A Serological Study of Rift Valley Fever Virus in Two Regions in Tanzania
En serologisk studie av Rift Valley Fever Virus i två regioner i Tanzania

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SUMMARY
Rift Valley Fever (RVF) is a disease caused by Rift Valley Fever virus (RVFV), which is an arbovirus. An arbovirus is a virus that is transmitted by an arthropod vector, in this case a mosquito. The virus is a member of the Phlebovirus genus in the family Bunyaviridae. It was first identified in the Rift Valley in Kenya in 1930. The disease is a zoonosis but mainly affects domestic ruminants inducing massive abortions and a high mortality among young animals. RVF has a significant negative socio-economic impact during outbreaks in affected countries. Sheep, horse, cattle, goat, camel, buffalo and human can be infected by the virus, which today is endemic to sub-Saharan Africa and the Arabian peninsula.

RVFV has two transmission cycles: the epidemic cycle and the enzootic cycle. During the enzootic cycle the virus circulates at a low level and there are no notable clinical signs of the disease. The epidemic cycle often reoccurs every 3-15 years and is often associated with periods of heavy rain. In Tanzania the latest outbreak was in 2006-2007.

This degree project has been performed as a Minor Field Study (MFS) investigating the seroprevalence of RVFV in serum from sheep and goats in three different areas (Mikumi, Ngorongoro and Mahenge) in two different regions of Tanzania: Arusha and Morogoro. The purpose of the study was to evaluate whether RVFV is active during inter-epidemic periods in these areas and to get a better understanding of the epidemiology of the virus. Totally 29 of 354 analysed samples were seropositive, which is an overall seroprevalence of 11.3 % in Mikumi, 1.0 % in Ngorongoro and 11.2 % in Mahenge. The majority of the seropositive sheep and goats from all the three sampling sites were younger than seven years, indicating that RVFV circulates in low levels in the Arusha and Morogoro region during the inter-epidemic period.
SAMMANFATTNING


Detta examensarbete har genomförts som en Minor Field Study (MFS) för att undersöka seroprevalensen av antikroppar mot RVFV hos får och getter från tre olika områden (Mikumi, Ngorongoro och Mahenge) i två olika regioner i Tanzania. Regionerna heter Morogoro och Arusha. Målet med studien är att undersöka om viruset är aktivt under inter-epidemiska perioder i dessa områden och därmed få en bättre förståelse för virusets epidemiologi. Totalt var 29 av 354 analyserade prover seropositiva, vilket ger en seroprevalens på 11,3 % i Mikumi, 1,0 % i Ngorongoro och 11,2 % i Mahenge. Majoriteten av de seropositiva fåren och getterna från alla tre provtagningsplatserna i den här studien var yngre än 7 år vilket tyder på att RVFV cirkulerar i låga nivåer i Arusha och Morogoro regionen under den inter-epidemiska perioden.
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INTRODUCTION

Aims

The aim of my study was to investigate the seroprevalence of RVFV in goats and sheep during the late dry season in Tanzania and to evaluate if the virus is active during inter-epidemic periods. This is of interest since studies indicate that the virus can circulate among animals and humans without causing clinical signs (Heinrich et al., 2012; Kifaro et al., 2014). My study will also look into the socio-economic impact of RVFV. This is a Minor Field Study funded by a scholarship from The Swedish International Development Association (SIDA).

Study region

The United Republic of Tanzania is a country in East Africa slightly south of the equator. It is a part of the Africa Great Lake Region since three of Africa’s great lakes are partly in Tanzania and are connected to the Great Rift Valley in the west. The Great Rift Valley is a continuous geographic trench from Asia to South Eastern Africa. Both the branches of the Great Rift Valley pass through Tanzania. Tanzania’s mainland is dominated by wide steppes covered by different kind of bushes and accacia trees. Agriculture is estimated to represent 28 % of the gross domestic products (NE, 2014). This study was carried out in three separate areas in two different regions, Morogoro and Arusha in Tanzania (Fig. 1). Both of the regions have a short mild rain season between October and December and a long heavier rain season between March and May. The altitude varies between 482-1368 m in the two different regions (Chengula, 2013).

Morogoro region has an average temperature that differs between 18.6°C- 30°C and the average annual precipitation is 935 mm (Climatedata, 2014). Two different areas were sampled in Morogoro. The first one was around Mikumi, which is a part of the district Mvomero. Mikumi is close to Mikumi National Park where impalas, buffaloes and giraffes are a few examples of wild animals that can be found. The second area was around Mahenge in the southern part of Morogoro, which is part of the district Ulanga. Agro-pastoral farming is practiced in both of these areas.

Arusha region has an average temperature that differs between 13.8-25.2°C and the average annual precipitation is 1052 mm. (Climatedata, 2014). In the North the region borders to Kenya. The sampling in this region was carried out in Ngorongoro Conservation Area, which is situated 180 km west of the city Arusha (Fig. 2). The Conservation area covers a territory of 8300 square kilometres and belongs to the district Ngorongoro (Ministry of Natural Resources and Tourism, 2014). Pastoral farming is practiced within the conservation area so the domestic animals graze together with wild animals.

These specific study areas were chosen for several reasons. First of all to get a broad perspective of the current seroprevalence of Rift Valley Fever Virus in Tanzania. Secondly both of these regions practice pastoral farming and they were affected by the large outbreak in 2007. Thirdly to be able to sample small domestic ruminants living in close contact with wild animals.
Figure 1: The yellow dots mark our 3 study areas in Tanzania: Mikumi, Ngorongoro and Mahenge.

Figure 2: A sampling area of sheep in Ngorongoro. Photo: Nica Wachtmeister.
LITERATURE REVIEW

History and Expansion

Rift Valley Fever Virus (RVFV) was first identified in the Rift Valley in the Naivasha region in Kenya 1930. The disease was characterised by a high mortality rate (95 %) among young lambs and an increase in the numbers of abortions in ewes. A majority of the carcasses had a rapid decomposition, probably due to liver failure prior death. During post mortem examinations the characteristic finding was a necrotic liver. Histologically the lesions were described as focal necrosis of the liver (Daubney et al., 1931). RVFV has two transmission cycles. One is an epidemic cycle and the other one is an enzootic cycle. During the enzootic cycle the virus circulates at a low level and there are no notable clinical signs of the disease (Bird et al., 2009). The epidemic cycle often reoccurs every 3-15 years (Sindato et al., 2014).

