Seroprevalence of Rift Valley fever in sheep and goats in Zambezia, Mozambique
and preparations for a metagenomic study of arboviruses in ticks

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Seroprevalens av Rift Valley fever hos får och getter i Zambezia, Mozambique – och förberedelser inför en metagenomik-studie av arbovirus i fästingar

Isabelle Scharin

SUMMARY

The virus-mediated disease Rift Valley fever (RVF) was discovered during an outbreak in Kenya in the 1930s. Since then it has spread to most parts of Sub-Saharan Africa, and in the last decades several outbreaks have caused economic and health issues in Africa, Yemen and Saudi-Arabia. The disease mainly affects domestic livestock, causing abortions, but is also a zoonosis. To be able to control the spread of the disease it is important with surveillance for better knowledge about the distribution and virus circulation even in inter-epidemic/epizootic periods.

The human population is growing, and people and their livestock constantly move closer to areas with wild animals which act as reservoirs for different viruses. Also, humans and their animals often get within reach for arthropod vectors, hosting or carrying viruses. Taken together, this increases the risk for new emerging infectious diseases.

This study consists of two parts. One is a seroprevalence study of RVF in sheep and goats in Zambezia, Mozambique. The other is a metagenomic study that aims to search for tick and mosquito borne viruses, including RVFV, in the same area.

The RVF-study was a cross-sectional survey in cooperation with the University of Eduardo Mondlane (UEM), Maputo, Mozambique. Between the 25th of September and 2nd of October in 2013, blood samples were collected from sheep and goats in 8 different farms. Blood-sera from 187 goats and 181 sheep were analysed at the veterinary faculty of UEM with a commercially available ID-vet competition ELISA-kit. On the same field trip ticks were collected from sheep, goats and cattle. The metagenomic study was however only initiated during the time of this project and the remaining work, as it involves several steps that take some time to perform, will be conducted at the Swedish University of Agricultural Sciences during early 2014.

The results from the seroprevalence study showed an overall prevalence of 25.1% for goats and 44.2% for sheep. The prevalence has increased compared to an earlier study in the same province in 2010. The precipitation was higher this year than during 2010 which agree with the hypothesis that increased precipitation favour the RVFV spread, as more vectors are hatched. The results from comparable farms showed an increase in seroprevalence for sheep, though not for goats. Older animals showed to have a higher seroprevalence than younger which was expected. The most remarkable finding was the significant differences in prevalence between different farms.

Interviews with the animal keepers did not indicate that RVF caused any noticeable health problems in their herds. This study indicates that the virus circulates and causes subclinical infections in sheep and goats in Zambezia. In future research it would be interesting to focus on the big differences between the farms.

SAMMANFATTNING

Virussjukdomen Rift Valley fever (RVF) upptäcktes vid ett utbrott i Kenya på 30-talet, och var då en "Emerging infectious disease". Sedan dess har smittan spridits till de flesta delar av Afrika söder om Sahara och har de senaste decennierna orsakat flera utbrott i Afrika, Yemen och Saudiarabien som orsakat allvarliga ekonomiska och hälsomässiga konsekvenser. Sjukdomen drabbar framför allt tamboskap för vilka den orsakar aborter, men är en också en zoonos. För att kunna kontrollera smittan är det viktigt att känna till dess utbredning och på vilken nivå den cirkulerar, även mellan epidemiska/epizootiska perioder.

I och med att humanpopulationen växer och inkräktar på områden med vilda reservoarer för virus ökar risken för att nya "Emerging Infectious Diseases" skall uppstå. Många av dessa är spridda med artropoda vektorer, såsom myggor och fästingar.

Denna studie består av två delar, varav den ena delen består av en seroprevalensstudie av RVF hos får och getter i Zambezia, Mozambique. Den andra delen är en metagenomik-studie som syftar till att söka efter mygg- och fästingburna virus, inklusive RVFV, i samma område.

Studien av RVF utgjordes av en tvärsnittsstudie i samarbete med Eduardo Mondlane Universitet i Maputo, Mozambique. Mellan den 25 september och 2 oktober år 2013 samlades blodprover från får och getter in från 8 olika farmer. Serum från 187 getter och 181 får analyserades sedan på laboratorium på universitetets veterinärfakultet i Maputo med ett kommersiellt ELISA-kit. Samtidigt som blodproven togs samlades fästingar från djuren in för metagenomikstudien. Denna studie hann endast påbörjas under tiden för detta projekt och resterande arbete kommer att utföras på Sveriges lantbruksuniversitet tidigt under år 2014.

Resultaten från seroprevalensstudien visar en övergripande prevalens på 25,1% för getter och 44,2% för får vilket är en ökning jämfört med en tidigare studie år 2010. Det skulle kunna härledas till att nederbörden varit kraftigare under år 2013 och därmed ökad insektsaktivitet. På de enskilda farmer som kunde jämföras med en studie år 2010 ser man att seroprevalensen ökat för får medan den ligger kvar på samma nivå för getter. Äldre djur visade sig hade högre seroprevalens än yngre vilket var väntat. Det mest anmärkningsvärda denna studie visade var att seroprevalensen skiljde sig signifikant mellan olika farmer.

Djurens skötare sade sig inte uppleva några problem med sjukdomen, vilket indikerar att cirkulerande virus orsakar subkliniska infektioner hos får och getter i Zambezia. Framtida forskning skulle vara intressant att rikta mot att utreda orsakerna till att seroprevalensen skiljer sig så kraftigt mellan olika farmer.

CONTENT

INTRODUCTION

Aims

There are two directions for this master thesis. The primary aim was to evaluate the seroprevalence of Rift Valley fever virus (RVFV) antibodies in sheep and goats during late dry season year 2013 in Zambezia, Mozambique. The study was a cross-sectional survey which is part of a longer surveillance project conducted by University of Eduardo Mondlane's (UEM), veterinary faculty in Maputo. The second project was a broad pilot study, searching for any known or unknown virus in arthropod vectors in the same area, using a metagenomic approach. The metagenomic study aim was to identify potential pathogens that may become emerging infectious diseases (EID: s) in the future.

Since there have been several severe outbreaks in countries adjacent to Mozambique, it is interesting to estimate the prevalence of the virus and investigate the inter-epidemic state of RVF in Mozambique. One aim was to investigate how the seroprevalence has changed from an earlier survey in the same province in 2010. The hypothesis was that the seroprevalence should be higher this year since there has been a higher amount of precipitation which should favour the mosquito population carrying the virus.

The aim of the metagenomic study was to investigate the viral flora in ticks found on livestock; sheep, goats and cattle. The purpose of the study was to identify viruses, known and unknown, to eventually be able to foresee a potential threat to humans and domestic animals in form of an emerging infectious disease. Since the tick samples and blood samples were individually marked, it will be possible to correlate results from the metagenomic study to the seroprevalence study (even for the cattle which were used in a seroprevalence study for Schmallenberg virus).

Study region

Zambezia is a province in central Mozambique. It borders to Malawi in the west and the Indian Ocean in the east. The province has large areas drained by the Zambezi River, forming a delta in its outlet to the ocean and the coast consists mostly of mangrove swamps. The average temperature differs between 20.2°C and 30.2°C and the average annual precipitation is 1482mm, measured from the province capital Quelimane (Climate Data, 2013). Zambezia was chosen as region for the seroprevalence study due to its agricultural and geological properties making it a suitable environment for circulation of RVFV. The area has many rivers, dambos (waterlogged depressions) and wetlands creating good breeding possibilities for the insect vectors (Fafetine *et al.,* 2013).

There are also reasons to believe that the region is a good study area to look for potential emerging infectious diseases. Most arboviral infections are found in tropical developing countries (Quinn *et al.,* 2009). Places where there is a relatively big risk for occurrence of EID:s can be called "emerging disease hotspots". An overview of incidence rate of EID:s between 1940 and 2004 shows that most cases seem to have occurred in areas with a dense animal and human population. It shows high numbers of cases in central Europe, the east coast of the USA, Australia and some parts of Asia (Jones *et al.,* 2008). According to Jones *et*

al., (2008) this is due to a more frequent reporting and greater surveillance from richer countries than developing countries. The analyses of where EID:s most likely will occur show hotspots in developing countries with low latitude (Jones et al., 2008) which makes it relevant to move the research focus from where most scientific resources are based to the high risk areas. Hotspot areas include tropical Africa, which means that Zambezia in Mozambique is a place suitable for conducting research in this topic.

LITERATURE REVIEW

Emerging Infectious Diseases

An emerging infectious disease (EID) is, according to the World Health Organisation (WHO), an infectious disease that has not earlier appeared in a population, or has existed earlier but is expanding vastly in incidence or geographic range.

During the past 30 years, WHO has reported over 50 new human pathogens. As many as 60.3% of the EIDs are zoonoses, and of them 71.8% has a wildlife origin (Jones *et al.,* 2008).

Viruses, endemic in wildlife in tropical countries, could as a consequence of some kind of disturbance in the environment, infect and adapt to humans and/or their domestic animals and cause disease. Some changes make it possible for the virus to spread and multiply quickly, and many tropical viruses have, after the adaption to its new host, shown to be able to cause epidemics even in temperate countries (Weaver & Reisen, 2010). Examples of factors that contribute to the start of an epidemic/epizootic are; increased susceptibility in humans/livestock due to factors in the hosts, the virus capacity of adapting by mutations or other ways of changing its genome, changes in the climate or ecosystem, changed land use systems causing people to come in contact with wild reservoirs and changes in human behaviour such as increased international traveling and transportations of goods and animals (Committee of Emerging Microbial Threats to Health, 2001).

Examples of earlier EIDs are Rift Valley fever, Nipah virus, Hantavirus, Hendravirus, Dengue virus, West Nile virus and SARS.

Arboviruses as EID: s

Arbovirus means "arthropod-borne virus". Their maintenance in nature depends on biological transmission between vertebrate hosts by blood-feeding arthropods such as mosquitos, sand-flies, midges and ticks. Biological transmission means the virus has to replicate in the vector before infecting the next host. If the virus is spreading via contaminated mouthparts, directly between the vertebrate hosts it is called mechanical transmission. Some factors that determinate the geographical distribution of a specific arbovirus includes temperature, amount of precipitation and occurrence and distribution of vertebrate and arthropod vectors (Quinn *et al.,* 2009). Arboviruses include mainly RNA-virus taxa such as members of the families Togaviridae, Flaviviridae, Bunyaviridae and Reoviridae. Only one known DNA-virus is an arbovirus, which is the African swine fever virus (Weaver & Reisen, 2010).

