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The impact and control of malignant catarrhal fever in Tanzania

Felix John Lankester BVSc MSc

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy Institute of Biodiversity, Animal Health and Comparative Medicine



College of Medical, Veterinary and Life Sciences University of Glasgow United Kingdom April 2016

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I Abstract

Malignant Catarrhal Fever (MCF), an often-lethal infectious disease, presents as a variable complex of lesions in susceptible ungulate species. The disease is caused by a γ -herpesvirus following transmission from an inapparent carrier host. Two major epidemiological forms exist: wildebeest-associated MCF (WA-MCF), in which the virus is transmitted to susceptible species by wildebeest calves less than approximately four months of age, and sheepassociated MCF (SA-MCF) in which the virus is spread by sheep (primarily adolescents). Due to the lack of an *in-vitro* propagation system for the causative agent of the more economically significant SA-MCF, and with the expectation that cross-protective immunity may be provided, vaccine development has focused on the more easily propagated alcelaphine herpesvirus-1 (AlHV-1) that causes WA-MCF. In 2008 a direct viral challenge trial showed that a novel vaccine, employing an attenuated AIHV-1 (atAIHV-1) 'C500' virus strain, protected British Friesian-Holstein (FH) cattle against an intranasal challenge with virulent AlHV-1 'C500' virus. For cattle keeping people living near wildebeest calving areas in sub-Saharan Africa an effective vaccine would have value as it would release them from the costly annual disease avoidance strategy of having to move their herds away from the oncoming wildebeest. On the other hand, an effective vaccine will release herd owners from the need to avoid MCF, allowing them to graze their cattle alongside wildebeest on the highly nutritious pastures of the calving areas. As such conservationists have raised concerns that the development of a vaccine might lead to detrimental grazing competition.

The principle objective of this study was to test the novel vaccine on Tanzanian shorthorn zebu cross cattle (SZC). We did this firstly using a natural challenge field trial (Chapter Two) which demonstrated that immunisation with the atAlHV-1 vaccine was well tolerated and induced an oro-nasopharyngeal AlHV-1-specific and -neutralising antibody response. This resulted in an immunity in SZC cattle that was partially protective and reduced naturally transmitted infection by 56%. We also demonstrated that non-fatal infections occurred with a much higher frequency than previously thought. Because the calculated efficacy of the vaccine was less than that seen in British FH cattle we wanted to determine whether host factors, particular to SZC cattle, had impacted the outcomes of the field trial. To do this we repeated the 2008 direct viral challenge trial using SZC cattle (Chapter Four). During this trial we also investigated whether the recombinant bacterial flagellin monomer (FliC), when used as an adjuvant, might improve the vaccine's efficacy. The findings from this trial indicated that direct challenge with pathogenic AlHV-1 is effective at inducing MCF in SZC cattle and that FliC is not an appropriate adjuvant for this vaccine. Furthermore, with less

control group cattle dying of MCF than expected we speculate that SZC cattle may have a degree of resistance to MCF that affords them protection from infection and developing fatal disease. In Chapter Three we investigated aspects of the epidemiology of MCF, specifically whether wildebeest placenta, long implicated by Maasai cattle owners as a source of MCF, might play a role in viral transmission. Additionally, through comparative sequence analysis, at two specific genes (A9.5 and ORF50) of wild-type and atAlHV-1, we investigated whether the 'C500' strain, the source of which was taken from Africa more than 40 years ago, was appropriate for vaccine development. The detection of AlHV-1 virus in approximately 50% of placentae indicated that infection can occur in-utero and that this tissue might play a role in disease transmission. And, despite describing three new alleles of the A9.5 gene (supporting previous evidence that this gene is polymorphic and encodes a secretory protein with interleukin-4 as the major homologue), the observation that the most frequently detected haplotypes, in both wild-type and attenuated AlHV-1, were identical suggests that AlHV-1 has a slow molecular clock and that the attenuated strain was appropriate for vaccine development. In Chapter Five we present the first quantitative assessment of the annual MCF avoidance costs that Maasai pastoralists incur. In particular we estimated that as a result of MCF avoidance 64% of the total daily milk yield during the MCF season was not available to be used by the 81% of the family unit remaining at the permanent boma. This represents an upper-bound loss of approximately 8% of a household's annual income. Despite these considerable losses we concluded that, given an incidence of fatal MCF in cattle living in wildebeest calving areas of 5% to 10%, if herd owners were to stop trying to avoid MCF by allowing their cattle to graze alongside wildebeest, any gains made through increased availability of milk, improved body condition and reduced energy demands would be offset by an increase in MCF-incidence. With the development of an effective vaccine, however, this alternative strategy might become optimal.

The overall conclusion we draw therefore is that, despite the substantial costs incurred each year avoiding MCF, the partial protection afforded by the novel vaccine strategy is not sufficient to warrant a wholesale change in disease avoidance strategy. Nonetheless, even the partial protection provided by this vaccine could be of value to protect animals that cannot be moved, for example where some of the herd remain at the boma to provide milk or where land-use changes make traditional disease avoidance difficult. Furthermore, the vaccine may offer a feasible solution to some of the current land-use challenges and conflicts, providing a degree of protection to valuable livestock where avoidance strategies are not possible, but with less risk of precipitating the potentially damaging environmental consequences, such as overgrazing of highly nutritious seasonal pastures, that might result if herd owners decide they no longer need to avoid wildebeest.

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IV List of Publications

Chapter Two

Lankester FJ, Russell GC, Lugelo A, Ndabigaye A, Mnyambwa N, Keyyu J, Kazwala R, Grant D, Percival A, Deane D, Haig D & Cleaveland S. A field vaccine trial in Tanzania demonstrates partial protection against malignant catarrhal fever in cattle. Vaccine 2016;34:831?8. doi:10.1016/vaccine.2015.12.009.

Chapter Three

Lankester FJ, Lugelo A, Mnyambwa N., Ndabigaye A, Keyyu J, Kazwala R, Grant D, Relf V, Haig D, Cleaveland S & Russell GC. Alcelaphine herpesvirus-1 (malignant catarrhal fever virus) in wildebeest placenta: genetic variation of ORF50 and A9.5 alleles. PLoS One 2015;10. doi:10.1371/journal.pone.0124121.

Chapter Four

Lankester FJ, Lugelo A, Werling D, Mnyambwa N, Keyyu J, Kazwala R, Grant D, Smith S, Parameswaran P, Cleaveland S, Russell GC & Haig D. The efficacy of alcelaphine herpesvirus-1 (AlHV-1) immunization with the adjuvants EmulsigenTM and the monomeric TLR5 ligand FliC in zebu cattle against AlHV-1 malignant catarrhal fever induced by experimental virus challenge. Vet Micro. 2016.

Chapter Five

Lankester FJ, Lugelo A, Kazwala R, Keyyu J, Cleaveland S & Yoder J. The economic impact of malignant catarrhal fever on pastoralist livelihoods. PLoS One 2015;10:1?18. doi:10.1371/journal.pone.0116059.

V Approval

Chapter Two

The research was carried out with the approval of the Tanzanian Wildlife Research Institute (TAWIRI), the Commission for Science and Technology (COSTECH, Tanzania) and the Tanzania Food and Drug Administration (permit nos. 2011-213-ER-2005-141 and 2012-318-ER-2005-141). The vaccination trial, including immunization, sampling, clinical scoring and criteria for euthanasia after onset of MCF (Russell et al., 2012) followed protocols established during trials at the Moredun Research Institute, UK and were compliant with the Home Office of Great Britain and Northern Ireland 'Animals (Scientific Procedures) Act 1986' under project licence PPL 60/3839.

Chapter Three

The animal ethics committees of the Tanzanian Wildlife Research Institute (TAWIRI) and the Commission for Science and Technology (COSTECH, Tanzania) approved all aspects of this study, including all sampling procedures and the animal research that was conducted according to international guidelines (permit nos.2011-213-ER-2005-141 and 2012-318-ER-2005-141). The Ngorongoro Conservation Area Authority and the Tanzanian National Parks Association approved immobilization (using etorphine hydrochloride loaded pressure darts) and sampling of wildebeest in the Ngorongoro Crater and Tarangire populations respectively.

Chapter Four

All animal experiments were approved by the University of Nottingham and the Moredun Research Institute's experiments and ethics committees. The vaccination trial, including immunization, sampling, clinical scoring and criteria for euthanasia after onset of MCF (Russell et al., 2012) followed protocols established during trials at the Moredun Research Institute, UK and were compliant with the Home Office of Great Britain and Northern Ireland 'Animals (Scientific Procedures) Act 1986'.

Chapter Five

This study was approved by both the Tanzanian Wildlife Research Institute (TAWIRI) and the Commission for Science and Technology (COSTECH, Tanzania). The human subject research was conducted according to relevant international guidelines and was approved by COSTECH (permit nos.2011-213-ER-2005-141 and 2012-318-ER-2005-141). The animal research, which was non-invasive and was conducted according to international guidelines, was permitted by the Tanzanian Wildlife Research Institute (TAWIRI).

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VII Author's declaration

The author undertook all of the field activities and, other than the *in-vitro* FliC efficacy assays (Chapter Four), the creation of the vaccine and the virus-positive and negative ELISA antigen, collaborated on all of the experimental and laboratory work described. Work that was not undertaken by the author has been identified using footnotes in the text. The analyses were undertaken with assistance from Prof. S. Cleaveland, Dr. G. Russell, Prof. D. Haig and Prof. J. Yoder. All of the figures, and all except one of the images (which is appropriately credited), were created, or taken, by the author.

VIII Abbreviations

AIHV-1	Alcelaphine herpesvirus-1
BCS	Body condition score
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CpG ODN	CpG oligodeoxynucleotide
CI-ELISA	Competitive-inhibition enzyme-linked immunosorbent assay
ECF	East Coast fever
ELISA titre	AlHV-1-specific antibody titre
FliC	Bacterial flagellin monomer
НЕК	Human embryonic kidney cells
HG	Heart girth
LD ₅₀	Lethal dose, 50%
MCF	Malignant catarrhal fever
NS	Nasal secretion
OvHV-2	Ovine herpesvirus-2
PAMPs	Pathogen-associated molecular pattern molecules
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SA-MCF	Sheep-associated Malignant catarrhal fever
SDS	Sodium dodecyl sulphate
SZC	Shorthorn zebu cross
TCID ₅₀	Tissue-culture-infectious dose, 50%

- TLR Toll-like receptors
- VNA Virus-neutralizing antibody
- WA-MCF Wildebeest-associated Malignant catarrhal fever

Chapter One A review of malignant catarrhal fever and African rangeland ecology

IX

Malignant catarrhal fever

Malignant Catarrhal Fever (MCF) is an infectious systemic often-lethal disease that presents as a variable complex of clinical signs in susceptible species of the order Artiodactyla. The disease, which has a worldwide distribution, ranges from the sporadic to epidemic depending on the species affected and occurs following transmission from an inapparent carrier host to an MCF-susceptible species. Defined by the reservoir ruminant species from which the causative virus arises, two major epidemiological forms of MCF exist, wildebeest-associated (WA-MCF) and sheep-associated (SA-MCF) MCF.

Aetiology

The MCF causing viruses belong to the γ -herpesvirus sub-family (genus *Macavirus*; family *Herpesviridae*), which are characterised by having one of the most complex pathogenesis of infection of any known virus. The nature of the diseases they cause is a product of an intricate interaction between inherent properties of the virus, genetically-defined host susceptibility factors, and antiviral immune responses of the host. Diseases caused by this group of viruses are manifest by a wide range of pathologies, from inflammation to neoplasia. Features common to the group include tropism for lymphocytes, the establishment of latent infections, their ability to replicate in lymphoblastoid cells in-vitro, a high level of adaptation to their natural hosts, and induction of lymphoproliferative diseases and tumours (Roizmann, 1995; Crawford et al., 2002).

Of the ten viruses within the MCF sub-group six are currently reported to be associated with disease:

- Alcelaphine herpesvirus-1 (AlHV-1) carried by wildebeest (*Connochaetes spp.*) (Plowright, Ferris, and Scott, 1960; Roizmann et al., 1992)
- Ovine herpesvirus-2 (OvHV-2), endemic in domestic sheep (Roizmann et al., 1992; Hart et al., 2007)

- Caprine herpesvirus-2 (CpHV-2), endemic in domestic goats (Chmielewicz, Goltz, and Ehlers, 2001; Li et al., 2001c)
- 4. Alcelaphine herpesvirus-2 (AlHV-2) found in aoudads (*Ammotragus lervia*), hartebeest (*Alcelaphus bucelaphus*) (Roizman et al., 1981) and topi (*Damaliscus lunatus*) (Mushi et al., 1981), has recently been shown to produce MCF-like clinical signs after challenge in bison (Taus et al., 2014). Furthermore recognition of an AlHV-2-like MCF virus in diseased Barbary red deer (*Cervus elaphus barbarus*) (Klieforth et al., 2002) also suggests that these viruses may be pathogenic for some species under certain circumstances.
- The virus of unknown origin (MCFV-WTD) that has been reported to cause a classic MCF syndrome in white-tailed deer (*Odocoileus virginianus*) (Li et al., 2000; Kleiboeker et al., 2002)
- 6. Recently linked to incidence of MCF in bongo antelope (*Tragelaphus euryceros*), a herpesvirus found in asymptomatic ibex (*Capra ibex*) (Okeson et al., 2007)

Several other γ -herpesviruses of the MCF virus group, which have not yet been linked to disease, have been recently described as occurring in exotic ruminant species:

- Oryx-MCFV in the musk ox (*Ovibos moschatus*) and the South African oryx (*Oryx gazella*) (Flach et al., 2002; Li et al., 2003);
- Hippotragine herpesvirus-1 (HiHV-1) in roan antelope (*Hippotragus equinus*) (Reid and Bridgen, 1991)

Susceptible species

Despite sporadic reports of clinical disease occurring in free-ranging wildlife (Vikøren et al., 2006; Neimanis et al., 2009), MCF is principally a disease of captive animals such as domestic cattle, water buffalo and Bali cattle (banteng) (Ramachandran et al., 1982), farmed red deer (*Cervus elaphus*) (Reid et al., 1987) and American bison (*Bison bison*) (Schultheiss et al., 2000). Disease has also been reported in the pig (*Sus scrofa domesticus*) (Loken et al., 1998; Gauger et al., 2010) and experimental transmission has been successful in hamsters (*Mesocricetus auratus*) and rats (*Rattus rattus*) (Jacoby et al., 1988).

Susceptibility to the disease varies. Cattle, for example, are generally more resistant than bison, and some deer species, notably white-tailed (*Odocoileus virginianus*), axis (*Axis axis*),

and Pere David's deer (*Elaphurus davidianus*) are highly susceptible whilst in others, such as fallow deer (*Dama dama*), susceptibility is considerably lower (Crawford et al., 2002).

Forms of the disease

With distributions essentially mirroring those of the carrier host species, two principle forms of MCF were originally described: sheep-associated MCF (SA-MCF)(Baxter et al., 1993) and wildebeest-associated MCF (WA-MCF) (Plowright, Ferris, and Scott, 1960).

Initially observed in Europe, SA-MCF is a worldwide disease that occurs wherever sheep and an MCF-susceptible species are kept in close proximity to one another (Reid et al., 1984). Frequently seen in Indonesia, where OvHV-2 susceptible Bali cattle (*Bos javanicus*) are common, SA-MCF is typically sporadic in countries where *B. taurus* and *B. indicus* cattle are the predominant sub-species. SA-MCF is also an economic and welfare concern in bison and deer farming (Reid et al., 1987; O'Toole et al., 2002; Russell, Stewart, and Haig, 2009).

WA-MCF occurs naturally in sub-Saharan Africa wherever the carrier species, wildebeest (*Connochaetes taurinus*), come into contact with susceptible species such as cattle (Plowright, Ferris, and Scott, 1960). The disease is a particular problem for cattle keeping people living in eastern and southern Africa (Bedelian, Nkedianye, and Herrero, 2007; Cleaveland et al., 2001). Additionally, WA-MCF has also been reported throughout the world in zoological collections where mixed artiodactyl species including wildebeest are kept (Flach et al., 2002; Whitaker et al., 2007).

In addition to these two common forms of the disease, two new forms of MCF have recently been described which are not associated with sheep or wildebeest: Caprine-associated MCF is associated with disease in a range of cervid species and appears to be endemic in at least some goat populations (Crawford et al., 2002; Vikøren et al., 2006; Chmielewicz, Goltz, and Ehlers, 2001; Li et al., 2001c) suggesting that keeping goats in close proximity to cervid species is a risk. A fourth form of the MCF syndrome has recently been reported in white-tailed deer and the virus, which is genetically distinct but closely related to the viruses that cause the more common forms of MCF, has been characterised by limited sequencing, but as yet its natural reservoir host has not been identified (Li et al., 2000; Klieforth et al., 2002).

Wildebeest-associated MCF

It is generally accepted that the incidence of WA-MCF varies widely and is understandably highest in areas where wildebeest calve. Estimates for the annual incidence rate of MCF in cattle herds living in wildebeest calving areas in eastern Africa range between 5% and 20% (Plowright, 1986; Bedelian, Nkedianye, and Herrero, 2007). In southern Africa incidence rates of up to 20% have been recorded (Barnard and Pypekamp, 1988).

Transmission

Given that the development of MCF within a species, and even within a susceptible herd, can be sporadic (Himsworth et al., 2008), the transmission dynamics of MCF are complex and not fully understood. Transplacental transmission from female wildebeests to their unborn calves does occur (Plowright, Ferris, and Scott, 1960) and the persistence of the AlHV-1 virus in this species may be dependent, partially at least, on this vertical transmission. Once born, the congenitally infected calves rapidly develop a viraemia and begin excreting the virus. This infects other, non-infected, calves that, in turn, transmit the virus to the rest of the wildebeest calves. Once wildebeest calves become infected the virus, typical of a herpesvirus, does not distribute itself equally around the body but becomes compartmentalised in certain tissues, such as the cornea and nasal turbinates. This opens up possible pathways for virus shedding (Rweyemamu et al., 1974; Mushi et al., 1981; Plowright, 1986). Being avascular the cornea provides a relatively immunologically deprived site, well suited to viral replication. Once replication has taken place the virus enters the ocular and nasal secretions which provide a possible transmission route (Mushi, Karstad, and Jessett, 1980; Barnard et al., 1989). Virus has also been demonstrated in the pulmonary alveolar cells of wildebeest calves (Michel et al., 1997) which suggests another mechanism for excretion by aerosolisation.

Excretion primarily occurs in the initial stages of infection and by seven months of age all calves in the herd will have developed virus-neutralising antibodies that control the primary infection and drives the infection to become latent. These antibodies persist throughout life. It is commonly understood by cattle owners living near wildebeest calving grounds that cattle can once again be safely grazed in close proximity to wildebeest after the calves reach this age (Plowright, 1965).

The virus shed by wildebeest calves is in a cell-free state that is relatively stable and can survive up to 13 days outside the host (Mushi et al., 1981; Whitaker et al., 2007), although

exposure to direct sunlight is thought to rapidly inactivate the virus (Rossiter, Jessett, and Karstad, 1983). As pasture contamination is presumed to play a role in transmission, the ability of the virus to survive in the environment will have a bearing on subsequent transmission dynamics. Likely because the cell-free form of the virus is not excreted by disease-susceptible hosts, infection cannot pass from one disease-susceptible host to another and as such these species are regarded as dead-end hosts (Plowright, 1968).

The transmission pathway of the SA-MCF virus OvHV-2 differs from that of the WA-MCF virus in that, unlike wildebeest calves that are infected in the perinatal period by horizontal and occasional intrauterine transmission, lambs rarely become infected with OvHV-2 until after two to three months of age (Li et al., 1998). Moreover, wildebeest are thought to only shed virus for a limited period whilst young whereas, despite virus shedding peaking at six to nine months of age, levels of OvHV-2 DNA in the nasal secretions of adult sheep appears relatively consistent. Furthermore, although a seasonal pattern of shedding in sheep has not been identified, individual sheep can have occasional periods when the levels are relatively elevated (Li et al., 2001b). Interestingly, OvHV-2 has been found in the ejaculate of breeding rams, suggesting a potential sexual transmission route (Hüssy et al., 2002).

Latency

Like other herpesviruses, MCF viruses establish lifelong persistent infections in their natural hosts through latency. In this state the virus infected cells remain protected from being destroyed by the immune system, whilst the host, despite being chronically infected and intermittently shedding the virus, remains disease free (Li et al., 2004; Ackermann, 2006). This is in contrast to non-reservoir host species that lose control over the number of latently infected cells resulting in the development of MCF.

The cell type hosting the latent MCF virus, and the actual mechanism by which it is maintained, has yet to be determined. However, given that the PCR signal detected in carrier hosts is found in the lymphocytes it seems likely that T- or B- cells provide the most likely location (Schock and Reid, 1996; Ackermann, 2006). Reactivation from this latent state to the lytic phase results in the production of infectious virus which is subsequently transmitted either to naive carrier hosts, without inducing disease, or to susceptible hosts with the resultant development of MCF (Li et al., 2004).

Recrudescence of latent infections

The occurrence of MCF in herds of cattle that for many months or years have had no contact with the reservoir host has led to speculation that lateral transmission events (Schultheiss et al., 2000; Holliman, 2005) or indirect transmission through the use of shared farm implements (Holliman et al., 2007) may rarely occur. Additionally, that incubation periods of longer than six months may be possible, or that insect vectors can play a role in transmission (Barnard and Pypekamp, 1988). The recent development of highly sensitive screening techniques, such as PCR and direct-ELISA, has revealed that significant levels of sub-clinical or latent infection are present in normal cattle and bison, prompting the explanation that the progression of sub-clinical infections in susceptible species may be responsible for these unusual epidemiologic patterns (Li et al., 2001b; Otter, Pow, and Reid, 2002).

Seasonality

The timing of WA-MCF varies according to regional variations in the rainy seasons that drive wildebeest calving. Large numbers of viraemic wildebeest calves can be expected in northern Tanzania from January, and in neighbouring Kenya from March, each year. As the minimum incubation period for the disease is approximately 30 days, MCF in cattle can be expected to occur between February and May in Tanzania, and between April and July in Kenya. In South Africa the seasonality of MCF outbreaks in cattle has been reported to follow a different pattern with two periods in which MCF outbreaks occur (Barnard and Pypekamp, 1988; Honiball, Van Essen, and Du Toit, 2008; Reid and Van Vuuren, 2004). The first, in April, occurs when the wildebeest calves are approximately four months old and is similar in its timing to the pattern seen in East Africa. This suggests it is a consequence of cattle becoming infected by virus transmitted by young wildebeest calves. The second occurs between September and November when the wildebeest calves are between nine and eleven months old. The reasons for the second period are not clear. Possible explanations are that the South African environment may be driving two rounds of wildebeest virus shedding, one from calves as seen elsewhere in East Africa and a second, from older animals (possibly calves and / or adults), driven by other factors such as climate, food supply, enforced migration and / or human - wildlife interactions. Furthermore, there are two species of wildebeest found in South Africa: the blue wildebeest, also found in East Africa, and the black wildebeest (Connochaetes gnou) found only in South Africa. Both species transmit the MCF virus and it is possible that species-specific differences exist that affect the timing

of virus excretion. Finally, it is not clear from the few reports found whether virus characterisation to identify the causative agent was performed. Consequently it is possible that some of the cases that occurred in the second period might have actually been caused by OvHV-2.

Incubation period

The incubation period for cell-associated WA-MCF in cattle is usually between 10 and 37 days, with a mean of 21 days (Plowright, 1968). However the delay between infection and the first overt clinical signs can be as long as 18 weeks (Barnard, Pypekamp, and Griessel, 1989) or even up to eight months (Whitaker et al., 2007). A recent study examining the relationship between infective dose and incubation period in SA-MCF has revealed that there is a relationship between the dose of OvHV-2 viruses infecting bison and the subsequent incubation period. This suggests that infective dose plays a role in determining the period between first infection and the development of clinical signs (Gailbreath et al., 2010).

Clinical Signs of MCF

MCF is a broad syndrome with a variety of different clinical presentations which can range from sub-clinical, peracute to chronic (O.I.E., 2008; Kahn and Merck.M., 2005). Typical clinical signs include: fever, which classically marks the end of the incubation period, lym-padenopathy, hyperaemic and necrotic lesions of the nasal and oral mucosa, serous (later becoming muco-purulent) ocular and nasal discharge often resulting in the nares and muzzle becoming encrusted (Figure 1), severe eye lesions (panophthalmitis, hypopyon, corneal erosions), with corneal opacity (Figure 2) often beginning at the corneo-scleral junction and progressing inward. Up to 25% of cattle experience chronic disease, and sometimes the disease waxes and wanes, with skin lesions (erythema, exudation, cracking, crust formation) being common in these animals (Plowright, 1986; O.I.E., 2008; Russell, Stewart, and Haig, 2009; Kahn and Merck.M., 2005). Despite the wide range of symptoms, all of the different clinical signs seen in MCF result from two primary histo-pathological features common to all forms of this disease: namely lymphoid hyperplasia and vasculitis (Penny, 2005).

Sub-clinical cases

Doubt exist as to whether a mild form of the disease occurs, in which few clinical signs are witnessed (Plowright, 1986). However reports (Otter, Pow, and Reid, 2002; Powers et al., 2005; Moore et al., 2010) in which cattle have been tested positive for both OvHV-2



Figure 1: **Image of a cow with MCF** Copious nasal secretion in a cow suffering from MCF



Figure 2: **Image of a cow with MCF** Corneal opacity in a cow suffering from MCF

DNA and virus-specific antibody yet remained asymptomatic indicate that silent infections can occur in outbreaks of SA-MCF. Whether these animals recover from infection, develop latent subclinical infections, or potentially recrudesce with clinical MCF at a later time is not known. These observations suggest that additional host or environmental factors are involved in the initiation of MCF after OvHV-2 infection (Powers et al., 2005).

Morbidity and mortality

The case-fatality ratio from WA-MCF is reported to be between 96 and 100% with an average survival of approximately 9 days (Plowright, Ferris, and Scott, 1960; Bedelian, Nkedianye, and Herrero, 2007). This is supported by reports of there being no serological evidence of infections in cattle living in an area of heavy wildebeest exposure in Kenya (Rossiter, Jessett, and Mushi, 1980). The most distinctive clinical feature in cattle that do recover from clinical MCF is the presence of persistent bilateral chronic corneal lesions called 'leucomata'. Typically following damage or disease the bovine cornea regenerates quickly with minimal scarring making the slow rate at which these lesions resolve distinctive (O' Toole et al., 1997). Additionally, obliterative arteriopathy lesions, which persist for extended periods, are distinctive post-mortem findings useful in identifying cattle that have recovered from a previous episode of MCF (O' Toole et al., 1997; Brenner et al., 2002).

Pathology and cell-biology

The life-cycle of OvHV-2 has been studied extensively in SA-MCF. In the carrier host the virus changes its cell-tropism at three different stages: firstly the virus gains entry into the host through infection of the lung tissue; secondly the virus establishes latency through infection of lymphocytes which then circulate around the body in the peripheral system; and thirdly, during episodes of shedding, the virus replicates in the nasal turbinates (Li et al., 2008).

Once the virus has gained entry into a disease susceptible host the pathogenesis is poorly understood. However, like other lymphotropic-herpesviruses including Epstein-Barr virus (EBV) and murine herpesvirus-68 (MHV-68), it appears to involve an auto-destructive pathology, whereby cytotoxic lymphocytes destroy a variety of tissues (Taus et al., 2006; Anderson et al., 2008; Nelson et al., 2010). The principle histo-pathogenic changes witnessed in cases of MCF are necrotizing angiitis (which mostly affects medium-sized arteries and is a diagnostic stigmata of the disease in cattle) with perivascular lymphoid accumulation and

necrosis of mucosal epithelium, and lymphoproliferation and infiltration (Plowright, 1968; Simon, 2003; Russell, Stewart, and Haig, 2009). Rather than neoplastic, the lymphoproliferation appears to be hyperplastic in origin and occurs predominantly in lymphoid organs and also in the interstitial spaces of non-lymphoid organs (Buxton et al., 1984). Following the administration of the immunosuppressive cyclosporine-A to MCF infected rabbits, the lymphoproliferative features of the disease were eliminated but the progression of the disease was unaffected (Buxton et al., 1984). The authors concluded, therefore, that the lymphoproliferation was essentially a benign component of MCF, not involved in the lethal pathology of the disease.

The absence of viral DNA detectable in tissues of animals suffering MCF led researchers to the proposition that direct viral cytopathogenic effects were unlikely to be the cause of the pathology. Rather, they hypothesized, the lesions were a result of an indirect pathogenesis ('bystander effect') caused by a cell-mediated immune-dysfunction of a secretory T-cell activator (Reid and Buxton, 1985; Plowright, 1986; Reid et al., 1989; Schock and Reid, 1996). Later studies, employing more sensitive methods of detecting viral DNA, such as *in-situ* PCR and hybridisation, have revealed that the predominant cells infiltrating lesions are, in contrast to these earlier findings, infected with viral DNA throwing the 'bystander effect' theory in to doubt. It now seems more plausible that the indiscriminate tissue damage witnessed in cases of MCF are either a direct result of virus infected CD-8⁺ cytotoxic T- cells (Thonur et al., 2006; Hart et al., 2007; Dewals et al., 2008) or immune-mediated responses to them (Simon, 2003).

Further studies are required to fully understand the cell biology and pathogenesis of MCF. In particular the receptors used by the virus to enter cells to establish a latent or lytic infection and the particular cell type within which latency exists. Additionally the cellular site essential for replication and cell-free virus production vital for transmission remains unknown. Such questions provide focus points for further study and highly susceptible domesticated species such as bison, in which experimental infection can be induced through nasal inoculation of the virus, are suitable targets for such research (Russell, Stewart, and Haig, 2009; O'Toole et al., 2007).

Diagnosis

The World Organisation for Animal Health (OIE) recognises histopathology as the definitive diagnostic test for MCF (Russell, Stewart, and Haig, 2009). However, as there are a number of different diseases that have similar histopathological lesions, it has been suggested that

a diagnosis of MCF is most reliably made when based on a combination of clinical signs, detection of virus-specific antibodies in blood, histopathological findings in key tissues, and identification of the presence of MCF viral DNA through PCR (Moore et al., 2010; Russell, Stewart, and Haig, 2009).

In the face of either a single sick animal or the start of an outbreak, often the first diagnostic evidence available is the appearance of clinical signs that, although suggestive of MCF, are not pathognomonic. Indeed, there are several viruses that cause clinically similar syndromes that should be considered within the differential diagnosis list: for example, foot and mouth disease viruses, bovine papular stomatitis virus, infectious bovine rhinotracheitis virus, vesicular stomatitis virus, rinderpest virus, bluetongue viruses, bovine viral diarrhoea virus, and malignant catarrhal fever-associated viruses.

The development of a competitive-inhibition enzyme-linked immunosorbent assay (CI-ELISA) based on a monoclonal antibody (mAb) to the 15A epitope widely conserved among MCF viral strains of alcelaphine and ovine origin (Li et al., 1994) provides a useful diagnostic tool. The speed with which samples can be assayed, the relatively high specificity and the fact that anti-species specific conjugates are not required makes this a useful test for the initial screening of large numbers of animals (Müller-Doblies et al., 1998). The relatively low sensitivity of the CI-ELISA test does, however, require that negative results are confirmed with more sensitive methods such as PCR and histopathological analysis. The low sensitivity of serological analysis has, in certain cases, been attributed to the rapid progression of the disease, where death occurs before a humoural response has been initiated. Indeed, even in non-acute cases antibodies may not be produced (O.I.E., 2008). Also it has been suggested that the low sensitivity could be due to some animals not producing antibodies that target the single 15A epitope exploited by the CI-ELISA. The usefulness of this assay was improved, however, by the development of a direct CI-ELISA test which was significantly more sensitive than the indirect CI-ELISA, and was effective in both clinical and sub-clinical cases of MCF (Li et al., 2001a).

As with all serological based tests a major limitation is that they can only diagnose prior exposure to the virus and cannot differentiate between previous exposure with clearance of the virus, active infection, and latency (Powers et al., 2005; Moore et al., 2010). Additionally, CI-ELISA cannot differentiate between the different MCF viruses, which in regions of the world such as South Africa where both major forms of the disease occur (SA- and WA-MCF), is of critical importance when designing preventative and control measures (Dungu et al., 2002).

