Nss and Nsm. However, this subset of neutralizing antibodies is relatively small as a high correlation between the titer of neutralizing antibodies and the corresponding reactivity of the Gn ELISA could be clearly demonstrated with the testing of almost 2000 field sera.

Inactivated whole virus antigen preparations derived from mouse brain or tissue culture instead of recombinant antigen were used in the first RVF ELISAs (Paweska et al., 2003, 2005a,b). The reliable inactivation of virus preparations is crucial for their safe use and can be done only in the context of a functioning BSL 3 laboratory. Moreover, whole virus suspensions may not properly adhere to ELISA plates or could be too crude; both effects can hamper the performance of such an ELISA (Jansen van Vuren et al., 2007). In contrast to other serological assays widely used the
recombinant Gn protein is produced under BSL2 conditions. This is a great advantage as the Gn ELISA can therefore be used for large scale serological surveys. As Gn protein is sitting on the virus envelope and is therefore among the first immunogens to stimulate an antibody response (Pépin et al., 2010) it may even be suitable for the early detection of RVFV infections.

In combination with a NP based ELISA the Gn ELISA may also become a useful tool for DIVA vaccine strategies (Differentiating Infected from Vaccinated Animals) as several of the recently developed vaccines contain the two glycoproteins or the nucleocapsid protein as immunogens (Faburay et al., 2014). Comparing Gn and NP based ELISA results would allow the differentiation of vaccinated from field virus infected animals. Naturally infected animals react in both assays whereas immunized animals would have positive results with either the Gn or the NP based ELISA (Bird et al., 2012; Indran et al., 2012). Recently, a multiplex bead-based suspension LUMINEX assay which is detecting antibodies against glycoprotein Gn as well as against nucleocapsid protein simultaneously (van der Wal et al., 2012) was introduced which should also facilitate the DIVA vaccination strategy.

As the use of the novel Gn based indirect ELISA is restricted to small ruminant samples so far, future work should focus on its adaption to bovine, cameld and other relevant species. Alternatively, a species independent competition ELISA (cELISA) should be developed for the detection of RVFV antibodies in serum samples as not only livestock, but also other species like camels or buffaloes played an important role in the transmission and amplification of the virus during RVF outbreaks in Africa (LaBeaud et al., 2011; El Mamy et al., 2011; Faye et al., 2014). Therefore, it is essential to have suitable serological methods for analyzing samples also from these species. Several protocols for competition and antigen capture ELISAs using monoclonal antibodies against N protein have been published or are even commercially available (ID Screen RVFV competition multispecies ELISA; ID Vet). In previous studies, monoclonal antibodies were generated by immunizing mice with either DNA, recombinant N protein or infectious mouse brain and virus antigen (Zaki et al., 2006; Martin-Folgar et al., 2010; Fukushi et al., 2012; Kim et al., 2012; Fafetine et al., 2013). In the here presented study, large panels of well characterized mabs
Directed against all three structural proteins of RVFV have been generated (Jäckel et al., 2014) followed by a comprehensive analysis of their suitability as competing antibodies in a competition ELISA. First assay runs used polyclonal rabbit serum against the three recombinant structural proteins as binding competitor. Interestingly, a strong competition between hyperimmune serum and monoclonal antibodies was only detected for Gn derived mabs. Mab Gn 123 raised against Gn protein showed the best performance in the cELISA with hyperimmune rabbit serum and was therefore tested in a proof of principle study with 20 field sera from small ruminants. Results of this in house cELISA showed a high correlation to those obtained with a widely used commercial NP based ELISA. It is the first report of a competition ELISA based on recombinant Gn protein up to date.