As with the EID:s even the majority of arboviral diseases are zoonoses. Commonly the virus circulates between wild animals and arthropods, and domestic animals and humans are dead end hosts since they do not often develop viremia enough for effective transmission of the virus (Quinn *et al.,* 2009). However, evolutionary genetic changes of the virus that can fortify its virulence to the vertebrate host, or make it more effectively spread by its vector (Weaver & Reisen, 2010), or via another infectious route, can make it a health threat to humans and domestic animals.

Many arboviruses from tropical areas have been transmitted and caused disease to humans and their domestic animals due to so called "spill over" transmission (Weaver & Reisen, 2010). Vectors attracted to both humans and animals pose a major risk for transmission of pathogens between wild animals, domestic animals and humans. The closer human populations get to wildlife reservoirs, and thus in reach of the vectors, the bigger the risk for emerging of new diseases in humans and their domestic animals (Daszak *et al.,* 2000). Arboviruses which thrive in vectors attracted to humans could be the greatest risk of upcoming human diseases. Such vectors are for example the mosquito species *Aedes aegypti*, *Aedes albopictus* and species in the genera *Culex* (Weaver & Reisen, 2010). For an arbovirus to be able to cause an epidemic there must be the right prerequisites, the environment must be suitable for both vectors and vertebrate host population. The hosts can include a variety of both arthropod and vertebrate species and all need not necessarily be there at the same time but at least so that transmission can occur within certain intervals (Weaver & Reisen, 2010).

According to review by Weaver and Reisen (2010), ongoing big worldwide changes like global warming and human populations' movement and expansion, makes it necessary to revaluate where the research of arbovirus-caused EID:s is focused. Factors in modern times that increase the risk for EID:s are great populations living close together making viruses spread easily, constant increase in transportations and travel, introduction of viruses to new niches, and expanding populations with encroachment in rural areas. Due to increase in human population, especially in tropical countries, increase in global traveling and transportations, and potentially due to global warming creating suitable habitats for vectors in new areas and prolonging of transmission seasons, arboviral EID:s are likely to be more common in future.

Some examples of emerging infectious diseases caused by arboviruses are Rift Valley fever, West Nile virus, Japanese encephalitis virus, Bluetongue virus, Venezuelan equine encephalitis virus, dengue viruses and Chikungunya virus.

Tick-borne viruses

Of the more than 500 known arboviruses about one third is tick-borne, even though less than 10% of the tick species are known to be virus vectors (Labuda & Nuttall, 2004). Most of the species known to spread viruses belong to the large tick genera; *Ornithodores* and *Argas* in the Argasidae family and *Ixodes, Haemaphysalis, Hyalomma, Boophilus, Rhipicephalus, Amblyomma* and *Dermacentor* from the family Ixodidae. There are about 200 viruses described to spread via ticks, and of them 80% belong to the genera Orbivirus, Nairovirus, Phlebovirus and Flavivirus (Labuda & Nuttall, 2004).

The "life-cycle" of tick-borne viruses differs between species of hosts and viruses. The ticks can be divided into primary and secondary vectors. The primary vector is a species necessary for the virus maintenance while the secondary vector can be different species, each one functional as a vector for virus transmission (Labuda & Nuttall, 2004).

An important feature for ticks in their role as vectors for viruses is their long life. They can often survive for a long time between blood meals (Sonenshine, 1991 in Labuda & Nuttall, 2004). This is of interest since several studies have shown that viruses can persist in the tick for its whole lifespan (Rehacek, 1965; Davies, Jones & Nuttall, 1986), and thus a long life for a tick means a long time of persistence for the virus, when they can bide their time to find a new vertebrate host.

When feeding blood, a tick excrete substances in its saliva which have several effects, among one is to modulate the hosts immune system. Viruses can benefit from this mechanism, helping it to be able to infect co-feeding ticks on the same host vertebrate (Labuda & Nuttall, 2004). What differ between viruses having insect vectors versus viruses having tick-vectors are probably their abilities to cope with the environment in the different arthropods midgut, where they enter their vectors body, since the insects and ticks do not have the same physiology for processing their blood meals (Sonenshine, 1991 in Labuda & Nuttall, 2004).

In Mozambique, Dias (1993) and de Matos (2008) have found 21 tick species on cattle and/or goats. Most of them belonged to the genera *Rhipicephalus*, but also species from *Amblyomma, Hyalomma* and *Ixodes* were found.

One examples of a zoonotic viral diseases spread by ticks in southern Africa is the Crimean-Congo haemorrhagic fever, which most important vector is *Hyalomma rufipes* (Horak *et al.,* 2002). *H. rufipus* was one of the species found on cattle in Mozambique by both Dias (1931) and de Matos (2008). Some other examples of tick-borne zoonotic viruses in Africa are Nairobi sheep disease Virus, Dugbe virus and Thogoto virus (Burt *et al.,* 1996).

Rift Valley Fever

History and expansion

The Rift valley fever virus (RVFV) was discovered in Rift Valley in Kenya in 1930 by Daubney *et al.,* (1931). They described a high rate of abortions in ewes, high mortality in neonatal lambs and managed to isolate the causing agent. It is believed that the RVFV strains circulating today has their origin in the late 19th century, when a virus from wild reservoirs emerged in domestic livestock when people started having large-scale cattle and sheep farming in Africa. It is also possible that the virus is even older (Ikegami, 2012).

The disease has since its origin spread to almost all sub-Saharan countries of Africa, including the island Madagascar (Gerdes, 2004). Outside of Africa there have been outbreaks in Saudi Arabia and Yemen with first cases in year 2000 (WHO, 2010; Ahmed *et al.,* 2009). Presumably the wide spreading of the virus is due to transportation of domestic animals and/or by infected mosquitos introduced by wind (Ikegami, 2012).

There have been many outbreaks affecting humans since the 70s. Following are examples of the ones with largest impact. An outbreak in South Africa during 1974-1976 caused the first documented human deaths (Grobbelaar *et al.,* 2011) see fig 1. The largest epidemic so far took place in Egypt 1977-1978 when approximately 600 people died, 18 000 got symptoms and up to 200 000 were infected. A later major outbreak occurred in east Africa; Kenya, Somalia and Tanzania in year 2007, with big losses in livestock production and many human cases (WHO, 2007). The eastern African outbreak spread in 2008 to Madagascar and South Africa (Ahmed *et al.,* 2009). The latest outbreak was according to WHO (2013) in Mauritania in September to October 2012.
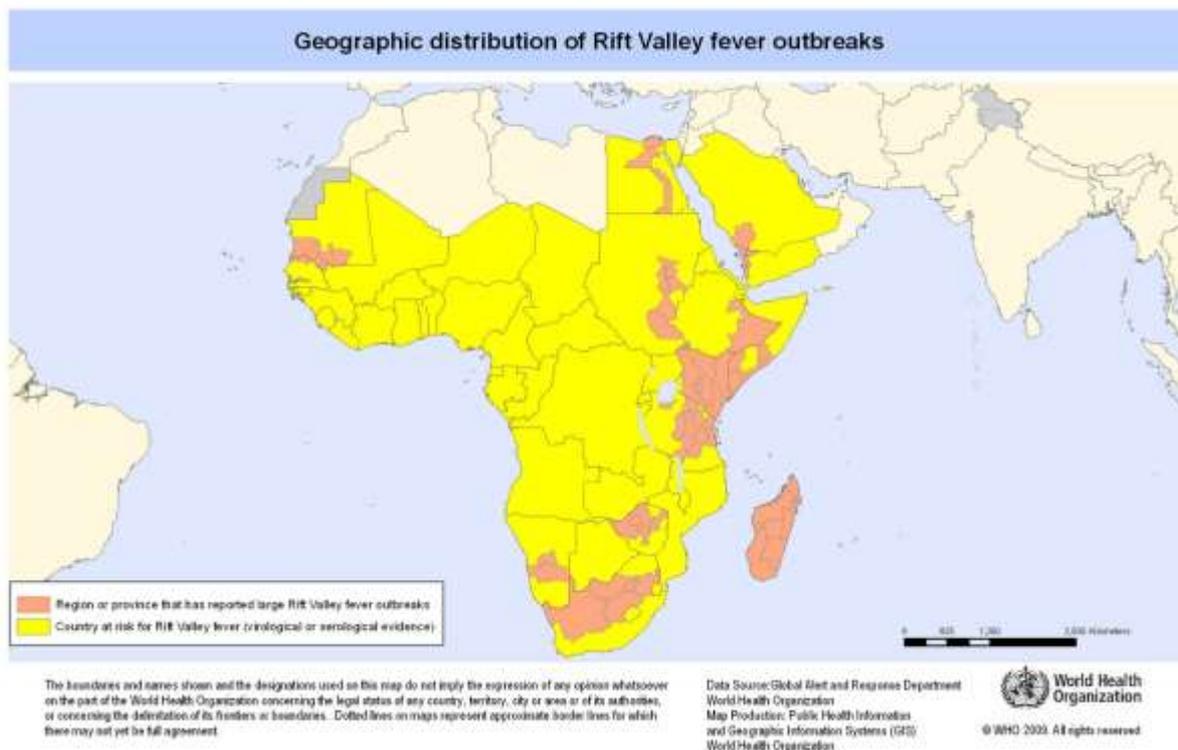
Figure 1. *Distribution of Rift Valley fever. Yellow countries are infected and orange areas have had reported large outbreaks (Reproduced, with the permission of the publisher: WHO, 2009).*

Impacts

Outbreaks of RVF can have big negative socio-economic impacts. The disease can bring problems with rural food security and nutrition and cause economic losses both for separate households and society. Since RVF is present in many poor countries, the disease does have severe effects for individuals and family, who depend on a small number of live animals.

In case of an outbreak several levels of production and commerce are affected. There will be reduced milk production due to abortions, losses of live animals and reduced prize on meat and livestock due to customers anxiety. Measures in order to control the disease cause additional losses, for example limitations in transport. An example is the big outbreak in Eastern Africa in 2007, which caused estimated losses of more than US$24.5 million for Kenya and US$3 million for Tanzania. The costs were due to preventions of further spreading

of disease and also further measures like surveillance and control, training of staff, identifying infected areas, vaccination and raising of public awareness (FAO, 2012).