The advent of PCR provided a diagnostic assay that was more sensitive than the CI-ELISA (Baxter et al., 1993; Li et al., 1995), and more sensitive and specific than histopathology, with the obvious additional advantage that diagnosis could be made ante-mortem. This particular attribute has enabled repeated longitudinal testing over time, revealing that some individual animals remain latently infected (Müller-Doblies et al., 1998). However PCR analysis can give false negative results particularly when limited tissue samples are available for analysis (Desmecht et al., 1999). This scenario likely reflects the tendency of MCF viruses to become compartmentalised within the body, underscoring the importance of effective tissue selection for diagnostic analysis. In addition, intermittent positive results have been recorded in longitudinal studies, indicating that latently infected animals may have a lymphocyte infection rate close to the lower detection threshold of PCR (Powers et al., 2005). Since γ -herpesviruses in general can not be propagated easily in cell-culture, PCR does represent the method of choice when a diagnosis of viral infection is required (Jacobsen et al., 2007), plus it has the advantage of enabling phylogenetic analysis to be performed (Russell, Stewart, and Haig, 2009).

Two types of PCR assays have been developed for both OvHV-2 and AlHV-1 viral DNA: nested and real-time (quantitative) PCR. Real-time PCR enables viral load to be quantified, whilst nested PCR can be up to ten times more sensitive and enables diagnoses to be made in cases when the viral load is very low, for example in latently infected animals, or when presented with difficult biological samples, such as paraffin embedded tissues (Russell, Stewart, and Haig, 2009). A limitation of the PCR assay has been that it is not capable of simultaneously differentiating among the five different pathogenic MCF viruses, requiring several time-consuming and expensive assays to be performed before the specific virus could be identified. The identification of unique sequences within the viral DNA polymerase gene for each of the different MCF viruses provided a solution to this problem and enabled the development of a multiplex real-time PCR that could rapidly and reliably differentiate between the pathogenic MCF viruses. This development is of particular use to zoos and game farms with mixed-species operations where specific viruses need to be quickly differentiated and a plan for control established (Cunha et al., 2009).

Vaccine development

Following infection of epithelial cells on mucosal surfaces, herpesviruses spread via intracellular bridges to a variety of virus-specific tissues wherein they can cause immediate disease and, if the host survives, they establish a latent infection that persists for life (Israel et al., 1992). This capacity to persist is a consistent feature of herpesvirus infections and reflects an ability to deter host immune defences. For example once latency has been established herpesviruses are able to express very few, if any, protein antigens that could alert immune mechanisms to their presence. Additionally they are able to down regulate the presentation of viral antigens in the context of class I major histocompatibility complex (MHC) proteins (Krause and Straus, 1999). For reasons such as these the control of herpesvirus infections has presented a particular challenge with development of vaccines being hampered by failure to effectively prevent infection, latency, reactivation from latency and transmission of virus to susceptible hosts (Israel et al., 1992).

Irrespective of these challenges attempts to develop an effective vaccination against MCF have been under way for many years. Early attempts utilized inactivated lymph node suspensions (Piercy, 1954), live virus passaged in rabbits (Plowright, 1968), and living or formalinised preparations of the infectious agent combined with an adjuvant (Plowright et al., 1975). None of the trials resulted in a vaccine that was able to consistently prevent disease. Later, progress was made when it was shown that rabbits inoculated with inactivated cell-free AlHV-1 (C500 strain) virus developed partial protection against subsequent challenge with AlHV-1 virus administered systemically (Russell, 1980). Further, inactivated cell-free AlHV-1 virus combined with Freund's adjuvant was shown to protect rabbits against cell-free virus challenge but, despite evoking a high titre of neutralizing antibody, did not protect the rabbits from challenge with cell-associated virus (Edington and Plowright, 1980). Later, it was demonstrated that rabbits would develop antibodies and subsequently resist challenge when hyper-immunized with live cell-associated virus, but failed to do so when immunized with inactivated cell-associated virus (Rossiter, 1982). This work was not transferred to cattle.

Further insights on the specific immunological features that are particular to MCF were provided by challenge studies performed on cattle that had either been immunized with trial vaccines or those that had naturally survived the disease (Piercy, 1954): of the cattle that received the trial vaccines very few survived subsequent challenge with MCF virus, and the few that did died after further challenges several months post-inoculation, suggesting that any immunity that had developed was short lived. In contrast a cow that had survived a natural infection resisted multiple MCF challenges carried out for more than one year, despite having lower titres of serum neutralizing antibody than the immunized cows. These findings suggested that the immune response resulting from a natural infection with MCF that was supported when the attempts to vaccinate against MCF failed despite high levels of circulating virus neutralizing antibodies being detectable (Plowright et al., 1975). In recent years attempts have therefore been made to develop vaccination strategies responsive to the natural method of transmission.

Due to the lack of an *in-vitro* propagation system for the causative agent (OvHV-2) of the more economically significant SA-MCF, vaccine development has focused on WA-MCF, the causative agent of which (AlHV-1) can easily be cultured *in-vitro*. As vaccines for γ herpesviruses have been found to provide cross-protective immunity against other closely related viruses (Gailbreath et al., 2010) it is possible that a vaccine developed against AlHV-1 may also provide cross-protective immunity to a closely related virus such as OvHV-2. For example, cattle immunized against bovine herpesvirus-4 (BoHV-4), a non-MCF virus, were partially protected when challenged with a virulent form of AlHV-1 (Rossiter, Gumm, and Mirangi, 1988).

The elucidation of the full sequence of the AlHV-1 genome (Ensser, Pflanz, and Fleckenstein, 1997) and the identification of genes involved in virulence raises the possibility of constructing recombinant vaccines. Progress was further enhanced by AlHV-1, like other herpesviruses, mutating and attenuating during serial passage, rendering the virus less virulent and increasingly cell-free (Wright et al., 2003). The genetic alterations that cause the attenuation are not entirely consistent but are not thought to result in any changes in the gross structure of the virus particle itself. Rather they involve altered gene expression and the translocation of DNA from the central unique sequence of the genome to areas either next to or in between terminal repeat elements at either end of the genome (Handley et al., 1995; Wright et al., 2003; Dry et al., 2008). These alterations result in the production of a 'high passage' attenuated form of the AlHV-1 virus that, benefitting attempts to develop a vaccine, is highly adapted to growth in cell culture but that can no longer cause disease.

The most recent attempts to design and test a vaccine effective against MCF have exploited this attenuated version of the virus (atAlHV-1) and, crucially, have involved the development and use of a fatal intranasal challenge system using cell-free virus. These laboratory trials showed that a two-dose inoculation of atAlHV-1 'C500' strain (administered by an injection in the cranial musculature of the neck on day zero and boosted at day 28) protected cattle when faced with an intranasal challenge with a virulent AlHV-1 C500 strain obtained from low pass tissue culture (Haig et al., 2008). This protection was shown to last for up to six months (Russell et al., 2012). When the virus challenge was administered via the intravenous route this immunisation regime, in-spite of high serum neutralizing antibody

titres, did not provide a protective effect. This suggested that the immunity engendered by the intra-muscular vaccination resulted from its ability to prevent primary viral infection at the site of the nasal mucosal epithelium. Given that the natural mode of transmission is believed to be through cattle inhaling the virus, the conclusions from these trials were that this vaccine strategy might be effective at protecting cattle following exposure to cell-free virus naturally transmitted from wildebeest calves. Field trials were therefore required to test this hypothesis.

Pastoralism, land use, conservation and cultivation

Pastoralism in the rangelands of Africa

In this thesis the term pastoralism and pastoralists are used generically to broadly describe people who, to a greater or lesser degree, use mobility to keep and maintain livestock herds.

Pastoralism is thought to have spread southwards into eastern, and then southern, Africa around 4000 BP (Marshall, 2000; Homewood, 2008). Today pastoralists represent a culturally and linguistically diverse range of societies with differences in livestock species kept (small ruminants, cattle, camels) and the degree to which hunting and small-scale, rain fed agriculture are practiced and relied upon (Galaty, 1993; Spear and Waller, 1993).

Treated as common property resources by pastoralists and sheltering a great diversity of free-ranging wildlife species (Bourn and Blench, 1999; Wambwa, 2005), the (semi-) arid 'rangelands' of Africa, which cover two-thirds of the land mass south of the Sahara, are characterized by seasonally available plant-associated water and nutrients (Homewood, 2004). In such 'dry-land' ecosystems, where dynamics are driven by unpredictable and extreme fluctuations in biophysical factors (rain, fire, disease, grazer populations and grazing pressure, plant population dynamics), and where vegetation productivity is more related to rainfall patterns than grazing intensity (Ellis, 1995; Noy-Meir, 1982; Caughley, Shepherd, and Short, 1987), mobility offers an efficient survival strategy to humans, their livestock and wild animals alike (Homewood, 2004). For example, pastoralists and large grazing herbivores, such as wildebeest and white-eared kob (*Kobus kob*) that cannot meet their metabolic water requirements solely from their forage, all exploit migration as a means of accessing seasonal rainfall driven water and food supplies (Maddock, 1979). Migration also benefits the land itself as it provides the vegetation time to recover from grazing each year (Sinclair and Fryxell, 1985).



Figure 3: Extent and distribution of sub-Saharan rangelands (Homewood, 2004)

Pastoralism, migration and conservation

By its very nature, however, migration is dependent on space to allow unrestricted movement and as a result both the migratory wildlife populations and the transhumant human populations are dependent on these rangelands remaining unfenced and uncultivated (Borner, 1985). This notion, that wildlife populations and traditional livestock systems are equally dependent on the preservation of the integrity of their common environment, is clearly at odds with the belief that livestock is a major factor in land degradation and loss of wildlife; a commonly held belief which itself is at odds with contemporary studies showing that pastoralism is an effective strategy in conserving the environment to the benefit of wild animal and plant species (Kock, 2005; Scoones, 1995). The fact that the last significant, unrestricted, wild ungulate populations surviving in Africa are associated with pastoral systems (Kock, 2005) also suggests a co-dependency unrecognised by many.

Indeed, it has been argued that the diversity of wild animal species in African rangeland ecosystems is less determined by the size of protected areas, but rather the extent of the wider savanna ecosystem within which the protected areas sit (Homewood, 2004). When range-land ecosystems surrounding protected areas are dominated by conservation-compatible land uses like herding and small-scale farming, mobility is not inhibited and the biodiversity of the whole landscape benefits (Reid, 2012; Homewood, 2004). Where large-scale land conversion for cultivation has taken place the protected area becomes isolated, surrounded by unusable habitat, and the impact on wildlife can be devastating (Homewood, 2004).

This balance between a traditional pastoral lifestyle and the preservation of a diverse rangeland ecology is, however, fragile. Due in part to institutional barriers and restrictions on land-use, conflict between pastoralists and wildlife over grazing areas and water resources has, in recent years, been increasing (Wambwa, 2005; Sachedina and Chenevix Trench, 2009). An often cited cause of conflict, particularly in northern Tanzania, is the success of the rinderpest eradication programme which resulted in a dramatic increase of the Serengeti wildebeest population (growing from 200,000 to current levels of approx.1.5 million) and the extension of their range into areas previously used by pastoralist cattle herders during the wet season. This population growth has not been matched, however, by increasing livestock numbers which in East African rangelands have for several decades fluctuated with drought and disease, showing alternate crash and recovery, but no overall trend (Homewood et al., 2001).

The economics of pastoralism

Due in part to changes in land tenure, conservation and land fragmentation, keeping livestock in the traditional pastoralist manner in East Africa has, in recent decades, become increasingly difficult. Modern economic demands require pastoralist households to have access to cash to pay for health, education and food, increasing the need for livelihood diversification to generate income (Homewood, Kristjanson, and Chenevix Trench, 2009; Homewood, Trench, and Brockington, 2012; McCabe, Leslie, and DeLuca, 2010). From wage labor in cities (May and McCabe, 2004), to involvement in gemstone mining and selling of beadwork and livestock products (Sachedina and Chenevix Trench, 2009; Smith, 2014), these shifts can be seen as coping strategies to protect households from having to sell livestock (O' Malley, 2003). One livelihood change that is steadily increasing and that has the potential to devastate pastoralism and wildlife, particularly in the critical buffer zones that surround protected areas in Kenya and Tanzania, is the conversion of lands for large scale cultivation (Homewood et al., 2001; Galvin, 2009).

Shifting from pastoralism to crop-based agriculture

In the past 50 years pastoralists in East Africa have become increasingly reliant on cropbased agriculture. This has been caused, in part, by growing preference for agricultural foods as well as the decline of livestock numbers relative to humans (McCabe, Leslie, and DeLuca, 2010), but also reflects state biases on agricultural development. For example, government policies in East Africa, while viewing transhumant pastoralism as incompatible with ecological health (Looloitai, 2014), modernity or wildlife conservation (Benjaminsen, Maganga, and Abdallah, 2009), have pressured pastoralists to reduce livestock numbers and take up farming (Looloitai, 2014). In Tanzania the 'Kilimo Kwanza' ('Farming First') policy has encouraged agricultural production over pastoralism, leading to land disputes and resettlement to facilitate large scale agriculture production (Mahonge, Sangeda, and Mtengeti, 2014). Reduction in the livestock per capita ratio, declining milk yields in the dry season, and overall increase in food insecurity following drought or outbreaks of livestock disease have also contributed to this growing reliance upon crop-based agriculture (McCabe, Leslie, and DeLuca, 2010). Cultivation is also viewed by pastoralists as a good way to stake a prior claim against threats of land being expropriated for conservation (Fairhead, Leach, and Scoones, 2012; Gardner, 2012; Benjaminsen and Bryceson, 2012) or conversion to industrial agriculture (Homewood, Kristjanson, and Chenevix Trench, 2009).

The impact of cultivation

Despite growing pressures to cultivate, in many areas of East African rangelands aridity and marginal soil fertility limit the potential for cultivated food production. Additionally, rangeland cultivation removes dry season pastureland, vital for wild and domestic herbivores. Furthermore, some predict that climate change will make East African rangelands warmer, increase rainfall unpredictability, reduce plant available moisture, and increase the frequency of extreme climatic events (Stige et al., 2006). These changes may favour mobile livestock keeping in non-fragmented rangelands, rather than cultivation which requires heavy inputs and leads to more fragmentation (Galvin, 2008; Hobbs et al., 2008; Davies and Nori, 2008).

Despite these concerns, conversion of rangeland for commercial cultivation has, in recent years, been ongoing and the impact on wildlife populations has been devastating. For example, in Kenya privatisation and conversion of formerly communal rangeland around the Maasai Mara National Reserve to commercial monoculture entailed drastic land cover changes resulting in significant wildlife declines (Homewood et al., 2001; Norton-Griffiths, 1996; Thompson and Homewood, 2002; Lamprey and Reid, 2004). A similar pattern is occurring around Tanzania's Tarangire National Park, where permanent subsistence and large-scale farming is increasingly isolating the park and leading to declines of large mammal species (Borner, 1985; T.C.P, 1998). These scenarios illustrate that conservation goals are often as dependent on the viability of community lands (that form buffer zones around national parks) as they are on the integrity of the state controlled protected areas that they surround (Sachedina and Chenevix Trench, 2009).

The impact of improved livestock health

The conversion of rangelands to agricultural plots also reduces the options for livestock to avoid infectious diseases (Reid, 2012). For example, with mobility increasingly restricted, traditional MCF avoidance strategies such as moving cattle away from wildebeest during calving season are difficult to practice (Reid, 2012). This has resulted, in recent years, in large numbers of cattle dying from MCF (Lyons, N. unpublished data). Additionally, as grazing options become increasingly limited, herds are forced to share pastures which increases the risk of transmissible and vector borne diseases (Reid, 2012; Cleaveland et al., 2001).

In the face of such pressures, improvements in livestock production systems will, it is argued, reduce incentives for non-wildlife-compatible conversion of rangeland to agriculture

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to be a key intervention in the improvement of livestock production systems will be the control, through veterinary interventions such as vaccination delivery, of infectious diseases (e.g. MCF, foot and mouth disease and East Coast fever) that impose significant costs on pastoralist livestock owners (Homewood and Rogers, 1991; Mizutani et al., 2005). Indeed, in a 23-year study, it was estimated that disease caused twice the losses that were incurred by predation from carnivore species (Mizutani et al., 2005), whilst recent work in Laikipia County (Kenya) puts the figure even higher (Amphlett, C. unpublished data). Such interventions, however, have the potential for complex ecological and economic implications (Homewood et al., 2006). For example, the availability of a highly effective MCF vaccine is likely to change traditional disease avoidance strategies, releasing cattle owners from the need to move their herds away from wildebeest each year, possibly resulting in detrimental grazing competition on key pastureland vital for wildebeest populations during their calving season. On the other hand, others predict that without the need to keep large herds as an insurance against drought and disease, improvements in livestock production systems will result in smaller, healthier herds. This in turn, it is argued, will result in healthier ecosystems (Kock, 2005). Others caution that increased profits generated through livestock may be invested in commercial cultivation, shifting the balance between cultivation and livestock with potentially complex implications for the regional ecology. A recent study, however, showed no evidence that the increased income generated from improved livestock survival was being invested in commercial mechanized cultivation. Rather, it suggested that livestock keeping could become profitable enough for pastoralists to focus their labour primarily on livestock production and trade, while keeping some subsistence level of cultivation (Homewood et al., 2006). Research is required, therefore, to test these hypotheses and to address the question of whether the delivery of improved livestock keeping, through interventions such as vaccine campaigns, will result in increased livestock herds, the shift of land-use patterns, and / or a change in rangeland habitat.

The following chapters begin to address some of these questions: in Chapters Two and Four we report the findings from two trials (a field trial and a direct viral challenge trial) which we employed to assess the efficacy of a novel vaccine strategy against MCF in Tanzanian shorthorn zebu cross (SZC) cattle. In Chapter Three we investigate whether the WA-MCF virus (AlHV-1) can be found in wildebeest placental tissues, and the implications this may have for transmission to cattle. Additionally we report a DNA sequence-based analysis of AlHV-1 diversity which allowed conclusions to be drawn regarding the suitability of the

AlHV-1 C500 strain for the development of the novel vaccine. In Chapter Five we report the first quantitative economic assessment of the impact that MCF has on pastoralist (Maasai) households in northern Tanzania. Finally in Chapter Six we provide a synthesis of our findings, we place the study within a broader scientific context, discuss some of the limitations and provide suggestions for future research.

Chapter Two

X

Malignant catarrhal fever - a vaccine field trial

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Introduction

Malignant catarrhal fever (MCF) is an infectious systemic disease of artiodactyls that is caused by γ -herpesviruses of the genus Macavirus. The disease occurs following crossspecies transmission from a carrier host that harbours the virus sub-clinically (Russell, Stewart, and Haig, 2009). Two major epidemiological forms of MCF exist, defined by the reservoir species from which the causative virus arises: i) wildebeest-associated (WA-MCF) (Plowright, Ferris, and Scott, 1960) and ii) sheep-associated (SA-MCF) (Reid et al., 1984). WA-MCF occurs primarily in sub-Saharan Africa wherever wildebeest come into contact with cattle. The causative pathogen in WA-MCF, alcelaphine herpesvirus-1 (AlHV-1) (Plowright, Ferris, and Scott, 1960; Roizmann et al., 1992), is excreted principally by wildebeest calves (Connochaetes taurinus) in the three months following the brief annual calving period. To avoid disease, pastoralists move their cattle from wildebeest calving grounds, often to more marginal land tens of kilometres away, at a time of year when the condition of cattle is most vulnerable. Consequently, the economic costs associated with MCF in high-risk areas can be significant (Bedelian, Nkedianye, and Herrero, 2007; Cleaveland et al., 2001; Lankester et al., 2015b). SA-MCF, caused by ovine herpesvirus-2 (OvHV-2), occurs worldwide wherever sheep and disease-susceptible animals are in proximity (Reid et al., 1984). OvHV-2 is phylogenetically related to AlHV-1 with significant DNA sequence identity (Hart et al., 2007). Typical clinical signs of both WA-MCF and SA-MCF in cattle include fever, lesions in the oral and nasal mucosa, mucopurulent nasal discharge, corneal opacities and, frequently, death (Russell, Stewart, and Haig, 2009; Penny, 2005). Histologically, MCF is characterised by vasculitis, epithelial damage and lymphocytic infiltration of tissues (Plowright, 1968). It is currently believed that virus-infected T-cells are responsible for MCF pathogenesis, although the specific mechanisms are not fully resolved (Dewals et al., 2008; Simon, 2003). Transmission from reservoir hosts to MCF susceptible animals is thought to be by aerosol transmission and contact with the virus on pasture. For AlHV-1 MCF, there is recent evidence that wildebeest placentae contain virus (Lankester
et al., 2015a), but its role in the epidemiology of MCF is not clear (Cleaveland et al., 2001; Rossiter, Jessett, and Karstad, 1983).

There have been several attempts to develop an effective vaccine against MCF (Plowright et al., 1975; Edington and Plowright, 1980). Recent success with an attenuated AlHV-1 vaccine that protected British Holstein-Friesian (FH) cattle from experimental intra-nasal challenge with AlHV-1 (mimicking a natural route of transmission) (Haig et al., 2008; Russell et al., 2012; Parameswaran et al., 2014), was based on the induction of a mucosal barrier of virus-neutralizing antibodies in the oro-nasal pharyngeal region (Haig et al., 2008; Russell et al., 2012). This vaccine was effective for six months, which should protect cattle during the wildebeest calving season (Russell et al., 2012). In this study the vaccine was tested for the first time under field trial conditions in northern Tanzania. The trial was timed to coincide with two consecutive annual wildebeest calving seasons (mid-February), with wildebeest calves expected to shed AlHV-1 virus until approximately three months of age (Plowright, 1965).

Materials and methods

Study site and baseline serological survey

The study site was the Simanjiro Wildlife Dispersal Area (Simanjiro Plain), a mixed-use livestock grazing and wildlife dispersal area 40 km east of Tarangire National Park in northern Tanzania (latitude -3.952239, longitude 36.47537) (Figure 4). Prior to the start of the trial, MCF seroprevalence was estimated in cattle herds around the study site. Households and the cattle therein were selected at random, with household names being drawn from a hat and, within each selected household herd, every n^{th} cow that passed through a gate being selected for sampling. As a result of this procedure a total of 362 cattle were sampled from 22 herds in four villages within 40 km of the national park (Figure 4). Serum samples were heat-treated (56°C, 30 minutes) to inactivate adventitious pathogens before being shipped frozen to the Moredun Research Institute, UK, for serological analysis (as described below).

Animals

A total of 200 shorthorn zebu cross (SZC) cattle of approximately six months of age were purchased at a local primary livestock market in the village of Sukuro. On arrival at the study site (at least one month before MCF vaccination), all cattle were fitted with ear tags and were



Figure 4: A map of the study site

Tarangire National Park, the Simanjiro Plain, the wildebeest migration routes (blue dotted line) and the direction that cattle are traditionally herded (black solid line) to find substitute grazing pastures (orange area) are indicated. The locations of the herds that tested MCF seropositive (black triangles) and seronegative (green circles) in the baseline serological screen are also shown (Source: Map created by Thomas Morrison and Felix Lankester)

immunized against East Coast fever (ECF), which is locally prevalent, using the infection and treatment immunization method (Di Giulio et al., 2008). Additionally, all cattle were treated for endo- and ectoparasites using 1 ml / 50 kg body weight ivermectin (IvomecTM, Merial Animal Health, Essex, UK), administered by subcutaneous injection. Thereafter the cattle were treated with the ecto-parasiticide alphacypermethrin (ParanexTM, Farmbase Ltd, Dar es salaam, Tanzania), administered at 100 mg / litre every two weeks.

Virus and vaccine ¹

The C500 strain of the AlHV-1 virus used for vaccination was prepared at the Moredun Research Institute (Haig et al., 2008). The attenuated AlHV-1 C500 strain, passaged more than 1000 times, was used as the source of virus for immunisation (Handley et al., 1995). This cell-free virus was obtained from bovine turbinate cell culture supernatants, clarified by centrifugation and stored in batches at minus 80 °C. Representative aliquots of attenuated AlHV-1 were titrated (50% tissue-culture-infectious dose (TCID₅₀)) as described for virulent AlHV-1 (Haig et al., 2008).

Field trial design

The field trial was a blinded randomized controlled trial. Sample size calculations indicated that groups of 43 cattle would enable, with 95% confidence and a power of 80%, the detection of a decline in the proportion of exposed animals succumbing to MCF from 30 to 5% (Plowright, 1964; Barnard and Pypekamp, 1988). To increase the power, groups of 50 vaccinated and 50 unvaccinated animals were used and the trial was carried out twice, firstly between December 2010 and July 2011 (2011 trial) and secondly between the same months the following year (2012 trial). This sample size gave us sufficient power to calculate a vaccine efficacy of 57% and above.

In each trial the cattle were randomly divided into two groups: a vaccinated group (n = 50) that received a prime and, four weeks later, a boost containing the attenuated AlHV-1 C500 virus mixed with the adjuvant EmulsigenTM (20% v/v) (an oil-in-water adjuvant containing micron-sized oil droplets with a high surface area available for antigen coating (MVP Technologies 2012)) administered intramuscularly in the upper neck region (Figure 5); and an unvaccinated group (n = 50) that received a mock prime and boost using a virus-free EmulsigenTM inoculum. In each trial, the challenge phase began in mid-February and lasted

¹The laboratory work described in this section was not carried out by F. Lankester



Figure 5: **Image of vaccine delivery** Administration of intramuscular inoculation in to the upper neck region



Figure 6: **Image of trial herding method** Trial cattle being herded close to grazing wildebeest and their calves

until the end of May. During this period, cattle were grazed as a single herd close to wildebeest and their calves (Figure 6).

To estimate the daily intensity of challenge during the 2011 trial a contact index (reflecting the intensity of contact between the trial cattle and wildebeest calves) was calculated using the following equation:

Daily contact index =
$$\alpha \mathbf{x} \beta \mathbf{x} \gamma$$

Where (α) is the duration (minutes) that the trial cattle spent grazing within 200 metres of wildebeest calves, (β) is the number of wildebeest calves present during these encounters and (γ) is an ordinal value that represents the minimum recorded distance (measured by BushnellTM golf range-finder; Bushnell-UK, Chessington, UK) between the calves and trial cattle during the period (zero to 50 metres = 3; 51 to 100 metres = 2; and > 100 metres = 1).

Clinical signs of disease (ocular / nasal discharge or lesions; changes in demeanour or



Figure 7: **Image of blood sampling** Blood samples for serological and molecular analysis were collected from the jugular vein

appetite) were recorded daily while rectal body temperatures were recorded every second day, or daily if sick. Cattle were classified daily as 'healthy', or, if they had temperature \geq 39°C or clinical signs as described above, 'sick'. Uncoagulated blood and nasal secretion (NS) samples were collected on a monthly basis from all cattle starting at the time of primary inoculation (month zero) and ending six months later (Figure 7). NS samples were collected using a tampon (Lil-letsTM, regular) inserted into one nostril for 10 minutes (Figure 8). Plasma and buffy coat cells (from uncoagulated blood) and NS samples were stored at -20°C. Prior to exportation to the UK cell-free samples were heat-treated at 56°C for 30 minutes.

Pathology and histopathology

In fatal cases a post-mortem examination was performed and tissue samples (kidney, liver, lung and lymph node) were collected and fixed in 10% formalin. Following export to the Moredun Research Institute (licence: POAO(S)/2011/54) the fixed samples were embedded in paraffin wax and stained with hematoxylin and eosin. Histopathological examination enabled the pathology of each case to be summarized as: Category A: MCF (most organs contained significant numbers of lesions consistent with a diagnosis of MCF)(Haig et al., 2008); Category B: non-specific infection, consistent with MCF (small number of lesions of



Figure 8: **Image of nasal secretion sample collection** Nasal secretion samples were collected using a regular tampon inserted into the nostril

mild intensity, without extensive infiltration of lymphocytes or clear vasculitis, in one or two studied organs); or Category C: negative (no significant lesions observed).

Antibody responses

MCF-affected cattle can develop virus-specific but not virus-neutralizing antibodies, while induction of neutralizing antibodies in plasma and NS is associated with vaccine induced protection (Haig et al., 2008; Russell et al., 2012). AlHV-1 virus-specific responses were measured in the monthly collected plasma and NS samples by ELISA following previously described protocols (Haig et al., 2008; Russell et al., 2012).

Briefly, virus from cell-free culture fluid of bovine turbinate cells infected with attenuated AlHV-1 C500 was concentrated by centrifugation (35,000 x g, 3 hours, 4 °C). Virus pellets were resuspended in phosphate-buffered saline (PBS) containing 0.8% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma-Aldrich Co. (www.sigmaaldrich.com))), and the insoluble virus pellet was collected by centrifugation as above. The virus pellet was then solubilised in 0.2% sodium dodecyl sulphate (SDS) before dialysing back into 0.2% CHAPS in PBS. This virus-positive ELISA coating antigen had a protein concentration of approximately 2 mg / ml. To produce a negative antigen for comparison, cell-free culture fluid from uninfected BT cells was treated according to the same protocol and the resulting solution used as negative ELISA antigen².

Pairs of adjacent rows of 96-well microtitre plates (Greiner, high protein binding (Sigma-Aldrich)) were coated with 50 μ l of 5 μ g/ml virus-positive or -negative antigen in 0.1 M carbonate buffer pH 9.6. Individual samples of blood plasma or sterile-filtered nasal secretion fluid diluted in PBS were then applied in duplicate to positive and negative antigen wells at serial dilutions from 1:100 to 1:4000. A standard curve, comprising 1:100 to 1:4000 dilutions of an AlHV-1-positive plasma pool was included on each plate to ensure reproducibility in the assays. A negative serum at 1:500 dilution was also included with each test sample series. Antibody bound in each well was detected using 1:1000 rabbit anti-bovine IgG-Horseradish Peroxidase conjugate (Sigma-Aldrich).

AlHV-1 virus-specific titre (hereafter termed ELISA titre) values for each sample were calculated as the difference between means of positive and negative antigen wells for each sample dilution. In the baseline sero-survey, positive samples were defined as those having an ELISA titre value greater than the cut-off value (mean plus $3 \times$ standard deviation of all samples). In the vaccine field trial, ELISA titre values were used to calculate a relative titre for each test sample, with respect to standard curves of pooled MCF-positive plasma or NS, with dilutions of 1/20 to 1/6400. ELISA titre values have been expressed as the reciprocal of the calculated titre (e.g. 20 - 6400). To reduce the likelihood that false positive titres were counted, any sample that gave a calculated titre of less than 20 (i.e. below the range of the standard curve) was not considered positive.

Plasma and NS AlHV-1 virus-neutralizing antibody (VNA) assays were also conducted. These assays were performed at two time points: i) the time point of primary vaccination and ii) the point of peak ELISA titre response. All available NS samples from vaccinated cattle (94) were tested, while a subset of these (23) was also tested for plasma VNA. All unvaccinated cattle with MCF-specific ELISA titre >20 were also tested for VNA in both NS and plasma. The selection for VNA analysis is shown in Table 1. Briefly, the VNA test was based upon inhibition of AlHV-1-induced cytopathic effect in BT cells by dilutions of plasma or nasal secretion fluid as described (Haig et al., 2008). Assays were carried out in 96-well tissue culture plates with BT cells at greater than 80% confluence. All assays used a high titre bovine anti-AlHV-1 serum as a standard and included non-specific toxicity control wells containing sample and cells without virus.

²The antigen preparation protocol described was not performed by F. Lankester

Sample	Trial	Group	Time	Number
type			point	of cattle assayed
Plasma	2011	Unvaccinated	Day zero	3
Nasal secretion	2011	Unvaccinated	Day zero	3
Plasma	2011	Unvaccinated	Two-month	2
Nasal secretion	2011	Unvaccinated	Two-month	11
Plasma	2011	Vaccinated	Day zero	8
Nasal secretion	2011	Vaccinated	Day zero	8
Plasma	2011	Vaccinated	Two-month	7
Nasal secretion	2011	Vaccinated	Two-month	48
Plasma	2012	Unvaccinated	Day zero	4
Nasal secretion	2012	Unvaccinated	Day zero	4
Plasma	2012	Unvaccinated	Two-month	4
Nasal secretion	2012	Unvaccinated	Two-month	10
Plasma	2012	Vaccinated	Day zero	13
Nasal secretion	2012	Vaccinated	Day zero	13
Plasma	2012	Vaccinated	Two-month	13
Nasal secretion	2012	Vaccinated	Two-month	46

 Table 1: Virus neutralizing antibody assay sample selection

The VNA analyses were conducted on a sample of the trial cattle at the time of primary vaccination and at the two-month time point. The vaccinated cattle selected for VNA analysis were chosen at random, while all of the unvaccinated cattle with ELISA titres >20 were tested.