RVF outbreaks have today been detected in 19 countries; there among Kenya, Tanzania, South Africa, Sudan, Egypt, Madagascar, Botswana, Somalia and the Arabian Peninsula. The most recent outbreaks were reported in South Africa (2008, 2009 and 2010) and there was a major outbreak in Kenya and Tanzania 2006-07 (Balenghien et al., 2013).

The virus

RVFV is a member of the Phlebovirus genus and the family Bunyaviridae (Pepin et al., 2010). The virus is about 100 nm in diameter and it assembles into an icosahedral particle (Sherman et al., 2009). It is spherical shaped and as other bunyaviruses it is an enveloped, singe-stranded RNA virus consisting of three segments of RNA with negative polarity S (small), M (medium) and L (large) (Ikegami, 2012). The S segment encodes nucleocapsid protein N and a non-structural protein (NSs). The NSs weakens the host’s immune response because it is an interferon antagonist (Pepin et al., 2010). The M segment encodes the glycoproteins on the envelope. The L segment encodes the L protein that is the RNA-dependent viral RNA polymerase (Schmaljohn, 1996).

Glycoproteins are important in the viral cycle and are responsible for the virus’s infiltration in the cell (Pepin et al., 2010). The virus matures by budding in the golgi compartment and all processes of replication are taking place in the cytoplasm of the infected cells (Pepin et al., 2010). After inoculation by the mosquito, the virus is transported in the lymph drainage to the regional lymph nodes where it replicates and produces a systemic viremia (EFSA, 2005).

The virus can be divided into 7 major genetic lineages based on the sequences of the S, M and L segment (Bird et al., 2007). Virus strains of RVFV that have been identified in different parts of Africa have sequences that can all be found in each of the 7 main lineages. This indicates that there is a movement of the virus through Africa (Bird et al., 2007). The nucleotides in RVFV’s genome has approximately 4 % diversity and the protein coding level has about 1 % diversity, which is a low genetic diversity and an important feature of the virus. It can indicate that RVFV has a low tolerance for mutation or that the virus strains have a relatively recent ancestor (Bird et al., 2007; Jenkins et al., 2002).

Transmission and epidemiology

Rift Valley Fever is an arbovirus, which is a virus that is transmitted by an arthropod vector, in this case by infected mosquitoes. The vector species can vary between regions but the most
common vector is a mosquito belonging to the genus *Aedes* (WHO, 2010). *Anopheles*, *Culex* and *Eretmapodites* are other genus of mosquitoes that can transmit the virus but they mainly transmit the disease during epizootic periods (House *et al.*, 1992). The vectors acquire the infection by feeding on infected animals (Fig. 3) (WHO, 2010). The virus can be dormant for several years in the mosquito eggs if the eggs are in dry soil. After heavy rainfall the soil gets flooded and the eggs hatch increasing the transmission of the virus (Linthicum *et al.*, 1985). The female mosquito can also pass the virus directly to her offspring. (WHO, 2010; Favier *et al.*, 2006). Animals can be infected by a bite from an infected mosquito, through infected animal tissues and infected body fluids. Foetal materials and placenta from animals that have aborted contain a large amount of virus particles and pose a great risk of infecting the environment or animals in close contact (Pepin *et al.*, 2010). Sheep and cattle are more susceptible to the virus than goats (Bird *et al.*, 2008).

Humans can be infected in the same way as animals but they can also acquire the infection from percutaneous or aerosol routes (Bird *et al.*, 2008). The risk group for humans being infected by RVFV are those having direct contact with animals in one way or another. Having the animals near by the house or practicing milking, slaughtering and other farming activities increases the risk of coming in contact with body fluids from infected animals and infected mosquitoes (Mohamed *et al.*, 2010). People working close to animals for example herdsmen, farm workers, veterinarians and butchers have an increased risk of being infected (Isaäcson, 2001).

RVF outbreaks are proven to be associated with heavy rainfall in Africa. Generally elevated water tables and flooded grassland areas, so-called dambos, favour the eggs of mosquitoes of the genus *Aedes* to hatch (Davies *et al.*, 1985). However not only the amount of rain, but also the rainfall variability, alternating rainy and dry periods, can be advantageous for the mosquitoes’ reproduction and consequently the generation of the virus (Soti *et al.*, 2012).

A study made by Evans *et al* (2008) in Kenya indicated that wild ruminants are infected with RVFV and play a part in the epidemiology during the inter-epidemic periods. In that study, African buffalo, black rhino, Thomson’s gazelle, lesser kudu, impala, African elephant and Waterbuck were positive for RVFV neutralizing antibodies. Bats may also be able to function as a reservoir for the virus and be a part of the endemic cycle. Several bats inoculated with RVFV did not show clinical signs of disease but the virus was detected in their urine up to 18 days after inoculation (Oelofsen and Van der Ryst, 1999).
Clinical Picture

RVFV primarily affect ruminants as sheep, goats, cattle and buffaloes (FAO, 2003). The clinical manifestation in domestic ruminants is epidemic abortions (abortion storms) in pregnant animals, fever, discharges from the eyes and nose, colic, vomiting, haemorrhagic diarrhoea and a high mortality rate among young animals (Chengula et al., 2014). Young lambs can catch a sudden high fever and die 36 hours after the first clinical sign (Pepin et al., 2010).

RVF in humans often results in an uncomplicated disease. The clinical picture of RVF in most humans is acute mild fever with influenza symptoms that the person can recover from spontaneously (WHO, 2010). Although in rare cases (< 8 %) infected humans can develop severe complications, there among retinitis, rhinitis, hepatitis, encephalitis, haemorrhagic fever and death (Mohamed et al., 2010). The incubation for RVF in humans is two to six days and the case fatality rate is 1 % (WHO, 2010). Humans are considered to be dead-end hosts (FAO, 2012).