All described methods were finally implemented during a RVFV outbreak in Mauritania in 2010. Repeated outbreaks of Rift Valley Fever occurred in this Western African country so far starting in 1987 with 220 deceased humans (Jouan et al., 1988; Digoutte et al., 1989). Subsequently, in the following years further RVF epidemics occurred in 1993 (Zeller et al., 1995), 1998 (Nabeth et al., 2001) and in 2003 (Faye et al., 2007). The 2010 outbreak in Northern Mauritania affected humans and livestock including small ruminants and camels likewise. In a study published by El Mamy et al., 2011, sampling was carried out between September and October 2011 and revealed a seroprevalence rate of about 42% in small ruminants and 33% in camels. In our study we analyzed samples that were collected in the same outbreak area in a time period starting in December 2010 and lasting until February 2011 and were provided to us by the Centre National d’Elevage et de Recherche Vétérinaires (CNERV) (Jäckel et al., 2013b). In total, 163 serum samples from camels, cattle and small ruminants were assayed for virus specific RNA by real-time PCR and analyzed for the presence of IgG and IgM antibodies using two different commercially available ELISAs and an in house indirect immunofluorescence assay. Furthermore, the sera from sheep and goats were tested with the novel Gn based ELISA. The overall seroprevalence rate for IgG and IgM antibodies in the investigated livestock and camels was slightly higher (57 %) as compared to recently published results of 37 % by El Mamy (2011), which indicates a growing infection
rate over time. In detail, the number of IgG/IgM positive sheep and goats deviated between 69% in our study and 43% in the previous study by El Mamy. Similar results could be shown for camels as the seroprevalence rates for IgG and IgM antibodies were about 33% at the beginning of the epidemic (El Mamy et al., 2011) and about 45% in samples taken later in the epidemic. In addition, there was an increasing number of IgM positive sheep and goats from 42% in September/October 2010 going up to 53% in animals bled between December 2010 and February 2011. By comparison of the three different serological assays used in this study (competition ELISA; IgM capture ELISA and indirect IgG ELISA) it could be shown that only 11 out of 93 tested small ruminants contained exclusively IgG antibodies. Therefore, a recent exposure of the majority of animals to the virus can be assumed which indicates an ongoing outbreak in Mauritania from December 2010 to February 2011. Furthermore, a novel indirect immunofluorescence assay (IIFA) was established to detect RVFV specific IgG antibodies in serum samples from sheep, goats, cattle and camels. The assay showed similar results when compared to the applied ELISAs. In detail, seroprevalence rates measured with the indirect IgG Gn ELISA (55%) and IIFA (61%) were almost the same for sheep and goats. The results for camels in the commercial competition ELISA detecting both IgG and IgM antibodies and the results obtained by IIFA were almost identical with 45 % (ELISA) and 44% (IIFA), respectively. In cattle, two out of eight samples were positives in the indirect immunofluorescence assay compared to one positive out of eight for the IDvet ELISA. Differences may result from the lack of a clear cut-off in the immunofluorescence assay and the higher number of inconclusive readings.

All samples were subjected to real-time PCR analysis. Two different PCR protocols - an already published (Bird et al., 2007) and a novel in house protocol- were used with different target sites at the L-segment (primer sequences provided by Ariel Vina-Rodriguez; Institute for Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institute, Germany). Four out of 163 samples were positive in both PCR assays displaying viral loads of 67,9 up to more than 14.000 copies per reaction. Quantification was carried out with synthetic RNA harboring the target region of the in house assay. Interestingly, all positive samples derived from camels. One partial
sequence could be recovered (GenBank accession number: JQ974833) which is the first camel-derived sequence that has been published on GenBank up to date. A comprehensive and detailed phylogenetic analysis was not possible due to the short length of only 859 nucleotides, but the sequence showed a close similarity to viruses isolated during the RVF outbreak in Mauritania in 1987. Similar results for camel samples collected earlier in the 2010 RVF outbreak in Mauritania were published recently; the generated sequences also showed the closest correlation to sequences from the 1987 outbreak (El Mamy et al., 2014). As two out of the four positive camelid samples were collected in January 2011 when the outbreak was officially declared to be over (February 28th 2011 as stated by the OIE), the outbreak was actually not resolved at this time. The circulation of virus even after the official declaration of the outbreak was shown by new outbreaks in 2012 (Sow et al., 2014; Promedmail, 2012) and 2013 (OIE, 2013; Promedmail, 2013). Starting point of the latest outbreak was Senegal from where the virus spread to southern Mauritania. Similar to the outbreak in 2010, affected species were again camels and small ruminants.