Detection of viral DNA in blood

AlHV-1 DNA was assayed at three time points during the challenge phase of the trial, following the period of highest contact with wildebeest. In addition, to test for pre-existing infections, a random selection of cattle from the 2011 trial were tested for AlHV-1 DNA in samples collected at the time of the primary booster.

Viral DNA was extracted from frozen buffy coat samples using the ZR Viral DNA KitTM (Zymo Research Corporation, USA) according to the manufacturer's instructions and was assayed by nested PCR as described previously (Russell et al. 2012). Briefly, following a first round of AlHV-1 specific hemi-nested PCR, performed using AHV-POL1 (5'-ggctcataatctatgctactccac-3') and AHV-POL2 (5'-attctccacaaactgttttgt-3') primers, a 2 μ l aliquot was used for a second round PCR performed using AHV-POL internal forward primer (5'-ccaaaatgaagaccatctta-3'), and the first-round POL2 as reverse primer. All PCR reactions were carried out using BIOTAQ DNA polymerase or HotStarTaq Plus DNA polymerase. Thermal cycling conditions were adjusted according to the polymerase used. Hemi-nested PCR products were analysed by electrophoresis on 1.8% agarose gel and visualized, photographed and documented using Bio-Rad Gel DocTM EZ system. Although the hemi-nested PCR used here did not distinguish between vaccine and challenge virus, previous studies

have shown that AlHV-1 DNA was not detected in the blood of any cattle vaccinated with the attenuated virus, even up to a year after vaccination (Haig et al., 2008). Therefore PCR positive samples from trial cattle were considered to contain pathogenic AlHV-1, and the respective animals considered infected, if one or more PCR analyses during the challenge phase were positive.

Case definitions

Three case definitions were used for this trial based on PCR detection of AlHV-1 DNA, histopathology and clinical signs:

- I **Not infected**: AlHV-1 DNA was not detected by PCR in any buffy-coat sample taken during the challenge phase of the trial.
- II **AlHV-1 infected**: AlHV-1 DNA was detected by PCR and the animal survived or, if the animal died, there were no histopathological lesions indicative of MCF.
- III Fatal MCF: the animal died following clinical signs typical of MCF. AlHV-1 DNA was detected by PCR and post-mortem histopathological findings were consistent with MCF (Categories A and B).

Exploratory analysis of non-fatal infections and recrudescence

To investigate the longer-term consequences of non-fatal infections, including possible recrudescence, 27 cattle from the 2011 trial (including 13 which had PCR or serological evidence of non-fatal AlHV-1 infection) were kept until the end of the 2012 trial.

Statistical analyses

All statistical analyses were performed using the R language for statistical computing (Team, 2013). Linear regression models were used to assess vaccine safety and to determine whether the results were consistent between the two phases of the trial. A Pearson correlation coefficient was used to examine the relationship between virus-specific and -neutralizing antibody titres. Logistic regression was used to analyse the predictive effect of i) antibody titres on survival and ii) vaccination status and antibody titres on AlHV-1 infection status. Vaccine efficacy (VE) is equivalent to the percentage reduction in the incidence of a specific outcome (or 'case') that is attributable to the vaccine and is calculated

using the notation and formula shown in Table 2 (Knight-Jones et al., 2014). Vaccine efficacy confidence intervals were calculated using established formulae (Orenstein et al., 1985).

Table 2: A vaccine efficacy calculation 2 x 2 table							
Metric	Group	Case	Non-case	Total	Risk	Efficacy	
	Vaccinated	a	b	a + b	$Rv = \frac{a}{(a+b)}$		
Case						$VE = (1 - \frac{Rv}{Ru}) \times 100$	
	Unvaccinated	с	d	c + d	$Ru = \frac{c}{(c+d)}$		

The number of 'cases' in vaccinated and unvaccinated cattle are given by a and c respectively; the number of 'non-cases' in vaccinated and unvaccinated cattle are given by b and d respectively; Rv and Ru are the relative risks of a case occurring in vaccinated and unvaccinated cattle respectively; the vaccine efficacy (VE) at reducing the incidence of 'cases' is equivalent to the percentage reduction in 'cases' that was attributable to the vaccine (Knight-Jones et al., 2014).

Results

Baseline seroprevalence survey

Of the 362 cattle sampled during the baseline survey, four cattle (from three separate herds) were seropositive for MCF, giving an apparent seroprevalence of 1%. The locations of the tested and MCF seropositive herds are shown in Figure 4.

Vaccine safety

In the 2011 trial, cattle were monitored closely during the two weeks following primary vaccination for signs of ill health. Although there were no adverse sub-cutaneous reactions at the inoculation sites, seven percent of cattle were recorded as being 'sick' during this two week period. However the percentage of sick cattle was the same in the vaccinated and unvaccinated groups. Furthermore, regression analysis, with vaccination status as the explanatory variable and daily body temperature as the response variable, indicated that vaccination status was not a predictor of daily body temperature (p = 0.93, t = 0.09, df = 698). Consequently we conclude that the mild sickness recorded during this period was unrelated to vaccination and that the vaccine is likely to be safe for use in SZC cattle.

Contact between wildebeest calves and trial cattle

The data used to calculate the daily contact index are shown in Table 3 and the temporal contact pattern is shown in Figure 9. Early in the challenge phase few wildebeest calves were born and the index was low (< 3000). Thereafter the number of calves in contact increased sharply, to a peak of 142 between day 26 and 30, (contact index > 23,000). Variation in calf numbers appears to drive the pattern of contact up to day 50, after which calf numbers reduce to below 100 and the increase in contact index is driven equally by increasing contact duration and proximity. This might reflect either that the wildebeest were becoming habituated to cattle grazing close by or that the Maasai herders, who had been instructed to graze the trial cattle as close as possible to the wildebeest, were improving their herding skills. Maximum contact index (40,824) occurred between days 81 and 85. The wildebeest began to move away from the Simanjiro Plain around day 100 and the daily contact index decreased to zero by day 106 (Figure 9).

Period	Duration	Wildebeest	Distance	Ordinal	Contact
(days)	(mins) (α)	calves (β)	(m)	value (γ)	index
1-5	108	12	82	2	2,584
6-10	85	5	31	3	1,268
11-15	282	8	57	2	4,512
16-20	190	36	70	2	13,739
21-25	186	85	121	1	15,810
26-30	166	142	287	1	23,564
31-35	175	53	197	1	9,262
46-50	177	112	77	2	39,360
51-55	331	19	84	2	12,578
56-60	258	20	110	3	15,480
61-65	249	25	58	2	12,177
66-70	134	33	85	2	8,844
71-75	331	25	91	2	16,525
76-80	172	60	70	2	20,640
81-85	324	42	44	3	40,824
86-90	320	22	98	2	14,058
91-95	193	36	83	2	13,896
96-100	211	25	68	2	10,339
101-105	111	9	116	1	999
106-110	0	0	NA	0	0

Table 3: Wildebeest calf contact data

 α is the duration (minutes) that the trial cattle spent grazing within 200 metres of wildebeest calves, (β) is the number of wildebeest calves present during these encounters and (γ) is an ordinal value that represents the minimum recorded distance between the calves and trial cattle during the period (zero to 50 metres = 3, 51 to 100 metres = 2, and > 100 metres = 1



Figure 9: Contact between field trial cattle and wildebeest calves

Contact index values are plotted in 5-day increments from the start of the challenge phase.

Field trial outcomes

Clinical, mortality and histopathological data

A summary of the outcomes of the 2011 and 2012 trials is shown in Table 4. Across both trials, three vaccinated and four unvaccinated cattle died after developing clinical signs consistent with MCF. Histopathological examination indicated that all seven cattle had pathology consistent with MCF (Categories A and B) and were PCR positive for AlHV-1 DNA. An additional vaccinated animal died peracutely in the 2012 trial with symptoms typical of black-quarter disease, a bacterial infection most commonly caused by *Clostridium chauvoei* (acute lameness, crepitus and sudden death). There were no lesions observed on histopathology (Category C) but PCR analysis performed on kidney and mediastinal lymph nodes was positive for AlHV-1 DNA.

Analysis of AlHV-1 infection by PCR

Viral DNA was not detected in any sample collected prior to the challenge phase. Postchallenge PCR results are summarized by vaccination group in Table 4 and Figure 10.

In total, 45% of unvaccinated cattle became AlHV-1 infected compared with 20% of vaccinated cattle. Modelling indicated that vaccination status was a significant predictor of infection with unvaccinated animals more likely to be infected (p < 0.001, z = -3.5, df = 185).

Trial	Group	Clinical ^a	Died	Histopath. ^b	PCR ^c	Case	Unvacc.
						$def.^d$	PCR neg.
							serocon. ^e
2011	Vaccinated	39	2	Cat. A: 2	Pos 10	I. 38	
	(n = 50)				Neg 38	II. 8	
					NT 2	III. 2	
						ND 2	
2011	Unvaccinated	46	2	Cat. A: 1	Pos 22	I. 24	13
	(n = 50)			Cat. B: 1	Neg 24	II. 20	
					NT 4	III. 2	
						ND 4	
2012	Vaccinated	50	2	Cat. A: 1	Pos 9	I. 38	
	(n = 50)			Cat. C: 1	Neg 38	II. 8	
					NT 3	III. 1	
						ND 3	
2012	Unvaccinated	49	2	Cat. B: 2	Pos 19	I. 27	12
	(n = 50)				Neg 27	II. 17	
					NT 4	III. 2	
						ND 4	

Table 1.	Crown specific	outcomes of	the 2011	and 201) field	triala
Table 4:	Group specific	outcomes of	the 2011	. and 201.	<i>2</i> neia	triais.

^{*a*}Clinical: Number of cattle that were recorded as being sick during the challenge phase

^bHistopath.: Number of fatal cases whose tissues were categorized as: Cat. A: pathology consistent with MCF; Cat. B: pathology consistent with a non-specific infection; and Cat. C: no pathology detected.

^cPCR: Pos = AlHV-1 DNA detected at one or more time points; Neg = AlHV-1 DNA not detected in any sample; NT = not tested or ambiguous result.

^dCase def.: I = not infected; II = AlHV-1 infected; III = fatal MCF; ND = not defined.

 e Unvacc. PCR neg. serocon. = Number of unvaccinated cattle that, despite being PCR negative, had serological evidence of AlHV-1 infection.



Figure 10: Field trial PCR results

For each of the 2011 and 2012 trials, the percentage (and the $\pm 95\%$ confidence intervals) of vaccinated (Vacc) and unvaccinated (Unvacc) cattle that tested PCR positive is shown.

Case definitions and vaccine efficacy

The clinical, mortality, histopathology and PCR data allowed every animal to be allocated to one of three case definitions summarized in Table 4. The numbers in each category did not differ significantly between the 2011 and 2012 trials (p = 0.54, t = -0.6, df = 185). In total, 19 of 95 vaccinated cattle became infected with AlHV-1, of which three developed fatal MCF, whilst 41 of 92 unvaccinated cattle became infected, of which four developed fatal MCF. One vaccinated animal was PCR positive for AlHV-1 but died peracutely of suspected black quarter and this was assigned to Case Definition II (AlHV-1 infected) due to the pathognomonic clinical signs of black quarter and the lack of MCF-specific histopathology.

To determine the VE for reducing AlHV-1 infection, PCR data from both the 2011 and 2012 trials were combined and used to populate Table 5 according to the method described in Table 2. The calculated vaccine efficacy for preventing infection with AlHV-1 was 56%. Given the low numbers of fatal cases the vaccine's efficacy at preventing fatal MCF has not been detailed in the table, however it would have given an efficacy of 25%.



Figure 11: Field trial AlHV-1 specific antibody results

Monthly geometric mean (and $\pm 95\%$ confidence intervals) nasal secretion (plots A and C) and plasma (plots B and D) AlHV-1-specific antibody titres in vaccinated (dashed line) and unvaccinated (solid line) animals. Both the 2011 and 2012 trials are plotted (P = primary vaccination, B = booster vaccination and C = beginning of challenge phase).

Serological analysis

The geometric mean NS and plasma AlHV-1-specific antibody titres (ELISA titres) (calculated at monthly time points) are shown in Figure 11. All vaccinated animals had positive plasma and NS ELISA titres with mean plasma ELISA titres peaking two months after primary immunization. NS ELISA titres also peaked two months after primary immunization in the 2012 trial but, unlike the plasma titres, showed a significantly lower response in the 2011 trial (p < 0.001, t = 13.2, df = 98). During the challenge phase, 40 of the 100 unvaccinated animals had at least one plasma or NS sample return a seropositive result suggestive of exposure to AlHV-1. Despite this, the mean plasma and NS ELISA titres of unvaccinated animals were essentially zero throughout both the 2011 and 2012 trials.

VNA analyses were also performed. At the time point of peak ELISA titres, 31 and 76% of vaccinated cattle from the 2011 and 2012 trials, respectively, had positive NS VNA titres. No unvaccinated cattle in either trial produced VNA. The geometric mean NS VNA titres



Figure 12: Correlation of ELISA and VNA serological responses

Correlation of nasal secretion AlHV-1 ELISA and VNA titres (log_{10}) . The line and the shaded area indicate the regression line and associated $\pm 95\%$ confidence interval (r = correlation coefficient). Subsequent infection status is shown as follows: light grey triangles = PCR positive cases; dark grey circles = PCR negative cases.

of vaccinated cattle in the 2011 trial were significantly lower than the 2012 trial titres (p < 0.001, t = 4.4, df = 92).

The effect of antibody titre on infection status

At peak serological response, ELISA and VNA titres in vaccinated cattle were significantly correlated in plasma (p < 0.02, r = 0.25, t = 2.4, df = 92) and NS (p < 0.001, r = 0.51, t = 5.6, df = 92). Figure 12 illustrates the correlation and how the titres of infected and uninfected cattle were distributed. Although we have shown that vaccination status, and thus presence of virus-specific and VNA antibodies, is a strong predictor of infection status, modelling indicated that the magnitude of ELISA and VNA titres did not have a significant influence on whether vaccinated cattle became infected (p > 0.3; Figure 13). However the presence of VNA antibodies in NS of vaccinated cattle was weakly associated with protection from infection in vaccinated cattle (p = 0.07).

Infection status determined by post-challenge seroconversion

In unvaccinated cattle seroconversion is an indicator of AlHV-1 infection and this was used in combination with PCR results to determine the proportion of unvaccinated cattle that became



Figure 13: Analysis of field trial serological responses by outcome

Nasal secretion and plasma geometric mean ELISA titres (and $\pm 95\%$ confidence intervals) from vaccinated animals are plotted for each month during the 2011 and 2012 trials. Dashed lines indicate infected (n = 19) and solid lines uninfected cattle (n = 76) (P = primary vaccination, B = booster vaccination and C = beginning of challenge phase).

infected during challenge. Of 97 initially seronegative unvaccinated cattle, 40 seroconverted during the challenge phase, while a further 26 remained seronegative but were PCR positive (Table 4). In total 66 out of 97 (68%) unvaccinated cattle showed evidence of post-challenge infection, of which four died of MCF. The proportion of infected unvaccinated cattle that died of MCF was 6%.

Exploratory analysis of non-fatal infections and recrudescence

To investigate the longer-term consequences of non-fatal infections, including possible recrudescence, 27 cattle from the 2011 trial were kept until the end of the 2012 trial. Despite 13 cattle having PCR or serological evidence of non-fatal AlHV-1 infection, none showed any signs of MCF during this extended period.

Discussion

This paper describes the first field trial of a vaccine for wildebeest-associated malignant catarrhal fever, previously tested using experimentally challenged British FH cattle (Haig et al., 2008; Russell et al., 2012; Parameswaran et al., 2014). The clinical data from this trial

indicate that the attenuated virus vaccine formulation was well tolerated, with no evidence of adverse effects. Importantly, vaccinated cattle were more than twice as likely as unvaccinated cattle to remain uninfected with MCF virus transmitted naturally from wildebeest. Due to the unexpectedly low number of fatal MCF cases, we cannot draw conclusions regarding the vaccine's efficacy at reducing lethal MCF. As we are not aware of any herpesvirus vaccines that prevent infection and the establishment of latency, we consider the proportion of vaccinated animals, in both laboratory trials (70 (Haig et al., 2008) and 81% (Russell et al., 2012)) and this field trial (80%), that did not become infected in the face of intense challenge as a promising indication for vaccine improvement strategies. Initial work to improve the response induced by the current vaccine strategy by the inclusion of specific toll-like receptor agonists with the adjuvant has been tested in other vaccine trials (Parameswaran et al., 2014) and Chapter Four. However, any further improvement of this vaccine should be done in the context of a natural challenge system that will demonstrate benefits for livestock keepers.

The number of cattle used in the trial enabled a vaccine efficacy of 57% and above to be calculated. However the efficacy of the vaccine at preventing infection with AlHV-1 was calculated to be 56%, which falls just below the limit of confidence provided by the sample size. This outcome is a reflection that the assumptions used to calculate the sample size were inaccurate. Specifically, many more vaccinated cattle than expected (20 as opposed to the assumed 5%) were subsequently infected by AlHV-1.

The serological analysis indicated that the vaccine was effective at eliciting a virusspecific immune response in all vaccinated cattle. ELISA titres peaked two months after primary inoculation. The NS results, however, varied between the trials, with both ELISA and VNA titres being significantly lower in 2011. As the plasma ELISA titres from both the 2011 and 2012 trials were very similar (Figure 11), it is likely that these results were caused by a sample processing issue in the 2011 NS samples rather than a failure of the vaccine itself.

The moderately strong correlation between the ELISA and VNA titres in NS and plasma samples (Figure 12) was expected, with all cattle that exhibited high VNA titres also having a high ELISA titre. There were, however, some cattle that, despite having relatively high ELISA titres, had low or zero VNA titres. It is unclear why these cattle failed to produce neutralizing antibodies but host genetic or immunological factors could focus virus-specific immune responses on antigens or epitopes that were non-neutralizing. Indeed, two AlHV-1 capsid proteins, which are unlikely to be neutralizing antigens, are strongly recognized by AlHV-1 vaccinated or infected cattle sera (Bartley et al., 2014). Analysis of NS VNA data

from the vaccinated cattle showed that the correlation between presence of VNA antibodies and protection from infection approached, but did not achieve, statistical significance (p < 0.07). This is illustrated in Figure 12, where nine of 60 cattle with VNA titre >10 were infected (grey triangles on the right of the figure). This lack of a significant association may indicate that the induction of mucosal VNA is not as strong a correlate of protection as previously thought and that other aspects of the immune response induced by vaccination may also contribute to protection from MCF. It may also reflect differences in the mode of challenge between this trial and previous trials where the source of virus, the dose and the timing of challenge were all controlled.

WA-MCF is reported to have a 'case-fatality ratio' of between 96 and 100% (Plowright, Ferris, and Scott, 1960; Bedelian, Nkedianye, and Herrero, 2007; Rossiter, Jessett, and Mushi, 1980). However, these reports may be based on observed progression of clinical MCF cases in the absence of capability to estimate sub-clinical infection rates. The baseline seroprevalence analysis of cattle provided preliminary evidence that non-fatal AlHV-1 infections do occur. Similarly, among the trial cattle, four animals had low ELISA titres in plasma at day zero, although none had detectable AlHV-1 DNA at any time point tested. Two of these cattle were subsequently vaccinated and developed high ELISA and VNA titres, while the other two were unvaccinated and had low positive ELISA titres but no VNA titre. It is unclear whether this apparent pre-exposure to AlHV-1 influenced the outcome of these cattle became infected during the challenge phase of the trial, however only 6% developed fatal MCF. This high frequency of non-fatal infections could reflect the true range of outcomes following AlHV-1 infection. Indeed, in SA-MCF, non-fatal infections have been reported (Otter, Pow, and Reid, 2002; Moore et al., 2010).

The East Coast fever vaccination, administered to all trial cattle before the MCF vaccine, may have influenced the post-infection outcomes seen in the trial. The ECF vaccine is thought to induce a cell-mediated immune response that suppresses the proliferation of CD8 T-cells (Di Giulio et al., 2008; Radley et al., 1975). The cell biology and pathogenesis of MCF are not fully understood, but the associated indiscriminate tissue damage is thought to involve virus-infected CD8 T- cells (Dewals et al., 2008; Thonur et al., 2006; Hart et al., 2007; Simon, 2003). Thus, the immuno-modulating effects of the ECF vaccination might have provided some protection from MCF pathology and warrants further study.

This trial also provided further insights into natural AlHV-1 infection. For example, most PCR-positive samples came from a time point close to challenge day 70 in both trials.

The estimated incubation period for MCF is about 21 days (Plowright, 1968), indicating an infection window close to day 49, which coincides with a peak in wildebeest contact index (Figure 9). These results show that herding cattle close to wildebeest calves of less than three months of age does expose them to AlHV-1 and underscores the accuracy of the timing of the traditional Maasai disease avoidance strategy.

Conservationists have often been concerned that an effective MCF vaccine may result in large-scale, unsustainable shifts in livestock grazing that could cause environmental damage in the important wildlife dispersal areas adjacent to parks and game reserves. However, the partial (56%) protection provided by this vaccine is probably insufficient for cattle owners to risk changing traditional avoidance strategies to graze cattle in productive lands alongside wildebeest during the calving season. Nonetheless, even partial protection would still be of value to protect animals that cannot be moved, for example, where some of the herd remain at the permanent family boma to provide milk for women and children attending school (Lankester et al., 2015b), or where land-use changes make traditional disease avoidance strategies difficult (Reid, 2012; Cook, 2015). A partially protective vaccine may therefore offer a feasible solution to some of the current land-use challenges and conflicts, providing some protection to valuable livestock where avoidance strategies are not possible, but with less risk of potentially damaging environmental consequences. More widely, the vaccine could also play a role around the world in disease prevention strategies where cattle live in close proximity to zoological gardens housing wildebeest calves (Meteyer et al., 1989; Whitaker et al., 2007).

Chapter Three

XI

Alcelaphine herpesvirus-1 in wildebeest placenta: Genetic variation of ORF50 and A9.5 alleles

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Introduction

Malignant catarrhal fever has been impacting the lives of cattle keeping people living in eastern and southern Africa for millennia (Gifford-Gonzalez, 2000). Yet, despite this long-term relationship, the epidemiology of MCF is not fully understood. Specifically questions remain regarding how frequently intra-uterine infection occurs in wildebeest (Plowright, Ferris, and Scott, 1960; Pretorius, Oosthuizen, and Van Vuuren, 2008) and, once infected, how AlHV-1 is transmitted from calves to susceptible species. For example Tanzanian pastoralists consider placenta tissue, foetal membranes, foetal fluid or calf hair (moulted at 3-4 months) as a major source of MCF virus transmitted from wildebeest calves (Cleaveland et al., 2001). Whilst these perceptions of placental involvement in MCF transmission are firmly held they are not supported by the available evidence (Rossiter, Jessett, and Karstad, 1983). The first objective of this chapter, therefore, was to collect and examine wildebeest placental tissue in order to determine whether AlHV-1 infects these tissues and, as a consequence, whether these tissues might be a source of infection for susceptible species.

The second objective of this chapter was to compare the strains of virus circulating in East Africa with the AlHV-1 C500 virus (originally obtained from an AlHV-1-infected ox in Kenya in the 1960s and since passaged in New Zealand White rabbits (Plowright et al., 1975)) which has been the strain of choice for recent vaccine development (Haig et al., 2008; Russell et al., 2012). Given that the protection afforded by the novel vaccine strategy to shorthorn cross (SZC) cattle under natural virus transmission (reported in Chapter Two), was less than that experienced by British Friesian Holstein (FH) cattle under experimental challenge, investigating the genetic diversity that might exist between the viral strains was of interest.

To investigate this a novel spliced gene (A9.5) was used. A9.5, which is predicted to encode a secreted glycoprotein (Russell et al., 2013), has a detectable homologue in OvHV-2 (Ov9.5) that has been shown to be both polymorphic and highly variable with nine alleles

showing as little as 50% predicted amino acid sequence identity (Russell et al., 2014). Thus far A9.5 has been analysed in samples from only two separate sources, the C500 strain of AlHV-1 (Russell et al., 2013) and a diagnostic sample taken from an Ankole cow with MCF (Whitaker et al., 2007). The two were found to have different alleles: A9.5*0101 in the C500 virus, and A9.5*0201 in the Ankole cow. Given the level of polymorphism found in Ov9.5, it is expected that analysis of the homologous A9.5 gene from a range of wildebeest and cattle samples will allow comparisons to be made between C500 and contemporaneous wild strains circulating in East Africa.

Materials and Methods

Biological samples for analysis of AlHV-1 infection were collected from four sources and are detailed in Table 6:

- 1. Wildebeest calf and dam: Uncoagulated blood samples were collected from two wildebeest populations resident in Tanzania: i. The Ngorongoro Crater population, which despite residing in the Crater for long periods of time are known to occasionally disperse and mingle with the population resident in the wider Serengeti ecosystem; ii. The Tarangire population, which live predominantly in the vicinity of Tarangire National Park. The two populations are separate, both in the geographical and evolutionary sense (Georgiadis, 1995). All sampled wildebeest were immobilized (between March and May 2012) using etorphine hydrochloride (CaptivonTM, Wildlife Pharmaceuticals, Karino, South Africa) and all blood samples were collected by jugular venipuncture directly in to EDTA VacutainerTM collection tubes (BD Diagnostics, New Jersey, USA). Wildebeest were not selected by random, with individuals that were easiest to immobilise being selected. Samples were collected from three dams and five approximately 32-day-old calves from the Ngorongoro Crater population and from five approximately 100-day-old calves from the Tarangire population. The ages of the calves could be accurately judged as the wildebeest calving season, for which the authors recorded the start date, is highly synchronised and only lasts a few days. Buffy coat cells prepared from the blood samples were stored on FTATM cards (Whatman FTA Classic, Thermo Fisher, UK) prior to DNA extraction, while blood plasma was stored at minus 20 °C.
- 2. Wildebeest placenta: Samples of freshly passed wildebeest placenta from the Ngorongoro Crater population, consisted of approximately 1 cm³ of full thickness tis-



Figure 14: **Image of field sampling** Field team collecting placenta tissue samples in the Ngorongoro Crater

sue. To collect these samples field teams followed calving wildebeest and waited for the placenta to be expelled. As soon as the placenta fell away from the dam, approximately 45 minutes after the calf was born, the team would drive up to the placenta and collect the tissue sample (Figures 14 and 15). None of the dams paid any attention to their placenta, and there was no licking or sniffing observed. Consequently we consider all samples to have been uncontaminated with the dam's ocular or nasal secretion. All samples once collected were frozen (at minus $20 \,^\circ$ C) prior to DNA extraction.

- 3. **MCF vaccine trial cattle:** Blood samples, collected by jugular venipuncture directly in to VacutainerTM collection tubes as part of a vaccine trial involving SZC cattle living near Tarangire National Park, were selected for analysis if they came from individuals that had become naturally infected with MCF virus transmitted by wildebeest calves. Buffy coat cells, prepared from the blood samples, were stored on FTA cards or frozen (at minus 20 °C) prior to DNA extraction.
- C500: The C500 strain of AlHV-1 in the UK, for which complete genome sequence is available (Ensser, Pflanz, and Fleckenstein, 1997). Purified AlHV-1 C500 DNA was used as a control in PCR and sequencing analyses.



Figure 15: **Image of placenta tissue collection** Sample of tissue being collected from a recently passed wildebeest placenta

Table	Table 6: Details of sample sets and summarized results of diagnostic assays							
Animal ^a	Population ^b	Sample	Sample	Real time	Nested	AlHV-1	A9.5	
	/ Location		Numbers	PCR	PCR	specific	sequence	
				positive	positive	antibody	obtained	
						positive		
Wb	N'goro	Placenta	94	1	50	NA	23	
	Crater							
Wb	N'goro	Buffy	3	0	1	3	0	
dam	Crater	coat						
Wb	N'goro	Buffy	5	0	5	4	3	
(32 do)	Crater	coat						
Wb	Tarangire	Buffy	5	0	1	5	1	
(100 do)		coat						
Cattle	Tarangire	Buffy	200	0	59	154*	6	
(adult)		coat						

^aAnimal: Wb = wildebeest; do = day old;

^bPopulation / Location: N'goro Crater = Ngorongoro Crater;

*This number includes 54 out of 100 *unvaccinated* animals with at least one sample of nasal secretion or blood plasma with MCF-specific ELISA titre > 10. Of these animals, 20 were found to be AlHV-1 positive by nested PCR.

AlHV-1 specific antibody reactivity

Duplicate samples of wildebeest blood plasma were tested twice using an AlHV-1 specific direct ELISA, essentially as described in Chapter Two (Russell et al., 2012), except that known positive and negative wildebeest serum samples from the UK were used as controls and to generate standard curves for determination of AlHV-1-specific antibody titre.

Detection of viral DNA

The presence of AlHV-1 was determined by PCR analysis of DNA extracted from buffy coat cells and placenta tissue samples. DNA was purified from the frozen cattle buffy coat samples using the ZR Viral DNA Kit; from the FTA card stored wildebeest buffy coat samples using QiaAmp Mini Kit (Qiagen, Crawley, UK); and from the frozen placental samples using the Qiagen DNeasy Blood and Tissue Kit. Thereafter viral DNA was detected and analysed by AlHV-1-specific duplex real-time PCR (Traul et al., 2005; Russell et al., 2012) (targeting the AlHV-1 ORF3 gene (Traul et al., 2005) and the genomic β -actin gene (Russell et al., 2012)) and nested diagnostic PCR (Flach et al., 2002). Each set of assays was controlled by the inclusion of known AlHV-1 positive and negative bovine genomic DNA samples and potential cross-contamination between reactions was controlled by the inclusion of template-free reactions. To study viral variation in positive samples, segments of the ORF50 gene, encoding the lytic cycle trans-activator protein, and of the spliced A9.5 gene (Russell et al., 2013) were sequenced and analysed as described previously for OvHV-2 (Russell et al., 2014). ORF50 encodes a transcription factor expected to be highly conserved, whilst A9.5 encodes a predicted glycoprotein of unknown function that has been demonstrated to be polymorphic in both AlHV-1 and OvHV-2 (Russell et al., 2014; Russell et al., 2013).

PCR amplification of A9.5 and ORF50 genes

Primers for nested PCR of the A9.5 gene were designed to target conserved areas flanking the predicted coding region, based on the available sequence of AlHV-1(Ensser, Pflanz, and Fleckenstein, 1997; Russell et al., 2013). Primers for amplification of ORF50 (AHVorf50-F, GCC AGG CAG AGG TAT GTG TT and AHVorf50-R, GGC CGT TGT GGG TAC TGT AT) were chosen within exon 2 of the ORF50 gene, to amplify a fragment of 543 base pairs for analysis of sequence variation. Primer pairs were designed using Primer3 (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser et al., 2007). The amplification of A9.5 used a nested PCR strategy (Russell et al., 2013) because a

non-nested approach amplified the gene poorly. This lack of efficiency probably reflects the constraints on the primer design aimed to amplify the entire A9.5 coding region. In contrast, the primer design for ORF50 required only amplification of a fragment of the gene. These primers amplified the ORF50 segment efficiently without the need for a nested approach. Amplification of ORF50 and A9.5 gene segments was attempted from genomic DNA samples in which AlHV-1 DNA had been detected by diagnostic nested PCR (Flach et al., 2002). For A9.5, a nested PCR approach was used with initial amplification performed in 25 μ l reactions using 1 unit KOD Hot Start DNA polymerase (Merck, Feltham, UK), 50-100 ng of genomic DNA, and 5 pmol each of primers A9.5geneF and A9.5ex6R (Russell et al., 2013). Amplification reactions consisted of a denaturation / activation step at 94 °C for 120 s; 30 cycles of $94 \,^{\circ}\text{C}$ for 30 s, $55 \,^{\circ}\text{C}$ for 30 s and $70 \,^{\circ}\text{C}$ for 60 s; and a final extension step at $70 \,^{\circ}\text{C}$ for 5 minutes. Aliquots of 2 μ l from this initial PCR were then used as templates in nested PCR amplifications using the same enzyme, buffer and PCR conditions but with 10 pmol per reaction of the nested primers A9.5cdsF and A9.5cdsR (Russell et al., 2013). The AlHV-1 ORF50 segment was amplified in a single reaction using the same conditions as described above for A9.5 except that 10 pmol per reaction of the AHVorf50-F and AHVorf50-R primers were used and reactions were amplified using 40 cycles of 94 °C for 30 s, 59 °C for 30 s and 68 °C for 60 s. The PCR products were analysed by agarose gel electrophoresis, stained with SYBR Safe DNA Gel Stain (Life Technologies, Paisley, UK) and visualized by UV transillumination before purification (QIAamp PCR purification system). PCR product concentrations were estimated after purification using a Nanodrop spectrophotometer (Labtech, Uckfield, UK). Approximately 300 ng of each PCR product was submitted for bidirectional nucleotide sequencing by Eurofins MWG Operon (Ebersberg, Germany), using the internal PCR primers as the sequencing primers. Electropherograms from each pair of sequencing reactions were assembled to produce sample consensus sequences for each gene segment amplified. To confirm that direct sequencing of PCR products produced an accurate representation of the target sequence in-vivo, PCR products of A9.5 from 4 samples were cloned into pGEM-T-Easy (Promega, Southampton, UK) and at least three clones representing each PCR product were sequenced.