Diagnostics

There are several possible methods to diagnose RVFV. The virus can be detected using virus isolation (can take a long time to get a result), RT-PCR and RT LAMP detection, which is a newer more efficient molecular diagnostic assay (OIE, 2014; Pepin et al., 2010). There are several serological diagnostic methods. Virus neutralization test is regarded as the gold standard but is not the most efficient method (Paweska et al., 2005). There are different kinds of enzyme linked immune assays (ELISA) detecting IgG, IgM and total antibodies (Pepin et al., 2010). In this study we used a competitive ELISA (ID-vet, Grabels, France), which detects IgG and IgM antibodies. This competitive ELISA has a sensitivity
between 91 and 100 % and a specificity of 100 % when using virus neutralization as the gold standard according to Kortekaas et al., (2013).

Neutralizing antibodies are produced rapidly after infection and can be seen in the blood 4-8 days post infection. IgM antibodies (acute-phase) are produced first and later on IgG antibodies (chronic). There are usually no traces of IgM antibodies in the blood 50 days after infection (Pepin et al., 2010). In animals RVFV creates a life-long virus neutralizing immunity (Barnard, 1979).

**Vaccine**

Today there are two different main types of vaccines that can be used for vaccinating animals: the inactivated whole-virus vaccine and the live-attenuated Smithburn vaccine (Indran and Ikegami 2012). Inactivated vaccines are expensive and require three inoculations and annual revaccination to obtain full protection, while the live attenuated vaccine provides a three-year immunity with one dose (Nachiket et al., 2010; Harrington et al., 1980; Pittman et al., 1999). However, the inactivated vaccine does not cause any abortions and it can be distributed to ruminants of all ages, which is convenient. If pregnant ruminants are vaccinated with the Smithburn vaccine it can lead to abortions and foetal abnormalities and as with all live attenuated vaccines there is a risk that it might reverse and become virulent again (Ikegami and Makino 2009; Indran and Ikegami 2012).

Clone 13 RVF is a new vaccine, which is a naturally attenuated vaccine. It has recently been introduced in South Africa. One dose is required and so far no side effects have been observed (OIE, 2014).

Today there are no RVF vaccines that have a DIVA (differentiating infected from vaccinated animals) marker (OIE, 2014). However, new more efficient and safe vaccines against Rift Valley Fever are being developed (Indran and Ikegami 2012).

**Prevention**

Vaccination can prevent the amplification of the virus and protect animals and humans from getting the disease. (FAO, 2012) There is however no point vaccinating during an outbreak, since it might intensify the outbreak due to the risk of iatrogenic transmission with reused needles (WHO, 2010). Vaccinating viremic animals with live attenuated vaccine might also result in a reassortant virus of field-strains and vaccine viruses (Grobbeelaar et al., 2011).

Things to consider for preventing RVFV outbreaks are to increase the level of surveillance in current high risk areas, have a vaccination strategy if necessary, inform the population about the disease and how to prevent transmission. The level of surveillance can include vector controls, to try to keep the animals from the vectors by for example using insect-proof stables or moving them to a higher altitude (FAO, 2012). At the same time it is desirable to not move infected livestock since they potentially can facilitate the transmission of the virus to non-infected areas (Carroll et al., 2011). Predicting the weather and potential heavy rainfall is also important in the increased surveillance (Jost et al., 2010).

The El Niño Southern Oscillation (ENSO) is a meteorological phenomenon that affects the sea surface temperature, which in turn causes climate changes (NE, 2014). Increased
Precipitation in East Africa is known to be connected with warm ENSO abnormalities (Joseph, 1991). It is possible to predict a RVF outbreak up to 5 months in advance by analysing the sea surface temperature together with index from satellites that record the vegetation’s response to heavy rain. In this way you can predict a possible outbreak and vaccinate animals to subdue an epidemic (Linthicum, 1999).

Rift valley fever in Tanzania

The major part of Tanzania’s livestock is located in the northern and central regions of the country (Chengula et al., 2013). In the whole country there are an estimated 17 million cattle, 11 million goats and 3.6 million sheep (Mohammed et al., 2010).

Since RVFV was first identified in Kenya 1930 (Daubney et al., 1931) outbreaks in Tanzania have been reported between December and June in 1930, 1947, 1957, 1960, 1963, 1968, 1977-1979, 1989, 1997-1998 and 2006-2007 in 39.2% of Tanzania’s districts. The outbreaks have varied in size and the inter-epidemic average period has been 7.9 years. Since the first outbreak was reported in Tanzania in 1930, the virus has spread southward from the Ngorongoro district in the north to the Ulanga district in the south (Sindato et al., 2014). Dense vegetation, advantaged temperature conditions and the presence of ruminants make it favourable for mosquitoes to breed, replicate the virus and pass it on to animals and humans in Tanzania (Heinrich et al., 2012). An observation made by Chengula et al., (2013) showed that a lot of the good pastures in Tanzania were found in low-land areas. In these areas there were also a lot of mosquitoes, which can increase the transmission of the disease.

It is reported that RVFV is present in different regions in Tanzania even during inter-epidemic periods and also in regions with no history of RVF (Heinrich et al., 2012). RVFV antibodies have been detected among domestic small ruminants younger than one year in several areas, which indicates an endemic presence of the virus (Kifaro et al., 2014).