The virus

Rift Valley fever virus is a RNA-virus within the genus *Phlebovirus* belonging to the family *Bunyaviridae*. The virion consists of an envelope covering the genome, packed as ribonucleocapsids (RNP). The virion is spherical and measures 80-120 nm in diameter. The envelope is constructed of capsomers made of glycoproteins, highly symmetric and arranged on an icosahedral lattice. The surface has spikes of surface projections embedded in a lipid bilayer (Sherman *et al.,* 2009). The ribonucleocapsid is different from other negative-sense RNA viruses in being string-like instead of helical (Raymond *et al.,* 2010).

The negative-sense genome of the virus consists of three single stranded RNA-segments. The segments differ in size called L (large), M (medium) and S (small). Segment L encodes for RNA-dependent RNA polymerase which synthesizes viral mRNA and genomic RNA (Ikegami, 2012). Segment M encodes for two big glycoproteins in the virus envelope and two smaller proteins, of those one none-structural which could possibly act anti-apoptotic (Won *et al.,* 2006). Segment S encodes for a nucleocapsid protein which associates with the RNA polymerase, and for a non-structural protein called NSs which constitutes the virus virulence factor through antagonising the host's immune system (Pepin *et al.,* 2010).

The RVFV replicates in the host's cell cytoplasm, and virions use the Golgi compartment to bud. The genome segments transcribe into mRNA and replicate using synthesis of cRNA (Pepin *et al.,* 2010). For starting transcription Bunyaviridae cleaves the hosts mRNA and use capped 5' fragments with 10-15 nucleotides as primers to synthetize its viral mRNA (Schmaljohn & Nichol, 2007 in Ikegami, 2012). The cRNA synthesis is however initiated with 5' nucleoside triphosphates (Pepin *et al.,* 2010).

The RVFV can be divided to 7 major genetic lineages. Although RVFV is widely spread geographically the strains in different areas are closely related at nucleotide and amino acid levels. In parts of central and eastern Africa, a large number of different strains circulate. A phylogenetic tree of segment M is seen in fig 2. The genetic diversity indicates that long-term enzootic circulation of virus in the environment causes development and maintenance of different lineages. When outbreaks have occurred in previously un-infected areas it is often due to one specific strain introduced via animals, humans and/or mosquitos (Ikegami, 2012).
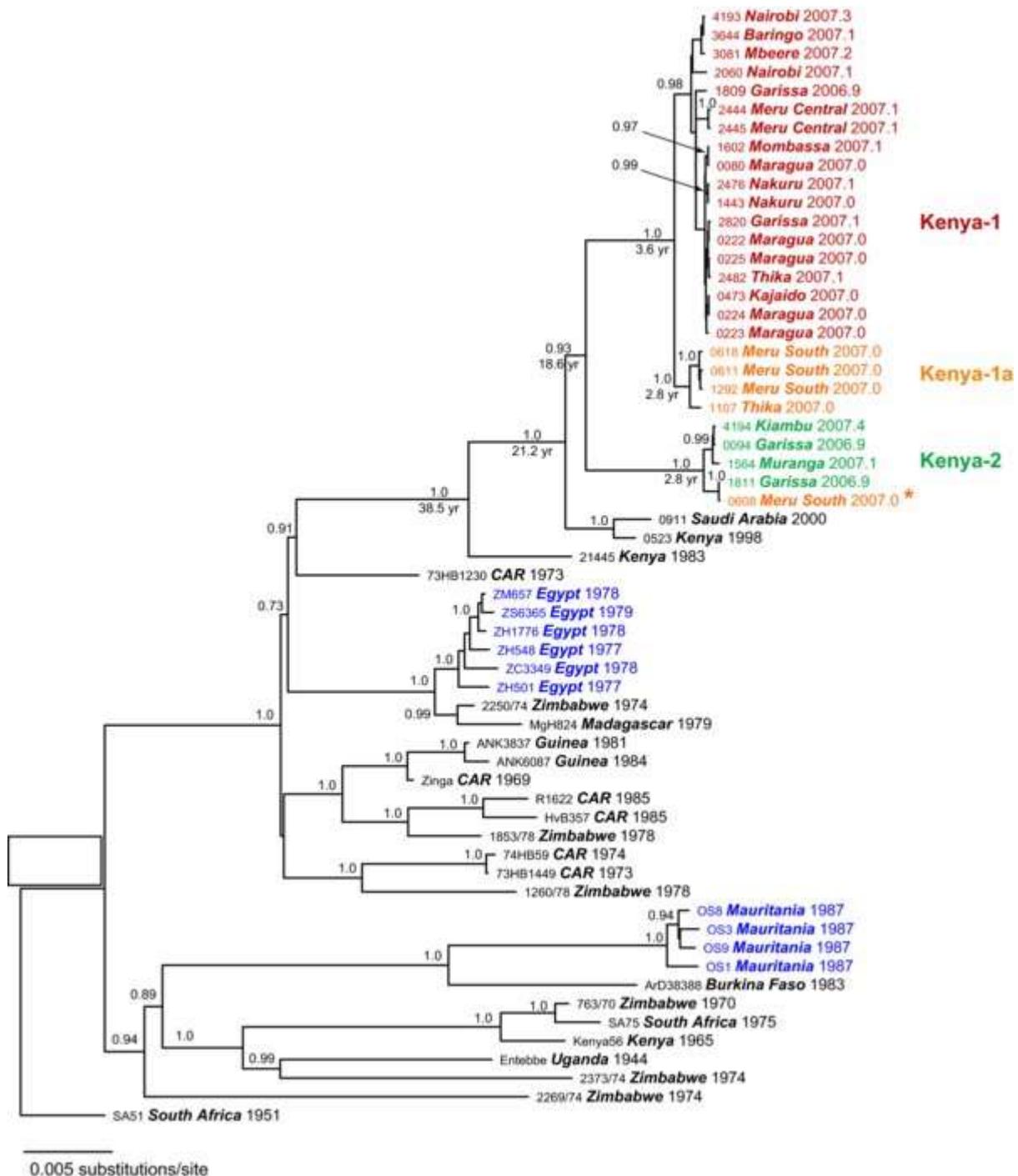
Figure 2. *An estimation of what the phylogenetic tree of Rift Valley fever virus segment M looks like, obtained from Bayesian studies (Pepin et al., 2010). Creative common license.*

Genetic analyses of virus strains from different geographical areas has shown that the strains have very similar parts of the genome even where it was expected to be most likely to change due to immune selection pressure (Grobbelaar *et al.,* 2011). The low genetic diversity could mean either that the virus has low tolerance for mutation within its own genome, or that the ancestor of the strains we today call RVFV are not too distant in time (Pepin *et al.,* 2010).

Findings of combinations of genome segments from virus-strains with different geographical distribution shows that the RVFV is capable of genome reassortment if viruses from different strains occur simultaneously in the same host (Sall *et al.,* 1999; Ikegami 2012) This suggests there is risk of reassortment between attenuated vaccine virus strains and wild virus strains (Grobbelaar *et al.,* 2011).

Pathogenesis

Rift Valley fever virus can cause disease in very many species, for example rodents, sheep, primates, goats, cattle, camels, dogs, cats and ferrets. During experimental infections also rabbits, guinea pigs, birds, horses, pigs and other animals have shown to be able to become infected, but do not develop symptoms (Ikegami & Makino, 2011). However the disease mainly causes problems for domestic livestock and humans (Olive *et al.,* 2012). The mechanism of species-specific susceptibility is still unknown. Sheep seems to be more susceptible as compared to cattle and camels (Ikegami & Makino, 2011) and young animals are much more susceptible and suffer harder from the disease than adults. This might be due to that the virus target-cells allow the virus to replicate in a larger extent in younger animals than in older. It could also be a matter of differences in the immune system between juveniles and adults (Pepin *et al.,* 2010). Aside from age differences the pathogenesis also differs between different species, probably because of the different antiviral responses of the immune system (Ikegami & Makino, 2011). Studies have shown that genetics play a big role in susceptibility to RVFV-infection among sheep, and the course of disease differs in severity between breeds. The differences in susceptibility are not only evident between breeds but also within the same breed (Olaleye *et al.,* 1996). Exotic breeds are more susceptible than native breeds and areas with large numbers of imported exotic bloodlines have often suffered harder from RVF than other areas (FAO, 2003). There are also differences in virulence between different lineages of the virus (Ikegami, 2012).

The virus primary target organ in all species is the liver. After entering the body it is thought that the virus enters the immune systems sentinel cells and is transported to the liver. In a severe infection it is possible to find the virus in all tissues (Pepin *et al.,* 2010). The virus enters the cells via a still unknown cellular receptor. The NSs (Non-structural protein from the S genome segment) gene products block the host-cell's antiviral response, being an interferon antagonist. The infection causes hepatic necrosis and attraction of inflammatory cells to the liver. In necropsy of infected animals it is typical to find widespread hepatic necrosis (Pepin *et al.,* 2010).

In laboratory experiments, using animal models, three different paths of disease have been seen. One is an acute lethal infection; the viremia is uncontrolled and causes a rapid death. The second is a mild infection with full recovery or asymptomatic infection. The third way is that the animals primarily recover but get a later onset of complications. The complications can be new episodes of fever and viremia which can cause e.g. ataxia and damage to the retina, and in the worst case be fatal (Pepin *et al.,* 2010).

Histopathological changes in lambs are extensive hepatocyte necrosis, predominantly centrilobular or midzonal. The amount of virus increases progressively and affects primarily

hepatocytes. In some lambs the virus also causes necrosis of villi in jejunum, ileum and depletion of lymphocytes in the spleen. The same pathological patterns have been seen in mice and hamsters exposed to experimental infections (Ikegami & Makino, 2011).

Transmission and epidemiology

Rift Valley fever virus transmission primarily occurs with arthropod vectors between its mammal hosts (fig 3). In endemic areas the vector borne transmission is the way the virus continues to circulate (Pepin *et al.,* 2010). The vector responsible for most virus transmissions is the mosquito of genus *Aedes,* but the virus has shown to be able to use many other species of insects as vectors. Other ways of transmission than via insect bites are by contact with infected animal tissues or body fluids via inhalation of virus. Material from abortions is highly contagious and can contaminate the ground. The virus is relatively stable in the environment, though destroyed by direct sunlight. In dry blood the virus persists for up to 3 months (FAO, 2001). The greatest risk for human infection is through inhalation of aerosol or via inoculation in case of a broken skin barrier, when having contact with infected animals or with blood and organs from infected animals (WHO, 2010).