Bioinformatics

Unless otherwise indicated, all DNA sequence analysis was carried out using DNASTAR Lasergene software (V8.0 and above; www.DNASTAR.com). DNA sequence information from each amplicon was assembled using the SEQMAN program and consensus sequences representing the region flanked by the PCR primers were derived. Any DNA sample that did not give good quality sequence traces on both strands was discarded. The consensus sequences for each sample and locus were aligned using the MUSCLE algorithm (Edgar, 2004) or MAFFT (Katoh and Standley, 2013). For A9.5, the positions of introns and exons were defined according to the annotation of A9.5*01 (Russell et al., 2013) and conservation of splice donor and acceptor sequences was confirmed by visual inspection, while the conservation of a continuous A9.5 open reading frame was confirmed by generation of predicted A9.5 cDNA sequences and their translation. Phylogenetic and evolutionary analysis of all sequences was done by maximum likelihood methods using the program MEGA (version 5 or above; megasoftware.net) (Tamura et al., 2013).

Nucleotide sequence accession numbers

Nucleotide sequences of the gene fragments amplified in this work have been submitted to the European Nucleotide Archive (ENA; www.ebi.ac.uk/ena) and have been assigned accession numbers as follows: AlHV-1 orf50*0101, LN823968; orf50*0102, LN823969; orf50*0103, LN823970; orf50*0201, LN823971; orf50*0301, LN823972; orf50*0401, LN823973; A9.5*0202, LN823974; A9.5*0203, LN823975; and A9.5*0301, LN823976.

Ethical clearance

Permission to immobilise wildebeest, to collect placenta and to carry out the MCF vaccine trial was provided by the Tanzanian Wildlife Research Institute and the Tanzanian Commission for Science and Technology (permit numbers 2010-259-NA-2005-141 and 2011-213-ER-2005-141). Permission to import inactivated blood plasma, serum and DNA on FTA cards into Scotland was provided by the Scottish Executive Rural Directorate (import licenses POAO(S)/2011/54, and POAO(S)/2012/37).

Results

Detection of AlHV-1 virus DNA in wildebeest samples

The nested PCR method detected viral DNA in buffy coat samples from one of the three dams, in six of the ten calves and in 50 of the placental samples (summarised in Table 6). The six positive wildebeest calf buffy coat samples consisted of all five of the 32-day old calves from the Ngorongoro Crater population and one of the five 100-day old calves from the Tarangire population. In contrast, the less sensitive duplex real-time PCR assay detected

AlHV-1 DNA in none of the wildebeest buffy coat samples and in only one of the placental samples. All samples showed amplification of the β -actin internal control (Russell et al., 2012) and therefore were suitable for PCR.

Antibody reactivity of wildebeest blood samples

AlHV-1 specific antibody titre in blood plasma was measured in all collected wildebeest blood samples. Results are summarised in Table 6. All but one of the animals tested were positive for AlHV-1 specific antibodies, with titre values >100. The seropositive individuals included two wildebeest dams and four 100-day-old calves that tested negative for viral DNA by nested PCR. Interestingly, the single animal that did not have a positive AlHV-1-specific antibody titre was a 32-day-old calf that tested positive for viral DNA by nested PCR.

Sequencing of AlHV-1 loci from wildebeest and cattle

In order to look at viral sequence variation within the AlHV-1 positive samples identified, PCR fragments representing the variable A9.5 locus were analysed. To determine whether variation at A9.5 represented a generally high level of sequence variation between AlHV-1 strains, the ORF50 locus, encoding the lytic regulator RTA, was also analysed.

The ORF50 locus

Amplification of ORF50 was attempted from all positive wildebeest samples (placenta and FTA card preserved buffy coat) and from FTA card preserved cattle samples (Table 6). For all FTA card preserved buffy coat samples, amplification was unsuccessful. In each case the amplification was repeated to confirm the result. ORF50 was successfully amplified from 38 of the 50 AlHV-1-positive placenta samples. Sequencing of these PCR products identified six distinct ORF50 alleles (Table 7) that shared more than 99% sequence identity. The majority of positive samples had a sequence identical to ORF50*0101 (Ensser, Pflanz, and Fleckenstein, 1997), the allele carried by the C500 AlHV-1 strain. The other five alleles had ORF50 sequences that differed at only one or two base positions from ORF50*0101 (Figure 16 and 17). Two of the novel alleles (termed ORF50*0102 and ORF50*0103) encoded ORF50 proteins with identical sequence to ORF50*0101, whereas the remaining three novel alleles (0201, 0301 and 0401) encoded ORF50 proteins that differed by one amino acid from ORF50*0101 and by two amino acids from each other (Figure 17).

Species			Cattle			
Provenance	Ngoi	rongoro Cra	goro Crater Tarangire $Colf^{a}$ Colf b			
Age		Dam	Calf ^{<i>a</i>}	Calf ^b	Adult	
Sample type	Placenta	BC FTA	BC FTA	BC FTA	BC	
Sample numbers	94	3	5	5	6	
A9.5 alleles						
A9.5*0101	13 (56%)	0	1	1	3	
A9.5*0201 ^c	0	0	0	0	0	
A9.5*0202	0	0	0	0	3	
A9.5*0203	5 (22%)	0	1	0	0	
A9.5*0301	5 (22%)	0	1	0	0	
ORF50 alleles						
ORF50*0101	29 (76%)	0	0	0	NA	
ORF50*0102	5 (13%)	0	0	0	NA	
ORF50*0103	1 (2.6%)	0	0	0	NA	
ORF50*0201	1 (2.6%)	0	0	0	NA	
ORF50*0301	1 (2.6%)	0	0	0	NA	
ORF50*0401	1 (2.6%)	0	0	0	NA	
A9.5-ORF50 hapl	otypes					
A9.5*0101	7 (41%)	0	0	0	NA	
ORF50*0101						
A9.5*0101	3 (17.6%)	0	0	0	NA	
ORF50*0102						
A9.5*0101	1 (5.9%)	0	0	0	NA	
ORF50*0301						
A9.5*0203	3 (17.6%)	0	0	0	NA	
ORF50*0101						
A9.5*0301	3 (17.6%)	0	0	0	NA	
ORF50*0101						

 Table 7:
 Summary of AlHV-1 genotyping results

^{*a*}Calves <30 days old

^bCalves <100 days old

^cAs previously described (Russell et al., 2013);

Sample type BC = Buffy Coat; FTA = stored on FTA cards



Figure 16: Phylogenetic analysis of AlHV-1 ORF50 gene sequences

Nucleotide sequences of the six variants ORF50*0101 to ORF50*0401 were aligned using MAFFT and phylogenetic analysis was done in MEGA version 6.0 using the Maximum Likelihood method, based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood is shown. There were a total of 503 positions in the final dataset. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

0RE50*0101 CTTTTCAACTTCTGTCTTTTCCC 0RE50*0101 CTTTTCAACTTCTGTCTTTTCCC 0RE50*0101 CTTTTCAACTTCTGTCTTTTCCC 0RE50*0101 CTTTTCAACTTCTGTCTTTTCCC 0RE50*0101 CTTTTCAACTTCTGTCTTTTCCC 0RE50*0401 CTTTTCAACTTCTGTCTTTTCCC 0RE50*0401 CTTTTCAACTTCTGTCTTTTCCC PheSerThrSetValPheSer-	ORF50*0101 AGCTACCAGTCAGACGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTGCCTCTCGTATAGATGCATCTGCCACAGTTATGGGTATCCAGGGCCCAGTCACTAATCACGG ORF50*0103 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTGCCTCTCGTATAGATGCATCTGCCACAGTTAATGGGTATCCAGGCCCAGTCACTAATCACGG ORF50*0201 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTTGCCTCTCGTATAGATGCATCTGCCACATCAACCAGTTATGGGTATCCAGGCCCAGTCACTAATCACGG ORF50*0301 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTTGCCTCTCGTATAGATGCATCTGCCACATCAACCAGTTATGGGTACCCAGGCCCAGTCACTAATCACGG ORF50*0301 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTGCCTCTCGTATAGATGCATCTGCCACATCAACCAGTTATGGGTACCCAGGCCCAGTCACTAATCACGG ORF50*0301 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTGCCTCTCGTATAGATGCATCTGCCACATCAACCAGTTATGGGTACCCAGGCCAGTCACTAATCACGG ORF50*0401 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTGCCTCTCGTATAGATGCATCTGCCACATCAACCAGTTATGGGTACCCAGGCCAGTCACTAATCACGG ORF50*0401 AGCTACCACTCAGACGGGATTCTACTATAACCAGCAGTCAAGTGATAATTTTGCCTCTCGTATAGATGCATCTGCCACATCAACCAGTTATGGGTACCCAGGCCACTCACT	0RF50*0101 AAGTACTGACTCCGACACCCATGCAGAGAGAGAGAGGATGATGTTCCTGAAGCACCCCAAGCAGCAGGAAGCCAAACTCAGCCAAGAGAGACACAAGAGTTGCTGCGAGTGTCTGCAGAGTGCTACAATCC 0RF50*0103 AAGTACTGACTCCGACACCCATGCAGAAGACGATGATGTTCCTGAAGCACCCCAAGCAGCAAGCCAAACTCAGCCAAGAACTACCCAAGAGACAACAGGTTGCTGCAGTGTCTACAATCC 0RF50*0102 AAGTACTGACTCCGACACCCATGCAGAAGACGATGATGTTCCTGAAGCACCCCAAGCAGCCAAACTCAGCCAAACTACCCAAGAGACAACAGAGTTGCTGCAGTGTTACAATCC 0RF50*0102 AAGTACTGACTCCGACACCCATGCAGAAGACGATGATGTTCCTGAAGCACCCCAAGCAGCGAGACAACTCAGCCAAAACTACCCAAGAGACAACAGAGTTGCTGCAGTGTCTACAATCC 0RF50*0102 AAGTACTGACTCCGACACCCATGCAGAAGACGATGATTCCTGGAAGCACCCCAAGCAGCCGAAGCCCAAACTCAGCCAAACTACCCAAGAGAACAACAGAGTTGCTGCAGTGTCTACAATCC 0RF50*0401 AAGTACTGACTCCGACACCCATGCAGAAGACGATGATTCTCGGAAGCACCCCAAGCAGCGCAAACTCAGCCAAAACTACCCAAGAGAACAACGAGGTTGCTGCAGATGTCTACAATCC 0RF50*0401 AAGTACTGACTCCGACACCCATGCATGATGTTCCTGAAGCACCCCAAGCCCAAGCCAAACTCAGCCAAAACTACCCAAGAGAACAACGAGGTTGCTGCAGATGTCTACAATCC 0RF50*0401 AAGTACTGACTCCGACACCCATGCATGATGTTCCTGAAGCACCCCAAGCCCAAGCCAAACTCAGCCAAACTACCTAACAACAACAACAAGAGTGCTGCTGCAGAGGTGCTACAATCC 0RF50*0401 AAGTACTGACTCCGACACCCATGCATGATGTTCCTGGAAGCACCCCAAGCCCAAGCCAAACTCAGCCAAACTACCCAAGAGACAACAGACTGCCGCGAGGCCAACACTACCCAAGGACACAAGAGTCGCGAGGCTGCTGCAGGTGCTGCAGTGCTACAATCC 0RF50*0401 SCTTACAATCCGACACCCATGCAGAAGCATGTTCCTGGAAGCACCCCAAGCCCAAGCCAAACTCAGCCAAACTACCCAAGAGACACAAGAGTGCCGAGGCTGCTGCAGTGCTGCAGAGACGATGATGCC 0RF50*0401 SCTTACAATCCGACCCATGCAGAAGCAAGTGCCTGAAGCTCAGCCAAGCCAAACTACCCAAGAGACACAACAACTACCCGAGGCTGCTGCAGAGACGATGCCAAGACTACCCAAGCACTGCCGAGCCAAGCAACTACCCAAGACTACCCAAGGACCCCAAGCCAAGCCAAGCTACCAAGACTACCCAAGAGTGCCAAGAGACGACGACGAGAGCAACTACCCAAGACTACCCAAGCACAACTACCCAAGACTACCCAAGAGTGCCAAGACTACCCAAGACTACCCAAGACTACCCCAAGCACAACTACCCAAGACTACCCCAAGCACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGCACACACCCAAGCACAACTACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGCACTACAACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGACTACCCAAGCAACTACACAACTACCCAAGACTCACCCAAGACTACCCAAGCAACTACACAACTACACTACCCAAGACTCAACCAAC	ORF50*0101 CCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGCCACAGCCTCAATCTGAATATGACCCCAGCCTGACCTCACCTGATTCGGACACTGAGTCATGTGATAGCCAGGTTCGTCCAGA ORF50*0103 TCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGCCACAGCCTCAATCTGAATATGACCCCAGCCCGACCTCTCCACCTGATTCGGACACTGAGTCATGTGATAGCCAGGTTCGTCCAGA ORF50*0201 CCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGGCCACAGCCTCAATCTGAATATGACCCCAGCCCGACCTCTCCACCTGATTCGGACACTGAGTCATGTGATAGCCAGGTTCGTCCAGA ORF50*0102 CCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGGCCACAGCCTCAATCTGAATATGACCCCAGCCCGACCTCTCCCACCTGATTCGGACACTGAGTCATGTGATAGCCAGGTTCGTCCAGA ORF50*0102 CCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGGCCACAGCCTCAATCTGAATATGACCCCAGCCCGACCTCCCACCTGATTCGGACACTGAGTCATGTGATAGCCAGGTTCGTCCAGA ORF50*0101 CCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGCCACAGCCTCAATCTGAATATGACCCCAGCCTCGACCTCGACCTGAATCTGGACACTGAGTCATGTGATAGCCAGGTTCGTCCAGA ORF50*0401 CCCGTGCTGCAGCAAGACTCTCAGCCCAGGTGTGCCCACGCCCAATCTGAATATGACCCCAGCCTCGACCTGACTCGAGCCAGGTCATGTGATAGCCCAGGTTCGTCCAGA ORF50*0401 CCCGTGCTGCAGCAAGACTCTCAGGTGTGCCCACGCCTCAATCTGAATATGACCCCAGCCTGACCTGACTCGACACTGAGTCATGTGATAGCCCAGGTTCGTCCAGA ORF50*0401 CCCGTGCTGCAGCAAGACTCTCAGGTGTGCCCACGCCCAAGCCTGAATAGCCCGGCCTGCACGTGACGTCAGGTCATGTGATAGCCCAGGTTCGTCCAGA ORF50*0401 CCCGTGCTGCAGCACAGCATCTGAATATGACCCCGACCTCCACCTGCCCGGCCTGACTCGAGCCAGGTCATGTGATAGCCCAGGTTCGTCCAGA ProCysCysSerLysThrLeuSerProGlyYalProGlnProGLAGCCTGAATTGGAATATGACCCCGGCCTGCACGTGACGTCAGGTCATGTCAGGACGTCAGGTAGCCAGGTTCGTCCAGA	0RE50*0101 ACCAGAAGTTTTACTGGAAGCAAGTGAACCACAGTCAACCCTAGCTTACGGGTGCCCTGACATTCATCTCTTTTGAGAGAGTTCATCTTCAACTCAAGCAAAATACAGACGAAGAATTCGG 0RE50*0103 ACCAGAAGTTTTACTGGAAGCAAGTGAACCACAGTCAACCCTAGCTTACGGGTGCCCTGACATTTCATCTCTTTTGAGAGAGTTCTATCTTCAACTCAAGAAAATACAGACGAAGATCGG 0RE50*0101 ACCAGAAGTTTTACTGGAAGCAAGTGAACCACAGTCAACCCTAGCTTAGGGGTGCCCTGACATTTCATCTCTTTTGAGAGAGTTCATCTTCTAACAAAAAATACAGACGAAGAACGAAGAACGA 0RE50*0102 ACCAGAAGTTTTACTGGAAGCAAGTGAACCACAGTCAACCCTAGCTTAGGGGTGCCCTGACATTTCATCTCTTTTGAGAGAGTTCATCTTCTAAGAAAAAAAA
0 0	4480 480 480 480	3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3	240 240 240 240 240 240	120 120 120 120 120 120

Figure 17: Alignment of ORF50 sequences

The ORF50*0101 allele has a sequence identical to that in the AlHV-1 genome (Ensser, Pflanz, and Fleckenstein, 1997). Nucleotide differences from the ORF50*0101 sequence are shown by grey highlights. The order of sequences in the alignment is optimised to reflect the major clades in Figure 16 defined by the C-T polymorphism at position 457. The three-letter translation of ORF50*0101 is shown below the alignment, with residues altered by base substitutions in alleles ORF50*0201, *0301 and *0401 indicated by underlining.

A9.5 could be amplified from 26 of 57 AIHV-1 positive wildebeest samples (three FTA card preserved buffy coat samples and 23 placenta) and from six of 59 AlHV-1 positive vaccine trial cattle samples (Table 6, 7). Four alleles of A9.5 were found among these samples, sharing at least 87% nucleotide sequence identity (Figure 18). The majority of samples carried the A9.5*0101 allele, whilst the remaining samples carried three alleles which have not been previously described. These novel alleles were named, in accordance with the previously published nomenclature, A9.5*0202, A9.5*0203 and A9.5*0301. The novel alleles A9.5*0202 and A9.5*0203 encoded identical proteins to the previously published A9.5*0201 allele (Russell et al., 2013), differing only at two positions in the last intron. Novel alleles (Russell et al., 2013). A9.5*0203 and *0301 were found only in wildebeest from the Ngorongoro Crater population, whilst A9.5 *0202 was only found in cattle living near Tarangire National Park (Table 7). The number of samples with these minority alleles was too low to allow statistical analysis. Phylogenetic analysis of the A9.5 genomic and putative spliced cDNA sequences yielded trees with the same topology (Figure 19).

To study possible functional consequences of sequence diversity, the A9.5 protein sequences were compared with previously established Ov9.5 sequences (Russell et al., 2014). Phylogenetic analysis indicated that the A9.5 alleles were less diverse than the Ov9.5 alleles (compare branch lengths in Figure 20). Within the protein sequence alignment, only 24 of 175 aligned residues were identical in all sequences, with a further 20 residue positions showing conservation of amino acid properties (size, charge, hydrophobicity). The fully conserved residues included six cysteines and five potential N-linked glycosylation sites (Figure 21).

The protein sequences aligned in Figure 21 were used to perform structure-based homology searching using the hhpred server [http://toolkit.teubingen.mpg.de/hhpred]. The top hit was interleukin-4 (p < 0.0001), with similarity extending over the majority of the mature polypeptide (residues 26 to 151 of the alignment). This is in accord with previous suggestions that the A9.5 and Ov9.5 proteins share structural similarities with four-helix-bundle cytokines, in particular IL-4 (Russell et al., 2013; Russell et al., 2014).

Virus haplotypes

For both ORF50 and A9.5 the most frequent allele detected among animals characterized was the *0101 allele, the variant carried by the C500 strain of AlHV-1. In the 17 samples

A9.5*0101	A9.5*0101	A9.5*0101	A9.5*0101	A9.5*0101	A9.5*0101	A9.5*0101	A9.5*0101
A9.5*0201	A9.5*0201	A9.5*0201	A9.5*0201	A9.5*0201	A9.5*0201	A9.5*0201	A9.5*0201
A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202
A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202	A9.5*0202
A9.5*0203	A9.5*0203	A9.5*0203	A9.5*0203	A9.5*0203	A9.5*0203	A9.5*0203	A9.5*0203
A9.5*0301	A9.5*0301	A9.5*0301	A9.5*0301	A9.5*0301	A9.5*0301	A9.5*0301	A9.5*0301
Exon 4 AspEroSeEProAsnHisthrargCysGlnAlaPheCysProLys GACCCAACCCTAACCATACAAGETETCTGCCCAAAGEG T19 GACACCACCCTAACCATACAA	Exon 4 Exon 4 Exon 4 SerTyrnAnyroysely tgattagttattataatttttttaggtagtcaaagtattattattattettacttcaatttattttttttccagTCTTNTGCATACCGATATTGGGC 718 tgattagttattataatttttttaggtagtcataattattattattettacttccagtCTTNTGCATACCGATACCGATATTGGGC 718 tgattagttattataatttttttaggtagtcataatgataattattattattetttettecagtCTTNTGCATACCGATACCGATATTGGGC 697 tgattagttattataatttttttaggtagtcataatgataattattattattetteccagtCTTNTGCATACCGATACCGATATTGGGC 696 tgatgagttattaataatttttttaggtaggtattattaattatt	Exon 4 GINVALSerGLYGINPheGLUAShThrThrCysSerSerDevTalshTellSerGLULeuLeuGlnGLYTpLeuSerMetLeuGlnArg AAGTANCGGGCAATTGAGAATACAACATGTAGTTGATAATTGTAAATGTAACTGTAGAGAGGCAGCTGGCAGTGTGCGATGCTAGAGAGgtarg 60 GAGTANCGGGGCAATTGAGAATACAACTTGTAAGTGATTGTAAACATGTAACTGTAACAGACTGCTAGAGCCGGCTGTCAATGCTAGAGAGgtarg 60 GAGTANCGGGGCAATTGAGAATACAACTTGTAAGTGATTGTAAACATGTAACTTCTAACAGACTGCTAGAGCCGGCTGTCAATGCTAGAGAGgtarg 60 GAGTAACCGGGGCAATTGAGAATACAACTTGTAAGCTGATTGTAAACATAACTTAACTCTAACAGACTGGTAGAGGCTGGCAATGCTAGAGAGGtarg 60 GAGTAACCGGGGCAATTGAGAATACAACTTGTAAGCTGATTGTAAACATAACTTAACTCTAACAGACTGGTAGAGGCTGGCGGCTGTCAATGCTAGAGAGGtarg 60 GAGTAACCGGGGCAATTGAGAATACAACTTGTAACTTGTAAACATAACTTAACTTCTAACAGACTGCTAGAGGCTGGCGGCTGCCAATGCTAGAGAGGTag 60 GAGTAACCGGGCAATTGAGAATACAACTTGTAACTTGTAAACATAACTTAGTAGAGCTGTAGAGGCTGGCGGCTGCCAATGCTAGAGAGGTag 60 GAGTACCGGGCAATTGAGAATACAACTTGTAACTTGTAAACATAACTTAGTAGAGCTGTAGAGGCTGGCGTGTCAATGCTAGAGGGTGG 60 GAGTACCGGGCAATTGAGAATGCATGTAGTGCTGATTGTAAACATAACTTAGTGTAGAGGCTGGCGGCGTGCGATGCTAGAGGGTGG 60 GAGTAGCGGGCAATTGGAGATGGTGGTGGCTGCTGTAGAGCATGTAGAGGCTGGCGGCTGCGAGGGTGGCAATGGTGGGGGTGTGGGGGGGTGGGGGGGG	Exon 3 Evon 3 Troacerreroxaaraarroroxeecaaareroxaacaaaaaxaacaaceecoroxaacaacerryraareaaarroxarroxeerraareerroxeeroxeerroxeerroxeerroxeerroxeerroxeerroxeerroxeerroxeer	Intron 2 Lysprodyspredysprodysprodysprodysprodysprodysprodysprodysprodyspr	Intron 1 Exon 2 Exon 2 Second	Exon 1 Intron 1 AsplieLevalanilesermire 1 GGACATTCTTAACABCTTTACAGATCTTTTG 219 GGACATTCTTAACABCTCTTACAGATCTTTTG 200 GGACATTCTTAACABCTCTTACAGATCTTTTG 200 GGACATTCTTAACABCTCTTACAGATCTTTTG 200 GGACATTCTTAACABCTCTTACAGATCTTTTG 200 GGACATTCTTAACABCATCTTTTG 200 GGACATTCTTAACAGATCTTTTG 200 GGACATTCTTAACAGATCTTTTG 200 GGACATTCTTAACAGATCTTTTG 200 GGACATTCTTAACAGATCTTTTG 200 GGACATTCTTAACAGATCTTTTG 200 GGACATTCTTAACAGATCTTTG 200 GGACATTCTTAACAGATCTTTG 200 GGACATTCTTAACAGATCTTTG 200 GGACATTCTTAACAGATCTTTG 200 GGACATTCTTAACAGATCTTTG 200 GGACATTCTTAACAGATCTTG 200 GGA	Exon 1 Metgluglyargargyalleuvalleusertieleuensertieleuensekargeusekargeusekargyallysekegyallysekegyalasekertisleuarg Anggargargyalleuvalleusertieleurensertieleurensekargeusekertisteristeristeristeristeristeristeris

Figure 18: Alignment of A9.5 sequences

The positions of exons (uppercase) and introns (lowercase) were defined by comparison with the A9.5*01 sequence (Russell et al., 2013). The flanking sequences in the A9.5*0101 allele that were used as primers are derived from the AlHV-1 genome sequence (Ensser, Pflanz, and Fleckenstein, 1997) and are underlined. In the other alleles, nucleotides that differ from the A9.5*0101 sequence are shaded. Gaps, inserted to maintain alignment, are shown as dashes (-) and are found only in the introns.



Figure 19: Phylogenetic analysis of A9.5 predicted cDNA sequences

Nucleotide sequences of the five variants (A9.5*0101 to A9.5*0301) were aligned using MAFFT and phylogenetic analysis was done in MEGA version 6.0 using the Maximum Likelihood method, based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 453 positions in the final dataset.



Figure 20: Phylogenetic analysis of A9.5 and Ov9.5 predicted protein sequences

Nine distinct Ov9.5 and three distinct A9.5 amino acid sequences were aligned using the Maximum Likelihood method based on the Whelan And Goldman model(Whelan and Goldman, 2001). The tree with the highest log likelihood is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 145 positions in the final dataset.

that could be genotyped at both loci, the most common haplotype was also that carried by the C500 virus (Table 7). All of the other combinations of ORF50 and A9.5 alleles that were detected contained either the ORF50*0101 or the A9.5*0101 allele in combination with a different allele at the second locus (Table 7).

Discussion

In this study we have shown for the first time that AlHV-1 viral DNA can be detected in about 50% of placentae shed by parturient wildebeest. However, the observation that only about 1% of the same samples had virus detectable by real-time PCR suggests that the viral load in the wildebeest placentae tested was very low. There are two likely sources of the detected virus: i) latently infected cells from the dam's blood, from which the MCF virus could not be transmitted, and which would indicate that the placenta do not play a role in transmission of MCF; and / or ii) cell-free infectious virus from the calf's circulation, which would support the view that these tissues may play a role in the transmission of virus to cattle, as considered by Massai pastoralists (Cleaveland et al., 2001). Further work to determine



Figure 21: Alignment of A9.5 and Ov9.5 predicted protein sequences

The alignment was done by the Muscle algorithm (Edgar, 2004) and conserved residues are highlighted as follows: residues identical in all sequences are shown in light face on black background; residues of conserved type (hydrophobic/charged/polar) are shown in black face with grey high-lighting. Residues identical in all sequences are also shown below the alignment on the line labeled 'conserved'.
which of these two viral states predominate is required before conclusions regarding the role that placenta play in transmission can be drawn. The finding that many wildebeest placenta were infected, however, does support observations that wildebeest calves can be infected *in-utero* (Plowright, Ferris, and Scott, 1960; Plowright, 1965; Pretorius, Oosthuizen, and Van Vuuren, 2008).

Among the wildebeest tested, all five of the 32-day old calves from the Ngorongoro Crater population were positive for AlHV-1 by nested PCR, whilst only one of five 100-day old calves from the Tarangire population and none of the Ngorongoro Crater dams tested positive. ELISA testing showed evidence of AlHV-1-specific antibodies in all of the wildebeest tested except one calf in the youngest group. Whether the antibodies detected in the calves were maternal in origin or not remains uncertain. These observations support the view that infection of most wildebeest calves occurs *in-utero* or within the first month of life. Furthermore, these observations are in accord with previous studies indicating that excretion of AlHV-1 by wildebeest calves peaks between one and two months of age and thereafter falls. By six months of age, despite being persistently infected, wildebeest have a very low level of circulating virus-infected cells (undetectable even by the sensitive nested PCR assay) and, consistent with latent infection, may no longer be capable of transmitting the virus to cattle (Plowright, 1965; Rweyemamu et al., 1974; Barnard et al., 1989).

Despite the identification of five new alleles, the ORF50 gene was highly conserved within the samples analysed, with the protein sequences encoded being identical or differing by up to two amino acids. In contrast, three new A9.5 alleles were characterised, and these encoded proteins that differed by approximately 20% of residues. It is notable that the A9.5 alleles differed from each other by less at the nucleotide level (9-13%) than at the amino acid level (19-20%). This is due to the presence of approximately two-thirds of the allelic differences in the exons and the occurrence of non-synonymous substitutions in more than half of the affected codons. This is suggestive of positive selection for variation in A9.5 but no clear evidence is available to support this (Russell et al., 2013). Therefore, variation at the A9.5 locus might not be representative of variation across the whole virus genome. Indeed, while ORF50 is conserved, the A9.5 gene, as has been suggested for Ov9.5 (Russell et al., 2014), appears to be subjected to selective pressures that result in the polymorphism seen. In OvHV-2, nine different alleles of Ov9.5 have been classified (Russell et al., 2014), compared to the five A9.5 alleles (Figure 19). The Ov9.5 alleles are more divergent from one another, with 50% amino acid identity between the most divergent alleles, compared to the A9.5 gene in which the most divergent alleles have 80% predicted amino acid identity (Figure 20). The

strong conservation of cysteine residues and potential glycosylation sites across all of the A9.5 and Ov9.5 alleles (Figure 21) suggests that this highly variable protein has conserved structure and function. Recent analysis of the A9.5 and Ov9.5 predicted protein sequences indicated similarity with the four-helix bundle cytokines, IL4 and IL21 (Russell et al., 2014). Structural homology searching performed with the new A9.5 alleles identified in this study adds power to this analysis, identifying IL4 as the major homologue with a *p*-value enhanced from the earlier study. It is currently unclear whether the similarity with IL4 indicates only a shared structural fold or additional functional similarity.

Among 33 samples for which A9.5 sequences could be derived, only the most frequent allele, A9.5*0101, was found in wildebeest samples from both locations and in samples from cattle. A9.5*0202 was found only in cattle samples collected near Tarangire National Park, while A9.5*0203 and A9.5*0301 were detected only in wildebeest samples from the Ngorongoro Crater population. These geographical and species related differences may simply be a consequence of the low number of samples analysed from each site but may also result from the known isolation of the two wildebeest populations (Georgiadis, 1995). Unfortunately, ORF50 could only be amplified from wildebeest placental samples, so it is not possible to make any inferences from this data. It will be of interest to gather larger numbers of samples from across the range of wildebeest-associated MCF to determine the overall extent of AlHV-1 diversity and the epidemiology of MCF viruses in reservoir and susceptible species.