The outbreak 2006-07

The latest major RVF outbreak was reported in Tanzania 2006-2007, which is seven years ago. It began in late December 2006. On January 21st 2007, local veterinarians reported cases of massive abortions and deaths around the Arusha area that possibly could be RVF. After the District veterinary office had made a clinical and epidemiological investigation, similar clinical signs were also reported from the Manyara, Kilimanjaro, Tanga, Dodomo, Iringa and Morogoro regions, however the time intervals differed. The outbreak was officially announced on February 7th 2007 and ended July 2007 (Chengula et al., 2013). At that point 45 of 120 districts (10 of 21 regions) in Tanzania had been affected (Mohamed et al., 2010; Sindato et al., 2014). Reports from The Tanzania Veterinary Laboratory Agency of how many livestock died and aborted during the outbreak in Tanzania 2006-2007 can be seen in table 1. (Chengula et al., 2013).
Table 1: Reports from The Tanzania Veterinary Laboratory Agency of how many livestock died and aborted during the outbreak in Tanzania 2006-2007. The percentage refers to Tanzania’s total population of the different species in 2010. n = number of individuals.

<table>
<thead>
<tr>
<th></th>
<th>Died n</th>
<th>Aborted n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>16 973 (0.10 %)</td>
<td>15 726</td>
</tr>
<tr>
<td>Goat</td>
<td>20 913 (0.18 %)</td>
<td>19 199</td>
</tr>
<tr>
<td>Sheep</td>
<td>12 124 (0.31 %)</td>
<td>11 085</td>
</tr>
</tbody>
</table>

Vaccine campaigns in Tanzania started in March 2007 (Chengula, 2013). During the outbreak 2006-07 the government spent approximately 4 million US dollars trying to control the disease (Sindato et al., 2014). Sheep, goat and cattle were all vaccinated except for pregnant individuals and those younger than 6 months of age. The campaign began in the high-risk areas and ended in the low risk areas according to old records (Chengula et al., 2013).

The first case of RVF in humans during this outbreak in Tanzania was detected in January 2007. Of 511 suspected human cases 144 died. Among the 511 suspected cases 186 individuals were confirmed through laboratory tests having RVF and 123 individuals were classified as probable. Case fatality rate among the confirmed and probable cases was 47 % (Mohammed et al., 2010).

Impact

During the outbreak 2006-2007 the socio-economic impact was comprehensive. Not only the high mortality among livestock but also restrictions concerning livestock markets, export and movement of animals and the risk for humans being infected had a negative effect. The livestock keepers were affected economically but it was also a psychological distress. Indirectly non-livestock keepers were affected since there was a lack of red meat, the demand for other protein sources for example chicken and beans increased and the price followed. This turned into a problem for people with limited income. During all this time the government was also negatively affected due to the negative socio-economic consequences of the disease (Chengula et al., 2013).

The economic loss due to the death of domestic ruminants during the outbreak in Tanzania 2006-2007 was estimated to 6 million US dollars. RVF also caused decreased food security and malnutrition for many people (Sindato et al., 2014).
MATERIAL AND METHODS

Animals and sampling

Blood samples were collected from 242 goats and 236 sheep among 39 different herds in three different districts in Tanzania during three separate field trips between September-November 2014 (Table 2). The blood was collected from the animal’s jugular vein using a syringe, vacutainer, and blood collection tubes (Fig. 4). One EDTA tube and one serum tube was collected from each individual. When possible, equal proportions of sheep and goats in each herd were sampled. If there were individuals younger than one year in the herd, a few young animals were sampled to get an as broad age variation as possible. The sample size was decided by calculating how many animals that was possible to sample during the nine sampling days. At the same time we wanted to sample as many different herds as we could. Since we had time to sample about 4 herds per day we chose to collect samples from approximately 15 animals in each herd. However the size of the herds could vary quite a lot and we did not have that information in advance, so we adjusted our sample size during the field days.

Table 2: Blood samples were collected from 478 individuals among 39 different herds in 15 villages. This table describes the distribution of species, sex and also how many of the sampled individuals that were equal or older than 7 years. n = number of individuals.

<table>
<thead>
<tr>
<th>Herds</th>
<th>Sheep n</th>
<th>Goat n</th>
<th>≥ 7 years n</th>
<th>Male n</th>
<th>Female n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mikumi</td>
<td>12</td>
<td>83</td>
<td>94</td>
<td>27</td>
<td>41</td>
</tr>
<tr>
<td>Ngorongoro</td>
<td>14</td>
<td>66</td>
<td>87</td>
<td>3</td>
<td>51</td>
</tr>
<tr>
<td>Mahenge</td>
<td>13</td>
<td>87</td>
<td>61</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>236</td>
<td>242</td>
<td>31</td>
<td>125</td>
</tr>
</tbody>
</table>

Each field trip was performed in three consecutive days and we collected about 50 samples each day. During these days, blood samples were stored in a styrofoam box with cold clamps, maintaining a temperature around 5°C in the box. Most of the collected blood samples were at the end of the day centrifuged and serum, buffy coat and plasma were contained in three different cryovials. However due to field conditions some of the samples were not separated until three days after they were collected. During this time they were stored in a 5°C fridge. After the separation all the cryovials were stored in - 45 °C until serum was used for detection of RVFV antibodies.
RVFV ELISA

ELISA was performed at the Genome Science Center, Sokoine University of Agriculture in Morogoro, to examine the presence of antibodies towards RVFV using a competitive ELISA (ID-vet, Grables, France). A subset of samples was selected. They were selected to try to get an equal proportion of goat and sheep from each herd. At first 50% of the samples from each herd were analysed. After the first analysis the remaining samples from the herds with a positive or doubtful result were also analysed. In total 354 samples were analysed for the prevalence of RVFV antibodies, corresponding to 74% of the total samples collected (Table 3). Of the 354 samples 330 individuals were younger than 7 years.

Table 3: Distribution of the 354 samples analysed by the RVFV-ELISA. The percentage shows the percentage of the sampled individuals that were analysed.