The reason for *Aedes* being the main reservoir vector is the genus ability to sustain droughts. When feeding on blood, *Aedes* adopt the virus from infected animals and the virus can thereafter be transovarially transmitted to the mosquitos' eggs. The eggs have capacity to endure long periods of draught in the soil and hatch when rain falls again, flooding the ground. This makes the infection enzootic since the virus can be stored in eggs during dry season until next period of precipitation (Pepin *et al.,* 2010; WHO 2010). Depressions in the ground that contains water-saturated soil between rain periods are common in Sub-Saharan Africa and are called "dambos". Such habitats are important for the *Aedes* life cycle and therefore of great significance for the Rift Valley fever virus maintenance (Pepin *et al.,* 2010; Fafetine *et al.,* 2013). Outbreaks often occur after heavy rain fall, when many resting eggs hatch at the same time and the mosquito population increases explosively (Linthicum *et al.,* 1999). According to eastern African retrospective reviews there have been epidemics linked to heavy rainfall associated with El Niño- Southern Oscillation (ENSO) in cycles between 5 and 15 years (Pepin *et al.,* 2010; FAO 2012). After rainfall and start of spreading, other vectors which are dependent on fresh water availability, continue spreading the virus from newly infected to uninfected animals, for example the mosquito genera *Culex, Mansonia* and *Anopheles.* Even other blood feeding insects can contribute to spreading mechanically, for example *Culicoides, Stomyxos* and Tse-tse flies (FAO, 2003).
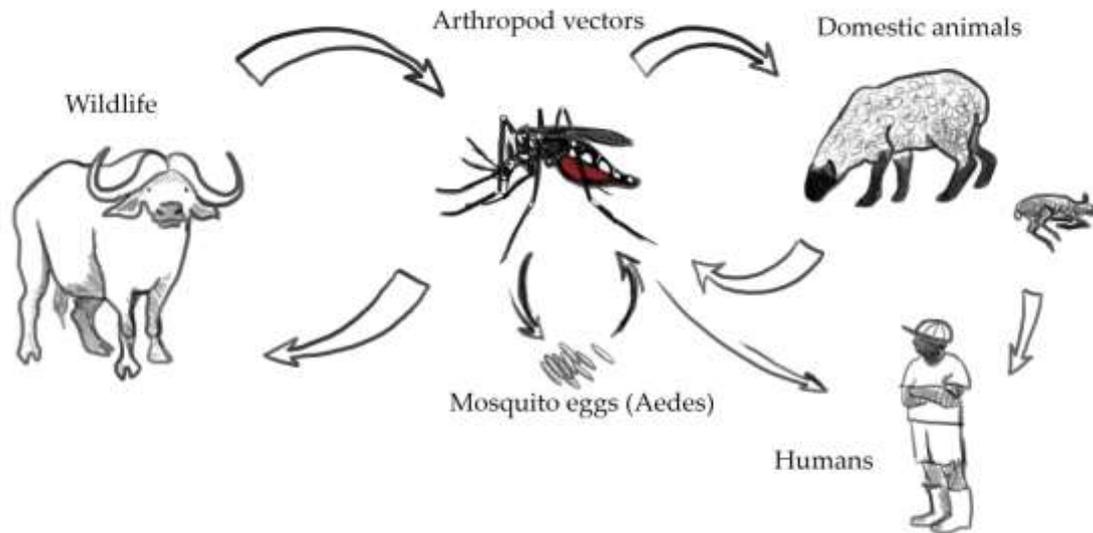
Figure 3. *Cycle of RVFV transmission. The virus is transmitted between wildlife, domestic animals and humans via arthropod vectors. In the eggs of the mosquito genus Aedes, the virus can persist through droughts. For humans a great risk for infection is contact with aborted and sick animals. Illustration: Isabelle Scharin.*

RVFV can expand to new areas with movement of infected animals and/or with windborne infected mosquitos, water or with animal products or infected material. If the new area contains vectors susceptible for the virus, an epizootic can occur when the virus spreads to the animals in the area. Outbreaks in new areas can be caused by that virus immediately infect livestock formerly free from the disease and without required immunity, spreading with mosquitos causing an epizootic/epidemic. Another possible course is that the virus first is introduced to a wild reservoir and circulates in wildlife before introduced to livestock, postponing the outbreak in livestock and humans. In the latter case, an outbreak is likely to evolve after large amounts of rain causing a rapidly increasing population of mosquitos (Ikegami, 2012; Pepin *et al.,* 2010). It seems the most important early factor in an epizootic are spreading via insects' bites, while in a later stage contamination via infected tissues could have larger impact (Pepin *et al.,* 2010). An area that has had an outbreak is considered endemic for RVF afterwards, if the environment is suitable for the virus vectors (FAO, 2012).

It has been shown that outbreaks do not only occur from one genotype of virus, but can be due to intensified transmission from several different strains already circulating in endemic areas. Translocated viruses do not have to cause an epidemic immediately but can circulate on subclinical levels and remain undetected until optimal climate conditions induce extensive proliferation (Bird *et al.,* 2008).

It is yet unknown which species constitute the main wild reservoirs for RVFV. Studies have detected antibodies in a number of wild species, but many are still left to be investigated. Since rodents are highly susceptible for the virus in laboratory experiments but do not die, they have been thought a possible wild reservoir. Data from different studies of prevalence in wild rodents in endemic areas contradict each other, why their role in maintenance of the

plague is uncertain. Wild ruminants are candidates but cannot be the only reservoir since they don't exist in the now endemic country Madagascar. Bats are reservoirs for other viruses in the family *Bunyaviridae* and would be of interest to perform further research on, according to Olive *et al.* (2010).

Clinical picture

The main symptom in ruminants in non-endemic areas is large numbers of abortions within a short time span, so called "abortion storms", in which the stage of pregnancy is irrelevant. Sheep are the most susceptible species and goats show similar symptoms. Infection of neonatal lambs is fatal in 95-100% (Ikegami, 2012). In indigenous breeds the rate of abortion rarely reaches above 30% while exotic introduced, more susceptible breeds of sheep have an abortion rate of almost 100%. The epizootic normally persists for 8-16 weeks. More symptoms of RVF can be fever, lymphadenitis, rhinitis, diarrhoea, vomiting, colic, constipation, dysgalactiae and jaundice (FAO, 2003).

In humans the vast majority of infections are asymptomatic. Of the clinical cases most people present 4-6 days after infection with influenza-like symptoms such as weakness, nausea, headache and muscle-pain. The fever lasts up to ten days and can come back several times before the person is completely recovered (Ikegami & Makino, 2011). In rare cases severe symptoms can occur, like hepatitis, chorioretinal damage, encephalitis and the often fatal course of haemorrhagic fever syndrome. Fatality of the disease in humans has differed a lot between different outbreaks but overall fatality in human is only 1-3% (Madani *et al.,* 2003; Ikegami & Makino 2011).

Diagnostics

A histopathological survey and the pattern of an outbreak can give strong indications of RVF, but since there is no unique clinical pattern for RVF, a correct diagnosis depends on reliable laboratory tests (Pepin *et al.,* 2010; Näslund 2010).

Laboraty methods for detecting the antigen are virus isolation, agar gel immunodiffusion, and different types of polymerase chain reaction (PCR). Of these, PCR is the most commonly used and quickest method. Serological tests include virus neutralisation, Enzyme-linked immunosorbent assays (ELISAs) and haemagglutination inhibition. Of these tests the virus neutralisation test is the one used to control animals and animal products for international transports (OIE, 2008).

Virus isolation gives reliable diagnoses but takes long time and is expensive, and is therefore rarely used in practise.

There are several highly sensitive PCR assays for RVFV, both RT-PCR (reverse transcription PCR) (Garcia *et al.,* 2001) real-time detection PCR (RTD-PCR) (Drosten *et al.,* 2002) and the newer technique RT-LAMP (real-time reverse-transcription loop mediated isothermal amplification) (Peyrefitte *et al.,* 2008). A diagnosis or exclusion of RVF should not rely on one single PCR-result but has to be confirmed in parallel tests, like antibody detection (Pepin *et al.,* 2010). There are several real-time RT-PCR:s developed for detection and quantification

of RVFV. Some target the NSs gene and can detect RVFV strains from all of Africa. Also broader and highly sensitive rRT-PCR which targets the L-segment has been developed. Since the amounts of virus particles in serum decreases by the time IgM appear the assay is most successful in detecting RNA if sample is taken before upcoming of IgM, which is day 1-4 of infection. There is another broadly reactive rRT-PCR targeting the S-segment (Ikegami, 2012). LAMP-assays can be useful for field diagnostics since they are performed at a constant temperature (60-65°C) and thus not require a thermal cycler. The existing LAMP-assays detect RVFV virus within 30 minutes and has shown to have very high diagnostic specificity and sensitivity (Ikegami, 2012; Peyrefitte *et al.*, 2008).

Serodiagnostics is safe to use in free areas since no active antigen is needed for the test. ELISAs and Virus Neutralization test differ to PCR in detecting which animals have been infected, even when the short viremia is over. This is convenient for monitoring the spreading of the disease (Kortekaas *et al.*, 2013). There are several different ELISA formats for detecting RVFV antibodies, and different commercial kits available (OIE, 2008). There are ELISAs detecting IgG, IgM and total antibodies. Recently developed ELISAs has been based on sucroseacetone-extracted antigens from mouse brain or tissue culture, and they have shown to be good diagnostic tools in disease surveillance and control programs, and for monitoring of immunoresponse from vaccines (Kim *et al.*, 2012; Pepin *et al.*, 2010).

To detect recent infections in animals the IgM-ELISA is sufficient. Studies have shown with experimental infections that IgM increases from day 3 and decreases after day 74. If searching for infections from a longer period IgG is more suitable since the levels rise from day 5 and still are high on day 77 after infection (Fafetine, 2007). Kortekaas *et al.* (2013) refers to many studies and conclude that IgM-ELISAs critically detect antibodies day four of infection while IgG-ELISAs can detect antibodies from day 8.