Herpesviruses, being DNA viruses, are typically stable with low rates of nucleotide substitution, as evidenced by the small numbers of base substitutions found among the ORF50 alleles sequenced. Thus, the alleles we have identified are likely to have been conserved in the population for a long period of time. This finding is supported by the fact that the previously characterized A9.5 alleles present in the C500 virus strain and in a diagnostic sample from wildebeest-associated MCF in the UK (Whitaker et al., 2007) encode A9.5 polypeptides that were identical to MCF virus fragments amplified from the cattle and wildebeest sampled in the two locations in Tanzania. Indeed, the most frequent haplotype identified among the Tanzanian samples was identical to the C500 virus at both the A9.5 and ORF50 loci, whilst all other haplotype combinations identified included either ORF50*0101 or A9.5*0101. This suggests that, like other herpesviruses which have relatively slow evolutionary molecular clocks (McGeoch, Gatherer, and Dolan, 2005), the C500 strain of AlHV-1 has remained closely related to AlHV-1 strains circulating in Tanzania, despite it having been propagated in African and European laboratories for approximately 50 years. This supports the view that C500 AlHV-1 remains a suitable strain for the development of a vaccine for wildebeestassociated MCF. Furthermore, similarity between the vaccine virus and Tanzanian strains of AlHV-1 reduces the risk of a novel, more pathogenic, virus mutant (derived by recombination between the vaccine virus and local strains of AlHV-1) escaping into the wild. This suggests that the AlHV-1 C500 strain could be safe for vaccination in MCF-endemic areas.

In summary, we have shown that about 50% of wildebeest placentae tested contained detectable AlHV-1 virus (measured as virus DNA), supporting evidence that wildebeest can be infected *in-utero*, and suggesting that placenta could play a role in MCF transmission. The higher AlHV-1 DNA detection rate in the younger cohort of wildebeest calves is consistent with previous observations that infection of wildebeest generally occurs within the first month of life and that viraemia peaks between one and two months. The discovery of new A9.5 alleles supports previous evidence that the gene is highly polymorphic and appears to encode a secretory protein with IL-4 as the major homologue. Differences in the alleles detected in Tarangire and Ngorongoro Crater suggest that geographical and evolutionary separation of wildebeest populations (Georgiadis, 1995) may have influenced the AlHV-1 alleles found. In contrast, the observation that the most frequently detected Tanzanian AlHV-1 haplotype was identical to the C500 virus suggests that AlHV-1 C500 is an appropriate strain for vaccine development and supports the view that AlHV-1 virus genotypes are stable over time. More extensive collection of samples from across the wildebeest range will enable the diversity of AlHV-1 strains to be further elucidated.

XII

Chapter Four Malignant catarrhal fever: a direct viral challenge vaccine trial

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Introduction

Malignant catarrhal fever (MCF), an often-fatal disease with a worldwide distribution, is caused by a γ -herpesvirus transmitted to susceptible ungulate species. Two principle forms of the disease exist, wildebeest- (WA-MCF) and sheep-associated (SA-MCF), that occur sporadically when ungulates such as cattle, deer, bison, water buffalo and pigs come in to contact with the carrier host (Russell, Stewart, and Haig, 2009). The clinical presentation is similar in both forms of the disease with affected animals suffering severe fever, depression, oral epithelial lesions, corneal opacities, ocular and nasal discharge and, frequently, death. The pathogenesis of MCF is poorly understood but it appears to involve an auto-destructive pathology resulting in lymphoid hyperplasia and vasculitis in a range of tissues (Russell, Stewart, and Haig, 2009; Penny, 2005; Taus et al., 2006; Anderson et al., 2008; Nelson et al., 2010).

WA-MCF, which is limited to the range of wildebeest (*Connochaetes spp.*) in sub-Saharan Africa and also occurs sporadically in proximity to zoological collections, is caused by the γ -herpesvirus, alcelaphine herpesvirus-1 (AlHV-1) (Plowright, Ferris, and Scott, 1960). SA-MCF, which is ubiquitous in domestic sheep and is caused by ovine herpes virus-2 (OvHV-2) (Hart et al., 2007), represents the more economically significant form of the disease because of its worldwide distribution. Due to the lack of an *in-vitro* propagation system for OvHV-2, however, vaccine development has focused on the wildebeest form of the disease, the causative agent of which can easily be cultured.

OvHV-2 is phylogenetically related to AlHV-1 with significant DNA sequence identity (Hart et al., 2007) and, although the potential for a vaccine based on AlHV-1 to provide cross-protective immunity against SA-MCF is likely to be slight (Rossiter, Gumm, and Mirangi, 1988; Gailbreath et al., 2010; Taus et al., 2015), an effective AlHV-1 vaccine may, in addition to providing benefits to livestock keepers living in proximity with wildebeest, provide a basis

for the development of a protective vaccine against OvHV-2.

United Kingdom based trials (Haig et al., 2008; Russell et al., 2012) of a novel vaccine strategy targeting British Friesian-Holstein (FH) cattle recently demonstrated that, following two intra-muscular inoculations of attenuated AIHV-1 mixed in the adjuvant EmulsigenTM, an effective barrier of AIHV-1-neutralising IgA and IgG1 antibodies can be induced in the mucosa of the oro-nasal pharynx, the presumed site for natural infection by the MCF viruses including AIHV-1. The vaccine strategy gave approximately 90% protection against AIHV-1 challenge (Haig et al., 2008; Russell et al., 2012) while the duration of protection was limited to around six months (Russell et al., 2012). However a subsequent field trial experiment (Chapter Two and Lankester et al., 2016) indicated that in shorthorn zebu cross (SZC) cattle, under conditions of natural transmission, the level of protection was only 56%. One explanation for the difference in the levels of protection seen in the UK- based experimental trial and the Tanzanian-based field trial is that FH and SZC cattle may respond differently to the vaccine. To explore whether such a difference exists, the same challenge trial as performed in the UK was repeated on SZC cattle and is reported here.

In response to the data generated in Chapter Two, a second objective of this challenge trial was to investigate whether the efficacy of the vaccine could be improved by exploiting the adjuvant properties of particular ligands / agonists for toll-like receptors (TLR) on immune system cells. TLRs, which form part of the innate immune system in animals and provide a first line of defense against infection, recognize pathogen-associated molecular pattern molecules (PAMPs) expressed by pathogens but not by mammalian hosts (Akira and Takeda, 2004; O'Neill, Golenbock, and Bowie, 2013). Up to thirteen TLRs are found in mammals, which recognize microbial and parasitic components such as unmethylated-CpG DNA (recognized by TLR9) and bacterial flagellin (recognized by TLR5). Importantly, engaging TLRs or TLR combinations on antigen-presenting cells (particularly dendritic cells that initiate and maintain immune responses) can generate inflammatory signals that influence the magnitude and type of the adaptive immune response that ensues (e.g. type 1 or type 2 helper T-cell responses (Th1 or Th2)). For this reason, TLR ligands have been the subject of much recent research into new generation adjuvants. For example the TLR9 ligand unmethylated CpG oligodeoxynucleotide (CpG ODN) has been shown to enhance Th1-mediated immunity and improve vaccine protection in cattle immunized with bovine herpesvirus-1 or foot and mouth disease virus (Ioannou et al., 2002; Ren et al., 2011). Additionally the recombinant bacterial flagellin monomer (FliC), that is recognized by TLR5, has demonstrated adjuvant properties for antibody- and cell-mediated responses in several mammalian and avian species, including mucosal immune responses (Akira and Takeda, 2004; McDermott and Huffnagle, 2014; Lee et al., 2015). The immunogenicity of TLR ligands does vary however. For example, in a recent study CpG ODN oligomer co-administered with EmulsigenTM did not improve the duration or magnitude of immunity of the AlHV-1 immunisation regime in FH cattle when compared to EmulsigenTM adjuvant alone (Parameswaran et al., 2014). In this study we aimed to compare the efficacy of the MCF vaccine formulation when attenuated AlHV-1 was combined with one of three different vaccine - adjuvant combinations: i) EmulsigenTM alone; ii) FliC alone; or iii) both adjuvants in combination.

Materials and Methods

FliC efficacy in-vitro ³

Prior to using FliC as an adjuvant in the vaccine challenge trial, an analysis was carried out (by co-investigators based at the University of Nottingham) in which the functional response of bovine TLR5 to ligand stimulation with FliC was assessed. Firstly, the pcDNA3-YFP plasmid containing wild type bovine toll-like receptor-5 (boTLR5) (Metcalfe et al., 2014) and a control pcDNA3-YFP plasmid with no insert were transfected separately into human embryonic kidney cells (HEK293) using a nucleofector kit (Annexa Biosystems, UK). Selective pressure was applied by addition of geneticin (G418, Invitrogen, UK, 600g /ml) until stably transfected cell lines were generated (2-3 weeks). Bovine TLR5 expression was confirmed by PCR, flow cytometry for YFP and fluorescence microscopy. HEK cells expressing human TLR5 (293-htlr5) were obtained from Invivogen (Toulouse, France).

For the FliC - TLR5 HEK assay, the TLR5 + HEK cells (human and bovine TLR5) and negative controls were seeded at 2 x 10^5 cells per well in a 24-well plate in 2 ml of Dulbecco's modified Eagle's medium (DMEM) + 10% foetal calf serum and incubated overnight at (37°C, 5% CO₂ in humidified air). Each cell line was then stimulated with four concentrations of two preparations of recombinant FliC: i) *Salmonella typhimiurium* Flagellin FliC (Enzo Life Sciences, Exeter, UK) < 0.05 EU/g endotoxin and ii) as a positive control, endotoxin-free FliC (tlrl-flic; Invivogen, Source Bioscience LifeSciences, UK). Cells were stimulated with each of the FliC preparations at 0.1, 0.3, 0.6 and 1 µg/ml concentrations and supernates were collected at both 24 and 48 hours post stimulation. All treatments were completed in duplicate. The 500 µl supernatants were cleared by centrifugation

³The laboratory work described in this section, which was not carried out by F. Lankester, took place at the University of Nottingham by a team led by Professor David Haig



Figure 22: **Image of traditional Maasai boma** At night the cattle were kept in a traditional Maasai boma (corral) as protection from predators

and stored at minus 20°C. The functional response of bovine and human TLR5 HEK cells, and control cells, to ligands was measured by their production of the chemokine CXCL8, using the Quantikine ELISA measuring human CXCL8 (RD systems, Abingdon, UK), as described recently (Willcocks et al., 2013).

Vaccine trial

Animals

Forty clinically healthy SZC cattle (31 entire male and 9 female) of approximately six months of age and weighing approximately 100 kg were purchased from livestock markets in the Simanjiro District in northern Tanzania. All cattle were immunized against the locally prevalent and often fatal lymphoproliferative cattle disease East Coast fever (ECF) (Homewood et al., 2006) using the infection and treatment immunization (ITM) method. This comprised an inoculation of *Theileria parva* sporozoites, combined with a treatment of a long-acting 30% oxytetracycline (Di Giulio et al., 2008). The animals were also given a single treatment against endo- and ectoparasites using 1 ml / 50 kg body weight ivermectin (IvomecTM, Merial Animal Health, Essex, UK) administered by a subcutaneous injection. Every other week thereafter the cattle were sprayed with the ecto-parasiticide alpha-cypermethrin (ParanexTM, Farmbase Ltd, Dar es salaam, Tanzania), administered at 100 mg / litre. All cattle were fitted with ear tags for identification. The cattle were housed at night in a traditional Maasai boma (corral) (Figure 22) and, during the day, were grazed on community pastureland in the village of Emboreet (latitude -3.952239, longitude 36.47537).

Virus and vaccine ⁴

The strains of the AlHV-1 virus used for vaccination and challenge were prepared at the Moredun Research Institute (Haig et al., 2008). Pathogenic virus challenge in this experiment was by intranasal inoculation of 10 ml of virus suspension with titre approximately 10^4 TCID₅₀ / ml. We were confident that this dose would provide a lethal dose in SZC cattle as it represented 50 x the LD₅₀ virus dose as determined on FH cattle (Haig et al., 2008). As described in Chapter Two, the attenuated AlHV-1 C500 strain, passaged more than 1000 times, was used as the source of virus for immunisation (Handley et al., 1995).

Study design

The trial took place between October 2012 and February 2013 at a time of the year when wildebeest were not calving and had yet to migrate out of the nearby Tarangire National Park. Natural exposure to AlHV-1 was therefore deemed unlikely during the course of the trial. The 40 cattle were randomly assigned to one of five experimental treatment groups (Table 8, n = 8 for each group) and injected intramuscularly in the upper neck (Figure 5) with:

- Group 1: attenuated AlHV-1 (atAlHV-1), 1 ml of 10⁷ TCID₅₀ per ml plus 20% v/v EmulsigenTM (MVP, Omaha, USA)
- **Group 2**: atAlHV-1 plus *S. typhimiurium* recombinant flagellin monomer FliC, 1 mg (Enzo Life Sciences, Exeter, UK)
- Group 3: atAlHV-1 plus EmulsigenTM and FliC (doses as in Groups 1 and 2)
- Group 4: EmulsigenTM alone, 20% v/v
- Group 5: FliC alone, 1mg

The five groups were primed as above on day zero and boosted (intramuscular, upper neck, same doses as for priming) as per their specified grouping on day 28. The group sizes were similar to those described in the two UK-based immunisation experiments upon which this trial was based (Haig et al., 2008; Russell et al., 2012). On day 77 after the primary inoculation all animals were challenged with 10 ml of 10^4 TCID₅₀ / ml of virulent AlHV-1 given intra-nasally (Figure 23). In accordance with the previous UK experimental trials, the endpoint of the trial was three months (90 days) after challenge to allow the development

⁴The work described in this section was not carried out by F. Lankester



Figure 23: **Image of direct viral challenge** Virulent AlHV-1 virus was inoculated intra-nasally on day 77 after the primary vaccination

of MCF in unprotected cattle. As the trial was held with the cooperation of local Maasai villagers in an area of Tanzania where MCF was endemic we decided, in recognition of local sensitivity toward the unnecessary slaughter of cattle, that the local community would retain all cattle that were healthy at the endpoint of the trial.

Sample collection and clinical analyses

Uncoagulated blood was collected in EDTA VacutainersTM from all animals at the day of primary and booster inoculation, and every two weeks thereafter (Figure 24) until the end of the trial on day 168 (24 weeks after primary inoculation). Nasal secretion samples were collected using a regular sized tampon inserted into one nostril for 10 minutes (Figure 8). Following removal, the tampon was squeezed inside the barrel of a 20 ml syringe and the extracted nasal secretion collected. Plasma extracted from the uncoagulated blood samples and nasal secretion samples were frozen at minus 20°C and, prior to being exported to the UK for serological analysis, were heat-treated at 56°C for 30 minutes. Buffy coat blood cells, also extracted from the uncoagulated blood, were stored frozen and on FTATM cards prior to DNA extraction for PCR detection of viral DNA. Clinical signs were monitored on a daily basis with animals recorded as 'sick' if there was evidence of fever (body temperature \geq 39°C), excessive ocular / nasal discharge or anorexia. Otherwise cattle were recorded as 'healthy'. Sick animals were scored using a clinical scoring matrix (Russell et al., 2012) that ensured euthanasia (which followed protocols established during trials at the Moredun Research Institute, and were compliant with Home Office of Great Britain and Northern Ireland

Group (<i>n</i>)	Primary	Immunisation	Virus challenge
	immunisation	boost	
	$(\text{week } 0)^{a,b}$	$(\text{week } 4)^{a,b}$	(week $10)^c$
	$10^{6.2}$ virus in	$10^{6.2}$ virus in	$10^4 \text{ TCID}_{50} \text{ virus}$
1 (8)	Emulsigen TM	Emulsigen TM	
	IM	IM	IN
	$10^{6.2}$ virus in	$10^{6.2}$ virus in	$10^4 \text{ TCID}_{50} \text{ virus}$
2 (8)	Bacterial flagellin	Bacterial flagellin	
	IM	IM	IN
	$10^{6.2}$ virus in	$10^{6.2}$ virus in	$10^4 \text{ TCID}_{50} \text{ virus}$
3 (8)	Emulsigen TM	Emulsigen TM	
	+	+	
	Bacterial flagellin	Bacterial flagellin	
	IM	IM	IN
4 (8)	Emulsigen [™]	Emulsigen TM	$10^4 \text{ TCID}_{50} \text{ virus}$
	IM	IM	IN
5 (8)	Bacterial flagellin	Bacterial flagellin	10 ⁴ TCID ₅₀ virus
	IM	IM	IN

Table 8: Challenge trial: Immunisation treatment groups and inoculations

^{*a*}The virus used for the primary and boost immunisation was attenuated AlHV-1 obtained from passage >1000. ^{*b*}The immunisation dose of virus is shown as TCID₅₀ titres (Haig et al., 2008). ^{*c*} The challenge virus was virulent AlHV-1 obtained from passage four (IM = intramuscular injection (upper neck region), IN = intranasal challenge).

'Animals (Scientific Procedures) Act 1986' under project licence PPL 60/3839) took place prior to the onset of severe clinical signs. While histopathology is recognised as the gold standard for MCF diagnosis, and a post-mortem examination was carried out on all animals that were euthanised due to onset of MCF, the tissue samples that were collected were lost in transit to the UK and could not be analysed. Infection status was therefore defined based on a combination of clinical signs, analysis of virus-specific antibody responses and PCR detection of AlHV-1 DNA.

Detection of viral DNA in blood

Detection of viral DNA in blood took place at the Nelson Mandela African Institution of Science and Technology (Tanzania). Viral DNA was extracted from the frozen buffy coat samples using the ZR Viral DNA KitTM and viral DNA was assayed by nested PCR as described in Chapter Two. AlHV-1 infection status during the challenge period was classified as positive if PCR analyses were positive at any of the three time points assayed (post-challenge day 28, 56 or 86). As described in Chapter Two, although the hemi-nested PCR used here did not distinguish between vaccine and challenge virus, previous studies have shown that



Figure 24: **Image of field sampling** Blood and nasal secretion samples were collected from each animal every two weeks

AlHV-1 DNA was not detected in the blood of any cattle vaccinated with the attenuated virus (Haig et al., 2008). Therefore PCR positive samples from trial cattle were considered to provide evidence of infection with challenge AlHV-1.

Analysis of antibody responses by ELISA

To quantify the systemic (plasma) and nasal mucosa total AlHV-1-specific and neutralising antibody titres (hereafter names ELISA titres and VNA titres respectively), the same ELISA and virus neutralization tests as described in Chapter Two were used respectively (Russell et al., 2012). ELISA titre values (difference between means of positive and negative antigen wells for each sample dilution) were used to calculate a relative titre for each test sample, determined with respect to a standard curve of pooled MCF-positive plasma diluted 1/20 to 1/6000. ELISA titre values have been expressed as the reciprocal of the calculated end-point dilution (e.g. 20 - 6000). To reduce the likelihood that false positive titres were counted, any sample that gave a calculated titre of less than 20 (i.e. below the range of the standard curve) was not considered positive.

Case definitions

Because histopathological analyses could not be performed on the post-mortem tissues of those cattle that did die, nor on the tissues of cattle that were alive (and were not sacrificed) at the end of the trial, we had only PCR, antibody responses and clinical signs to determine cases of MCF. We took a conservative approach, defining cases based on cattle being classified as symptomatic and the detection of AlHV-1 DNA by PCR or, in the case of unvac-

cinated cattle, an AlHV-1 specific antibody response following virus challenge. The various combinations of diagnostic evidence were classified as follows:

- I **Not infected**: Cattle remained asymptomatic, were PCR negative, survived and, if unvaccinated, had no antibody response following challenge.
- II **Fatal AlHV-1 infection**: Cattle were PCR positive post-challenge, had clinical signs compatible with MCF and subsequently succumbed to disease.
- III **Non-fatal AlHV-1 infection**: Cattle survived and were either PCR positive postchallenge or, if unvaccinated, showed an antibody response following challenge.
- IV Possible AlHV-1 infection: Cattle had clinical signs indicative of MCF but no PCR or antibody evidence of infection.

These case definitions allowed further division of cattle into those for which there was evidence of infection, hereafter termed 'infected' (case definition II and III) and those for which there was none, hereafter termed 'uninfected' (case definition I). Cases classified as definition IV were, based on clinical signs only, termed 'possibly infected'.

Vaccine efficacy and inter-trial regression analysis

An efficacy calculation (for preventing infection) was performed for the atAlHV-1 + EmulsigenTM vaccine formulation (Group 1) using the formula shown in Table 2 (Knight-Jones et al., 2014; Orenstein et al., 1985). The same calculations were also made for this formulation using data from the UK-based experimental trial (hereafter termed Russell) (Russell et al., 2012) and the Tanzanian-based field trial (Chapter Two and Lankester et al., 2016). Additionally an efficacy calculation was made for the formulation (atAlHV-1 + Freund's adjuvant) tested in the first UK-based trial (hereafter termed Haig) (Haig et al., 2008).

In addition the final AlHV-1 infection statuses ('infected' or not) of all participating vaccinated cattle was compiled from this and the three aforementioned trials. Logistic regression analysis was performed, with trial identity and subsequent infection status of vaccinated cattle as the explanatory and dependent variables respectively.

Statistical analyses

All plots and statistical analyses were performed using the R language for statistical computing (Team, 2013). A linear regression model was used to compare i) the effect that vaccination grouping had on ELISA titre and ii) the serological responses of vaccinated cattle that were classified as 'uninfected' or 'infected' with AlHV-1 challenge virus. Fisher's Exact Test for Count Data was used to test the differences in the proportions of cattle in i) each treatment group that were either 'uninfected' or 'infected' and ii) the relationship between prior exposure and survival in control group cattle. Vaccine efficacy calculations were made using standard formulae (Knight-Jones et al., 2014; Orenstein et al., 1985) and a logistic regression model was used to compare the results of this trial with three previous vaccine trials (Haig et al., 2008; Russell et al., 2012; Lankester et al., 2016).

Results

Bovine TLR-5 response to FliC in-vitro ⁵

Figure 25 shows that HEK cells expressing bovine TLR5 responded in a dose-dependent way to the FliC used as an adjuvant, although the response was stronger in HEK cells expressing human TLR5 (p < 0.02). This is in-line with recently published data on boTLR5 (Metcalfe et al., 2014). Despite the fact that the response pattern of both TLR5 was similar, the response of HEK cells transfected with either bovine or human TLR5 to 0.3, 0.6 and 1 μ g ml-1 FliC were significantly different from their respective control cell responses (p < 0.02 and p < 0.01 respectively). The positive control FliC (Invivogen) gave similar results to the adjuvant FliC in both cell types (not shown). Control HEK cells transfected with plasmid only did not respond to either FliC.

Baseline serological and PCR screen

At the start of the experiment (day zero) all 40 trial cattle were serologically tested by ELISA. Thirty-eight were seronegative and two (901 and 926) exhibited low, but positive, ELISA titres. Four animals (914, 928, 936, 937) showed evidence of AlHV-1 DNA in blood mononuclear cells by PCR. These results are shown in Table 9 and 10.

Group	ID	Baseline ¹	\mathbf{Died}^2	Clinical	PCR	\mathbf{Ab}^3	\mathbf{Def}^4	Status ⁵
1	903		Survived	Healthy	Neg	Fall	Ι	Not inf

Table 9: Challenge trial outcomes

Continued on next page

⁵The results described in this section were provided by Prof. David Haig (University of Nottingham). A *t*-test was used to determine if there were differences in the response of HEK cells expressing TLR5 to FliC ligands.

Table 9 – Continued from previous pageTable 9: Challenge trial outcomes

Group	ID	Baseline ¹	\mathbf{Died}^2	Clinical	PCR	\mathbf{Ab}^3	\mathbf{Def}^4	Status ⁵
1	912		Survived	Healthy	Neg	Level	Ι	Not inf
1	913		Survived	Healthy	Neg	Rising	III	Inf
1	918		Survived	Healthy	Neg	Level	Ι	Not inf
1	923		Died(22)	Sick	Pos	Rising	II	Inf
1	927		Survived	Healthy	Neg	Level	Ι	Not inf
1	928	Pos	Survived	Healthy	Neg	Level	I	Not inf
1	930		Died(38)	Sick	Pos	Rising	II	Inf
2	906		Survived	Healthy	Neg	Level	Ι	Not inf
2	908		Died(35)	Sick	Pos	Rising	II	Inf
2	915		Survived	Healthy	Neg	Level	Ι	Not inf
2	925		Died(66)	Sick	Pos	Rising	II	Inf
2	929		Died(38)	Sick	Pos	Rising	II	Inf
2	936	Pos	Died(56)	Sick	Pos	Rising	II	Inf
2	938		Survived	Healthy	Neg	Level	Ι	Not inf
2	940		Survived	Sick	Neg	Rising	III	Inf
3	905		Died(31)	Sick	Pos	Rising	II	Inf
3	907		Died(59)	Sick	Pos	Rising	II	Inf
3	910		Survived	Healthy	Neg	Level	Ι	Not inf
3	916		Died(50)	Sick	Pos	Rising	II	Inf
3	920		Died(38)	Sick	Pos	Rising	II	Inf
3	922		Died(38)	Sick	Pos	Rising	II	Inf
3	924		Died(88)	Sick	Pos	Rising	II	Inf
3	935		Survived	Healthy	Neg	Level	Ι	Not inf
4	904		Died(59)	Sick	Pos	Pos	II	Inf
4	909		Survived	Healthy	Neg	Neg	Ι	Not inf
4	914	Pos	Survived	Sick	Pos	Pos	III	Inf
4	917		Died(38)	Sick	Pos	Pos	II	Inf
4	926	28	Survived	Healthy	Neg	Neg	I	Not inf
4	931		Died(35)	Sick	Pos	Pos	II	Inf

Continued on next page

Group	ID	Baseline ¹	Died ²	Clinical	PCR	Ab ³	Def ⁴	Status ⁵
4	933		Died(35)	Sick	Pos	Pos	II	Inf
4	937	Pos	Survived	Sick	Neg	Pos	III	Inf
5	901	35	Died(28)	Sick	Pos	Pos	II	Inf
5	902		Died(22)	Sick	Pos	Pos	II	Inf
5	911		Died(31)	Sick	Pos	Pos	II	Inf
5	919		Survived	Sick	Neg	Neg	IV	Poss
5	921		Died(26)	Sick	Neg	Neg	IV	Poss
5	932		Died(35)	Sick	Pos	Pos	II	Inf
5	934		Died(38)	Sick	Pos	Pos	II	Inf
5	939		Died(38)	Sick	Pos	Pos	II	Inf

Table 9 – Continued from previous pageTable 9: Challenge trial outcomes

This table contains:

¹Baseline: Animals with AlHV-1-specific antibodies at day 0 of the experiment (pre-vaccination) are shown by the measured ELISA titre value (cut off value of 20), while animals that had detectable AlHV-1 DNA are indicated as 'Pos'.

²Died: Whether an animal survived or died is indicated, with the number of days that an animal died post-challenge given in parentheses.

³Ab: Summary of plasma ELISA titres post-challenge. For vaccinated animals (Groups 1, 2, 3), 'Fall' indicates a falling titre post-challenge, 'Level' indicates the titre was stable, whilst 'Rising' indicates the titre rose post-challenge (suggestive of active infection with AlHV-1). For control animals (Groups 4 and 5), sero-conversion with detectable AlHV-1-specific antibodies ('Pos') indicates infection with AlHV-1.

⁴Def: Cases (as described in the text) are defined as: not infected (I), fatal AlHV-1 infection (II), non-fatal AlHV-1 infection (III) and possible AlHV-1 infection (IV).

⁵Status: Fatal AlHV-1 infection (II) and non-fatal AlHV-1 infection (III) cases are defined as infected ('Inf') whilst not infected (I) cases are classed as uninfected ('Not inf'), and possible AlHV-1 infection (IV) cases as possibly infected ('Poss').



Figure 25: FliC efficacy assay

The functional response (and $\pm 95\%$ confidence intervals) of bovine and human TLR5 HEK cells, and control cells, to ligands measured by their production of the chemokine CXCL8 (IL-8 ELISA O.D.) is shown. HEK bov (plot A) and HEK hu (plot B) are HEK cells expressing bovine and human TLR5 respectively. HEK bov ctrl and HEK hu ctrl are HEK cells containing control pcDNA3-YFP plasmid. The HEK bov and HEK hu cell responses to 0.1, 0.3, 0.6 and 1 µg/ml FliC were significantly different (p < 0.02). The HEK bov and the HEK hu cell responses to 0.1, 0.3, 0.6 and 1 µg/ml FliC were significantly different from their respective control cell responses (HEK bov ctrl; HEK hu ctrl) (p < 0.02 and p < 0.01 respectively).

Group	ID	Baseline ¹	Day 105 ¹	Day 133 ¹	Day 163 ¹	Terminal $(days)^2$
1	903	Neg	Neg	Neg	Neg	
1	912	Neg	Neg	Neg	Neg	
1	913	Neg	Neg	Neg	Neg	
1	918	Neg	Neg	Neg	Neg	
1	923	Neg				Pos (99)
1	927	Neg	Neg	Neg	Neg	
1	928	Pos	Neg	Neg	Neg	
1	930	Neg	Neg			Pos (115)
2	906	Neg	Neg	Neg	Neg	
2	908	Neg	Pos			Pos (112)
2	915	Neg	Neg	Neg	Neg	
2	925	Neg	Neg	Neg	Pos	Pos (143)
2	929	Neg	Pos			Pos (115)
2	936	Pos	Neg			Pos (133)
2	938	Neg	Neg	Neg	Neg	
2	940	Neg	Neg	Neg	Neg	
3	905	Neg	Pos			Pos (108)
3	907	Neg	Neg	Neg	Pos	Pos (146)
3	910	Neg	Neg	Neg	Neg	
3	916	Neg	Pos			Pos (127)
3	920	Neg	Pos			Pos (115)
3	922	Neg	Pos			Pos (115)
3	924	Neg	Neg	Neg	Pos	Pos (165)
3	935	Neg	Neg	Neg	Neg	
4	904	Neg	Neg	Pos		Pos (136)
4	909	Neg	Neg	Neg	Neg	
4	914	Pos	Pos	Pos	Neg	
4	917	Neg	Pos			Pos (115)
4	926	Neg	Neg	Neg	Neg	
4	931	Neg	Pos			Pos (112)

Table 10: Challenge trial: PCR results

Continued on next page

Group	ID	Baseline ¹	Day 105 ¹	Day 133 ¹	Day 163 ¹	Terminal (days) ²
4	933	Neg	Pos			Pos (112)
4	937	Pos	Neg	Neg	Neg	
5	901	Neg	Pos			Pos (112)
5	902	Neg				Pos (99)
5	911	Neg	Pos			Pos (108)
5	919	Neg				Neg (103)
5	921	Neg				Neg (112)
5	932	Neg	Pos			Pos (115)
5	934	Neg	Pos			Pos (115)
5	939	Neg	Pos			Pos (115)

Table 10 – Continued from previous pageTable 10: Challenge trials: PCR results

This table contains:

¹Baseline and Day: The PCR results from samples collected at the start of the trial (day zero) and Day 105, 133 and 163 are shown.

²Terminal (days): The number of days to death and the result of the PCR assay performed on blood samples collected at this time (Pos = Positive, Neg = Negative).

The effect of standard AlHV-1 vaccination and FliC supplemented vaccine formula on protection

The details of individual animal and treatment group survival or acquisition of MCF are shown in Table 9 and are summarized in Table 11.

Serological responses to immunisation and virus challenge

Between the boost immunisation and the virus challenge, all the cattle in the vaccinated Groups 1, 2 and 3 showed a rise in nasal secretion ELISA titres, whilst cattle in Groups 1 and 3 showed a rise in plasma ELISA titres. ELISA titres peaked between weeks seven and eight before declining again. None of the cattle in either adjuvant control Group 4 or 5 had an ELISA titre following immunisation (Figure 26). When geometric mean titres were compared across the groups for all pre-challenge sampling time-points, Group 1 had significantly higher plasma ELISA titres than all other groups (p < 0.001, t < -3.4, df = 4 and 114), and significantly higher nasal secretion titres than all groups (p < 0.001, t < -3.4, df =

Table 11. Chanenge that. Case definition results							
Group	Vaccination	Ca	ase I	Defini	Total		
		I	II	III	IV	MCF cases	
1	atAlHV-1+	5	2	1	0	3	
	Emulsigen						
2	atAlHV-1+	3	4	1	0	5	
	FliC						
3	atAlHV-1 +	2	6	0	0	6	
	Em+FliC						
4	Em	2	4	2	0	6	
	only control						
5	FliC	0	6	0	2	6	
	only control						

Table 11: Challenge trial: Case definition results

Case definition I = not infected, II = fatal ALHV-1 infection, III = non-fatal AlHV-1 infection, IV = possible AlHV-1; Total MCF cases = case definition II and III; atAlHV-1 = attenuated AlHV-1 virus, $Em = Emulsigen^{TM}$, FliC = flagellin monomer.