<table>
<thead>
<tr>
<th></th>
<th>Sheep n</th>
<th>Goat n</th>
<th>Total analysed n</th>
<th>≥ 7 years n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mikumi</td>
<td>57 (69%)</td>
<td>67 (71%)</td>
<td>124 (70%)</td>
<td>20 (74%)</td>
</tr>
<tr>
<td>Ngorongoro</td>
<td>41 (62%)</td>
<td>64 (74%)</td>
<td>105 (69%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Mahenge</td>
<td>70 (80%)</td>
<td>55 (90%)</td>
<td>125 (84%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Total analysed</td>
<td>168 (71%)</td>
<td>186 (77%)</td>
<td>354 (74%)</td>
<td>24 (77%)</td>
</tr>
</tbody>
</table>

The enzyme-linked immunosorbent assay (ELISA) was an ID. Vet Screen for “Rift Valley Fever Competition Multi Species”. This kit is a competitive ELISA detecting anti-RVFV nucleoprotein antibodies (both IgG and IgM) in serum samples. ID. Vet is a French company founded in 2004 that develop and manufacture immunodiagnostic tests for the detection of infectious diseases in farm animals (id-vet.com, 2015).

The competitive ELISA was performed according to the instructions of the manufacturer and all the samples were run once. In brief the competitive ELISA has wells that are coated with
recombinant RVF nucleoprotein. The samples that are going to be analysed are added to the microwells. If there are anti-nucleoprotein antibodies these form an antibody-antigen complex and these hide the nucleoprotein epitopes. An anti-nucleoprotein-peroxidase (HRP) conjugate was added to the microwells. This HRP conjugate attached to the residuary nucleoprotein epitopes and formed an antigen-conjugate-HRP complex. The plate was washed to remove excessive conjugate. Next the substrate solution was added, which bound to the antigen-conjugate-HRP complex. If antibodies were present, the colour would remain the same. However if there were no antibodies in the tested sample the solution turned into blue and later yellow when stop solution was added. (ID Screen® Rift Valley Fever Competition manual, 2012). The plate was read at 450 nm.

To control the validity of each plate, the mean value of the two negative controls (OD_{NC}) was calculated and the plate was considered valid when OD_{NC} > 0.7. For a valid plate, the mean value of the two positive controls divided by OD_{NC} should be < 0.3. For each sample the competition percentage was calculated by dividing (OD_{sample}/OD_{NC}) x 100. If the value was equal or less than 40 % the sample was considered positive. A value greater than 50 % was a negative result and the values in between 40- 50% indicated a doubtful result.

Statistical analyses from the results of the serology were processed in Graphpad software. It was used to compare the seropositivity between sheep and goats. A confidence interval of 95 % was used.

**Questionnaire**

At the sampling missions a questionnaire was used to estimate the socioeconomic impact of infectious diseases. The questions were asked to each livestock keeper through an interpreter that most of the time was the local veterinarian or the assistant of the veterinarian. For the last field trip the questionnaire was modified to make it easier for the farmers to understand the questions. The questions were as following:

1. What kind of animals do you have?
2. Have your animals got any vaccinations?
   b. If yes, when?
3. Which disease do you find most harmful for your livestock?
4. Which clinical sign has caused the biggest problem in your livestock?
5. Have you had an increased amount of malformed kids/lambs during the last year?
6. Does your livestock have contact with wild animals?
7. Have you changed your habits around your livestock to prevent contamination?
8. If your livestock would get sick, how would that affect your family?
9. How many animals do you have?
RESULTS

Questionnaire

The livestock species in the three different areas were mostly sheep, goat, cattle and chickens. All the villages we visited also had dogs. Nineteen of the herds (48.7%) were not vaccinated for any disease during the two last years and none of the herds had been vaccinated against RVFV. Around Mikumi the sheep and goats had been vaccinated against PPRV (Peste des Petits Ruminants virus). In herds around Mahenge most of the cattle were vaccinated for Lumpy Skin disease in 2014 and some of the sheep and goats were vaccinated against PPRV 2013. The herds in the Ngorongoro Conservation area were vaccinated against anthrax 2014. In a few herds only the sheep were vaccinated.

The livestock keepers in all three districts found PPR (Peste des Petits Ruminants) to be the most harmful disease of their livestock; however it was only in Mikumi PPR was the main concern of the livestock keepers. In Ngorongoro the most feared disease was Anthrax, while in Mahenge they said it was parasites during the rainy season. The livestock keepers in Mikumi also mentioned several tick-borne diseases, for example anaplasmosis. Regarding which clinical signs that caused the biggest problem in the herd, in Mikumi and Ngorongoro the livestock keepers answered diarrhoea and pneumonia. In Mahenge the livestock keepers answered diarrhoea but specified it to diarrhoea during the rainy season.

All the herds had contact with some kind of wild animals. Around Mikumi one herd had contact with bigger wild animals like impala, wild dogs and lion, while the 11 remaining herds had contact with dik dik, a small antelope. In Mahenge the sheep and goats had contact with buffaloes, bush pigs and some of the cattle had contact with impalas. In Ngorongoro the sheep and goats co-existed with a variety of wild animals for example impala, buffalo, jackal, hyena and zebra.

Four of thirty-nine livestock keepers said that if a big amount of their animals get sick or die they would change boma, burn the faeces and the dead animals to prevent contamination. A boma is a smaller fenced area close to the family’s house and the livestock keepers keep their animals here during the night to keep them safe. All the livestock keepers explained that a massive loss of animals due to disease or other causes would affect their family, health and economy very negatively.

Questions were also asked about the general health and mortality in the herds. Eighteen of thirty-nine of the herds had during the last two years had an outbreak and 17 of the animal owners claimed it was due to PPRV. The clinical signs observed during the outbreaks were diarrhoea, pneumonia, coughing, nasal discharge, high rate of abortions and mortality among the young animals.

The number of animals in each heard varied between the different study areas as can be seen in Table 4.
Table 4. The approximate number (n) and mean of animals in each herd

<table>
<thead>
<tr>
<th>Study Areas</th>
<th>Type of livestock</th>
<th>Sheep n (mean)</th>
<th>Goat n (mean)</th>
<th>Sheep + Goat n (mean)</th>
<th>Cattle n (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mikumi</td>
<td></td>
<td>60-200 (130)</td>
<td></td>
<td></td>
<td>60-200 (130)</td>
</tr>
<tr>
<td>Ngorongoro</td>
<td></td>
<td>50-200 (150)</td>
<td>50-200 (125)</td>
<td></td>
<td>6-600 (303)</td>
</tr>
<tr>
<td>Mahenge</td>
<td></td>
<td>3-50 (26.5)</td>
<td></td>
<td></td>
<td>0-50 (25)</td>
</tr>
</tbody>
</table>

Seroprevalence of RVFV

We visited 39 herds in 15 different villages and 13 of these herds had at least one seropositive animal (Fig. 5). The herd-prevalence varied between the different herds in Mikumi, Ngorongoro and Mahenge; 0-55 %, 0-10 % and 0-25 % respectively.