The findings of our study with four real-time PCR positive camelid samples underline their role in virus transmission and amplification. Camels live in close contact to humans and are used for transboundary transportation which means substantial transmission risks. Indeed, most recently human infections were linked to camels affected with Rift Valley fever (Faye et al., 2014). Involvement of camels in RVF outbreaks in Mauritania were already seen in 1987 leading to a seroprevalence rate of about 33 % (Saluzzo et al., 1987). In 1998, there was one IgM positive sample among 39 tested camels (Nabeth et al., 2001). During the first RVF outbreak in Egypt in 1977, RVFV was most likely introduced by camels from northern Sudan (Davies et al., 1985; Ahmed Kamal, 2011). Additional reports came from RVF outbreaks in Kenya (Scott et al. 1963; Davies et al., 1985), Nigeria (Davies et al., 1985) and Sudan (Eisa, 1984). Finally, the detection of RVFV specific antibodies in camelid samples in Morocco (seroprevalence rate of about 15%) demonstrated the spread of RVFV even to North African countries (El Harrak et al., 2011).
In conclusion, several novel molecular and serological assays were established and tested with field samples from a recent RVFV outbreak in Mauritania in 2010. All diagnostic tools were suitable for the analysis of African field samples and gave reliable and reproducible results about the seroprevalence rate and virus circulation among sheep, goats, camels and cattle during a RVF outbreak in western Africa. It could be demonstrated that camels and small ruminants play an important role in the transmission and amplification of the virus which confirmed the observations made in earlier RVF outbreaks. A novel indirect IgG ELISA, an indirect immunofluorescence assay and a novel real-time PCR protocol for the molecular detection and quantification of RVFV were established which can be used for molecular and seroprevalence studies in RVF epidemics or endemically affected countries. These tests offer the fast, safe and reliable diagnostics of field samples in case of a RVF outbreak in Africa and elsewhere.
Chapter 6  Summary

Development of novel diagnostic assays for the detection and surveillance of Rift Valley fever virus infections in ruminants and camels

Susanne Jäckel

Rift Valley fever virus belongs to the family Bunyaviridae and is a vector-borne zoonotic RNA virus that can cause a severe and sometimes even fatal hemorrhagic fever, retinitis and meningoencephalitis in 1 % of the clinical human cases. In livestock, especially small ruminants and cattle, as well as in camels, the infection leads to fever associated with a high neonatal morbidity and mortality. Characteristic indications for a RVFV infection are so-called abortion storms among pregnant sheep and goats. Humans can become infected by direct contact to infectious body fluids or tissues of infected livestock or wildlife animals. Up to date, the virus is found in Africa and on the Arabian Peninsula. As more than 30 different mosquito species are possibly competent transmission vectors for RVFV and are also found in more moderate climate, there is a standing risk for an incursion and spread of the virus to currently non-affected countries including Europe. In endemic regions with regular epizootic outbreaks as well as in currently RVFV-free countries, fast and well functioning serological and molecular diagnostic assays are needed to facilitate quick and precise control measures in present or future acute outbreak situations.

The aim of this study was therefore to establish and validate novel diagnostic tools and assays for RVFV. The applicability of the methods was demonstrated in a recent RVF outbreak when a comprehensive serological analysis of serum samples from infected animals was carried out as well as a molecular characterization of RVFV isolates.

Structural proteins (nucleocapsid protein NP, glycoprotein Gn and Gc) were expressed in E.coli and used as immunogens for the production of poly- and monoclonal antibodies as well as utilized as antigens in diagnostic assays. All three proteins were highly immunogenic in rabbits and mice which allowed the generation
of poly- and monoclonal antibodies. In total, 45 ELISA-reactive murine monoclonal antibodies were further characterized by Western blot and with an adapted indirect immunofluorescence test. 32 of them also recognized conformational epitopes of virus proteins so that they can potentially be used in immunohistochemistry for antigen detection. One of the mabs directed against glycoprotein Gn also showed promising results in a competition ELISA with polyclonal rabbit serum as well as with serum samples from RVFV infected sheep and goats.