The method of diagnostics used for this seroprevalence study was a competition ELISA produced by ID-vet, detecting both IgG and IgM. Kortekaas *et al.* (2013) compared 5 commercially available ELISA-kits among one was the kit used in this study. The conclusion was that all the tests showed high sensitivity and specificity and that they were all adequate to use for serological confirmation. The sensitivities and specificities were calculated using comparison with the gold standard test – virus neutralization. In the ring study performed by Kortekaas *et al.* (2013) the ID-vet competition ELISA's sensitivity was calculated to between 91 and 100% and the specificity were 100%.

Prevention and vaccine

There is a widespread use of vaccine for animals, both from modified live attenuated virus and from inactivated viruses. One dose of attenuated vaccine is enough for life long immunity; however the liability of the live vaccine lies in that it may cause abortion in gestational animals and disease in neonates (Grobbelaar *et al.*, 2011). On the other hand the vaccine with inactivated viruses needs to be administered multiple times for adequate protection which can be an administrative problem. The so called Smithburn strain vaccine was the first attenuated vaccine. Other vaccines have been developed afterwards but even if

they are safer than the Smithburn vaccine they can still cause abortions in sensitive animals (Pepin *et al.,* 2010).

When vaccinating a population at risk it is important that the vaccination takes place before an outbreak. If vaccinations are performed during an outbreak it is likely to inflame the situation due to the risk for transmission between animals when veterinarians move between them to vaccinate, spreading virus with needles and syringes (WHO, 2010; Grobbelaar *et al.,* 2011).

Besides from the risk of abortions when using attenuated live vaccines there is a risk of the vaccine virus strain reassorting with wild virus strain if inoculated in an animal with viremia. This event adds to genetic diversity in the wild gene pool and may cause additional virulence (Ikegami, 2012). During the years of outbreaks 1969 to 1979 this seems to have happened. Studies have discovered segments with origin from the vaccine and from circulating wild lineages in the same virus proving reassortment between vaccine and wild virus (Grobbelaar *et al.,* 2011).

There is a new vaccine called Clone 13, now available for livestock in South Africa, which has shown to be safer than many earlier vaccines. Studies have shown that the new vaccine seems safe concerning abortions, teratogenic factors and pyrexia (Dungu *et al.,* 2010). Some other new attenuated vaccines which are being investigated, but not available at the market yet, have the important feature of being possible to differentiate infected from vaccinated animals (DIVA) (Wilson *et al.,* 2013). Potentially even the Clone-13 virus and two existing strains called MP-12ΔNSm and ΔP-12ΔNSm can be used as DIVA-vaccines if ELISAs targeting NS and NSs become available (FAO, 2011).

One single vaccine should be able to provide equally good protection from all current strains of RVFV if targeted on the M-segment, because of the genetic similarity of the strains (Ikegami, 2012; Grobbelaar *et al.,* 2011). This also implies for one antiviral drug targeting the RNA-dependent RNA-polymerase (L-segment), since the enzyme is similar through all strains of the virus (Ikegami, 2012). There are today no sufficient antiviral drugs for RVFV.

Other measures than vaccination can be to slow down or prevent spreading of the disease by stopping transports of animals from infected to free areas. During outbreaks, regulations and prevention of moving livestock are very useful. Another measurement to control the disease is to limit breading possibilities for the vectors. This is however very hard in times of flooding (WHO, 2010). The Food and Agriculture Organisation have objectives to set up early warning systems for Rift Valley fever, to estimate big outbreaks before they occur. That could help countries to get time to take precautions against the disease spreading. The warning system would include local surveillance and monitoring climate data, such as weather forecasting, estimating rainfall, monitoring ENSO (El Niño Southern Oscillation is a disruption in the tropical Pacific Ocean atmosphere which affects weather and climate) and even monitor vegetation dynamics for better knowledge about wildlife distribution, including arthropods and vectors of diseases (FAO, 2012).

Rift Valley Fever in Mozambique

There were reports of RVF outbreaks in Mozambique in the 60s (Valadão, 1969). After that there has been poor surveillance due to the political situation in Mozambique, having war of independence followed by civil war. Niklasson *et al.* (1987) proved endemicity of RVF in their report 1987, showing 2% seropositivity in Mozambiquan women, and in the 1990s antibodies were found in cattle (National Directorate of Livestock 2002 in Lagerqvist *et al.*, 2013).

Since there are no regular surveys performed to map and follow the spreading of RVF in Mozambique the disease is likely under-reported (Fafetine *et al.*, 2013). It is however likely that RVF is endemic in some parts of Mozambique and that sheep, goat and cattle get infected during the inter-epidemic periods (DINAP 2002 in Fafetine *et al.*, 2013).

There have been studies of seroprevalence of antibodies in goat and sheep in the Zambezia Province, conducted by the veterinary faculty of University of Eduardo Mondlane, since the conditions in the area are suitable for the ecology of RVFV (Fafetine *et al.*, 2013). The studies were cross-sectional and longitudinal. In 2007 and 2010 cross-sectional studies from five districts in Zambezia showed that the adjusted overall seroprevalence of IgG antibodies in sheep was 35.8% and in goats 21.2% in 2007 compared to 9.2% in sheep and 11.6% in goats in 2010. Samples in 2010 were taken from two districts out of five that had the highest seroprevalence in 2007. Why the seroprevalence turned out to be lower in the later study might be due to less precipitation. The average amount of rain per month in 2007 was 150mm compared to 80mm in 2010 (Fafetine *et al.*, 2013). Other factors that may have caused the difference could be climate, agro-ecological conditions and/or sampling strategies. The diagnostic procedure was the same in both studies (Fafetine *et al.*, 2013).

The study by Fafetine *et al.* (2013) in 2007 and 2010 showed that infections had occurred even during dry season, but that they had probably been mild or subclinical since no symptoms had been noticed by the farmers. The presence of antibodies in sheep and goats proved inter-epidemic circulation of RVF in Zambezia, Mozambique (Fafetine *et al.*, 2013).

Metagenomics

Since arthropod vectors have an important role in spreading viruses which can cause EID:s it is of interest to investigate their viral flora. Metagenomics is a good method to search for all known and unknown viruses in samples such as vectors. Chen and Patcher (2005) has in their review article defined metagenomics as the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species.

The great advantage with using metagenomics is that it does not require previous knowledge of the genome searched for. Compared to other methods for sample analyses, the perspective is wide. You don't have to use specific sequences to detect a target as with PCR, and there's no need for being able to cultivate the pathogen. Instead metagenomics make it possible to study all genomes in a sample and find known as well as yet unknown DNA and RNA viruses (Delwart, 2007).

Metagenomics techniques make it possible to take a sample from the environment, extracting the DNA/RNA from the sample, sequence all genetic material in the sample and thus investigate genetic composition of the sample. The technique has during the last ten years been used to study viral populations in several different environments, for example seawater (Breitbart *et al.,* 2002), faeces (Breitbart *et al.,* 2003) and bats qua virus reservoirs (Li *et al.,* 2010).

The basic principles of Metagenomic techniques used in this study are as follow:

1. Sample preparation

Preparation of the samples is the first step before sequencing is possible. It is crucial to prepare the samples to get rid of host nucleic acid to be able to find viral nucleic acid. There is need for separation of the virus particles from the cells, and/or to degrade the host's nucleic acid and concentrate the viral genome. This can be done using centrifugation, filtration and nuclease treatment (Thurber *et al.,* 2009; Delwart, 2007). The samples can be pooled to increase the chance to find virus and ultracentrifugation can be used for concentration of virus (Blomström, 2010). If the scope is to find viruses only, a filter can be used to remove larger cells such as bacteria. That was not done in this study since bacteria also are of interest, and there are some viruses that are almost as large as bacteria (Van Etten *et al.,* 2010). Nucleases (DNase and sometimes RNase) and are often used to degrade the hosts nucleic acid. The method depends on that the viral nucleic acids are protected by a viral capsid, which is the case for most viruses but not all (Allander *et al.,* 2001). It is difficult to degrade the host ribosomal RNA and there are different methods to achieve this, although all have their drawbacks. With nuclease treatment it is possible to lower the amount of host nucleic acid enough to get the viral nucleic acid part large enough for detection (Blomström, 2010).

2. Sequence-independent amplification: Random PCR

This step aims to multiply all nucleic acid from the sample with the intention to show the genetic composition of the whole sample (Ambrose & Clewley, 2006). There are different methods for achieving this, in this study Random PCR was used. Before the random PCR is run, the extracted DNA and cDNA (from RNA) need to be labelled. The label is a known adaptor sequence tagged in each end of the RNA/cDNA and these tags are the targets for the random PCR's primers (Blomström, 2010).

3. Large scale sequencing

Sequencing is the way to annotate the nucleic acid in the samples. The method used for sequencing data in metagenomics has to be able to sequence on a very large scale to find the viral nucleic acid, since it is likely that the host nucleic acid left in the sample still are a much bigger part of the genetic material than the viral nucleic acid. Several different technologies exist for this step. Traditionally the PCR-products have been cloned into bacteria for creating libraries which can be characterized with different methods. The last decade more efficient methods have been developed, these are called next-generation sequencing or high

throughput-sequencing and they outcompete earlier techniques by far if comparing daily through put of sequencing and costs of the sequencing (Kircher & Kelso, 2010).

4. Bioinformatics

This step refers to analysing the vast amount of data, received from the sequencing. This is one of the most difficult parts in metagenomics since the information given can include different species, incomplete genomes and the sequences can be diverged compared to sequences in the database. Wooley *et al.* (2010) call the data "noisy and partial". It can also be difficult to find new viruses since they cannot be compared to anything in the database. Different programs exist and many new are being developed as this paper is written.

5. Follow-up of findings and causations

A follow-up of a metagenomic study is often desirable. Depending on what was found, the follow up can involve different methods. Indications of discoveries need to be followed up to be confirmed or further investigated. This can include further genetic characterisation, prevalence studies and different diagnostic methods, many mentioned in the previous chapter "Rift Valley fever – diagnostics".