In total we analysed 74 % of the animals we sampled. In the first step 272 samples were analysed (Table 4) and the remaining 82 individuals were analysed during step two. Of the 354 analysed samples totally 29 individuals were positive (22 + 7) and 6 individuals were doubtful. When the seroprevalence was calculated the doubtful individuals were considered as negative (Table 5). All the ELISA plates used in the study were valid.
Table 4: Results from the first step ELISA, 272 animals were analysed, and 22 animals were seropositive.

<table>
<thead>
<tr>
<th></th>
<th>Sheep % (n)</th>
<th>Goat % (n)</th>
<th>Total % (n)</th>
<th>≥ 7 years % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mikumi</td>
<td>4.7 (2)</td>
<td>18.4 (9)</td>
<td>12.0 (11)</td>
<td>6.7 (1)</td>
</tr>
<tr>
<td>Ngorongoro</td>
<td>2.5 (1)</td>
<td>-</td>
<td>1.1 (1)</td>
<td>-</td>
</tr>
<tr>
<td>Mahenge</td>
<td>12.0 (6)</td>
<td>9.8 (4)</td>
<td>10.9 (10)</td>
<td>-</td>
</tr>
<tr>
<td>Total (%)</td>
<td>6.7 (9)</td>
<td>9.4 (13)</td>
<td>8.1 (22)</td>
<td>6.7 (1)</td>
</tr>
</tbody>
</table>

Table 5: Results from ELISA showing the total seroprevalence and number of positive animals. In total 354 individuals were analysed.

<table>
<thead>
<tr>
<th></th>
<th>Sheep % (n)</th>
<th>Goat % (n)</th>
<th>Total % (n)</th>
<th>≥ 7 years % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mikumi</td>
<td>3.5 (2)</td>
<td>18.0 (12)</td>
<td>11.3 (14)</td>
<td>30.0 (6)</td>
</tr>
<tr>
<td>Ngorongoro</td>
<td>2.4 (1)</td>
<td>-</td>
<td>1.0 (1)</td>
<td>-</td>
</tr>
<tr>
<td>Mahenge</td>
<td>14.3 (10)</td>
<td>7.3 (4)</td>
<td>11.2 (14)</td>
<td>-</td>
</tr>
<tr>
<td>Total (%)</td>
<td>7.7 (13)</td>
<td>8.6 (16)</td>
<td>8.2 (29)</td>
<td>25.0 (6)</td>
</tr>
</tbody>
</table>

The total seroprevalence for all the analysed samples was 8.2 %. Mikumi and Mahenge had a similar seroprevalence, 11.3 % and 11.2 % respectively which differed slightly between the first and second analysis step. Ngorongoro had the lowest seroprevalence of the three study areas; only 1 of 105 animals was seropositive. In total the seroprevalence in goats was slightly higher than in sheep. However in Mahenge the seroprevalence in sheep was higher than in goats and in Mikumi it was the other way around. Of the animals older or equal to 7 years of age 25 % were seropositive for RVFV-antibodies, while 7 % of the analysed animals younger than seven years were seropositive. The seropositive animals younger than seven years (n= 23) did not live during the major outbreak in 2006-2007. In Mikumi five of the six seropositive animals older than seven years were found. Mikumi also had the highest seroprevalence among the herds, one herd had a seroprevalence of 55 %.
DISCUSSION

In this study the aim was to investigate the seroprevalence of RVFV among sheep and goats and to evaluate if the virus is active during the inter-epidemic periods (IEP). The results indicate that 8.2% of the goats and sheep in the study districts have encountered RVFV sometime during their life. Since the competitive ELISA used in this study detects both IgM and IgG it is not possible to estimate when the animals were infected. This would have been possible if it was possible to differentiate IgG from IgM, due to the fact that IgM levels increase before IgG do in the blood (Pepin et al., 2010). Of the 8.2% that were positive 21% were older than 7 years. The latest outbreak in Tanzania with a subsequent vaccination campaign was in 2007, which is seven years ago (Chengula, 2013). Hence one cannot exclude that the older animals are positive due to previous vaccination since there today is a lack of DIVA compatible vaccine (OIE, 2014).

In this study 79% of the seropositive animals were younger than seven years, which is an overall majority of the seropositive animals. This strongly indicates that RVFV is circulating in low levels in the three districts during IEP, since the last outbreak was seven years ago and animals younger than seven years did not live during that outbreak. Since the seroprevalence between Ngorongoro differed a lot from Mikumi and Mahenge; 1.0%, 11.3% and 11.2% respectively, the Ngorongoro district might be an exception to this statement.

Ngorongoro had a low seroprevalence (1.0%), which is unexpected since the Ngorongoro district has been involved in all RVF outbreaks that have occurred in Tanzania (Sindato et al., 2014). I cannot find a good explanation for the result. We did not ask the livestock keepers if they had an RVF outbreak seven years ago, so it might have been a coincidence that we sampled herds that had not been involved in the outbreak 2006-2007 or that the virus is not present in this area during IEP. However the only individual that was seropositive in the Ngorongoro district was younger than seven years, which indicates that the virus is circulating in this area during IEP too.

The way we selected our animals for this study might have contributed to a selection bias especially the way we selected which individuals to analyse. Since the analysis was done in two steps and in the second step we analysed all the animals that were in the herds with at least one seropositive individual from the first step, this might have led to the fact that we got a false high seroprevalence in certain herds. In total we only analysed 74% of the animals we sampled so there have been multiple steps of selection in this study. All selections that are not randomly done can be a source of bias and since the animals selected for sampling and analysis in this study were not done randomly one must consider this while interpreting the result. Apart from the result from Ngorongoro the seroprevalence in Mikumi and Mahenge corresponds to other serological studies mentioned in the next paragraph (Sumaye et al., 2013). Therefore I do believe that the result from Mikumi and Mahenge is credible. Also the seroprevalence was rather consistent in both of the steps, which reduces the issue of bias in the selection of which animals we analysed. Mikumi had the highest seroprevalence (55%) among the herds. In the first step 7/11 animals were analysed from this herd and five of these were positive. During the second step the remaining 4 animals from this herd were analysed and one more was then seropositive, so the second analysis did not cause a false high seroprevalence. There were no clinical signs or results from the interview with the livestock
keeper that could explain the high seroprevalence from this certain herd, apart from that this herd had more contact with wild animals than the other herds around Mikumi. The livestock keeper mentioned impala, wild dogs and lions and some of the animals his herd had contact with. Another interesting point is that 5 of the 6 seropositive animals ≥ seven years were from Mikumi, however they were not from the same heard. It is difficult to know the vaccination status of RVF since we only asked if the animals had been vaccinated during the two recent years and the vaccination campaign for RVF was seven years ago. Further studies of the prevalence, especially around Mikumi are desirable.

Previous studies have shown positive seroprevalence of antibodies to RVFV in other parts of Tanzania during the IEP. Our study area Mahenge was in the Ulanga district. A study performed by Sumaye et al., (2013) including serum from cattle, sheep and goats in Ulanga 2011 had a seroprevalence of 13 % compared to 11.2 % in our study from the same district. In the study by Sumaye et al., (2013), one must consider that cattle also were included in the seroprevalence, but the prevalence is almost the same indicating a consistent circulation of RVFV. In the same study the district Kilombero was also investigated. Both of these districts are in Morogoro region and the overall prevalence in Morogoro region was 11.3 % compared to our overall prevalence in the same region that was 11.2 %. An interesting and expected fact in that study, was that the seroprevalence was 5.5 % in animals born after the outbreak in 2006-2007 and 22.7 % in animals that had lived through the outbreak. Even IgM antibodies were found in some of the samples indicating a more recent infection in 2011, four years after the major outbreak (Sumaye et al., 2013). The region Kigoma in northwestern Tanzania has not experienced an RVF epidemic, however serum samples collected from sheep and goats in September 2011 had an overall seroprevalence of 5.4 % and antibodies were also detected in animals younger than one year (Kifaro et al., 2014). During the outbreak 2006-2007 serum samples from cattle, sheep and goats were collected in the regions Muanza, Arusha, Tabora, Dodoma, Tanga, Mbeya, Iringa Pwani, Dar es Salaam, Lindi and Mtwara. ELISA analysis was performed on 200 of these samples and the overall seroprevalence was 39.5 % (Chengula et al., 2014). It is not surprising that the seroprevalence was higher when the sampling was associated with an outbreak. Although compared to the seroprevalence during the endemic period, which is approximately 10 %, the seroprevalence during the epidemic period is not that high. It may be due to that a lot of animals were sampled before they had produced antibodies or another strong indication that the virus is very active during inter-epidemic periods.

Domestic camels have recently been introduced into Tanzania. In 2010 109 camels were sampled in the Northern part of Tanzania and 27.5 % of these were IgG seropositive. The seropositivity was higher in the camels older than 10 ≥ years and lower in camels ≤ 5 years (Swai and Sindato, 2014). This can be due to the fact that the older camels experienced the outbreak in Tanzania 2006-2007 while the younger camels only have encountered RVFV during the IEP when the levels of the virus is low. Camels have played a major part in the amplification of the virus in previous RVF outbreaks and might in the future also do so in Tanzania (El Mamy et al., 2011). RVFV antibodies have also been found in humans during IEP. In 2004 seropositive humans were found in Tanga region (Swai and Schoonman, 2009). Mbeya region in the southwestern part of Tanzania has not reported RVF outbreaks, however
the human population seroprevalence of RVFV was 3.2 % in 2008 (Heinrich et al., 2012).
The conclusion of the comparison of these different studies performed in Tanzania is that
encountered antibodies in animals and humans in areas with no history of RVF outbreaks and
in individuals younger than seven years strongly indicate that RVFV is endemic end
circulating in low levels in several regions in Tanzania.

When we were out in the field sampling the animals, owners were asked how old each animal
was. Some knew exactly how old every individual was while some said that the animal was
around a certain age. In several herds the livestock keepers could only tell which animals that
were younger and older than one year, so we looked at the teeth of the sheep and goats to
estimate the age (ESGPIP, 2009). This method is subjective and could contribute to the fact
that many of the animals got an approximate age. Some were estimated to be older than four
years while in fact they might have been older than seven. This can influence the
interpretation of the result if the animals have antibodies due to vaccination or true infection.
We chose to divide the animals in younger and older than seven years when presenting the
result since giving the animal an exact age would not have been credible.

It would be desirable to do a molecular study on the samples in this study to confirm that the
virus is circulating in silence during IEP in these areas during the late dry season. Also if the
virus is found, it would be advantageous to analyse the virus to see which strain it is and to
see if it has developed, changed or become more virulent and compare it to other analysis to
get a better understanding of the virus evolution (Bird et al., 2007; Nderitu et al, 2011).
However the viremia only lasts 1-6 days post inoculation so during the enzootic cycle when
the virus is circulating at low levels the chances of sampling viremic animals during the
correct five days are very low since they probably will not have any clinical signs of the
disease (Weingartl et al., 2014).

Our plan from the beginning in the study was to carry out RT-PCR on the positive samples
but due to several unfortunate happenings (reagents stuck in customs in Dar es Salaam, power
cuts and a broken RT-PCR machine) this was not possible. In a study made by Bird et al.,
(2008) they emphasize the importance of combining molecular and serlogic methods to get a
true picture of the actual RVFV situation in a certain area, since antibodies can be found
without the animal having the virus and vice versa (Bird et al., 2008).

A lot of the animals we sampled did have contact with wildlife. On a couple of farms the
sheep and goats did not have direct contact with wild animals. However their boma was next
to the cattle’s boma. Humans owning cattle were associated with higher seroprevalence of
RVFV in a study made by Heinrich et al., (2012). It might be because mosquitoes prefer to
feed on cattle, that cattle are one of the main amplifying hosts, or another hypothesis might be
that cattle have more contact with wild animals because they graze further from the boma. In
that way sheep and goats might have indirect contact with wild animals and can possibly get
infected indirectly by the wild animals.

In 1991 House et al., (1996) sampled 16 lions in the Ngorongoro Crater in Tanzania and 6 of
these were seropositive for RVFV. At that time the Masai people were allowed to graze their
livestock in the crater so not only the wild ruminants but also wild carnivores might have a
part in the epidemiology and transmission of the virus since carnivores also seem to be able to
carry the virus. To date this is one of the two publications I have found about the seroprevalence in wild animals in Tanzania. The second one performed by Sindato et al., (2013) shows a positive seroprevalence among buffalo and elephant in the Northern part of Tanzania during IEP between 2002-2006. Several studies on wildlife have been performed in Kenya (Bird et al., 2008; Britch et al., 2013; Evans et al., 2008). These studies show presence of antibodies among impala, buffalo, waterbuck, giraffe and warthog. The seroprevalence among these animals was low during the IEP and high during epizootics (Britch et al., 2013).

In Central African Republic RVFV antibodies have been detected among wildlife in areas where outbreaks have not been reported (Pourrut et al., 2010). It would be interesting for further studies to sample wild ruminants and do a molecular and serological study to get a better understanding of the RVFV in Tanzania during IEP.

Other studies have observed a higher seroprevalence of RVFV in sheep (Rostal et al., 2010; Jeanmaire et al., 2011). However no significant difference was found between sheep and goats in a study performed by Sumaye et al., (2013). In this study goats (8.6 %) had a higher seroprevalence than sheep (7.7 %) although it was not statically significant. It would be interesting to have the difference of seroprevalence between sheep and goats in mind when a new screening is performed.

It is important to have an active surveillance regarding RVFV in endemic countries to prevent new outbreaks and to avoid the subsequent negative effect it has on the livestock, population, welfare and economy. Even though the outbreaks reoccur in an irregular pattern it is worth to keep track of varieties in ENSO that might lead to an increase in precipitation in a few months (Joseph, 1991). When an increase in precipitation is expected it is possible to vaccinate livestock in advance and inform the population on how to prevent transmission and infection. While we were preforming our fieldwork in Tanzania and interviewing the livestock keepers, nobody mentioned RVF as being one of the diseases they feared the most for their livestock and family. It made it feel like we were searching for the prevalence of an out-dated virus or that there is a lack of knowledge about RVFV. Chengula et al., (2013) pointed out that a majority of the people interviewed in the regions Arusha, Morogoro and Manyara did not know that RVF was an outbreak disease. There have also been indications that a big part of the population does not know that RVF is a zoonosis (Swai and Schoonman, 2009).

We must keep in mind, and also inform that major outbreaks can reoccur every 3-15 year and if the authorities are not prepared, RVF can cause severe socio-economic losses, disturb the animal welfare and spread to larger areas in Tanzania and the world (Chengula et al., 2013). The clinical symptoms of RVF in humans can resemble those of malaria and other febrile diseases. In the beginning of an outbreak it might therefore be easy to miss the diagnosis (Koram and Molyneux 2007). If a malaria test is negative but the patient still has fever it is worth considering RVF as a differential diagnosis especially since both the diseases increase when the mosquito population increases which often is associated with heavy rainfall.

Of the herds we visited, 44 % said they had had an outbreak during the last two years and claimed it was due to PPRV. In most of the cases the diagnosis is made only by the clinical signs and PPR is a differential diagnosis to RVF (Bird et al., 2009), which is good for local veterinarians to keep in mind as it can function as a warning signal for a new RVF outbreak.
During IEPs RVFV can be detected at low levels and that is why it is important to have accurate and highly sensitive diagnostic methods. Maquart et al., (2014) compared a new highly sensitive real-time RT-PCR with the reference RT-PCR. The reference PCR only tested positive on 13% of the samples that the new RT-PCR identified. Since RVFV can circulate without clinical manifestations it is important to have sensitive diagnostic methods to be able to have an early-warning system (Maquart et al., 2014). The virus may be in countries no one today is aware of and it might by accident be introduced to a new naive country and develop into an epidemic. As mentioned earlier, infected livestock can facilitate the spread of the virus (Carroll et al., 2011). Infected mosquitoes can spread the virus even further if they are accidently transported in a plane because they can survive long flights (Russell, 1987).

In the US, RVF is considered as a potential threat as a biological weapon and is number three on the list of the most dangerous animal threats (Mandell and Flick, 2010). Europe should also take caution regarding the virus since it is possible for RVFV to spread to Europe and theoretically the virus can survive in Europe and cause an epidemic too, since possible vectors and ruminants exist (EFSA, 2005). After my study I do not think RVFV is the primary pathogen to be most concerned about causing an epidemic in Europe today. However conditions can change very fast in our globalised world. Therefore it is desirable that more research should be done to get a better understanding of RVFV during the IEP and in the future hopefully be able to prevent new epidemics of RVF reoccurring in Tanzania and other parts of the world.

**CONCLUSIONS**

Since the last outbreak of RVFV in Tanzania was seven years ago and the majority of the seropositive animals in this study are younger than seven years, it suggests that RVFV is circulating in low levels in the Arusha and Morogoro region during the IEP. It would be desirable for future studies to sample wild small ruminants and do a molecular and serological study to get a better understanding of the epidemiology of RVFV.
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Lastly I want to thank my colleagues and true friends Lovisa Levin and Ida Herbe that carried out this study with me. You are irreplaceable.
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