As antibodies against both glycoproteins can already be detected four up to eight days after an infection with RVFV, a novel indirect ELISA based on glycoprotein Gn was established as an alternative to the already published or commercially available ELISAs using nucleocapsid protein as antigen. A direct detection of Gn- as well as NP-specific antibodies can facilitate the use of DIVA vaccines (Differentiating Infected from Vaccinated Animals) which are usually composed of RVFV subunits. The new Gn based ELISA was eventually validated with almost 2000 field samples from four African countries (Senegal, Mozambique, Uganda and Yemen). These sera had been tested earlier by virus neutralization test which is considered as the gold standard method. The revealed sensitivity of 94,56% and specificity of 95,57% of the Gn ELISA is suitable for its use as diagnostic assay in monitoring studies. Interestingly, this close correlation between the Gn ELISA and VNT results indicates that neutralizing antibodies are primarily directed to the Gn protein.

The new Gn ELISA was eventually used for the analysis of 163 serum samples collected from small ruminants, cattle and camels from December 2010 to February 2011 during the late phase of a RVFV outbreak in Mauritania.

The overall RVFV antibody prevalence in these animals was 57%. Interestingly, the IgM prevalence for small ruminants was almost 53% which is indicative for a continuing outbreak and virus transmission events. Gn ELISA results were compared to data obtained by an adapted indirect immunofluorescence assay (IIFA) and to data obtained with commercial NP based ELISAs. Excellent correlations between the results of all assays used were found.

Moreover, in four of the camel sera viral RNA was detected by real-time PCR which underlines their importance for the amplification and transmission of the virus. As
camels are living in close contact to humans in Mauritania and are important for transboundary transport, they can pose a considerable transmission risk.

In summary, the here described newly developed diagnostic tools and assays are a useful addition to already existing methods as could be shown in a RVF epidemic in Mauritania. As the novel test systems are robust, rapid and reliable they are suitable for the use in endemic African regions as well as in non-affected countries in Europe and North America.
**Chapter 7 Zusammenfassung**

Entwicklung neuer diagnostischer Methoden zum Nachweis und zur Überwachung von Rifttal-Fieber-Virus Infektionen in Wiederkäuern und Kamelen

Susanne Jäckel


Bis zum jetzigen Zeitpunkt ist das Virus in Afrika und auf der Arabischen Halbinsel aufgetreten. Mehr als 30 Stechmückenarten, die auch in gemäßigten Klimazonen vorkommen, sind mögliche kompetente Vektoren für das Virus, sodass die ständige Gefahr einer Viruseinschleppung und -ausbreitung in nicht-endemische und/oder bislang nicht betroffene Länder besteht. Sowohl in Endemiegebieten mit regelmäßigen epizootischen Ausbrüchen als auch in bislang Rifttal-Fieber-freien Ländern müssen schnelle und gut funktionierende serologische und molekulare Nachweismethoden verfügbar sein, um zielgerichtete und schnelle Bekämpfungsmaßnahmen in aktuellen und zukünftigen Ausbrüchen durchführen zu können.

Ziel dieser Arbeit war es daher, neue diagnostische Nachweismethoden zu etablieren und zu validieren. Die Methoden konnten erfolgreich in einem aktuellen
Ausbruchsgeschehen eingesetzt werden. Dabei wurden Serumproben von infizierten Tieren sowohl umfassend serologisch untersucht als auch RVFV-Isolate molekular charakterisiert.

RVFV-Strukturproteine (Nukleokapsidprotein NP sowie die Glykoproteine Gn und Gc) wurden bakteriell exprimiert, aufgereinigt und als Immunogene für die Herstellung von poly- und monoklonalen Antikörpern sowie als Antigen für serologische Testverfahren verwendet. Alle drei Proteine waren hoch-immunogen in Kaninchen und Mäusen, was die Generierung von poly- und monoklonalen Antikörpern ermöglichte. Insgesamt 45 ELISA-reaktive murine monoklonale Antikörper wurden mit Hilfe des Western Blots sowie eines adaptierten indirekten Immunfluoreszenztests weiter charakterisiert. 32 der Klone detektierten konformationelle Epitope der Virusproteine, was auch einen Einsatz der Antikörper für immunhistochemische Untersuchungen zur Antigendetektion ermöglicht. Ein gegen das Gn-Protein gerichteter monoklonaler Antikörper zeigte außerdem vielversprechende Ergebnisse in einem kompetitiven ELISA sowohl mit einem polyklonalen Kaninchenserum als auch mit Serumproben von RVFV-infizierten Schafen und Ziegen.

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