-5.6, df = 4 and 155) except Group 3. Following virus challenge, geometric mean plasma and nasal secretion ELISA titres increased in all three vaccinated groups, but error bars were large indicating a wide range of individual variation. Both control groups also had a rise in titres after virus challenge, however these were very low compared to the immunized groups.

The VNA titres in nasal secretion and plasma were low in all five groups following primary immunisation (Figure 27), but, following boost immunisation, the titres of cattle in vaccinated Groups 1 and VNA titres than both Groups 1 and 3 (p < 0.001, t = -3.4, df = 74 (plasma); p < 0.001, t = -3.5, df = 73 (nasal secretion)). Both control Groups 4 and 5 remained seronegative for neutralising antibodies, whilst all of the animals in Groups 1, 2 and 3 seroconverted.

PCR results

To identify animals that had become infected, blood samples from all cattle were assayed for AlHV-1 DNA at three post-challenge occasions: week 15, 19 and 23. Where available, PCR was also performed on terminal blood samples taken from animals that succumbed to MCF. The full set of results are shown in Table 10. In Groups 1 to 5, AlHV-1 virus DNA was detected in two (25%), four (50%), six (75%), five (62,5%) and six (75%) out of eight cattle respectively. The post-challenge PCR status of each animal is summarized in Table 9.



Figure 26: AlHV-1-specific antibody titres

The geometric mean nasal secretion (plot A) and plasma (plot B) total AlHV-1-specific antibody titres (and $\pm 95\%$ confidence intervals) for Groups 1 to 5 are shown for each sampling time point (atAlHV-1 = attenuated AlHV-1 virus, Em = EmulsigenTM, FliC = flagellin monomer, P = primary vaccination, B = booster vaccination, C = virus challenge, and * = PCR assay time-points). For group descriptions see Table 8. The wide confidence intervals after virus challenge (C) indicate large individual variations (see text and Figure 28).





The geometric mean AlHV-1-neutralising antibody titres (and $\pm 95\%$ confidence intervals) in nasal secretion (plot A) and plasma (plot B) for each of Groups 1 to 5 are shown (atAlHV-1 = attenuated AlHV-1 virus, Em = EmulsigenTM, FliC = flagellin monomer). The primary inoculation occurred in week one, the booster in week four and the challenge in week eleven.

Case descriptions

The majority of animals were classified as either case definition I (not infected) or II (fatal ALHV-1 infection), with a small number of animals showing more complex combinations of signs (Table 9). In Group 1, two animals succumbed to MCF with clear clinical signs and detection of virus DNA in the blood post-challenge and were classified as case definition II. Six animals survived with no clinical signs and no detection of AlHV-1 DNA in the blood at any time point after challenge. Of these one animal (number 913), despite being asymptomatic and PCR negative, had a rising antibody titre following challenge and was classified as case definition III (non-fatal AlHV-1 infection). The other five were classified as case definition I. In Group 2, three animals survived with no clinical signs and no detection of virus DNA post-challenge and were classified as case definition I. Four animals succumbed to MCF with clinical signs and virus DNA in the blood post-challenge and were classified as case definition II. One animal (number 940) showed clinical signs indicative of MCF and, although virus DNA was not detected, had a rising MCF-specific antibody titre in the blood after challenge and was therefore classified as case definition III. In Group 3, two animals survived with no clinical signs and no detection of virus DNA post-challenge and were classified as case definition I, and six animals succumbed to MCF with clinical signs and virus DNA detected post-challenge and were classified as case definition II. In Group 4, four animals succumbed to MCF with clinical signs and virus DNA detected post-challenge and were classified as case definition II, while two animals survived with no clinical signs and no detection of virus DNA in the blood post-challenge. These animals also had no MCFspecific antibody response and were classified as case definition I. A further two animals (numbers 914 and 937) survived but had clinical signs indicative of MCF. Both developed MCF-specific antibody responses after challenge but only one had detectable virus DNA in the blood post-challenge. These were classified as case definition III. In Group 5, six animals succumbed to MCF with clinical signs and virus DNA detected post-challenge and were classified as case definition II. The remaining two animals (numbers 919 and 921) showed clinical signs of MCF but had no detection of virus DNA in the blood post-challenge and no MCF-specific antibody response. One animal died and the other survived, these were classified as case definition IV (possible AlHV-1 infection). The numbers of each case type per group are summarized in Table 11.

Comparison of groups

When infection status ('uninfected' versus 'infected') of Groups 1 and 2 were compared to their respective adjuvant-only control Groups 4 and 5, the difference in the total number of infected versus uninfected cases was not significant (p = 0.13 and 0.2 respectively). When infection status of Groups 1 and 2 combined were compared to unvaccinated cattle Groups 4 and 5 combined the difference in the number of infected cattle was just outside the conventional threshold of significance (p = 0.06). Cattle classified as 'possibly infected' were not included in this analysis. The results were not substantively different when cattle with evidence of prior exposure to AlHV-1 were removed from the analysis. FliC appeared to have a negative effect on post-challenge survival (comparing Groups 1 and 3) but this effect was not significant (p = 0.06).

The relationship between serological response and protection

We calculated the geometric mean nasal secretion and plasma ELISA titres for 'infected' and 'uninfected' cattle in each treatment group for each time point. The results are illustrated in Figure 28. In the pre-challenge titres of 'infected' and 'uninfected' cattle there was no difference within any of the treatment groups. In contrast, in Groups 1, 2 and 3, the post-challenge plasma and nasal secretion titres of 'infected' cattle were all significantly higher than 'uninfected' cattle (p < 0.04).

The relationship between prior exposure and survival in control group cattle

Of the sixteen control group cattle four had evidence of prior exposure to AlHV-1 through baseline seropositivity (numbers 901 adn 926) or evidence of AlHV-1 DNA in blood mononuclear cells (numbers 914 and 937). Of these cattle, three survived (numbers 914, 926 and 937) (Table 9). A comparison of the proportions of pre-exposed and non-pre-exposed cattle that survived and died was just outside the conventional level of significance (odds ratio = 11.8; p = 0.06).

Vaccine efficacy and inter-trial regression analysis

An efficacy calculation (for preventing infection) was performed for the formulations used in this trial and the field, Russell and Haig trials. The results, which are shown in Table 12 and Figure 29, indicate that the calculated efficacies in the UK trials (80-90%) were higher



Figure 28: AlHV-1-specific antibody titres and outcome

The geometric mean nasal secretion (plots A - E) and plasma (plots F - J) AlHV-1-specific antibody titres (and $\pm 95\%$ confidence intervals) were plotted for each treatment group according to the outcome following challenge (uninfected = solid line; infected = dashed line; P = primary vaccination, B = booster and C = challenge).

than the Tanzanian trials (50-60%), however the wide confidence intervals indicate that these differences were not significant. The efficacy of the atAlHV-1 + EmulsigenTM vaccine formulation following experimental virus challenge was 50% in this trial in which SZC cattle were used whilst it was 81.5% in the Russell trial in which FH were used.

Logistic regression analysis was performed with trial identity (this trial (DVCT), the field trial, Russell and Haig trials) and final infection status of vaccinated cattle as the explanatory and dependent variables respectively. There was no evidence of a significant difference in the proportion of vaccinated animals that were protected from infection between the four trials (p > 0.18).

Table 12: Vaccine efficacy calculations								
Trial	Group	No.	No. not	Risk	Vaccine			
		infected	infected		efficacy			
					(95% CIs)			
Haig	Vacc	1	9	0.10	90%			
	Unvacc	6	0	1.00	(35.8 - 98.4%)			
Duccell	Vacc	3	15	0.17	81.5%			
Russell	Unvacc	9	1	0.90	(46.9 - 93.5%)			
Field	Vacc	19	76	0.20	56%			
rieid	Unvacc	41	51	0.45	(29.5 - 72%)			
DVCT	Vacc	3	5	0.38	50%			
	Unvacc	6	2	0.75	(-33.2 - 81.2%)			

Vaccine efficacy calculations for four vaccine trials testing the attenuated AlHV-1 vaccine are shown (Haig = Haig et al., 2008; Russell = Russell et al., 2012, Field = Chapter Two and Lankester et al., 2016 and DVCT = this trial). The number of vaccinated (Vacc) and unvaccinated (Unvacc) animals that became infected, the calculated risk of becoming infected in each group (Risk) and the resultant vaccine efficacies (and $\pm 95\%$ confidence intervals) are shown.

Discussion

This was the first trial to investigate, in an East African breed of cattle, the efficacy of the new immunization strategy against MCF (Haig et al., 2008; Russell et al., 2012) under experimental viral challenge. In addition, we wished to determine whether FliC used as an additional adjuvant could improve the magnitude of the protective immune response against AlHV-1 infection.

In this study, all three immunization regimes (Groups 1, 2 and 3) stimulated 100% seroconversion, generating local (nasal secretion) and systemic (plasma) AlHV-1-specific and neutralizing antibodies in all immunized cattle. Despite the 100% seroconversion in the vaccinated cattle, when infection status and survival were compared, between vaccinated and



Figure 29: Vaccine efficacy for four trials testing the attenuated AlHV-1 vaccine

Haig = Haig et al., 2008; Russell = Russell et al., 2012, Field = Chapter Two and Lankester et al., 2016 and Tz challenge = this trial. Black whiskers represent the $\pm 95\%$ confidence intervals.

their respective unvaccinated control groups, the differences were not significant. Despite the lack of significance, Group 1 had the smallest number of animals that became infected with AlHV-1, the fewest fatal cases and significantly higher antibody titres than the other vaccinated groups.

The comparison of vaccine efficacies (Table 12 and Figure 29) between the field trial (Chapter Two and Lankester et al., 2016) (56%) and this trial (50%) suggests that the mode of challenge did not greatly effect the efficacy of the vaccine, even though direct intra-nasal administration of a very high virus dose is likely to represent a more severe challenge than most field exposures. Despite no FH cattle being used in this trial, which makes direct comparison difficult, the comparison of vaccine efficacies across this study and the Russell trial allows us to speculate whether the sub-species of cattle used (*Bos taurus* (FH) or *B. indicus* (SZC)) might impact efficacy. Indeed, the calculated efficacy was 31.5% less when the vaccine was used in SZC. Vaccine efficacy will decrease following a reduction in either the number of protected vaccinated animals or the number of cases in unvaccinated animals. Notably the risk of vaccinated SZC cattle becoming infected (38%) was more than double that of FH (17%). This could suggest that the vaccine is not as effective in SZC at stimulating the appropriate immune response to protect against AlHV-1 infection. Conversely the risk

of unvaccinated SZC cattle becoming infected following experimental viral challenge (75%)was considerably lower than for FH cattle (90%). It is possible that differences between the trials (e.g. animal husbandry, co-infections, deterioration of the vaccine en route to Tanzania and the treatments given to the SZC cattle) may have contributed to a reduction in the effectiveness of the viral challenge. It is also possible that, following repeated annual exposure to migrating wildebeest, the African breed is more resistant to AlHV-1. This view is supported by recent genetic studies on the closely-related East African Shorthorn Zebu, which provide evidence of selection for traits related to survival in the African environment, including factors such as resistance to endemic pathogens (Bahbahani et al., 2015; Murray et al., 2013). Additionally the increased survival of SZC seen could be the result of natural adaptive immunity following previous exposure. Indeed, in this trial, post-hoc analysis of baseline samples collected before challenge indicated that three of the five surviving control group cattle had evidence of a prior exposure to AlHV-1. Thus previous exposure to MCF virus might have influenced the outcome of subsequent infection. This speculation, which remains to be addressed experimentally, suggests that sufficient time should be provided to allow baseline serological and PCR testing to identify, and exclude, all animals that have been pre-exposed to AlHV-1 naturally. The two animals that were sero-positive in the pretrial sample could also be the result of infection with viruses that cross-react with the ELISA used here. While it is likely that other MCF virus infections would induce cross-reactive antibodies detectable by AlHV-1 ELISA, it has also been reported that bovine herpesvirus-4 (BoHV-4) antibodies can detect AlHV-1 infected cells by immunofluorescence (Dewals et al., 2005). However previous testing did not show cross-reactivity with BoHV-4 specific sera in the direct MCF ELISA (Fraser et al., 2006).

The unexpected survival of control group cattle could also be related to the immunological effects of the ECF vaccination, which was administered to all trial cattle one month before the MCF vaccine and is thought to manifest its protective immunity through a cellmediated effect that suppresses the proliferation of infected CD8 T-cells (Di Giulio et al., 2008; Radley et al., 1975). While the pathogenesis of MCF is not fully understood, the indiscriminate tissue damage caused by MCF is thought to be either a direct result of virus infected CD8 cytotoxic T- cells (Dewals et al., 2008; Thonur et al., 2006; Hart et al., 2007), or immune-mediated responses to them (Simon, 2003). Thus, immuno-modulating effects of the ECF vaccination could provide some protection from the pathogenesis associated with lethal MCF for both vaccinated and unvaccinated trial cattle. This hypothesis will be investigated in a subsequent field trial. The vaccine's efficacy as determined by this challenge trial and the preceding field trial is estimated to be within the range of 50 - 56%. As discussed in Chapter Two, this level of efficacy is likely to be too low for the annual vaccination of cattle to replace the traditional MCF avoidance strategy. However the partially protective vaccine may have an important role to play providing protection to valuable cattle which are kept back at the permanent household to provide milk to the family (Lankester et al., 2015b), or to cattle that, because of recent changes in land-use and ownership, are restricted from moving away from the oncoming wildebeest. Furthermore, given there are very few, if any, herpesvirus vaccines that effectively prevent infection and the establishment of latency, we consider the proportion of SZC vaccinated animals that did not become infected in the face of intense challenge in this and the field trial as a promising indication for future MCF vaccine improvement strategies.

The second objective of this study was to assess the effect, on serological response and protection, of flagellin (FliC) as an adjuvant, given either alone or in combination with EmulsigenTM. The rationale was that FliC has demonstrated adjuvant properties for antibody- and cell-mediated responses in several mammalian and avian species, including mucosal immune responses (Akira and Takeda, 2004; McDermott and Huffnagle, 2014; Lee et al., 2015). The *in-vitro* analysis of FliC reactivity with bovine TLR5 on HEK cells indicated that there was a CXCL-8 response but not as great as FliC with HEK cells expressing human TLR5. Furthermore, there have been reports that, compared to human TLR5, bovine TLR5 responds poorly to FliC (from S. typhimiurium (Metcalfe et al., 2014)), and a recent study has indicated that this was due to differences in amino acids present in the intracellular region of the molecule (Osvaldova et al., 2014). We decided to proceed with the trial, however, as our analyses did show FliC stimulated bovine TLR5 (Figure 25). Moreover, there may be other FliC sensing molecules in cattle. Indeed, in mice and humans the Naip5/6 NIrc4-inflammasome as well as the intracellular sensor Ipaf also detects FliC and flagellin can induce antibody responses independent of TLR5 and the Naip5/6 NIrc4-inflammasome (López-Yglesias et al., 2014). This indicated a complex pathway for flagellin recognition.

In the immunization Group 2 (atAlHV-1 + FliC), ELISA and VNA titres in nasal secretion and plasma were significantly lower compared to those in Group 1 (atAlHV-1 + EmulsigenTM) (Figures 26 and 27). Additionally, although not significant, fewer animals were protected in Group 2 than Group 1 (Table 9 and 11). In the Group 3 animals (atAlHV-1 + EmulsigenTM and FliC) the presence of FliC was associated with significantly reduced plasma ELISA and VNA titres, and there was an apparent reduction in the number of animals

protected from MCF when compared to Group 1. The reduced antibody responses associated with FliC as an adjuvant does suggest it is unlikely to enhance the correct protective immune response to prevent MCF. This lack of efficacy could be due to poor TLR5 stimulation of dendritic cells and other antigen-presenting cells at the injection site or in the draining lymph nodes. It may also indicate that, if FliC is engaging other flagellin / FliC recognition molecules and pathways in cattle (as described above), they do not support an MCF protective response. On the other hand the reduction in ELISA and VNA titres in Group 2 and 3 animals supports the notion that FliC has a *suppressive* effect on the antibody response to AlHV-1. In support of this, there have been recent reports that FliC and TLR5 engagement can generate and activate regulatory T cells (Tregs) (Hao et al., 2014). These cells inhibit ongoing immune responses to prevent tissue damage and are particularly important in maintaining peripheral tolerance to antigens and infections and suppressing autoimmune disease (Josefowicz, Lu, and Rudensky, 2012). This might explain the inhibition we see. We conclude, therefore, that addition of TLR agonists can modulate the immune response to attenuated AlHV-1 vaccine. In the case of FliC, however, this modulation appeared to cause immuno-suppression, and this is a phenomenon worth looking into in more detail.

Wildebeest-associated MCF has been described as a disease with a case-fatality ratio greater than 96% (Plowright, Ferris, and Scott, 1960). The finding that 15% of the trial cattle had evidence of prior exposure to, and infection by, AlHV-1 was therefore surprising. Non-fatal infections have been reported in sheep-associated MCF (Otter, Pow, and Reid, 2002; Moore et al., 2010) and serological evidence of non-fatal infections with AlHV-1 was described in the field trial (Chapter Two). The findings from this viral challenge trial add further evidence that non-fatal outcomes might be common in the wildebeest-associated form of the disease and that the case-fatality ratio could be considerably lower than previously described.

The cell biology and pathogenesis of MCF are also poorly understood. The fact that four cattle were PCR positive at baseline suggests that, following initial infection, virus was not eliminated from cattle that survived the infection. It is not clear whether the virus in these cases became latent, residing in certain body tissues as it does with the carrier host species, nor whether it might cause disease symptoms at a later stage.

In Groups 1, 2 and 3 the post challenge nasal secretion and plasma antibody titres were significantly higher in animals that became infected with AlHV-1 than those that did not. This boosted (anamnestic) response, which resulted from the virus managing to infect these animals, was also detected in the UK-based trial (Russell et al., 2012). Regardless of the rise

In summary, the findings from this trial indicate that following immunization with the attenuated AlHV-1 C500 + EmulsigenTM formulation SZC cattle respond in a similar manner to FH cattle: the vaccine has been shown to induce an oro-nasopharyngeal AlHV-1-specific and neutralizing antibody response and there is evidence that, when combined with EmulsigenTM as an adjuvant, the vaccine formulation induces a partial protective immunity. A larger study is required to better quantify this effect. We have shown that direct challenge with the pathogenic AlHV-1 virus is effective at inducing MCF in SZC. We have also provided evidence that the atAlHV-1 + EmulsigenTM formulation may be less effective at stimulating a protective immune response in SZC cattle than FH cattle. Furthermore, and in support of the field trial, we have provided evidence that non-fatal AlHV-1 infections are relatively common and we speculate that there could be resistance to fatal MCF in SZC cattle, possibly through genetic background, previous (sub-clinical) exposure to AlHV-1 or alternative acquisition of a level of inherent immunity. Finally, we demonstrated that FliC is not an appropriate adjuvant for the atAlHV-1 vaccine.

XIII

Chapter Five

The economic impact of

malignant catarrhal fever on pastoralist livelihoods

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Introduction

Wildebeest-associated malignant catarrhal fever (MCF) is an often-lethal disease that affects cattle and is a particular problem for cattle owners living in eastern and southern Africa (Bedelian, Nkedianye, and Herrero, 2007; Cleaveland et al., 2001). The disease is caused by a γ -herpesvirus, Alcelaphine herpesvirus I (AlHV-1), which is excreted by wildebeest calves (Connochaetes taurinus) in the three months following the brief annual calving period (Plowright, Ferris, and Scott, 1960). Consequently being in proximity to wildebeest is only a risk to cattle for a specific and limited period each year. Although sedentary populations do exist, the wildebeest is predominantly considered a migratory herbivore that specialises on feeding on short, green grass and, in many populations, it returns annually to the same pastures to give birth and suckle its young, with the timing of its arrival being linked with seasonal rainfall (Estes, 1976). If located outside of a national park these ancestral calving ground pastures may also be utilized by domestic livestock in mixed-use buffer zones (Figure 4). Because wildebeest calves pose a risk of MCF, livestock owners living in these zones face a dilemma: to move their cattle away from the wildebeest calving grounds on to more marginal land (substitute pastures) at a time of year when the new pasture is most nutritious and the health of cattle most vulnerable (the traditional, and current, disease avoidance strategy), or to stay and risk infection and disease. The potential impacts of the traditional disease avoidance strategy vary in different areas but include a) an increased disease burden from vector-borne and directly transmitted diseases due to the confinement of large numbers of cattle herds, often in woodland areas where vectors of disease are more concentrated, b) reduced access to salt, c) losses resulting from the energy demands associated with traveling large distances away from the wildebeest calving grounds and d) the impact of grazing poorer quality forage at a critical time of year for recovering body condition (Cleaveland et al., 2001). Consequently, the economic costs associated with the disease have been reported

to be significant in regions where MCF risk to cattle is high (Li et al., 2008; Russell, Stewart, and Haig, 2009; Bridgen and Reid, 1991). These costs, however, have never been quantified.

Following recent advances in the development of a vaccine (Haig et al., 2008), a field trial was carried out to determine the efficacy of a new MCF immunisation strategy. For livestock owners the direct and indirect benefits of an effective vaccine could be significant: fewer cattle would die from MCF each year and, rather than having to move away, immunized cattle could continue to graze on the nutritious pastures of the wildebeest calving grounds at a time when the grass is at its most nutritious.

This vaccine trial provided an opportunity to evaluate the potential economic benefits that could accrue from an effective vaccine, through (1) avoiding household resource and management costs incurred moving cattle away during the wildebeest calving season, and (2) being able to graze cattle on the higher quality pasture of the wildebeest calving ground.

To assess the management and resource costs associated with moving, we used household survey data. To estimate the costs associated with changes in livestock body condition that result from the traditional MCF avoidance strategy we a) compared body condition of cattle moved away to substitute pastures (cattle owned by Maasai householders - the *control* herd) with cattle belonging to the vaccine field trial that were allowed to graze alongside wildebeest on their calving ground pastures (vaccine trial animals - the *treatment* herd) and b) used a hedonic price regression (a statistical model that allows estimation of the marginal contribution of a good's attributes to its market price (Coatney, Menkhaus, and Schmitz, 1996; Rosen, 1974; Scarpa et al., 2003)). We hypothesize that livestock condition and value will increase more (or decrease less) for cattle which are able to graze alongside wildebeest calves on higher quality forage, than for cattle that have been moved to substitute pastures in the traditional way. Based on the output of the hedonic price regression, we monetize these comparative physical impacts in terms of their effects on the estimated market price of animals that are able to remain on the calving area and those that are moved away during calving season.

Materials and Methods

The human subject research was conducted according to relevant international guidelines and was approved by the Tanzanian Commission of Science and Technology (permit nos.2011-213-ER-2005-141 and 2012-318-ER-2005-141). Informed oral consent was given by all participants. Written consent was not given as many of the participants were not literate.

To ensure that the participants were consenting, a well respected local elder was present for all interviews. Before proceeding the elder explained the purpose of the study to the participants and translated all questions in to the vernacular language. Only when the elder was satisfied that the participant had fully understood the implications of our study, and was happy to be interviewed, could we proceed. When oral consent had been given the name of the respondent was written onto the interview sheet and the elder checked a box that stated that the interviewee was consenting to be interviewed.

Trial setting

The study took place, with the approval and co-operation of the community, on communally owned land (latitude -3.952239, longitude 36.47537) in a mixed-use buffer zone 25-40 km east of Tarangire National Park in a village called Emboreet in northern Tanzania's Simanjiro District (Figure 4). The buffer zone is inhabited by transhumant pastoralists and agropastoralist communities and is an important dispersal area for wildlife such as wildebeest and zebra (*Equus quagga*) that seasonally move out of the national park during the wet season (November to April) to calve. The migration of these herbivores is driven by seasonal water resources and by the high levels of nitrogen and phosphorus in the area's vegetation that result from underlying volcanic soils and which are important for lactating females (Voeten and Prins, 1999; Sachedina, 2010; Holdo, Holt, and Fryxell, 2009). These high quality pastures are referred to in this study as the wildebeest calving grounds.

MCF avoidance - herd management and associated costs

The first part of the economic impact assessment aimed to assess MCF avoidance practices and the associated costs incurred by livestock herd owners. A questionnaire survey (shown in Appendix One) targeted 16 Maasai livestock owners, selected because they lived close to the wildebeest calving ground and their herds grazed these pastures when wildebeest were not present (Figure 4). The survey was carried out four times between 2011 and 2012. The questionnaire, which was delivered in the Maasai language before being translated to English and was trialled with bilingual participants not involved with the trial, asked the respondents about specific MCF-related management decisions and the impact that these decisions had on their households. The data from this survey are summarized both graphically and in tabular form to provide a basis for a qualitative discussion of the impact of management activities on households, including the distributional impacts of MCF avoidance within the household. Part of this discussion concerns income loss due to lost opportunities to sell milk. To estimate an upper bound on this cost we used existing data on the proportion of Maasai household income that is typically derived from milk. In lieu of any data being available for the Simanjiro District, we used a study of the Maasai community of Kitengela (Kenya) (Homewood, Chenevix Trench, and Kristjanson, 2009).

MCF avoidance - health and condition associated costs

The second part of the study aimed to estimate the impact of MCF avoidance on cattle market value by comparing the difference in the market value of animals that were moved to avoid MCF (Figure 4) against cattle that were not moved. A four-step strategy was followed, involving sample identification, data collection, regression analysis, and market value inference.

- 1. Sample identification: We use data from three sources:
 - (a) A trial herd (n = 100) from the vaccine field trial (hereafter called the treatment herd) which, in contrast with traditional Maasai herding practices, was grazed on the wildebeest calving area during the period February May. All other husbandry conditions were locally typical.
 - (b) Two privately owned control herds (n = 100 & 150) selected on the basis of their location (based in the village of Emboreet) and representativeness (locally typical breed and husbandry conditions and, importantly, moved away from the wildebeest calving ground during MCF season).
 - (c) A market sample (n = 185) which represented a set of cattle individually sold at a primary livestock market in the Simanjiro District. Selection was determined by owner willingness to participate. Animal attribute data, analogous to that recorded for the treatment and control herds, was recorded as described below. In addition, the sale price of each animal was collected. All cattle in (a), (b), and (c) were shorthorn zebu cross (SZC).
- 2. **Data collection:** At four seasonally relevant time points, a broad set of physical attribute data were collected for all cattle belonging to the treatment and control herds and the market sample. The survey instruments are shown in Appendix Two and Three. The recorded attribute data included heart girth (cm); wither height (cm); body condition score (1 (thin) - 5 (fat)); colour; age (months); the number of pairs of incisors; the vaccination status (with respect to east coast fever (ECF), anthrax / black quarter,

and lumpy skin disease); sex; if female, whether a heifer; the outcome of the cow's last calving; if male, whether castrated. All animal attribute data was collected by the same enumerator and, based on preliminary analysis, only a selection of the variables were used in the final regression. The attribute data collection time points were as follows:

- Time point 1: Early February when typically the rains have begun and fresh new grass has started to grow. This was the beginning of the period when cattle were moved away from the wildebeest calving ground to substitute grazing pastures and represented the start of the trial period and the base-line for this study;
- Time point 2: Early March, just after the wildebeest calving season, marked the mid-point of the period in which cattle were kept away from the wildebeest calving ground pastures;
- Time point 3: Early May, the risk period for MCF transmission had passed and cattle managed in a locally typical manner had just returned to graze on the wilde-beest calving ground pastures;
- Time point 4: Mid-November, represented the end of the long dry season.

Collection of data at these specific points enabled changes in non-fixed attribute data to be quantified across three time periods:

- Time period I (time point 1 to 2): The control herds were moved away from the wildebeest calving ground to substitute pastures; the end of this period marked the beginning of MCF risk.
- Time period II (time point 2 to 3): The control herds were grazed on substitute pastures throughout this period, whilst the treatment herd remained on the wildebeest calving ground; this was the high risk period for MCF.
- Time period III (time point 3 to 4): The control herds have returned to graze alongside the treatment herd on the wildebeest calving ground for the duration of the long dry season; this was the post-risk MCF period.

Disease data were also collected: Control herd owners were asked which of their cattle had suffered from ill-health during the preceding time period (or preceding two months for the first time point). Being an experimental herd for which health data was already being recorded, disease data were compiled for the treatment herd every two or three days.

- Hedonic price estimation: Based on the attribute and sale price data from the market sample we estimated the parameters of a hedonic price regression (Coatney, Menkhaus, and Schmitz, 1996; Scarpa et al., 2003).
- 4. Market value inference for control and treatment herds: The attribute data from the treatment and control herds were inserted into the hedonic regression, which provided an estimate of the market value of each animal in each of the herds based on its physical condition (Rosen, 1974). From these estimates, the average differences in market value across herds and across time periods were calculated. Consequently we were able to infer the herd-level impact of having to move cattle away from the wildebeest calving ground.

The attrition rate of cattle in the control herds was substantial with only 141 out of 250 cattle making it through to time point 4, with most (65%) being lost to follow up after time point 3. Because we were focusing on changes in cattle condition over time, our results (shown in Table 18) utilized only those animals who remained in the herd across all three time periods (those who did not disappear from our sample prior to time point 4). However, attrition could in principle affect the outcome of our analysis if animals died or were sold or given away due (at least in part) to differences in condition, which is likely. To assess this we repeated the means tests with the data on lost cattle included in our calculations until lost. For example, if an animal was sold from the control herds in time period 2, we included the changes in its condition in the first time period. We found that the results were not different qualitatively than if all data for lost animals were excluded, and therefore the inferences do not change.

MCF avoidance - impacts on value

The hedonic price regression relates the market sale price (P_i) to a set of the animal's attributes X_i for a set of cattle i = 1...N sold at market. Hedonic price theory provides little guidance on the specific functional form of the regression relationship, except that market prices for goods are positive. A general linear price function can be written as

$$g(P_i) = f(\mathbf{X}_i; \beta) + \varepsilon_i, \tag{1}$$

where $g(\cdot)$ and $f(\cdot)$ are transformation functions and $\mathbf{X}_i \beta = \sum_{k=1}^K X_{ik} \beta_k$ is an index function, linear in K attributes and associated parameters β , including an intercept term. For example, if $g(P_i) = P_i$ and $f(\mathbf{X}_i; \beta) = \mathbf{X}_i \beta$, equation 1 is a linear regression $P_i = \mathbf{X}_i \beta + \varepsilon_i$. Nonlinear Box-Cox formulations of $g(\cdot)$ and $f(\cdot)$ are possible (Davidson and MacKinnon,
1993; Cook and Weisberg, 1982; Box and Cox, 1964), of which linear or log-linear functional forms are the most commonly applied special cases. Likelihood Ratio tests based on preliminary Box-Cox regressions rejected the linear form and failed to reject when the log of price was used as the dependent variable (p = 0.60). We therefore applied a log-linear model of the form

$$\ln(P_i) = \mathbf{X}_i \beta + \varepsilon_i, \tag{2}$$

where the continuous variables in X may in general be natural logarithms of the original variables (which applies in our specific case), and quadratic interaction terms as described below are included to allow for interaction effects of the explanatory variables. Ordinary Least Squares applied to this regression using the market data (after transforming P to $\ln(P)$) provides consistent parameter estimates $\hat{\beta}$ assuming a correctly specified model.

A full range of explanatory variables (attribute data) were included in $X_i\beta$ for the first iteration of the hedonic regression. The heteroskedasticity-robust Huber/White/sandwich covariance estimator was used to calculate standard errors and a final hedonic regression specification was selected based on economic theory and step-wise deletions guided by a combination of F- and T- tests for significance, AIC and BIC values. The most important modelling decisions in this regards were as follows:

First, livestock attributes *observable* at the market are most likely to impact price (unsubstantiated claims of high milk production or vaccination are likely to be discounted by buyers). This hypothesis was supported from initial regressions. We therefore focused on observable attributes: (i) Heart girth (*HG*), a summary statistic that correlates strongly with skeletal size, is a statistically important explanatory variable for explaining price variation (Ayele et al., 2006; Getachew, Teshale, and Ketema, 2012); (ii) body condition score (*BCS*), a summary measure for the visual determination of nutritional status of an animal (Evans, 2010); and (iii) *Age*, a potentially important characteristic because, even if all physical characteristics are perfectly controlled for, the temporal distribution of costs and benefits from owning an animal vary over its lifetime and benefits or costs accrued in the distant future tend to be discounted relative to those accrued in the near future (Frederick, Loewenstein, and O'donoghue, 2002). Indicator variables for sex (*male* = 1 if male, zero otherwise) and whether a female is a heifer (*heifer* = 1 if a female has not calved, zero otherwise) were also included.