MATERIAL AND METHODS

Blood sampling

The samples for this study has been taken in two of the province Zambezia's 16 districts; Nicoadala and Mopeia, and close to the capital of the province; Quelimane. Of the 8 farms sampled, 3 were in Nicoadala, 3 in Mopeia and 2 adjacent to Quelimane (fig 4).
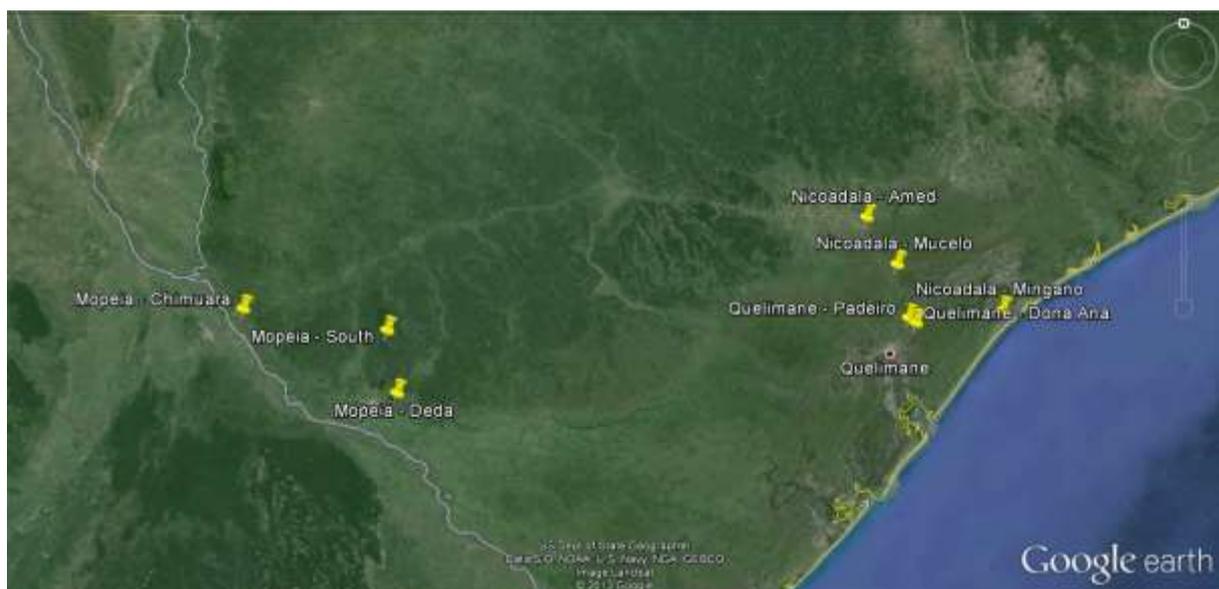

Figure 4: *Map over Zambezia with yellow pins showing the location for each sampled farm.*

The farms chosen for sampling had more animals than average farms of the province. This made it possible to gather more samples during the time-limited field trip. Data from the farms Amed and Chimuara were possible to compare to earlier studies from year 2010. The other farms were chosen by the Mozambiquan Veterinary Services of Zambezia Province. Before starting the field study we visited the Official Veterinary Service of the province to get allowance to perform studies in their area. They provided us with a technician who directed us to farms and helped with some practical work.

Blood were collected from 335 goats and 401 sheep in total. The animals were bled from the jugular vein into serum tubes, using vacutainer (fig 5). The serum was later transferred from the serum tubes into Eppendorf tubes which were marked with a number for each individual animal.

No blood from bovines was collected for the RVF study, since most cattle is vaccinated in Zambezia and the seroprevalence cannot be differentiated between natural infection and antibodies from vaccine strains of virus.

Figure 5. *Blood sampling at two different farms. Photo: Isabelle Scharin and Hedvig Stenberg*

At all visited farms the animal keeper was interviewed about the animal health in the farm. They were asked about mortality during the last year in different age categories, if they had noticed any specific symptoms and some more specific questions about symptoms of RVF, like abortions and foetal malformations. They were also asked about vaccination routines in the cases they had cattle.

Laboratory analyses - serology

Serum from 187 goats and 181 sheep were analysed with a competitive ELISA, thus searching for both IgG and IgM. It was not possible to test all samples due to the number of available ELISA-kits. 50 µl undiluted serum was used for the analyses. The analyses were performed in the serology lab at UEM: s veterinary faculty. "ID Screen Rift Valley Fever Competition Multi-species" (ID-Vet) was used according to the manufacturer's instruction (fig 6). This ELISA detects antibodies directed against the RVFV nucleoprotein and indicates antibodies caused by natural infection as well as vaccination.

Figure 6. *ELISA-plate as used in this study. Photo: Isabelle Scharin.*

The serum samples were only tested in one well each. Optical density of 450 nm was used to read the results. The results were analysed in Excel and validated and interpreted according to the protocol provided by the manufacturer.

Statistical analyses of results from serology

The results were organized and processed in Excel. Chi-square tests were used to compare the differences in seroprevalence between this and an earlier studies in the area, between genders, between age groups and between different farms. A confidence interval of 95% was used.

Vector sampling

Mosquito collection was performed at the farm Chimuara, which lay close to fresh water and constituted a suitable environment for mosquitos. There were also trials on other areas for mosquito collection, though they turned out futile due to too much wind during the time the traps were set.

The traps were active from dusk to dawn and all mosquitos were collected during 5 nights. The traps used were battery driven light-traps of the brand "Clarke", designed for adult mosquitos, with preference for Anopheles (personal communication Ana Paula Abílio, entomologist at National Institute of Health, Maputo). When turned on, a lamp attracted the mosquitos and a fan blew them down into a net cage beneath. Between 3 and 4 traps were used simultaneously and put up in different places, some in houses of humans, and some by the animal houses where the goats and sheep stayed during night time. Except from the first night, the traps were only put up by the animal houses since that showed to give the by far biggest catch. The mosquitos were identified and divided between the genus *Culex, Mansonia* and *Anopheles* within days after capture.

Ticks were collected by hand from the bodies of cattle, goat and sheep during the episodes of blood sampling. Ticks from each animal individual got its own number, and blood from the same animal was dropped onto an FTA-card, making it possible to correlate tick to animal individual. The ticks were identified to species at the veterinary faculty of EMU.

Both mosquitos and ticks were stored in ethanol 99.8% until time for preparation.

Laboratory analyses – preparation for metagenomics

The sequencing and bioinformatic analysis of the DNA and RNA prepared from the Mosquitos and ticks were not done before this report was written. However the preparation of the samples was performed at the UEM:s laboratories.

- Pre-preparation

20 mosquitos of each genus: *Anopheles, Culex* and *Mansonia*, were put in 3 different Eppendorf tubes. The ticks were put in a separate Eppendorf tube from each host (n 1-6). 47 samples of ticks were chosen due to the number of templates we had access to for DNA extraction. One sample derived from water buffalo, 9 from Zebu cattle, 16 from goat and 21 from sheep. Favoured were samples with many ticks for the purpose of getting as much viral material as possible from each sample, second to that care was taken to choose samples from different farms.

Before the ticks and mosquitos were transferred to the new tube they were washed in distilled water. To each tube between 500 and 900 µl DNase buffer (1x) was added and the samples were homogenised with a battery driven homogeniser (VWR). After homogenisation the samples were centrifuged for 5 minutes at 4000 rpm. In some samples with a big volume solid material, more DNase buffer was added and the samples centrifuged once more. The supernatant were then transferred to a clean tube and centrifuged again in 10 minutes at 4000 rpm.

The supernatant was then divided into 4 new tubes for each mosquito species, and 2 new tubes for each tick sample. Half of the tubes contained 250 µl and 750 µl trizol were added and saved in a freezer that holds -80° C, these were sent to Sweden for later RNA-extraction. The reason not to extract the RNA in Maputo was that RNA is too unstable to transport. The other half of the tubes contained 200 µl and was prepared for DNA extraction. To each of these tubes 2µl RNase A and 100 U DNase I were added. The tubes were shortly vortexed before incubation in 37° C water bath in 1, 5 – 2 hours.

- DNA-extraction

A protocol for QIAamp DNA mini kit was followed. At the elution step the samples were eluted in 50 µl elution buffer. The DNA was stored in $-20°C$ until further use.

- DNA labelling

All DNA from the mosquito samples and some of the DNA from the tick samples were labelled at the veterinary faculty of the UEM. Due to problems with equipment at the laboratory the rest of the DNA were sent to Sweden before labelling for continued progress at the Swedish University of Agricultural Sciences. For labelling, a mastermix was made of 2 µl FRoV26/N (10 µM) (Allander *et al.,* 2005), 1.5 µl dNTP (10mM) and 1.5 µl NEB2 buffer (10X) for each sample. The mix was blended by pipetting and added 10 µl DNA of each sample. The samples were stored in 94° C for 2 minutes, then put on ice and 0.5 µl Klenow Fragment (3'-5' exo-) were added. The samples were stored in 37° C for 60 minutes, then

94°C for 2 minutes and then on ice where the same amount Klenow fragment was added once more. Last step in labelling was storing in 37° C for 60 minutes, finishing with 75° C for 10 minutes. The labelled DNA was incubated in – 20°C until further use.

- Random amplification of labelled DNA

2,5 µl of the previously labelled DNA were added to a mastermix of 5 µl buffer (10x), 5 µl MgCl$_2$ (25 mM), 1 µl dNTP (10mM), 4 µl FR20 (10mM), 0,5 µl AmpliTaque Gold Polymerase (5U/ µl) and 32 µl H$_2$O. For each labelled DNA sample 5 separate reactions were run. The samples were put in a PCR machine in UEM:s PCR laboratory and run with following program: 95°C 12 min; 40 cycles of - 95°C 30 sec, 58°C 30sec, 72°C 90 sec; and a final 10 min elongation step at 72°C.

Gel electrophoresis: The received PCR-products were then run on 1.5% gel in 130V for 25 minutes as a test to see if the PCR has amplified enough DNA.

- Still to be done before large scale Sequencing

For complete preparation for metagenomics, RNA need to be extracted from the samples stored with trizol and all RNA and remaining DNA need to be labelled and amplificated in prior to purification and cleaving of the PCR products. This will take place at the Swedish University of Agricultural Sciences in Uppsala in early 2014.

RESULTS

Rift Valley Fever Serology

The number of samples analysed from each farm, and the distribution in sex and age categories are shown in table 1 and 2. The districts names Mopeia, Nicoadala and Quelimane, are put in front of the farm-names.

Table 1. *Number of goats in different categories for each farm. Only farms with tested n > 30 are included*

Farm	n	Gender		Age distribution (month)		
		Female	Male	0-6	6-12	>12
Mopeia - Chimuara	47	31	16	2	25	20
Nicoadala - Amed	40	35	5	10	11	19
Nicoadala - Mingano	33	25	8	5	8	20
Quelimane - Dona Ana	32	18	14	3	8	21
Quelimane - Padeiro	30	23	7	7	3	20

Table 2. *Number of sheep in different categories for each farm. Only farms with tested n > 30 are included*

Farm	n	Gender		Age distribution (month)		
		Female	Male	0-6	6-12	>12
Mopeia - Chimuara	42	32	10	3	15	24
Mopeia - South	33	16	17	1	7	25
Nicoadala - Mucelo	66	46	20	24	18	24
Quelimane - Padeiro	32	23	9	2	6	24

Of the total 368 animals tested with the ELISA-kit, 127 tested positive for antibodies against RVFV, which gives an overall seroprevalence for all sampled animals of 34.5%. The seroprevalence in goats were 25.1% and in sheep 44.2%. Table 3 shows the total numbers of tested animals divided into sex and age groups, and the seroprevalence for each group.

Table 3. *RVF seroprevalence divided by species, sex and age. All samples analysed are included. The difference between genders were not statistically significant while the differences between age categories were significant (p<0.05)*

			Total sampled	No. Positive	Seroprevalence (%)
Goats	All		187	47	25.1
	Sex	Female	135	33	24.4
		Male	52	14	26.9
	Age	0-6 months	29	6	20.7
		6-12 months	55	4	7.3
		>12 months	103	37	35.9
Sheep	All		181	80	44.2
	Sex	Female	123	60	48.8
		Male	58	20	34.5
	Age	0-6 months	31	5	16.1
		6-12 months	46	11	23.9
		>12 months	104	64	61.5

The seroprevalence varied between 7.5% and 41.9% in goats if excluding farms with less than 30 animals tested. The seroprevalence in sheep varied between 19.7% and 90.9% between the different farms (table 4).

Table 4. *RVF seroprevalence by farm. The differences between the farms with ≥30 samples showed to be highly significant (p<0.05)*

Farm	No. goats	Seropositive (%)	No. sheep	Seropositive (%)
Mopeia - Deda	5	60.0	-	-
Mopeia -Chimuara	47	21.3	42	40.5
Mopeia - South	-	-	33	90.9
Nicoadala - Mucelo	1	0.0	66	19.7
Nicoadala - Amed	40	7.5	8	37.5
Nicoadala - Mingano	33	26.5	-	-
Quelimane - Dona ana	32	40.6	-	-
Quelimane - Padeiro	30	30.0	32	53.1

When comparing with the results from the earlier studies by Fafetine *et al.* (2013) from the farms Chimuara and Amed, the results in this study show that the seroprevalence in goats have not changed significantly since 2010 (fig 7). In the district Nicoadala (farm Amed) the seroprevalence have dropped from 61.9% (n=42) in 2007 to 10.6% (n=66) in 2010 to 7.5% (n=40) in this study. In the farm Chimuara no study was performed 2007, but the results in seroprevalence 2010 were 21.4% (n=140) and in 2013 21.3% (n=47).
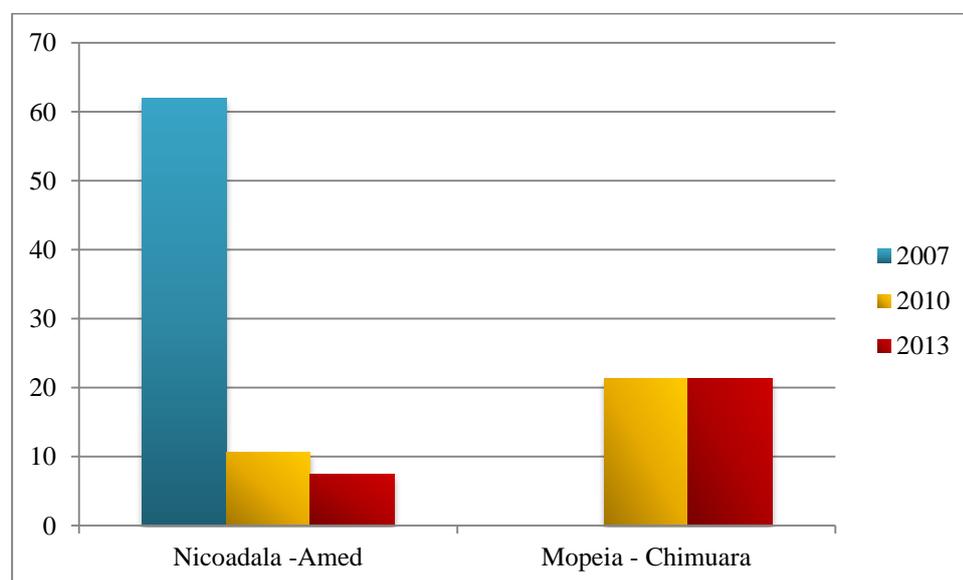

Figure 7. *Percentage of seropositive goats in 2007 where data exists, 2010 and 2013. Data from 2007 and 2010 are taken from Fafetine et al. 2013. The differences between 2010 and 2013 could not be proven statistical significant in any of the farms (p<0.05).*

In district Mopeia, farm Chimuara, the seroprevalence have increased in sheep from 21.7% (254) in 2010 (Fafetine et al., 2013) to 40.5% (n=42) in 2013 (fig 8).
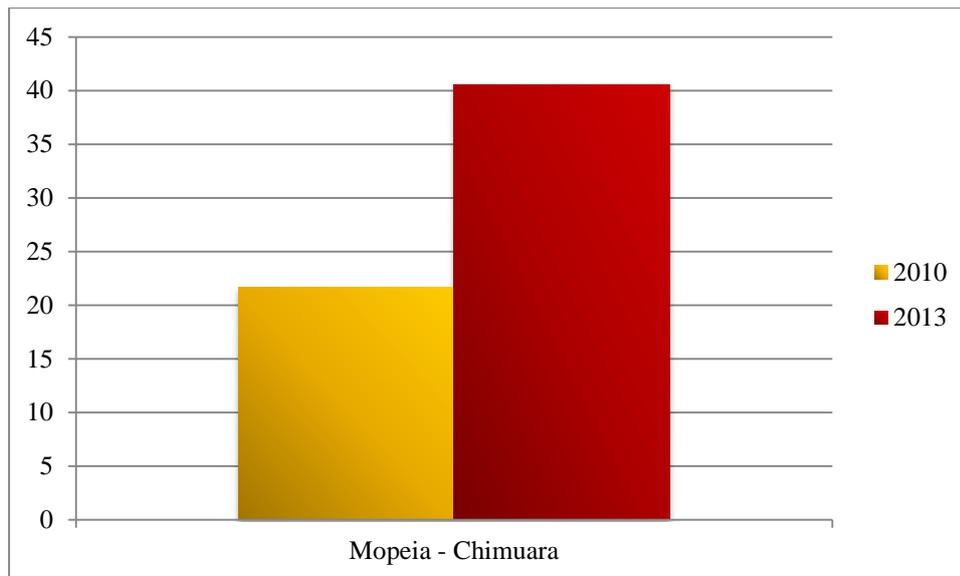
Figure 8. *Percentage of seropositive sheep in 2010 and 2013. Data from 2010 were taken from Fafetine et al. 2013. The differences between 2010 and 2013 proved to be statistical significant (p<0.05).*

Results from the interviews

All farms had some cases of death among their animals during 2013, both in sheep and goats, and in mixed ages. The animal keepers had different explanations to the causes of the mortality and disease seen in their herds; most of them did not seem to match the symptoms characterizing RVF. Only three farms reported any cases of abortions the last year; Nicoadala- Mucelo had late abortions in 5 sheep and 4 cattle, the animal keeper thought it was due to starvation. In Nicoadala – Mingano, the animal keeper told of 4 early abortions in goats and 2 in cattle and that some neonatal goats have died, presumably due to lack of milk from the mother. In Quelimane- Dona Ana, one cow had an abortion the week before the interview. No farm had seen any malformed foetuses. Of the farms with abortions the farm Mucelo and Mingano vaccinates their cattle for RVF, though not this year, and the farm Dona Ana vaccinates against something but they were unsure of what.

Preparations for metagenomics

Though not many results from this study can be presented in this paper due to lack of time for the lab-work, the results from the gel electrophoreses after random PCR can be shown.

This thesis focuses on the diseases and results of the tick-samples, and my co-worker in this project, Hedvig Stenberg, presents the results from the mosquito samples (see her MSc Thesis). Gel electrophoresis was run on the 4 samples amplified with PCR; two from cattle, and two from sheep. Every sample showed a somewhat short smear (fig 9).
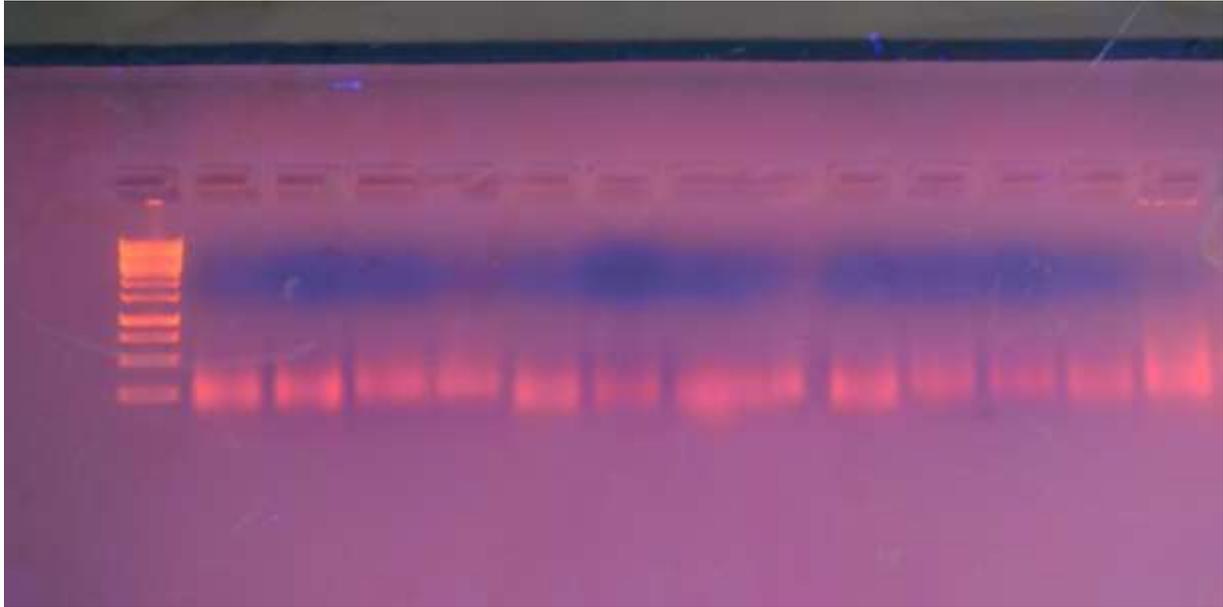
Figure 9. *Gel electrophoresis on PCR-products from tick samples. From the left:*
1: Ladder 100bp
2-14: Samples from ticks, nr 2,5,6,7 from one sample, nr 2, 8, 9 from one, nr 3, 10, 11, 13 from one and 4 and 12 from one.

The samples were sent to Sweden in the middle of December 2013. Researchers at Swedish University of Agricultural Sciences, at the Section of Virology, Department of Biomedical Sciences and Veterinary Public Health, will complete the metagenomic study in the beginning of 2014.

DISCUSSION

Rift Valley Fever

The serological study of Rift Valley fever antibodies in goats and sheep show some changes compared to earlier studies in the area. It does also show differences between different groups of sampled animals in this study. The differences and changes in seroprevalence and some possible causes are discussed below.

The overall seroprevalence from this study in Zambezia was 25.1% in goats and 44.2% in sheep. In 2010 the seroprevalence was 11.6% in goats and 9.2% in sheep (Fafetine *et al.,* 2013), and the seroprevalence thus seems to have increased with 13.5% and 35% for goat respectively sheep. The chi-square test on the difference in seroprevalence between 2010 and this study 2013 showed high significance with $p<0.001$. All samples in 2010 were taken in the Mopeia and Nicoadala districts (Fafetine *et al.,* 2013). Compared to the studies in Zambezia in 2007 with the results of 21.2% seroprevalence in goats, and 35.8% in sheep (Fafetine *et al.,* 2013), the prevalence seems to have increased with a few percent in both species.

The precipitation average in Quelimane in 2013 (not including December) has been 117mm per month, compared to 80mm in 2010, and 150mm in 2007. The average for 2013 will probably be higher when December can be included since it is a month in the rain period. The average monthly precipitation for January to April in 2013 was 333mm compared to 114mm in 2010 and 210mm in 2007 (Instituto Nacional de Meterologica, Maputo Mozambique, 2013). This indicates that the hypothesis of a rise in seroprevalence connected to more precipitation, and thus more vectors and viral circulation, could be true. Comparing the seroprevalence in Zambezia with a recent study of inter-epidemic transmission of RFV in Kilombero River Valley, Tanzania, Zambezia seems to have a rather high seroprevalence. Sumaye's *et al.* (2013) study showed an overall prevalence in goats of 11.9% and in sheep 11.4%.

The farms Chimuara and Amed sampled in this study were also sampled in 2010, and Amed even in 2007 (Fafetine *et al.,* 2013). Figure 7 and figure 8 show the changes in seroprevalence between the studies. Concerning goats, the figure 7 shows a big drop in seroprevalence between 2007 and 2010 in farm Amed, which in Fafetine *et al.* (2013) was explained as a possible cause of decrease in precipitation from 2007 to 2010. The drop with 3% from 2010 to 2013 does not show statistical significance. In the farm Chimuara the seroprevalence is almost exactly the same in goats between 2010 and 1013. Thus it seems the seroprevalence in goats in the two comparable farms had been rather stable the last three years. The situation is somewhat different concerning sheep. The figure 8 shows a rise in seroprevalence with 19%, proven statistically significant. One explanation for the rise in seroprevalence in sheep could be the earlier mentioned increased precipitation. It is difficult to explain why the seroprevalence has not changed in the same direction for goats but a theory could be that since sheep have shown more sensibility to the infection (Woods *et al.,* 2002) they are more easily affected by a higher infection pressure.

In the studies in 2007 and 2010 an IgG ELISA were used. It should be feasible to compare with the competitive ELISA used in this study that detects both IgG and IgM. If anything, this study would detect some more infected animals due to that this method also detect the newly infected which has not yet developed IgG antibodies. It is unlikely though, that this should affect the figures significantly, especially since the samples were taken in the end of the dry period when the transmission rate presumable is in a low stadium and thus the animals probably mostly have IgG antibodies.

The seroprevalence in this study is higher in sheep than in goat in every farm sampled, and hence show the same tendency as the studies of Fafetine *et al.* (2013) in Zambezia. From past epizootics, the experience is that sheep are the most susceptible species (Woods *et al.,* 2002). Engströms (2012) seroprevalence study in Gaza, Mozambique in 2011 was showing the opposite. In that study 11% of sheep and 21% of goats were positive, Engström mentions that the higher seroprevalence in goats might have to do with the higher number of goats in the sampled area.

In both goats and sheep this study shows the highest seroprevalence in the adult animals, thus showing the same results as earlier studies (Fafetine *et al.,* 2013; Engström, 2012) which can

be explained by that the animals risk of receiving the virus increases with time. In sheep the seroprevalence follow the pattern of increasing with age, although in goats the age category

0-6 months has a higher seroprevalence than the category 6-12 months. The higher percentage of seroprevalence in the youngest goats than in the ages 6-12 months could be related to maternal antibodies (FAO, 2011). It is unclear what makes the sheep and goats different in this subject. One theory is that the goats in the category 0-6 months were mainly younger individuals than the young tested sheep, and thereby most of the maternal antibodies in the young sheep depleted.

A remarkable thing seen when comparing seroprevalence in the different farms was the significant big differences between the farms, even within the same districts. This study showed a span between 7.5% and 40% between different farms for goats, and between 19.7% and 90.9% for sheep, not including farms with less than 30 samples (table 4). This is not the only study to notice a big difference in prevalence between different farms. Cêtre-Sossah *et al.,* (2012) saw prevalence between herds of goat vary between 35 and 88% in Mayotte, an Island between Mozambique and Madagascar. Some of the differences can be explained by having a different proportion of young and adult animals in the different farms, but not all. Even when taken into account this factor there are big differences between many farms. The differences does not either follow a geographical pattern, comparing table 4 with the map (fig 4) shows that the farms closer to each other do not have a more similar seroprevalence results. Possible reasons for the big differences between farms would be interesting to investigate further.

According to the interviews with the animal keepers, they have not experienced problems with symptoms typical for RVF. The farms with some cases of abortions did not have higher seroprevalence than the rest of the farms. One sampled goat had recently had an abortion before the blood sampling, and she tested negative for RVF in the ELISA. This study indicates that RVFV circulates mostly on a sub-clinical level in sheep and goats in Zambezia. All sampled animals in Zambezia seemed to be of indigenous breeds, which could contribute to a low display of symptoms even if the transmission rate is rather high. Imported breeds, exotic for the area, are in general much more susceptible for the RVFV than breeds from the tropical and subtropical areas of Africa (FAO, 2003).

Suggestions for further research of Rift Valley fever in Zambezia

It would be easier to declare significant differences in prevalence over time if following the same farms to a bigger extent than done in this study. It would also help to use a more strict protocol of how many animals in each age group that is to be tested from each farm.

As earlier mentioned it would be very interesting to investigate why the seroprevalence differs so much between the sampled farms. The result seems to indicate that neither distance between farms, nor geographical area can predict seroprevalence of a randomly selected farm. Likely, there are more important factors probably overlooked in the present study, influencing the results in a more significant way. Examples of such factors could be closeness to fresh

water in the dry period, or "dambos" i.e. a good environment for vectors of RVF, if vaccination of cattle in the neighbourhood matters for transmission to goats and sheep, size of herds, closeness to wild fauna etc.

Metagenomics

The only results of the metagenomic study of tick-borne viruses presentable at time for presentation of this thesis, is the gel electrophoresis of the received PCR-product of four samples of ticks. The smears seen in figure 9 are about 100bp in molecular weight, which is of lower molecular weight than expected. This could be due to somewhat degraded genetic material. Ticks among other arthropods are known to have some PCR-inhibiting substances.

Sources of errors

A source of error concerning the study of RVFV was the uncertainty in age in some of the animals. It happened regularly that the animal keepers argued about some animals age, and the feeling during the field trip was that the judgement of age differed between different farms and that several of the animals were indiscriminately put in an age category. This could affect the statistics of prevalence in the different age categories negatively.

One Source of error in the metagenomic study were when labelling the DNA gained was about half as big a volume as it should be. Most likely the cause was not calibrated, or of other reasons not functional, pipettes. This was the reason for sending the rest of the tick-DNA samples to Sweden for continued lab work. Another source of error was during the random PCR of the four labelled tick samples, the lids of some tubes opened during the PCR process and some of the PCR product were lost to steaming. This was probably caused by a mistake when adjusting the settings of the PCR machine its lid were closed in a setting for another size of tubes.

Conclusions

In conclusion, this study of seroprevalence of RVFV in Zambezia, Mozambique shows an existing inter-epidemic circulation of the virus. The disease seems to have a subclinical course in most animals and causes, according to interviews, in present no obvious problems for farmers and people in the area. It seems that the overall prevalence could be related to the amount of precipitation, the infection rate increasing with a higher amount of rain, although this should be regarded with caution since the studies were not performed at the exact same places in the province and other factors may be overlooked.

This study raises some new questions about RVF in Zambezia and Mozambique. It does not explain why the prevalence has increased for sheep when remaining constant in goats in the comparable farms, or why there was such a big difference in seroprevalence between different farms. It would be interesting to perform further research based on these questions. It is also important to continue surveillance of RVFV in Mozambique to see if it expands, to be able to foresee and possibly prevent outbreaks. Concerning the metagenomic study, different kinds of follow-up studies could be in order, depending on the findings when the study is complete.

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