Aggregate market prices naturally vary over the course of the year, so we included datespecific indicator variables to account for aggregate market price fluctuations. Logarithmic transformations of the continuous variables and a parsimonious set of linear and quadratic terms minimized the AIC and BIC information criteria.

A description of the retained attribute data is given in Table 14. The final regression used for price estimation was:

$$\ln(P_{i}) = \beta_{0} + \beta_{1} \ln(HG)_{i} + \beta_{2} \ln(HG)_{i}^{2} + \beta_{3} \ln(HG)_{i} \ln(BCS)_{i} + \beta_{4} \ln(BCS)_{i}^{2}$$
(3)
+ $\beta_{5} \ln(Age)_{i} + \beta_{6} \ln(Age)_{i}^{2} + \beta_{7} \ln(Age)_{i}^{3} + \beta_{8} I[Male_{i}] + \beta_{9} I[heifer_{i}]$
+ $\beta_{10} I[Feb] + \beta_{11} I[Mar] + \beta_{12} I[Apr] + \beta_{13} I[Nov].$

A cubic polynomial for age was included to best allow for hypothesized curvature in price due to age, because lower-order polynomials alone did not conform to theory, and because the visual relationships between age and market price indicated a sinusoidal relationship.

This final hedonic regression allowed the calculation of an estimated market price for any given animal on any given day. To do this we simply entered the cattle specific attribute values recorded for the treatment and control herds into Equation 3.

Our principle objective, however, was to quantify the impact on price that was caused by a change in those specific attributes that could potentially be affected by disease avoidance strategies. Consequently, from the list of attributes that made it through to the final hedonic regression, the attributes of interest to our analysis were *BCS* and *HG*.

The percentage change in price with respect to a percentage change in *BCS* (also known as the *elasticity* of price with respect to BCS) is

$$\frac{\partial \ln(P)}{\partial \ln(BCS)} = \beta_3 \ln(HG) + 2\beta_4 \ln(BCS)$$

$$\approx \frac{\% \Delta P}{\% \Delta BCS}.$$
(4)

where $\%\Delta X = (X_j - X_i)/X_i$ for a change of any variable from X_i to X_j , and $\frac{\%\Delta P}{\%\Delta BCS}$ represents the percent difference in P with respect to a percent difference in BCS holding all else constant. The relationship between $\frac{\partial \ln(P)}{\partial \ln(BCS)}$ and $\frac{\%\Delta P}{\%\Delta BCS}$ is asymptotically exact as changes in BCS approach zero, but is approximate for relatively larger changes in BCS (hence the use of \approx in Equation 4). The coefficients on the indicator variables such as Male, Heifer and the date variables require careful interpretation as well. An unbiased estimator of the percentage change in the transformed dependent variable P with respect to a change in an indicator variable I from 0 to 1 is $\Delta \ln P/\Delta I = e^{\hat{\beta} - v[\hat{\beta}]/2} - 1$, where $\hat{v}[\hat{\beta}]$ is the estimated variance of $\hat{\beta}$, calculated using the Delta Method (Kennedy, 1981; Garderen and Shah, 2002; Greene, 2012).

An estimate of percent change in price due to a percentage change in BCS from one time point, t to the next t + 1 (holding all else constant) is derived by multiplying both sides of Equation 4 by $\% \Delta BCS$:

$$\%\Delta_t P \approx \left(\frac{\partial \ln(P)}{\partial \ln(BCS)}\right)\%\Delta_t BCS,$$
(5)

where $\frac{\partial \ln(P)}{\partial \ln(BCS)}$ is defined in Equation 4. Analogous elasticities for *HG* and *Age* can be derived (as first derivatives) from equation 3.

Equation 5 amounts to a difference equation and is useful for estimating the effect of $\ln(HG)$, $\ln(BCS)$, and $\ln(Age)$ on price holding all else constant. Note that *Male* and *Heifer* do not vary over time and so drop out, and that the date indicators, which were necessary only to control for market conditions for estimation, are set to zero for the following analysis. Consequently the estimated market price correspond to the base case which is the 1^{st} market sample time point.

As all three variables vary simultaneously, the difference in estimated $\ln(P)$ from one time point to the next, due to simultaneous changes in $\ln(Age)$, $\ln(HG)$, and $\ln(BCS)$, is:

$$\Delta_t \ln(\hat{P}_i) = \hat{\beta}_1 \Delta_t \ln(HG)_i + \hat{\beta}_2 \Delta_t \ln(HG)_i^2 + \hat{\beta}_3 (\Delta_t \ln(HG)_i \ln(BCS)_i) + \hat{\beta}_4 \Delta_t \ln(BCS)_i^2 + \hat{\beta}_5 \Delta_t \ln(Age)_i + \hat{\beta}_6 \Delta_t \ln(Age)_i^2 + \hat{\beta}_7 \Delta_t \ln(Age)_i^3.$$
(6)

Were we to apply equation 6 to estimate the change in price over time for an animal it would include not only the effects of changes in HG and BCS but also the effects of ageing on price. As animals simultaneously age and change body composition over time the effects of Age should be netted out of the price changes to avoid conflating age and condition effects (HG is strongly correlated with total body weight, which tends to increase more for younger cattle in a given environment (Wanderstock and Salisbury, 1946)). As the age structure of the treatment and control herds were very different (the treatment herd was selected for a vaccination trial, while the control herds were maintained for the benefit of households), netting out the affect of differences in age on price is important for isolating the effects of HG and BCS. To do this we subtracted the age effects from both sides, which provides:

$$\Delta_t \ln(\hat{P}_i^*) = \hat{\beta}_1 \Delta_t \ln(HG)_i + \hat{\beta}_2 \Delta_t \ln(HG)_i^2 + \hat{\beta}_3 \Delta_t (\ln(HG)_i \ln(BCS)_i) + \hat{\beta}_4 \Delta_t \ln(BCS)_i^2,$$
(7)

where $\Delta_t \ln(\hat{P}_i^*) = \Delta_t \ln(\hat{P}_i) - (\hat{\beta}_5 \Delta_t \ln(Age)_i + \hat{\beta}_6 \Delta_t \ln(Age)_i^2 + \hat{\beta}_7 \Delta_t \ln(Age)_i^3)$ is the estimated percent change in expected price due solely to changes in *HG* and *BCS*. Consequently the right hand side of equation 7 amounts to the difference equation for $\ln(P)$ with respect to changes in $\ln(HG)$ and $\ln(BCS)$ holding *Age* constant.

As described above, for any variable X, log-differences are approximately equal to percent changes: $\Delta_t \ln(X)_i \approx \% \Delta_t X_i$ (the approximation is asymptotically exact as $X_{i,t} - X_{i,t-1}$ becomes small). Therefore, including the percent changes $\% \Delta_t HG$ and $\% \Delta_t BCS$ across time points $t = (t = 1 \rightarrow 2, 2 \rightarrow 3, 3 \rightarrow 4)$ for each animal *i* in equation 7 along with the estimated coefficients provides an estimated percent change in price $(\% \Delta_t \ln(\hat{P}_i^*))$ due to changes in cattle condition. Comparison of the sample means $(\overline{\%} \Delta_t \hat{P}_{i;h}^* = (1/n_h) \sum_{i=1}^{n_h} \Delta_t \ln(\hat{P}_{i;h}^*))$ for each herd (h = control, treatment), where n_h is the number of animals in herd h, allows a herd-level comparison of changes in price due to changes in the condition of animals in each of the two herds.

Results

We present results in relation to i) the management costs associated with MCF avoidance, and ii) the impact of MCF avoidance on the predicted market value of cattle.

MCF avoidance - herd management and associated costs

Table 13 provides an overview of the questions and summary statistics that emanated from the herd management questionnaire. Ninety percent of herd owners moved their cattle away from the wildebeest calving grounds to substitute pastures to avoid MCF during the calving season. The owners who did not move their cattle away had small herds consisting of less than seven head of cattle and they cited wanting to keep any milk produced at the boma (a traditional Maasai household unit) as the reason for not moving their herds. The substitute pastures to which cattle were moved were on average 21.3 km away from the boma (Figure 30), took 2.2 days to reach and the cattle remained there for an average of 88 days (Figure 31).

There was a significant positive association between the distance a herd was moved away from the wildebeest calving grounds to reach a substitute pasture area and the total time it spent away from the boma (p < 0.03, r = 0.4) (Figure 32). Only 10% of herd owners employed non-family members to move and manage their cattle away from the boma, with 90% of households involving only members of the family unit. Within these households

				95%	6 CI
Variable	Results	SE	Ν	LL	UL
Prop. of herds moved away to avoid MCF	0.90	0.05	31	0.79	1.01
Prop. of each herd moved away	0.82	0.07	28	0.67	0.96
Prop. total cattle moved away	0.90	0.005	*3858	0.89	0.91
Mean no. of cattle remaining at the boma	14.76	2.72	25	9.43	20.1
Prop. of total lactating cattle moved away	0.61	0.02	*503	0.57	0.65
Prop. of total cattle not moved away	0.74	0.12	*237	0.51	0.97
that were lactating					
Prop. total daily milk from cattle	0.71	0.02	*792	0.68	0.74
herded away from boma					
Prop. daily milk that was produced away from	0.10	0.08	*356	-0.05	0.25
boma, returned to family at boma					
Mean time herd spent away (days)	88	2.60	30	82.97	93.16
Mean distance herd moved away (km)	21.3	3.10	30	15.20	27.37
Mean journey to substitute grazing (days)	2.2	0.38	30	1.43	2.91
Prop. that gave 'to avoid wildebeest'	0.94	0.04	30	0.86	1.02
as reason for moving					
Mean no. family members moved with herd	2.27	0.21	30	1.85	2.69
Prop. total family members that moved	0.19	0.10	*189	0.00	0.38
away with herds					
Prop. used non-family members to move cattle	0.10	0.05	30	-0.01	0.21
Perception of calving ground pasture: Good	0.52	0.09	31	0.34	0.70
Perception of calving ground pasture: Med	0.48	0.09	31	0.30	0.66
Perception of substitute pasture: Good	0.42	0.09	31	0.25	0.59
Perception of substitute pasture: Med	0.55	0.09	31	0.37	0.73
Perception of substitute pasture: Low	0.03	0.03	31	-0.03	0.09
Practise differ if MCF not a problem: 'Yes'	0.90	0.05	31	0.79	1.01
How would practice differ: 'Would not move'	0.96	0.04	28	0.89	1.03
How would practice differ: 'Treat less for tryps'	0.11	0.06	28	-0.01	0.23
How would practice differ: 'Cultivate more'	0.04	0.04	28	-0.03	0.11
How would practice differ: 'Dip less'	0.07	0.05	28	-0.02	0.16

Table 13: MCF management response

This table contains an overview of the questions and summary statistics that emanated from the herd management questionnaire (Prop. = proportion, SE = standard error, LL and UL = lower and upper 95% confidence limit, respectively and N = sample size. For most questions the sample size was the number of respondents that answered the particular question, in other instances (*), the sample size was the total number of cattle, lactating cattle, litres of milk or family members.



Figure 30: Density plot of distance travelled avoiding wildebeest calves

The distance (km) that cattle herds travelled to avoid wildebeest calves and MCF virus is shown. Dashed red line indicates the mean.



Figure 31: Density plot of time spent avoiding wildebeest calves

The length of time (days) cattle herds spent avoiding wildebeest calves and infection with the MCF virus. Dashed red line indicates the mean.

an average of 2.3 members (19% of the family unit) were recruited to move the cattle. Of the 90% of herds that moved away to avoid MCF, an average of 82% of each herd was moved, whilst 18% (an average of 15 cattle) were kept at the boma. Of the 18% of cattle that remained at the boma, an average of 74% were lactating (Figure 33). Despite lactating cattle being kept disproportionately at the boma and therefore at greater risk of MCF, 61% of all lactating cattle were moved to substitute pastures, resulting in an average of 71% of the total daily milk being produced by cattle that had been moved away. Of this milk only 10% was returned to the bomas. Therefore, on average, 64% of the total daily milk was not available to be used by the 81% of each family unit that remained at the bomas. The respondents reported that all of this milk was drunk by the herders or the calves, with none being sold or discarded. This constraint on milk allocation between herders and boma imposes costs on the household. To estimate an upper bound on this cost we used Maasai household income data derived from the Kitengela study (Homewood, Chenevix Trench, and Kristjanson, 2009) which found that 52% of the average gross annual household income is derived from milk, of which 27% comes from sales and 25% from consumption(Homewood, Chenevix Trench, and Kristjanson, 2009). Assumine that none of the milk from the moved cows is consumed or sold, the loss of 64% of the household's milk, which, if the Kitengela population is comparable, amounts to a loss of 33% of the household's income during the calving season. Assuming that the cattle are away for 88 days (or 24% of the year), this upper-bound represents a loss of about 8% of the household's annual income. Because the herders and calves do consume the milk the actual loss of income will be less than this. The calculation is useful, however, as it provides a limit above which the economic loss is unlikely to be.

When asked for reasons why the herders had chosen to move their cattle away from the wildebeest calving grounds (and their village pasturelands), 94% said that they had moved away to avoid wildebeest, whilst 6% said they moved their cattle to seek better pasture. The quality of pasture at the substitute grazing areas was perceived by the respondents to be moderately lower than that of the wildebeest calving grounds (substitute — high 42%, medium 55% and low 3%; and calving grounds — high 52%, medium 48% and low 0%). If MCF were no longer a health risk for their cattle 90% of herd owners say they would change their management practises, with 96% stating that they would no longer move their cattle away from the wildebeest calving grounds.



Figure 32: Time - distance scatter plot

A scatter plot showing the length of time (days) cattle spent away and the distance they travelled (km). The blue line and the grey areas indicate the regression line (p < 0.03, r = 0.4) and the $\pm 95\%$ confidence interval respectively.



Figure 33: Image of Maasai woman milking a cow

Lactating cattle, like the one that this Maasai woman is milking, are disproportionately kept back at the boma during the MCF season and are therefore at a greater risk of disease (source T. Lembo).

	Table 14: Data description	
Data variable	Description	
Age	Age of cattle in months	
BCS	Body condition score (1 (thin) - 5 (fat))	
HG	Heart girth (cm)	
Male	Male =1, female = 0	
Heifer	Female before first calving = 1; otherwise = 0	
Time point 1	6th February 2012	
Time point 2	4 March 2012	
Time point 3	1st April 2012	
Time point 4	24th November 2012	

This table contains a description of the retained attribute data collected from cattle sold at livestock markets and from the control and treatment herds.

MCF avoidance - health and condition associated costs and the impact on value

We now present the hedonic price regression results, a comparative summary of the impacts of body condition score (*BCS*) and heart girth (*HG*) on market price, and a comparison of the changes in cattle condition and market value between the treatment and control herds. Table 14 provides a description of the attribute data retained in the hedonic price regression. Table 15 provides summary statistics and price effects for all these variables, whilst Table 16 contains the final hedonic regression results.

Only cattle characteristics that were immediately verifiable by the buyer had a significant impact on price and were retained in the final regression. Other unverifiable characteristics, including whether or not the individual had been immunised against the locally prevalent disease east coast fever (ECF), the reported daily milk yield or number of previous calves, had no effect. The results show that, with all else constant, the market value of an animal begins to decline at about 86 months (7.2 years) and that the characteristics *HG*, *BCS*, *Age*, *Male* (castrated and entire males) and *Heifer* were statistically important determinants of market value. Regarding age, the visual relationships between the variable *Age* and market price indicated a sinusoidal relationship with the three age-associated coefficients being jointly significant (F = 4.49, p = 0.0047). Of these variables, only *HG* and *BCS* can be impacted by husbandry conditions and are of interest to the discussion regarding impacts of disease avoidance on value. Table 17 provides the elasticities of market price with respect to *BCS*, *HG*, and *Age*, evaluated at the means of the market data. Both *HG* and *BCS* have positive effects on price, but the effect of *HG* on price is ten times larger than that of *BCS*, and is more strongly significantly different from zero.

The percentage change in the mean values of HG and BCS and mean predicted

Herd	Variable	Mean	sd	min	max	Ν
Market sample	Price	235.2	88.7	100	575	185
	HG	124.7	15	90	162	185
	BCS	2.8	0.5	1	4	185
	Age	35.8	23.5	11	120	185
	Male	0.6	0.5	0	1	185
	Heifer	0.2	0.4	0	1	185
Control herds	HG	132.8	19.5	63	180	836
	BCS	3.1	0.5	1	5	839
	Age	42.4	26.6	6	166	1000
	Male	0.4	0.5	0	1	1000
	Heifer	0.4	0.5	0	1	996
Treatment herd	HG	124.9	9.1	100	154	393
	BCS	2.9	0.4	2	4	393
	Age	17.4	5.4	6	40	400
	Male	0.7	0.4	0	1	400
	Heifer	0.3	0.4	0	1	400

Table 15: Summary statistics for market sample, treatment and control herds

This table provides summary statistics for all variables used in the final regression and price effect estimation. Price is in Tanzanian shillings (x 1,000).

Table 10. Heudine price regression								
Variable	Estimate	<i>t</i> -stat						
$\ln(HG)$	-26.464	3.81***						
$\ln(HG)^2$	2.901	3.98***						
$\ln(HG) \times \ln(BCS)$	0.241	5.81***						
$\ln(BCS)^2$	-0.483	3.95***						
$\ln(Age)$	-2.920	1.54						
$\ln(Age)^2$	0.875	1.56						
$\ln(Age)^3$	-0.081	1.51						
Male	0.090	1.23						
Heifer	0.202	2.68***						
I[Feb]	67.649	4.12***						
I[Mar]	0.073	1.77*						
I[Apr]	0.218	4.57***						
I[Nov]	0.178	4.27***						
Constant								
R-sq	0.78							
N	185							
p < 0.1; **p < 0.05; ***p < 0.01								

Table 16. Hedonic price regression

(The heteroskedasticity-robust Huber/White/sandwich covariance

estimator was used to calculate standard errors)

Joint significance of Age: $F_{171}^3 = 4.5$, p=0.0047. This table contains the results of the final hedonic price regression.

		Table of I	ai circus		
	Variable	Estimate	z-stat	<i>p</i> -value	
-	$\ln(BCS)$	0.177	1.62	0.105	
	$\ln(HG)$	1.742	9.47	< 0.001	
	$\ln(Age)$	0.133	3.39	0.001	

Table 17: Table of marginal effects

This table contains the marginal effects of *BCS*, *HG*, and *Age*, evaluated at sample means from the market dataset.

Table 18: Means test by herd and time period for treatment and control herds

Time period	Name	Control	Treatment	t-stat	df	<i>p</i> -value
1	% change in BCS	-5.4	0.8	-3.4	140	0.0010
1	% change <i>HG</i>	0	3.3	-3.4	180	0.0009
1	% change in \hat{P}_i^*	-0.9	5.0	-3.2	215	0.0015
2	% change in BCS	11.9	4.2	4.2	196	< 0.0001
2	% change in HG	3.4	6.5	-4.0	219	0.0001
2	% change in \hat{P}_i^*	6.8	13.4	-3.9	221	0.0001
3	% change in BCS	-11.8	-23.8	4.3	220	< 0.0001
3	% change <i>HG</i>	-1.3	-8.6	10.2	223	< 0.0001
3	% change in \hat{P}_i^*	-6.4	-22.2	9.2	221	< 0.0001

¹Satterthwaite's approximate degrees of freedom given unequal variances.

The table contains the herd specific percentage change in mean *HG*, *BCS* and net predicted price (\hat{P}^*) that occurred in time periods 1, 2 and 3.

price (\hat{P}^*) that occurred in time periods 1, 2 and 3 are recorded for each herd in Table 18. Below we present the results including only cattle that made it through to the end of the study.

Time period 1: In this period, during which the treatment herd remained on the pastures of the wildebeest calving grounds whilst the control herds were moved approximately 50 kilometres to substitute grazing areas, the change in the control herds' mean *BCS* (- 5.4%) was significantly different (p = 0.001, t = -3.37, d.f = 140) from that of the treatment herd (+ 0.8%). The mean *HG* for the control herds remained constant, in comparison to the treatment herd which increased by 3.3% (p < 0.001, t = -3.39, d.f = 180). The combined effect of changes in *BCS* and *HG* are reflected in the differences in the change in mean predicted price, \hat{P}^* , which, in time period 1, increased by 5% for the treatment herd compared to a 0.9% decrease for the control herds (p = 0.0015, t = -3.22, d.f = 215). (These sample means and the *t*-tests for $\overline{\hat{P}_t^*}$ do not account for the variance in the predictions themselves, so while the *t*-tests test for differences in average predicted prices, they do not account for the sampling variation in these individual predicted prices themselves.)

Time period 2: In this period, during which the control herds were grazed entirely on substitute pastures, the treatment herd's mean *BCS* increased by 4.2% whilst the control

herds' mean *BCS* increased significantly more, by 11.9% (p < 0.0001, t = 4.24, df = 196). In the same interval, the control herds' mean *HG* increased by 3.4% whilst the treatment herd's mean *HG* increased significantly more, by 6.5% (p = 0.0001, t = -3.98, df = 219). The mean price of cattle for both herds increased in this period, however the price increase experienced by the treatment herd (13.5%) was more than twice that of the control herds (6.8%) (p = 0.0001, t = -3.86, d.f = 221).

Time period 3: In this dry season period, during which the control herds had returned from the distant pastures to graze alongside the treatment herd, the mean *BCS* of the control and treatment herd decreased by 11.8% and 23.8% respectively (p < 0.0001, t = 4.28, df = 220), whilst the *HG* decreased by 1.3% and 8.6% respectively (p < 0.0001, t = 10.23, df = 223). The mean price decreased for both herds, however the mean price of a cow in the treatment herd decreased much more, decreasing by 22.2% compared to the 6.4% drop in the control herds (p < 0.0001, t = 9.2, d.f = 221).

Occurrence of disease

During the entire study period 3% of cattle from the control herds were reported sick, compared to over 90% of the treatment herd. Focussing only on the treatment herd, in time period 1 the mean daily percentage of the herd reported sick was 4.5%. By the end of time period 2, however, a respiratory infection had entered the herd and the daily mean percentage of the herd reported sick had increased to 7%, and in time period 3 (until the end of the vaccination trial in early July) the mean rose again to 24% (Figure 34). The cause of the respiratory infection remains unknown, however analysis indicates that it was not MCF. This clinical data is relevant to the discussion that follows regarding the changes in body condition score and predicted price.

Discussion

This study, which builds on and supplements existing qualitative findings (Bedelian, Nkedianye, and Herrero, 2007; Cleaveland et al., 2001), provides the first quantitative assessment of the annual costs that Maasai pastoralists incur as a result of the implementation of their traditional strategy to avoid the fatal livestock disease, wildebeest-associated malignant catarrhal fever. The economic costs assessed in this study can be categorized as (1) travel and other management costs and (2) livestock-productivity losses, which we discuss in turn.

In order to avoid contact with wildebeest and transmission of MCF most Maasai house-



Figure 34: Proprtion of treatment herd cattle recorded sick

A bar chart of the proportion of the treatment herd cattle recorded sick during the study period. Data collection points were either 2 or 3 days apart and this accounts for the variation of the spacing of the bars. The plot is annotated with lines indicating the position of time periods 1 - 3.

holds moved their cattle away from the wildebeest calving ground during the infectious period, February to May. Because the distances moved were frequently > 20 km, most of the daily milk yield produced by cattle that had moved away was not returned to the boma. As a consequence of this, not all cattle within a herd tended to be moved, with most owners choosing to keep a small number of cattle back at the boma. Approximately three quarters of the cattle that remained at the boma were lactating, reflecting the important role that milk plays both as a component of the diet and of the overall household income. As a consequence some of the most valuable animals faced the greatest risk of disease. However, in order to reduce the likelihood of these valuable animals becoming sick with MCF, only a small number were kept at the boma by the family during the MCF season; few enough to allow grazing on the limited pastures immediately proximate to the boma where the risk of exposure to wildebeest was lower.

Despite this preference for keeping lactating cattle back at the boma nearly two-thirds of all lactating cattle were moved during MCF season. As a result most of the daily milk yield was not available to be used by the majority of the family unit that remained at the boma. In traditional Maasai households, many of which survive on an energy intake of approximately 70% of the international recommended threshold (FAO/WHO, 1973; Homewood, 1992), most of the protein intake, and between between one third and a half of the energy intake, is derived from milk (Homewood, 1992). This reliance on milk has become more

precarious recently with an expanding human population, a declining human / livestock ratio and increased sedenterization all decreasing the animal milk supply per person (Homewood, Chenevix Trench, and Kristjanson, 2009; Fratkin, Abella Roth, and Nathan, 1999). Consequently the proportion of milk not returned to the boma during the three month MCF season is likely to represent a considerable seasonal nutritional loss to some households. Importantly, and because herders are the young men, this loss will predominantly affect the most economically vulnerable members of the household that remain at the boma, the women, the very young, and the elderly. Furthermore, the period that the cattle are moved to avoid MCF is the time of year when rainfall is at its peak and the fresh pasture at its most nutritious (Voeten, 1999) and, as a consequence, milk supply usually at its highest (Nicholson, 1984). Therefore being unable to return 64% of the milk to the boma at this time of year will compromise a family's capacity to convert the seasonal glut of milk into a form of preserved dairy product, such as cheese, or to sell it.

Conversely, while the household members at the boma have less than they would have otherwise, the calves and herders receive a relative glut of milk to consume. Milk deflected to human consumption, though benefitting the family in the short-term, will depress the survival and the rate of maturation of young animals and therefore impair herd replacement and growth in the long-term (Western and Finch, 1986). Rather than being lost, therefore, the proportion of this glut of milk which is not returned to the family but is drunk by the calves themselves is literally being fed in to the household's most secure long term investment. Such a scenario makes economic sense for better-off, calorifically sufficient, households with a relatively large number of livestock of which only 35-45% are typically milked. However, for poorer households that have fewer livestock and reduced consumption options and that rely disproportionately on their livestock for milk (typically milking 65-75% of their herds) (Lybbert et al., 2004), investing the glut of milk in the health of their calves may be a constraint that imposes substantial costs in terms of current milk consumption for the family.

Milk has also been shown to play a significant role in the income stream of the average Maasai household (Bedelian, Nkedianye, and Herrero, 2007; Homewood, Chenevix Trench, and Kristjanson, 2009). Using milk sales data from the Kitengela Maasai population, we have calculated that the loss of household milk during the calving season represents an upper bound of about 8% of the household's annual income. Income elasticities for milk have been estimated to range around one for sub-Saharan Africa (Jahnke, 1982) and rural northern Tanzania in particular (Mdoe and Wiggins, 1997). This implies that, for the upper bound milk production assumption, both milk consumption in the household as well as consumption

of all other goods (as an aggregate category) would each respectively decline by a similar percentage when the household are constrained from selling milk by it being produced far away from the boma and markets (Jehle and Reny, 2011).⁶

The use of milk sales data from Kitengela, as an estimate of the proportion of household income generated by the sale of milk in Simanjiro, warrants some discussion: it is possible that the Maasai population of Kitengela, through its proximity to Nairobi, has more income generating options than the Maasai population in Simanjiro. Consequently the proportion of a household's total income that is generated by milk sales might be lower in Kitengela than Simanjiro. This would imply that our estimates, of the proportion of income lost due reduced opportunities to sell milk, are conservative. Conversely, given that the rural Simanjiro population supplies the town of Arusha compared to the peri-urban Kitengela population that supplies the demands of the larger city of Nairobi, it is possible that the proportion of income derived from the sales of milk is lower in Simanjiro. Further research is required to shed light on this question. Because of this uncertainty we have taken a conservative approach and have assumed that the 8% figure represents an upper bound on milk value loss. Given that, although the milk produced away from the boma is not sold, none is discarded either (as it is consumed by the herders or the calves), this approach seems sensible.

In summary, the primary impact of MCF avoidance on milk usage is through household distribution effects with the distance between the majority of milk production, the boma and point of potential sale representing an economic constraint. This constraint induces a reallocation of milk relative to how households, particularly the most impoverished, would choose to use the milk given the "unconstrained" case with zero MCF risk (and therefore no avoidance).

MCF avoidance induces further production losses through impacts on cattle body condition. We estimated these losses as reductions in market value, because market value represents the net value of the income stream from the cattle asset over its lifetime, and also represents the opportunity cost of holding (keeping) an animal rather than selling it.

In time period 1, the treatment herd, which grazed on the wildebeest calving ground, gained condition (both *BCS* and *HG*) whereas the control herds, moved to substitute grazing areas, lost condition (Table 18, row 1). As a result the change in predicted value, directly attributable to these condition differences, was 6% more in the treatment than the control

⁶The inference that consumption of all other goods as an aggregate category falls by about 8 percent in this case follows from the Engel aggregation property of demand (Jehle and Reny, 2011). With only two categories of goods, given that the income elasticity of milk is one, the income elasticity of the other category of goods must also equal 1, which means that an 8 percent decrease in income leads to an 8 percent decrease in consumption of both milk, and all other goods as a group.

herds. The loss of body condition of the control herds was likely due to both the energy requirements of movement (with herds moved on average 23 km and maximally 88 km away from the wildebeest calving grounds), as well as the lower quality of pasture in the substitute areas where they grazed. While pasture quality was not measured directly, most of the Maasai in this study considered that wildebeest calving grounds comprise the higher quality pasture. This is consistent with known drivers of wildebeest movement, with wildebeest in the Serengeti ecosystem known to move to high-quality pastures that are rich in nutrients and minerals during calving and lactation periods (Ben-shahar, Coe, and Url, 2014; Holdo, Holt, and Fryxell, 2009; Morrison and Bolger, 2012). Should an effective vaccination allow all the local cattle herds to remain on the wildebeest calving grounds then grazing competition might arise and the associated benefit of remaining on the pasture could decrease. An ecological impact assessment carried out in the neighbouring Ngorongoro Conservation Area, however, predicted that forage availability was not a major factor in limiting cattle numbers (Boone et al., 2002). Although this study was carried out some time ago, and livestock numbers may have changed since then, it suggests that future pasture competition, following effective vaccination, may be relatively minor.

In time period 2, the *BCS* of the control herds rebounded, increasing by three times more than that of the treatment herd. Conversely, the treatment herd HG increased by almost twice as much as that of the control herds. Considering the different grazing strategies experienced by the two herds during this period, and the reported superior quality of pasture on the wildebeest calving ground, we expected that the changes in both BCS and HG would be greater for the treatment herd, but this was not the case. We hypothesize that this pattern of outcomes could be due to three factors: (a) compensatory growth (accelerated growth following a period of slowed development, particularly as a result of nutrient deprivation (Leonard, Lawrence, and Fowler, 2002)), (b) unforeseen supplemental feeding by the owners of the control herds which was reported after the end of the trial, and (c) the outbreak of a non-MCF respiratory infection that affected most of the treatment herd in time periods 2 and 3 (Figure 34). During the same time period little or no illness was reported in the control herds. Thus the combination of supplemental feeding of the control herds and illness in the treatment herd, together with the physiological phenomenon of compensatory growth, may explain the result that BCS increased more in the control herds during this period. Despite the impact of this respiratory infection, the estimated change in value (% change in \hat{P}_i^*) of the treatment herd attributable to condition changes during this period increased by twice as much as that of the control herds, reflecting the fact that HG affects price by one order of magnitude more than BCS (Table 17).

During time period 3, which was also the dry season when livestock are expected to lose condition, the *BCS* and *HG* in both herds decreased, although the treatment herd lost more. This was likely the result of the high incidence of respiratory disease in this herd. As a consequence of these losses the portion of price attributable to condition decreased for both herds during the final time period although the loss in value experienced by the treatment herd was considerably higher than the control herds.

Given the disproportional incidence of the respiratory infection, it is difficult to estimate the full impact that traditional MCF avoidance strategies have on cattle value through changes in condition parameters. However, if we exclude time period 3, during which the sickness was most prevalent, and consider only periods 1 and 2, the increase in conditionassociated value of the treatment herd (18.4%) was three times greater than that of the control herds (5.9%). We attribute this differential to the contrasting MCF disease avoidance strategies employed by the two herds. Given the dual impacts of the supplemental feeding of the control herds and the respiratory infection, which started to impact the treatment herd approximately three weeks before the end of time period 2, and that the calculation does not include any condition losses associated with the control herds returning to the wildebeest calving ground in May, we consider this differential conservative.

It has been reported that moving cattle to avoid MCF forces herds to graze in wooded areas, increasing exposure to insect vectors and associated diseases (Bedelian, Nkedianye, and Herrero, 2007; Cleaveland et al., 2001). This assertion is supported by some of the cattle owners in our sample who anticipate that, in the absence of MCF, they would spend less on medical treatment for trypanosomiasis and who predict that the frequency of dipping cattle would decrease. However in this study it was the treatment herd, grazing alongside wildebeest, that suffered more disease specifically because of the non-MCF respiratory infection that affected the herd. Without knowing the aetiology of the respiratory infection it is difficult to draw conclusions about its source however it is possible that the increased contact between the treatment herd and wildebeest was a factor. For example, wildebeest could transmit other respiratory pathogens that infect cattle and impact health. If this is the case then the increased risk of non-MCF respiratory infections affecting MCF vaccinated herds needs to be included in future economic assessments of MCF vaccination. However, given that cattle frequently graze alongside wildebeest at other times of year and reports of wildebeest transmitting non-MCF respiratory diseases to cattle could not be found, this seems unlikely.

Examination of the changes across the time points in Table 17 shows how fleeting *BCS* is, and it is therefore not surprising that differences in *BCS* have a smaller price effect than *HG*. If an animal is purchased thin or fat *BCS* can be altered by feeding or poor environment relatively rapidly. Whereas *HG*, which although affected in part by fattening, short-run nutrition and health fluctuations, primarily represents skeletal size and therefore productive capacity of an animal (Wanderstock and Salisbury, 1946). *HG* is therefore less fleeting, and this difference is reflected in the market price of the animal.

Finally, the labour and travel associated costs of MCF avoidance are also not insignificant. Most households send their cattle away to find substitute pastures to avoid the wildebeest and this requires several family members to spend on average three months away from home. Although the social costs of this reallocation of resources were not specifically characterised, the impact, on the family and the young men themselves, of being away from the household for approximately a quarter of the year could be considerable. Moreover there are additional costs associated with both the setting up and running of a second boma (that is needed to house the herd and the travelling family members), and the intermittent travel that must be undertaken by foot between this and the primary boma (Bedelian, Nkedianye, and Herrero, 2007). Given the average distance moved (21.3 km), these ancillary travel costs are likely to be considerable.

Conclusion

MCF presents the Maasai with an epidemiological and economic dilemma. To herd their cattle away from wildebeest calves to avoid disease, and incur costs from lost opportunities to consume and sell milk and the energy and labour input required to move the cattle, or choose to remain on the wildebeest pastures which, without an effective vaccine, also incurs costs through a higher risk of disease. Given the incidence of MCF in cattle living in wildebeest calving areas in East Africa is 5% to 10% (Plowright, 1986; Bedelian, Nkedianye, and Herrero, 2007), the increase in disease costs associated with the alternative strategy will potentially offset any gains made through increased availability of milk, improved body condition and reduced energy demands from movements. This suggests that the traditional strategy is currently the least costly option. With the development of an effective vaccine, however, the alternative strategy might become optimal. Before this can be determined the aetiology and impact of non-MCF respiratory infections would need to be understood. In particular whether there are infections associated with increased contact with wildlife and,

if there are, whether these occur regularly, as seasonal outbreaks, during the MCF season. Other areas of study include measuring the impact that a reduction in opportunities to consume milk for approximately three months might have on the health of women and young children, and quantifying the burden of vector-borne and directly transmitted diseases associated with the confinement of large numbers of cattle herds on substitute grazing areas. Finally it would be of interest to characterise the social costs associated with young adult, typicaly male, family members being required to herd cattle for several months of each year away from the permanent household.

XIV Chapter Six Conclusion

Employing a field-based natural exposure trial and a direct viral challenge trial, this study (Chapters Two and Four) investigated the efficacy of a novel vaccine strategy against, and aspects of breed specific resistance to, wildebeest-associated malignant catarrhal fever (WA-MCF). These trials showed that, when used in shorthorn zebu cross (SZC) cattle, the vaccine provided partial protection against MCF. An unexpected result of the trials was the proportion of unvaccinated control group animals that became sick with MCF, which was less than that seen in British Friesian-Holstein (FH) cattle. This finding, which impacted efficacy calculations, suggested that SZC may have a greater resistance to WA-MCF than FH cattle. There were also questions concerning the suitability of the attenuated alcelaphine herpesvirus-1 (ALHV-1) C500 strain for vaccine development. This was investigated in Chapter Three through the comparison, of genetic differences at two loci, of C500 with strains of wild-type virus circulating in East Africa. Encouragingly, our sequence analysis suggested that C500 is a suitable strain for WA-MCF vaccine development. In addition, aspects of the epidemiology of MCF have been investigated, specifically whether wildebeest placenta, long implicated by Maasai as a source of MCF (Cleaveland et al., 2001), might play a role in viral transmission. The finding, of AlHV-1 virus in approximately 50% of placentae, indicate it may. And, with virus detection decreasing between 32 and 100-day old wildebeest, we have provided evidence to support the consensus that viral transmission from wildebeest wanes considerably as calves age. Finally, through examination of the economic and health impacts that traditional MCF avoidance has on pastoralist livelihoods (Chapter Five), we have provided the first quantitative evidence of the costs that WA-MCF imposes on livestock keepers living in proximity to mixed-use buffer zone areas where wildebeest calve in East Africa.

Infectious diseases endemic to wild ungulates indigenous to sub-Saharan Africa have plagued the cattle-keeping people of Africa for millennia with five diseases (MCF, East Coast fever, trypanosomiasis, Rift Valley fever and foot and mouth disease) considered to have had a more significant impact than others (Gifford-Gonzalez, 2000). Indeed, such was the impact of these diseases that they are thought to have delayed the establishment of domestic cattle in eastern and southern Africa by one thousand years *after* that of sheep and goats, which arrived approximately 4500 BP (Gifford-Gonzalez, 2000). With a mode of transmission not

In modern times the reduced range of the wildebeest has limited the impact of WA-MCF. Nevertheless, as the economic assessment demonstrated, it continues to exert livelihood costs for owners whose cattle graze the nutritious pastures of the wildebeest calving grounds. As a consequence many livestock owners view the development of an effective vaccine as a priority (Cleaveland et al., 2001). Looking farther afield an effective vaccine against WA-MCF might provide opportunities for a vaccine to be developed against sheep-associated MCF (SA-MCF). This disease has a much wider geographical range and a more significant economic impact than the form spread by wildebeest, however efforts to control SA-MCF have been stifled by the lack of an *in-vitro* propagation system for the related causative agent OvHV-2. Consequently hope for the development of a SA-MCF vaccine could be dependant on the success of vaccines developed from the more readily propagated virus, AlHV-1.

The findings from the two vaccination trials reported in this study have moved these developments forward. Firstly the trials demonstrated that immunisation with attenuated AlHV-1 C500 is safe and well tolerated in Tanzanian shorthorn cross cattle; secondly, that when combined with EmulsigenTM as an adjuvant, the vaccine induces an oronasopharyngeal AlHV-1-specific and neutralising-antibody response, both correlates of protection; and thirdly the results of the field trial indicated that vaccination reduces infection in SZC cattle by 56%. This degree of protection from infection is less than the level of protection suggested by the UK-based experimental trials (Haig et al., 2008; Russell et al., 2012) and is probably insufficient to allow cattle owners to risk a wholesale change to their traditional MCF avoidance strategies. However, this partial protection could still be of value to herd owners who are not able to move all of their cattle away. For example, whilst most of the herd is traditionally moved to avoid the oncoming wildebeest, the results of the economic impact assessment in Chapter Five showed that productive animals are frequently kept back at the permanent household to provide milk to women and children throughout the three month MCF risk period (Lankester et al., 2015b). Additionally, land-use changes are increasingly making traditional disease avoidance difficult (Reid, 2012; Cook, 2015) resulting in entire herds being unable to move away from wildebeest and their newly born calves. In circumstances such as these a partially protective vaccine could be of considerable value in augmenting disease avoidance strategies. Furthermore, the availability of a partially protective vaccine that would help to protect cattle that cannot move, but would not trigger large-scale

land-use changes, would alleviate concerns of conservationists that a MCF vaccine may have harmful environmental consequences (over-grazing in areas grazed by wildebeest, increasing competition and conflict between wildlife and livestock, and demands for more permanent infrastructure in critical wildlife dispersal areas). Finally, given the challenges faced in the development of effective vaccines targeting herpesvirus infections (we are not aware of any herpesvirus vaccine that prevents infection), the level of protection demonstrated in these trials is encouraging for future WA- and SA-MCF vaccine development programs.

It is worth considering what new questions have emanated from the study and what aspects of the study, if done differently, would have improved outcomes:

The calculation of the sample size for the field trial was based on reports of the incidence of MCF under traditional disease avoidance conditions being between 5% and 20% (Plowright, 1986; Bedelian, Nkedianye, and Herrero, 2007), a case-fatality ratio of at least 95% (Plowright, 1968), and our prediction that, with intentional grazing of cattle close to wildebeest calves, the incidence would increase to approximately 30%. Repeating the trial in 2012 meant the power was effectively doubled, which we felt was sufficient to determine the efficacy of the vaccine at protecting cattle from fatal MCF. The finding that, respectively, 45% and 20% of the unvaccinated and vaccinated cattle showed evidence of infection suggests that, had the predicted case-fatality ratio been realised, the sample size would have been sufficient to fulfill this objective. However, with very few cattle in the field trial dying, we were only able to draw conclusions regarding the vaccine's efficacy at preventing infection.

The case-fatality ratio warrants discussion: Given that 45% of unvaccinated cattle were infected yet only 2% died, suggests that the case-fatality ratio (95%) used to estimate the sample size is likely to be an overestimation. To calculate a case-fatality ratio the total number of deaths from a disease is divided by the total number of cases (Garske et al., 2009). Calculating the numerator is relatively straightforward, however ascertaining an accurate denominator is rather less easy: if asymptomatic or clinically mild cases occur it is likely that the number of cases will be under-estimated and, as has been reported for other disease outbreaks such as the novel influenza A/H1N1 pandemic in 2009 (Garske et al., 2009), the case-fatality ratio for WA-MCF were based, in the absence of capability to estimate subclinical infection rates, on observed progression of clinical MCF only. This will have resulted in the overestimation of the severity of the disease seen in the literature to date. Although questions were raised regarding the potential confounding effect that the East Coast fever vaccination may have had, the case fatality ratio that resulted from the field trial was only

4.4%.

The sample size of the direct viral challenge trial was a limiting factor. We based the sample sizes of the groups for the study on the two previous UK-based trials (Haig et al., 2008; Russell et al., 2012), which both showed a significant difference between the survival of the vaccinated and unvaccinated control groups. In the Emulsigen[™] only control group of the reported direct viral challenge trial (Chapter 4) the incidence of AlHV-1 infection and fatal MCF was, however, much lower than expected and this compromised the power of the subsequent group comparison analyses. Whether this low fatality rate was due to resistance in SZC cattle or the confounding effects of the ECF vaccine is uncertain. Investment in larger group sizes might have helped to provide the necessary power to overcome these effects.

In both the field and direct viral challenge trial, therefore, the number of MCF fatalities seen in the control groups was much less than expected. We have speculated that SZC cattle may have a resistance to MCF that afforded them a greater degree of protection from infection and developing fatal disease than was seen in British FH cattle (Haig et al., 2008; Russell et al., 2012). We have also speculated that acquired resistance from prior exposure might impact disease outcomes. It would be of interest to investigate these hypotheses further. Briefly, this would require a series of exposure trials to determine how infection and survival differs between unvaccinated and vaccinated B. indicus and B. taurus cattle. Additionally comparison of outcomes in groups previously unexposed to natural MCF with cattle that have evidence of previous exposure (sero-positivity and / or detectable AlHV-1 DNA) would be of interest. In addition, through the incorporation of comparison groups, investigating whether the ECF vaccine does provide a degree of protection against MCF would be of value. Given the prevalence of ECF in the region (Homewood and Lewis, 1987; Di Giulio et al., 2003) cattle taking part in any future trial would still require protection from this fatal disease, and this could be achieved without vaccination through regular applications of topical ecto-parasiticides. Despite these considerations, the trial does provide valuable information about the efficacy of the vaccine under prevailing systems of pastoral livestock management in East Africa, where ECF vaccination is widely implemented and a proportion of cattle likely to have experienced previous natural exposure to AlHV-1.

The dose of virus administered to the SZC cattle in the challenge trial is also worth consideration. If local cattle do have a degree of resistance to MCF, it could be argued that the dose administered in the direct viral challenge trial was insufficient and that a sensitivity analysis, to determine a minimal lethal dose, should have been carried out in advance of the study. However, as the dose of virus administered represented fifty times the LD₅₀ dose (as

determined on British FH cattle), we were confident a sensitivity analysis was not necessary and that the dose would be lethal to SZC cattle. That six out of the eight SZC cattle in control group 5 died of fatal MCF, suggests the dose used was sufficient.

The duration of immunity provided by the vaccine has been investigated experimentally in British FH cattle and is estimated to be six months (Russell et al., 2012). It would also be of interest to investigate the duration of immunity under natural conditions and this could be achieved in future research by keeping vaccinated cattle for an extended period and challenging them through close grazing with wildebeest calves seasonally over a number of years.

Broader insights of animal vaccination field trials

Comparison of the results of the direct viral challenge trials carried out in Tanzania (Chapter Four) and the UK (Haig et al., 2008; Russell et al., 2012) with the field trial (Chapter Two) raises some interesting issues regarding the value of employing field trials to assess veterinary vaccines. Whether for human or veterinary use, vaccines are typically evaluated during development to determine safety, immunogenicity and efficacy, with a variety of trials (challenge studies, randomized controlled trials, observational studies, serological evaluation etc.) employed. The reliance on which trial is used typically varies depending on whether the vaccine is for veterinary or human use. For example, like the three direct viral challenge trials, the evaluation of veterinary vaccines relies heavily on *in-vivo* challenge studies, which require a relatively small number of animal subjects to be exposed to high doses of pathogens. In contrast, evaluation of human vaccines in which subjects are experimentally exposed to pathogens are, for ethical reasons, rarely acceptable. Conversely field trials, which are routinely used to investigate 'vaccine effectiveness' in human medicine, are less commonly used to evaluate the efficacy of veterinary vaccines. One reason for this is that, when judged against the highly controlled conditions of the direct challenge trial, developers of veterinary vaccines consider the variable conditions of the field trial as inferior. Furthermore field trials are costly and, when used for assessing veterinary vaccines, their scale tends to be limited (Knight-Jones et al., 2014). As a consequence the function of the veterinary field trial is usually limited to the assessment of safety (O.I.E., 2012).

The results of the field trial in this study, and the variation in efficacy as compared to the UK based trials, challenge the assumption of inferiority and underscore how important it is that a wide range of factors, which will vary beyond the controlled conditions of a direct challenge trial, are considered. For example vaccine factors (such as sensitivity to fluctuations in cold-chain conditions), host factors (such as genetics and immuno-responsiveness to the vaccine), pathogen factors (such divergence between the wild-type pathogen and that used for vaccine development) and environmental factors (such as seasonal impacts on nutritional stress) are all likely to vary and warrant consideration (Knight-Jones et al., 2014). A number of these factors have been considered within this study: for example, we have examined the level of divergence between the AlHV-1 strains of virus used in vaccine development and those naturally circulating in wildebeest in Tanzania and we have considered whether concurrent ECF vaccination may confound efficacy. Other factors, such as the ease with which the vaccine can be inoculated by local operators and its sensitivity to fluctuations in the cold-chain, also require investigation before the vaccine's effectiveness under true field conditions can be quantified.

Rangeland management: Livestock and ecosystem health

Brief mention has been made of the impact that an effective vaccine against WA-MCF might have on mixed-use 'buffer zones' areas and wildlife conservation. These issues require a broader discussion. Within this discussion the terms 'pastoralism' and 'rangeland' are used. As defined in Chapter One the term 'pastoralism' is used generically to refer to people who, to a greater or lesser degree, use mobility as a means for keeping livestock. Whilst 'rangeland', which is also defined in Chapter One and shown in Figure 3, refers to the arid and semi-arid lands that cover two-thirds of the land mass south of the Sahara, are characterized by seasonally available plant-associated water and nutrients (Homewood, 2004), are treated as common property resources by pastoralists and, historically at least, have sheltered a great diversity of free-ranging wildlife species (Bourn and Blench, 1999; Wambwa, 2005).

WA-MCF has been plaguing the cattle keeping people of Africa for millennia with those living near wildebeest calving areas having little choice but to move their herds away from the pastures on which the wildebeest calve. As a result, during the months when wildebeest suckle, they experience little grazing competition from cattle for the highly nutritious mineral rich pasture of their calving areas. For this reason some conservationists have tended to view WA-MCF as a force for good and the development of a vaccine as a reason for concern.

The relationship between wildlife and livestock is, however, complex, co-dependant and long-standing (Reid, 2012), and arguing that one needs to be kept away from the other risks over simplifying the issue. For example, wildlife populations need to range widely, and often move outside of protected areas and into buffer zones to find forage and water. When these buffer zones areas are dominated by conservation-compatible land uses like herding

and small-scale farming, mobility is not inhibited and the biodiversity of the whole landscape benefits (Reid, 2012; Homewood, 2004). However, where buffer zones have become fragmented by large-scale land conversion for cultivation, the impact on wildlife can be devastating (Homewood, 2004). For example, in Kenya significant wildlife declines were recorded following the privatization and conversion to commercial monoculture of formerly communal rangeland around the Maasai Mara National Reserve (Homewood et al., 2001; Fairhead, Leach, and Scoones, 2012; Benjaminsen and Bryceson, 2012). A similar pattern is occurring around Tanzania's Tarangire National Park, where permanent subsistence and large-scale farming is increasingly isolating the park and leading to declines of large mammal species (Borner, 1985; T.C.P, 1998). Consequently conservation goals are not only dependent on the integrity of state-controlled protected areas but also on the viability of mixed-use buffer zone areas, and on local people's tolerance of wildlife on those lands (Sachedina, 2010; Hulme and Murphree, 2001). Given that these rangelands have been managed for millennia by pastoralists using a wildlife-compatible system, that enables access to pasture and water across a wide area, one might argue that, rather than trying to exclude cattle from particular pastures, greater conservation value could be achieved through the development of progressive policies that preserve traditional mobile livestock keeping systems.

These traditional systems, however, are rapidly changing as, in the past 50 years, pastoralists in East Africa have become reliant on agricultural products and are increasingly participating in farming. This has been caused, in part, by growing preference for agricultural foods as well as the decline of livestock numbers relative to humans (McCabe, Leslie, and DeLuca, 2010), but also reflects state biases on agricultural development (Looloitai, 2014). For example, Tanzania's 'Kilimo Kwanza' ('Farming First') policy encourages agricultural production over pastoralism, leading to land disputes and resettlement to facilitate large scale agriculture production (Mahonge, Sangeda, and Mtengeti, 2014). Reduction in the livestock per capita ratio, declining milk yields in the dry season, and overall increase in food insecurity following drought or outbreaks of livestock disease have also contributed to this change (McCabe, Leslie, and DeLuca, 2010). Cultivation is also viewed by pastoralists as a good way to stake a prior claim against threats of land being expropriated for conservation (Fairhead, Leach, and Scoones, 2012; Benjaminsen and Bryceson, 2012) or conversion to industrial agriculture (Homewood, Kristjanson, and Chenevix Trench, 2009).

Despite growing pressures to cultivate, in many areas of East Africa rangeland aridity and marginal soil fertility limit the potential for cultivated food production. Additionally, cultivation of rangelands removes dry season pastureland, which is vital for wild and domestic herbivores. Furthermore, some predict that climate change will make East African rangelands warmer, increase rainfall unpredictability, reduce plant available moisture, and increase the frequency of extreme climatic events (Stige et al., 2006). These changes may favour mobile livestock keeping in non-fragmented rangelands, rather than cultivation which requires heavy inputs and leads to more fragmentation (Galvin, 2008; Hobbs et al., 2008).

The conversion of rangelands to agricultural plots also reduces the options for livestock to avoid infectious diseases (Reid, 2012). For example, with mobility increasingly restricted, traditional strategies like grazing cattle away from wildebeest during calving season to avoid MCF are difficult to practice (Reid, 2012). This has resulted in significant losses, for example in cattle herds living near Lake Naivasha, Kenya (Lyons, N. unpublished data, 2015). Additionally, as grazing options become increasingly limited, herds are forced to share pastures which increases the risk of transmissible and vector borne diseases (Reid, 2012; Cleaveland et al., 2001).

A precarious future, therefore, awaits pastoralism and wildlife. The co-dependancy of both suggests that there is a need to develop new policies for rangeland management that support both wildlife populations and traditionally managed livestock herds. While many perceive the conservation of wildlife as having an intrinsic value for humanity, it is also undoubtedly important for economic development. In East Africa wildlife tourism is estimated to contribute 7-18% of GDP (United Republic of Tanzania, 2015; Korir, Muchiri, and Kamwea, 2013). Similarly, pastoralism contributes significantly throughout East Africa to national GDPs, potentially equivalent to that of agriculture (Behnke and Muthami, 2011). Further, if human rights, cultural identities and marginalized communities are to be upheld and supported, the protection of pastoralist livelihoods is critical (Benjaminsen and Bryceson, 2012; Behnke and Muthami, 2011; Brockington, Igoe, and Schmidt-Soltau, 2006). Consequently there are both economic and culturally compelling reasons to ensure that the management of pastoral lands, particularly those surrounding protected areas, remains socially equitable and environmentally sustainable. To achieve this, progressive pastoral policies are required that respect local knowledge, land-use, and livestock management practices and that balance the objectives of national conservation agendas with the development requirements of pastoral communities. If interventions are to succeed, so that buffer zone areas pay both biodiversity and livelihood dividends, they need to be built upon an understanding of the dynamics of the target systems on which they will be imposed. Given that ecological perturbations such as droughts are likely to occur with an irregular frequency, they will also need to retain an extensive scale.

Within any progressive pastoral policy, in addition to recognising and supporting mobility as a key strategy, the improvement of the efficiency of livestock production systems needs to be described. An example is the provision of improved veterinary care to control infectious diseases that impose significant costs on pastoralist livestock owners increasingly unable to exploit traditional avoidance strategies (Mizutani et al., 2005; Homewood and Rogers, 1991). If successful, such interventions could result in considerable economic benefits for the pastoral sector from improved marketing for local consumption of livestock meat and products. An example is the implementation of the 'infect and treat method' to protect cattle from East Coast fever, shown to be popular locally, to result in reduced calf deaths and increased milk production and significant economic benefits to pastoral communities (Marsh and Yoder, 2013). An effective vaccine against WA-MCF will likely lead to similar benefits.

Conclusion

MCF has been impacting cattle owning people in sub-Saharan Africa for millennia and continues to exert a significant economic impact on those herd owners whose cattle still share productive pastures with calving wildebeest. The novel attenuated AIHV-1 vaccine assessed in this study does confer a partial protection to SZC cattle, however this protection is likely to be insufficient for livestock owners to risk changing their traditional MCF avoidance strategies to graze cattle in productive lands alongside wildebeest during the calving season. Nonetheless, even a partially protective vaccine will be of value to owners who have cattle that they are unable to move and that risk suffering inadvertent exposure. Going forward, a community-wide cluster randomized trial would be the logical next step. This would allow a thorough assessment of the vaccine's effectiveness under true field conditions and to determine how useful it could be to people, many of whom still play a vital role in preserving the integrity of buffer zones that surround protected areas, yet whose cattle still remain exposed to this ancient, and fascinating, disease.

XV Bibliograpghy

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XVI Appendix One

Herd management questionnaire

- PERIOD IN QUESTION:
- HERD OWNER NAME:
- HERD LOCATION:
- No. OF CATTLE:

SECTION A: (Ask herd owner for broad description of what they did with the herd during this specific period. Be sure to cover issues such as if they moved the herd, why they moved, where they have come from and gone to and how far they travelled)

SECTION B: MCF SPECIFIC QUESTIONS: (to be asked for Jan - April period only; in all other periods go straight to Section C)

- 1. Did you move your herd this season to avoid MCF?
- 2. Did you move the entire herd?
- 3. If the answer to Q2. is NO, then how many cattle did you move and how many stayed behind?
- 4. Of the cattle that stayed behind, give reasons for each cow being left behind:
- 5. How many lactating cattle remained at the boma during the wildebeest calving season?
- 6. What was the typical milk production (not consumed by the calves) of these cattle that remained at the boma?
- 7. How many lactating cattle were moved away from the boma during the wildebeest calving season?
- 8. What was the typical milk production (not consumed by the calves) of these lactating cattle that moved away from the boma?
- 9. What happened to the milk produced by the cattle that moved away from the boma?
 - litres / day are CONSUMED BY THE HERDERS
 - litres / day are CONSUMED BY THE FAMILY AT THE BOMA

- litres / day are DISCARDED
- litres / day are SOLD
- 10. Of the lactating cattle that moved away from the boma, how did the typical amount of milk produced each day change (stayed the same, increase or decrease) in the period that they were moved away?
- 11. If it changed, by how many litres / day?
- 12. What was the average price of milk per litre during this period? TSH/Litre
- 13. How many calves remained at the boma during the wildebeest calving season?
- 14. How many of these calves died during this period?
- 15. How many calves were moved away from the boma during the wildebeest calving season?
- 16. How many of these calves died during this period?
- 17. When (what date) did you move your cattle?
- 18. When did you return (date)?
- 19. How far did you travel to reach the new destination away from the wildebeest calving area? KM
- 20. How long does it take to move your cattle herd away (to the new destination) from the wildebeest calving area? DAYS
- 21. Why did you choose the area where you moved your cattle to?
- 22. Did you move your entire family for the duration of the wildebeest calving season?
- 23. List the number of family members that moved with the cattle (ie. How many children/wives etc).
- 24. Did you use non-family members to herd the cattle?
- 25. How many non-family herders moved the cattle?
- 26. Did you pay these non-family members?

- 27. If YES then how much did you pay in total for the entire period that the herd was away? TSh
- 28. Does the herder(s) stay with the herd until after the wildebeest calving season?
- 29. Between Jan and Apr, how far do you usually move your cattle daily for water when away from the wildebeest calving area?
 - hours
 - km
- 30. Between Jan and Apr, how far do you usually move your cattle daily for salt when away from the wildebeest calving area?
 - hours
 - km
- 31. How far do you move your cattle daily for water during the rest of the year?
 - hours
 - km
- 32. How do they rate the pasture quality in the destination area?
 - High
 - Medium
 - Low
- 33. How do they rate the pasture quality in the wildebeest calving area?
 - High
 - Medium
 - Low

If MCF was NOT a problem...

- 34. Would your management practices during the wildebeest calving season (Jan to Apr) differ compared to what you do now during the wildebeest calving season?
- 35. If the answer to Q.a. above is YES, describe how the management practices would differ:

- 36. Are cattle in their first experience of a wildebeest calving season equally likely, more likely or less likely to die of MCF as cattle that have experienced more than one wildebeest calving season?
 - Equally likely
 - More likely
 - Less likely

SECTION C: general questions (to be answered in all periods)

- 37. How many lactating cows are in the herd at the present time?
- 38. For the past 2 weeks, what is the average amount of milk produced each day by the herd that is not drunk by the calves? LITRES / DAY
- 39. Have you fed your herd concentrates in this period?
- 40. Have you wormed your cattle in this period?
- 41. If answer to Q.a is Yes, then what estimated percent of the herd were wormed?
- 42. Have you dipped your cattle in this period?
- 43. If answer to Q.a is Yes, then what estimated percent of the herd were dipped?
- 44. How often have you dipped your cattle in this period?
- 45. Vaccinations: Please detail which diseases your cattle are vaccinated for (number of cattle):
 - ECF
 - FMD
 - Black quarter
 - Lumpy skin

• Ear tag number

- CBPP
- Other:
- 46. Has your herd suffered from any sickness, deaths/losses in this period?

- Date
- If sick/lost/died, give cause:
- If disease, what were the primary clinical signs?
- If disease, what do they think the disease was?
- Was the disease diagnosed from a known test protocol?
- Which?
- Was medical treatment given?
- How much did the treatment cost?
- Did the animal die?
- 47. How many abortions have occurred during this period?
- 48. Total management costs (TSh) for livestock owned and managed by the household: (*Calculate for each of cattle, goats, sheep, poultry, other*)
 - Hired labor (outside of household)
 - Feed/fodder
 - Supplements (salts or minerals)
 - Health and Vet Services
 - Other

XVII Appendix Two

CATTLE HEALTH / CONDITION SURVEY

1. TIME POINT:

- JAN
- MARCH
- MAY
- NOVEMBER

2. EAR TAG NUMBER

- 3. Cow is:
 - IN THE HERD
 - SOLD/GIVEN AWAY
 - DEAD

If cow is still in the herd then proceed:

- 4. Wither height: cm
- 5. Heart girth: cm
- 6. If female, how many calves has she had?
- 7. For latest pregnancy was the calf:
 - aborted
 - still born
 - died after birth
 - alive
- 8. What was the date of calving?
- 9. What was the date of the previous calving?

If female: Udder conformation (potential predictor of milk production)

10. Is udder healthy?

- 11. If NO, how many quarters of the udder are diseased?
- 12. Describe any udder lesions:
- 13. If female: amount of milk produced per day in litres (not consumed by the calf)?
- 14. Are there any health concerns with the cow?
- 15. Has this cow ever been wormed?
- 16. How many times in the last 12 months has the cow been wormed?
- 17. What was the approximate date of the last worming?
- 18. Has this cow ever been dipped?
- 19. How many times in the last 12 months has the cow been dipped?
- 20. What was the approximate date of the last dipping?
- 21. Has this cow been fed concentrates in this time period?
- 22. Vaccination status/evidence [list known vaccinations]
 - ECF
 - ANTHRAX / BLKLEG
 - CBPP
 - LUMPY SKIN
- 23. Body condition index score: (1 (thin) 5 (obese))
- 24. Describe any unusual features of the cow:

XVIII Appendix Three

LIVESTOCK MARKET SURVEY

- 1. Name of livestock market
- 2. GPS location of market
- 3. Price cow was bought/sold at (Tsh)
- 4. Estimated Age:
- 5. Permanent incisors: Number of pairs/
- 6. How many pairs have fully erupted?
- 7. If a pair is erupting, which pair is it?
- 8. If there is noticeable wear, on which pairs?
- 9. Gender:
- 10. If male: Castrated?
- 11. Breed category:
 - SAHIWAL BREED (FROM KENYA)
 - BORAN
 - TZ SHORT HORN ZEBU
 - HYBRID OF THESE
- 12. Describe the colour of the cow:
- 13. Wither height: cm
- 14. Heart girth: cm
- 15. If female, how many calves has she had?
- 16. For latest pregnancy was the calf:
 - aborted
 - still born

- died after birth
- alive
- 17. What was the date of calving?
- 18. What was the date of the previous calving?

If female: Udder conformation (potential predictor of milk production)

- 19. Is udder healthy?
- 20. If NO, how many quarters of the udder are diseased?
- 21. Describe any udder lesions:
- 22. If female: amount of milk milked per day in litres (not consumed by the calf)?
- 23. Are there any health concerns with the cow?
- 24. Has this cow ever been wormed?
- 25. How many times in the last 12 months has the cow been wormed?
- 26. What was the approximate date of the last worming?
- 27. Has this cow ever been dipped?
- 28. How many times in the last 12 months has the cow been dipped?
- 29. What was the approximate date of the last dipping?
- 30. Has this cow been fed concentrates in this time period?
- 31. Vaccination status/evidence [list known vaccinations]
 - ECF
 - ANTHRAX / BLKLEG
 - CBPP
 - LUMPY SKIN
- 32. Is the seller a) livestock trader, b) owner, c) relative of owner, d) if other describe
- 33. Is the buyer a) livestock trader, b) processor, c) butcher, d) household owner, e) if other describe

- 34. How far did the livestock travel to market? Km
- 35. How did the cow travel to the market?
- 36. How far is the buyer going to transport the cow? Km
- 37. How will the cow travel from the market?
- 38. Body condition index score: (1 (thin) 5 (obese))
- 39. Describe any unusual features of the cow: