SASVEPM CONFERENCE PROGRAMME

Proceedings of a meeting held at Lord Charles Hotel,
Somerset West, Western Cape, SouthAfrica
22-24 August 2007

Edited by SASVEPM Executive Committee

The views expressed in these proceedings are not necessarily those of the Editor or of the Executive Committee of the Society

The Acknowledgements : The following bodies provided support for the conference:

Pfizer Animal Health

Onderstepoort Biological Products

Dr. Roy Bengis provided the Continuing Education Programme

ISBN:

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OPENING ADDRESS

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PAPERS

CLASSICAL SWINE FEVER VIRUS INFECTIONS IN AFRICAN WILD SUIDS

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SUMMARY

Classical swine fever (CSF), a highly infectious virus belonging to the Pestivirus genus, was re-introduced in South Africa after an absence of 96 years. In Europe, South America and Asia, where the CSF virus causes frequent outbreaks, the only known hosts are domestic pigs (*Sus scrofa domesticus*) and the wild boar (*Sus scrofa ferus*). In South Africa wild suidae such as warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus* spp.) occur and it is unclear whether these species could play a role in the epidemiology of CSF should the disease become endemic in the domestic pig populations. An initial study was performed to determine whether these animals could become infected via intranasal infection with the virus strain that caused the outbreak in South Africa and whether they can transmit disease to in-contact cohorts of the same species. The results indicated that both warthogs and bushpigs can transmit the infection but that only the latter showed clinical signs typical of CSF. Viraemia was present in both species but seemed to be of shorter duration in warthogs. Preliminary results using PCR indicated the presence of viral RNA in nasal secretions and saliva of warthogs for periods up to 42 days post infection. This is the first study to demonstrate susceptibility to CSF virus infection in wild African suidae, and their potential role in the epidemiology of the disease still needs to be ascertained.

INTRODUCTION

Classical swine fever (CSF), or hog cholera is an OIE listed disease because of its potential to spread across borders and its high pathogenicity. In addition, it may cause widespread mortality that jeopardizes international trade of pigs and their products and leads to substantial economical losses for the pig industry. It is a highly contagious viral disease that manifests itself in acute, sub acute, chronic, congenital or clinically inapparent forms and pigs infected with highly virulent CSF virus strains may shed high amounts of virus before showing clinical signs of the disease. Clinically, it is indistinguishable from African swine fever (ASF).

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The causative agent is a virus of the genus *Pestivirus* of the family *Flaviviridae* and is closely related to the two other members of the genus, viruses causing bovine viral diarrhoea (BVD) and border disease (BD) (Darbyshire 1960). Cross-infection between these viruses occurs readily and they are now grouped by their reactivity to monoclonal antibodies (Cay, Chappuis, Coulalibaly, Dinter, Edwards et al. 1989) and their nucleotide sequences at selected genomic regions (Vilcek, Herring, Herring, Nettleton, Lowings & Paton 1994).

The role of the major member of wildlife suidae, the wild boar (*Sus scrofa ferus)* as a reservoir of classical swine fever (CSF) virus is well documented (Artois Depner, Guberti, Hars, Rossi & Rutili 2002; Lipowski 2003)□FQUOTE "" indicating at least the possibility for these animals to be susceptible to CSF virus. For bushpigs, there is much less known, but susceptibility to ASF has been demonstrated (Anderson et al. 1998).

This study aimed to determine the susceptibility of wild pigs (warthogs and bushpigs) to the CSF virus that was isolated in South Africa in 2005 and to determine the clinical course of disease, virus excretion and viral loads in various tissues after infection.

MATERIALS AND METHODS:

Experimental design:

Free-living warthogs (7) and bushpigs (6) were brought to the high containment facility at the Exotic Diseases Division, Onderstepoort Veterinary Institute. Five warthogs and four bushpigs were infected intranasally with 10^4 log $TCID_{50}$ virus in two separate challenge trials. For each challenge, two domestic pigs were inoculated intranasally with the challenge virus to control for virulence. Two uninfected bushpigs were kept apart from the inoculated animals and introduced as in-contact controls after 48 hours. The two uninfected control warthogs remained with infected animals for the duration of the trial. Animals were monitored for clinical signs and daily temperatures were taken. Serum and heparin blood samples as well as nasal, salivary and faecal swabs were collected as indicated in results. The experiment was terminated by euthanasia of the surviving animals 6 weeks post infection, whether or not symptoms occurred.

Serology and antigen detection:

IDEXX Laboratories HerdCheck antibody and antigen ELISA kits were used to detect antibodies to CSF virus in sera and antigen in heparinised blood respectively.

Detection of viral RNA:

Samples were frozen at –70°C within 8 hours of sampling and held at a maximum temperature of –40°C (Cardice) during transit. The processing and PCR were carried out essentially following the method of McGoldrick et al. (1999). RNA was extracted from the swabs using the QiaGen Mini viral RNA kit and resultant RNA was used as template in the diagnostic real-time nested RT-PCR.

RESULTS

The preliminary results are presented in this paper and the full data set will be published soon. During the warthog challenge, antigen could be detected in both the domestic pigs on day 7, 10, 12, 14 and 16 when they were euthanised. Antibody was detected in Pig 1 only on day 14 (results not shown). The two domestic pigs that were used as controls during the bushpig infection were sero-negative for all 12 days sampled post infection, and both were positive for antigen in heparinised blood on days 6, 8, 10 and 12 (results not shown).

Warthog infection and transmission experiment:

Warthogs (WH) 1-5 were infected via intranasal infection while WH6 and 7 were the in-contact controls. WH1 died shortly after infection, while WH2 was euthanised at day 14 by which time it had sero-converted (Table 1). Although no antigen could be detected in the blood of this animal, both the nasal swab and saliva were positive for genomic material on PCR at days 10 and 14. Viremia could be detected with certainty using the IDEXX kit in only two of the six animals, *viz*. WH5 (days 7 and 10) and WH7 (day 17). These results will have to be confirmed by virus isolation to determine whether the ambiguous results presented by the ELISA are indicative of a true viremia (Table 1). The intranasally infected animals all sero-converted between days 7-14 while the in contact animals did so at days 17 and 21 and all animals stayed sero-positive for the duration of the experiment.

Viral genomic material could first be detected in the nasal swab of WH3 at day 7 and at day 10 both nasal swabs and saliva were positive for WH2, WH3, WH5 and WH6, the latter being an in-contact control (Table 1). Only the nasal swab was positive for WH4 at day 10 post infection. Either samples or at least the nasal swabs were positive until day 42 in all 5 remaining animals. WH3-5 demonstrated viral material more constantly in the nasal swabs than the saliva samples.

The in-contact controls were not removed for the first 48 hours due to stress factors but the results indicated that no mechanical transmission of virus had taken place since the viremia, excretion of virus and sero-conversion were delayed in comparison with the experimentally infected animals.

Table 1: Summary of the serological results and antigen detection in the blood of the warthogs using the IDEXX kits as well as the PCR results on the saliva and nasal swabs

DPI	WH 1			WH 2			WH 3			WH 4			WH 5		
	Ab	Ag	PCR	Ab	Ag	PCR	Ab	Ag	PCR	Ab	Ag	PCR	Ab	Ag	PCR
0		D		-	-	--	-	-	--	-	-	--	-	-	--
3				-	-	--	-	-	--	-	-	--	-	-	--
7				-	-	--	-	-	-+^{N}	+	-	--	-	+	-
10				-	-	+^{S}+^{N}	+	Amb	+^{S}+^{N}	+	-	-+^{N}	-	+	+^{S}+^{N}
14				+	-	+^{S}+^{N}	+	-	+^{S}+^{N}	+	-	+^{S}+^{N}	+	Amb	-+^{N}

17		D	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
21			+	-	$+^{S}+^{N}$	+	-	$-+^{N}$	+	-	$+^{S}+^{N}$
24			+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
28			+	-	$+^{S}+^{N}$	+	-	$-+^{N}$	+	-	$+^{S}+^{N}$
31			+	-	$-+^{N}$	+	-	$-+^{N}$	+	-	$+^{S}+^{N}$
35			+	-	$+^{S}+^{N}$	+	-	$-+^{N}$	+	-	$+^{S}$?
38			+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
42			+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$

DPI	WH 6*			WH7*		
	Ab	Ag	PCR	Ab	Ag	PCR
0	-	-	- -	-	-	- -
3	-	-	- -	-	-	- -
7	-	-	- -	-	-	- -
10	-	-	$+^{S}+^{N}$	-	-	- -
14	-	-	- -	-	Amb	$+^{S}$ -
17	-	-	$+^{S}+^{N}$	+	+	$+^{S}+^{N}$
21	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
24	+	-	$+^{S}+^{N}$	+	Amb	$+^{S}+^{N}$
28	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
31	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
35	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
38	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$
42	+	-	$+^{S}+^{N}$	+	-	$+^{S}+^{N}$

+ = Positive, - = Negative, $+^{S}$ = Saliva positive, $+^{N}$ = Nasal swab positive, DPI = Days post infection, Ab = Antibody, Ag = Antigen, Amb = Ambiguous, ? Not done, * = In contact control, D = deceased

Bushpig infection and transmission experiment:

Six bushpigs (BP) were available for the experiment and BP1 and BP2 were randomly allocated as the in-contact controls. The PCR results are not yet available. BP1 was euthanased at day 19 by which time it had sero-converted and BP2 at day 33. The latter demonstrated antibodies to CSF virus by day 14 (Table 2). Sero-conversion in the intranasally infected animals occurred between days 10 and 12 (BP5 and PB4 respectively) while BP3 remained sero-negative until the time of its death at day 19.

Viral antigen could be detected in the blood of the infected animals from days 6 to 14 for BP5, days 10-19 in BP3 and 10 to 14 in BP4. The ambiguous results using the test need to be confirmed by virus isolation and if proved true positive, the viremia in the latter two animals will be increased. Of the two in-contact animals, BP1, had viral antigen in the blood on days 14-19 and BP2 demonstrated antigen from days 14 to 33.

Table 2: Summary of the serological results as well as the detection of antigen in the blood of the bushpigs using the IDEXX kits.

	BP1*		BP2*		BP3		BP4		BP5		BP6	
DPI	Ab	Ag	Ab	Ag	Ab	Ag	Ab	Ag	Ab	Ag	Ab	Ag
0	-	-	-	-	-	-	-		-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-		-	-	-	+	D	
8	-	-	-	-	-		-		-	+		
10	-	-	-	-	-	+	-	+	+	+		
12	-	-	-	-	-	+	+	+	+	+		
14	-	+	+	+	-	+	+	+	+	+		
19	+	+	+	+	-	+	+	-	+	-		
22	D		+	+	D		+	-	+	-		
26			+	+			+	-	+	-		
29			?	?			+	-	+	-		
33			-	+			+	-	+	-		
36			D				+	-	+	-		
40							+	-	+	-		
44							+	-	+	-		

+ = Positive, - = Negative, $+^{S}$ = Saliva positive, $+^{N}$ = Nasal swab positive, DPI = Days post infection, Ab = Antibody, Ag = Antigen, Amb = Ambiguous, ? Not done, * = In contact control, D = deceased

CONCLUSIONS

It was possible to infect the warthogs via the intranasal route and transmit disease to in contact animals of the same species. None of the animals demonstrated any clinical disease and no gross pathological changes were observed on post mortem. The viremia was transient and could be detected in only two of the six warthogs with certainty and in one animal, it lasted for 3 days. These results need to be confirmed by virus isolation and other more sensitive techniques. Viral RNA could be detected in the nasal swabs and saliva of all animals up to the termination of the experiment at day 42, which could indicate a carrier state. Chronic infections are a feature of CSF infections in the domestic pig and may indicate that these animals could be infectious for prolonged periods of time, but this will have to be experimentally confirmed.

The bushpigs similarly became infected and transmitted disease to in contact bushpigs. In the younger animals, symptoms indicative of CSF occurred and certain gross pathological changes on post mortem could indicate infection. The PCR results are not available yet. A more pronounced viraemia occurred in these animals that lasted between 4 and 9 days and could be demonstrated in all the animals, except one (BP6) that died during the course of the experiment. All animals sero-converted by day 19, except animal BP3, one of the infected animals, that was still negative by day 19 when it died.

Sero-conversion in the experimentally infected warthogs (days 7-14) was somewhat

sooner than what was observed in the bushpigs (days 10 and 12 and one animal still negative at day 19), as well as for the in-contact animals (warthogs between days 14 and 19 and bushpigs between days 17 and 21) but the number of animals used in these experiments are too small to attribute any statistical significance to this observation.

It is regrettable that the sampling frequency between the two experiments were not similar, which was due to the poor adaptation of the warthogs to the high containment facilities. It is possible that viraemia was missed in the infected warthogs which seems from this experiment to be less pronounced and likely of lesser duration. This should be confirmed in later experiments where animals could be kept under more suitable conditions.

The final conclusions will only be available when all the results have been analysed, but it has been shown with certainty that both species included in this experiment can become infected with CSF virus and transmit disease to in contact animals of the same species. The fact that warthogs did not become ill, yet CSF viral RNA could be detected in the saliva and nasal swabs of the warthogs up to day 42 is of great concern and needs to be confirmed by virus isolation. In both the case of warthogs and bushpigs, the spread of the disease to in contact animals indicates that there is a real possibility that CSFV could become a self-sustaining infection in these African wildlife species.

ACKNOWLEDGEMENTS

This work was funded by the Directorate of Animal Health, Department of Agriculture. Our gratitude to Jan Kgosana, Elias Magwai, Peter Modiba and Christoffel Twala for their excellent assistance in caring for the animals.

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THE ROLE OF WILD HOSTS (WILD PIGS AND TICKS) IN THE EPIDEMIOLOGY OF AFRICAN SWINE FEVER IN WEST AFRICA AND MADAGASCAR

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SUMMARY

African swine fever (ASF) adversely affects pig production in Sub-Saharan Africa. In Southern and Eastern Africa, the virus is maintained in populations of wild pigs (*Phacochoerus africanus*) and soft ticks from the Genus *Ornithodoros*. The bushpig (*Potamochoerus larvatus*) has also been showed to be asymptomatically infected by the virus. However, in other parts of the African continent such as West Africa or Madagascar, the role of wild hosts (wild pigs and soft ticks) has not been demonstrated. The objectives of this study were to investigate the existence of a sylvatic cycle and its potential impact on the epidemiology of ASF in Senegal and Madagascar. Samples from Malagasy bushpigs and Senegalese warthogs have been collected from areas of interface between domestic and wild pigs where ASF had been reported and analyzed for anti-ASF antibodies (ELISA) and viral DNA (PCR). In Senegal, ticks were collected on pig farms and near wild suids resting areas, and their infection status with ASFV was determined using PCR. In addition, sera from wild suids and domestic pigs were tested for anti-tick antibodies (ELISA). The preliminary results obtained so far suggest that the epidemiologic cycle of ASF in Senegal and Madagascar does not involve the warthog-tick-domestic pig transmission described in East and South Africa.

INTRODUCTION

African swine fever (ASF) adversely affects pig production in the African continent and Madagascar. In Southern and Eastern Africa, the virus is maintained in populations of warthogs (*Phacochoerus africanus*) and soft ticks from the Genus *Ornithodoros moubata* (Thomson 1985). The bushpig (*Potamochoerus larvatus*) has also been shown to be asymptomatically infected by the virus in several African sites (Anderson, Hutchings, Mukarati & Wilkinson 1998, Luther, Majiyagbe, Shamaki, Lombin, Antiagbong, Bitrus & Owolodun 2007) and is present in large numbers in Madagascar although its role

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in the epidemiology of the disease is uncertain (Roger, Ratovonjato, Vola & Uilenber 2001). In Senegal and Madagascar, the role of wild pigs in the epidemiology of ASF has never been demonstrated (Taylor, Best & Colquhoun 2007). *Ornithodoros moubata* is present in Madagascar but not in Senegal. However, an ecologically and taxonomically similar soft tick, *O. sonrai*, is present in that country in human settlements (Vial, Diatta, Tall, Ba, Bouganali, Durand, Sokhna, Rogier, Renaud & Trape 2006), and could act as a reservoir of the virus. In addition, some populations of warthogs, although progressively decreasing, remain in some areas of pig production in Senegal. The objective of this study was to investigate the existence of a sylvatic cycle and the potential role of wild hosts for ASF virus in these two countries.

MATERIALS AND METHODS

For the Senegal study, we chose the Sine Saloum region (Central Western region from Senegal), since the disease had been reported and was recently confirmed to be highly prevalent in domestic pigs (Etter, Ndiaye, Calderon, Seck, Laleye, Duteurtre, Mankor, Akakpo, Lo, Jori, Vial & Roger 2007). We inspected 101 pig farms and 48 warthog burrows for soft ticks in four different areas of the Sine Saloum region. More than 1000 specimens of *Ornithodoros sonrai*, the local vector of tick borne relapsing fever were found mainly in human settlements.

Sampled ticks were analysed by PCR amplification of VP72 gene B646L (Basto, Portugal, Nix, Cartaxeiro, Boinas, Dixon, Leitao & Martins 2006).

In addition, 162 warthog samples (serum, filter paper and tissue samples) were collected in hunting camps and nature reserves from six different areas of Senegal and Mauritania over three years and analysed by a Blocking ELISA test (Ingenasa, Madrid, Spain).

In Madagascar, 27 sera samples and 35 blood and tissue samples were obtained from bushpigs (*P. larvatus*) in the region of Marovoay (North Western side of the island, see Figure 1) with the help of local traditional hunters. In that area, this species is abundant

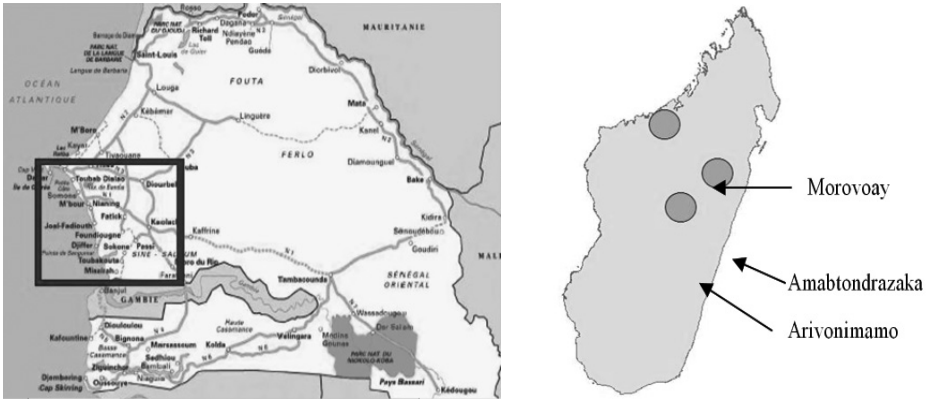

Figure 1: Study areas in Senegal and Madagascar

and ASF had recently been reported by farmers. Samples were analyzed by different serological and molecular methods (Blocking ELISA, p30Recombinant ELISA and Western Blott and PCR).

In addition, the 27 sera from bushpigs and 273 sera from domestic pigs collected in three different areas of Madagascar were tested for antibodies against *O. moubata* salival glands using an ELISA test (Canals, Oleaga, Pérez, Domínguez, Encinas & Sánchez-Vizcaíno 1990).

RESULTS

In Senegal, among the 101 inspected pig farms, *O. sonraï* was found on 44 farms. In 95% of cases, soft ticks were found inside rodent burrows situated close (25% at less than 10 m) to pig pens. In forested areas, no ticks were found within warthog burrows (n=48). However, some ticks were found in rodent burrows close to warthog resting sites and are pending to be analysed. Preliminary analysis of those tick samples found on pig farms in Senegal by PCR, allowed the detection of ASFV DNA in three of them. Among the 162 sera from warthogs, none was found positive to the Blocking ELISA test.

In Madagascar, all samples from bushpigs were found negative for antibodies (6 different methods tested) or direct detection of ASFV by PCR. The 27 bushpig sera were found negative for *O. moubata* salival gland antibodies.

Out of the 273 pig sera tested against *O. moubata* salival gland antibodies from the whole country, 51% were found positive, among which 7% were highly positive.

DISCUSSION

Although these results are only preliminary, they suggest that the epidemiologic cycle of ASF in Senegal and Madagascar does not involve the warthog-tick-domestic pig transmission otherwise described in East and South Africa (Plowright, Thomson & Neser 1994, Thomson 1985). No virus has been detected in wild pigs in any of these two countries. Nevertheless, in the case of Senegal, the ELISA test used has not been validated for warthogs and the results were not evaluated using a positive warthog reference serum, but only pig reference sera. Since pigs and warthogs are different species, their immune response to ASFV might be different or react to different antigenic proteins than those used in the ELISA test for pigs. Very few reports are found regarding the involvement of wild pigs in the epidemiology of ASF in West Africa and those are contradictory. While no proof of infection was found in warthog populations in Nigeria (Luther, Majiyagbe, Shamaki, Lombin, Antiagbong, Bitrus & Owolodun 2007, Taylor, Best & Colquhoun 2007), ASFV was recovered from a red river hog (*Potamochoerus porcus*) carcass in a protected area in the same country (Luther, Majiyagbe, Shamaki, Lombin, Antiagbong, Bitrus & Owolodun 2007) providing evidence that ASFV can circulate among West African populations of wild pigs. Therefore, further analysis of the samples collected in this study should be undertaken with more specific serological tests (PCR, Western Blott) and using warthog reference sera, in order to definitely

exclude an hypothetic role of warthogs in the epidemiology of ASFV in Senegal.

The role of *O. sonrai* in the epidemiology of ASF in Senegal has not been confirmed. The occurrence of *O. sonrai* in human settlements near pigsties, suggests that occasional contacts can occur between *O. sonrai* and domestic pigs. To the contrary, this soft tick was not present in warthog burrows in Senegal and only in some rodent burrows near warthog resting areas, suggesting that contacts between wild pigs and soft ticks, if they occur are probably very occasional. This suggests that soft ticks in Senegal have adapted to a different host than in Southern or East Africa. A large sample of 1000 ticks still has to be analysed to assess the prevalence of infection of ASFV in ticks collected in our study. Despite preliminary results suggest that ASFV could occasionally circulate in *O. sonrai,* experimental infections of that species of tick with ASFV are necessary to confirm a potential role as a host. Therefore our preliminary results suggest that compared to the importance of the domestic pig cycle, the role of wild hosts in the epidemiology of ASF in Senegal if not excluded, is probably negligible and does not play an important role in the maintenance of the disease.

To this day in Madagascar, ASFV has never been detected in buhspigs. No major mortalities have been reported since the introduction of the disease in the island, despite the fact that previous contacts between malgasy buhspig populations and ASFV were unlikely. It is known that the African bushpig can be infected with ASF virus and become viraemic without showing clinical signs (Anderson, Hutchings, Mukarati & Wilkinson 1998, Roger, Ratovonjato, Vola & Uilenber 2001). Moreover, occasional screening of bushpig samples for detection of ASFV antibodies have always failed to demonstrate a possible circulation of the virus in buhspigs and this was also the case in our survey. Generally speaking, data on prevalence of ASF virus in bushpig populations are scarce in ASF literature. It is estimated by some authors to be 10 times lower than in the case of buhspigs (Plowright, Thomson & Neser 1994, Thomson 1985). Our sample (n=35) was calculated to detect a prevalence of 8 to 10% with a 95% confidence interval, since we expected high levels of prevalence in domestic pigs and possible contacts with bushpigs were reported by local farmers. However, observed prevalence of ASFV in domestic pigs in the Morovoay area have been found much lower than expected (lower than 2%). In the case of the wild boar in Europe, it has been observed that ASF virus tends to disappear from wild pig populations in areas where prevalence of the disease in domestic pigs is low or nil. This has been observed in Sardinia where soft ticks are not present (Laddomada, Patta, Oggiano, Caccia, Ruiu, Cossu & Firinu 1994, Perez, Fernandez, Sierra, Herraez & de las Mulas 1998). Considering that the epidemiology of ASFV in the bushpig could be similar to the one of wild boar, the expected prevalence in bushpigs in the Morovay region could be negligible. Therefore the probability of being undetected with our sample size could be considerable (higher than 80%). In that case, further sampling would be necessary in order to confirm the hypothesis that the bushpig also plays a negligible role in the maintenance of the disease in Madagascar and that its contribution to the endemicity of the disease in this country might be irrelevant.

The detection of antibodies against *O. moubata* in 51% of the pig sera tested by the anti-tick ELISA test suggests that contacts between pigs and soft ticks might be more frequent than expected (Roger, Ratovonjato, Vola & Uilenber 2001). This test has been

used in Spain for the identification of farms infested with *O. erraticus* and validated with field data, suggesting a specificity of 95%(Canals, Oleaga, Pérez, Domínguez, Encinas & Sánchez-Vizcaíno 1990). However, this test has never been validated for *O. moubata* and its specificity and sensitivity are so far ignored. Further studies are needed to confirm the presence of soft ticks on farms where anti-tick antibodies were detected in pig sera. In addition, the presence of ASFV in *O. moubata* ticks in Madagascar is currently being analysed.

In Madagascar, no contacts between soft ticks and bushpigs are likely to occur since bushpigs do not live in burrows. In that sense, bushpig ecology regarding soft ticks is probably similar to the one of wild boars. This is also suggested by the fact that all our samples of bushpig were negative for detection of anti-Ornithodoros antibodies, while half of pig sera showed some level of reaction. Portuguese researchers failed to demonstrate any possible contacts between wild boars and local *O. erraticus* soft ticks in the Iberian Peninsula (Louza, Boinas, Caiado & Vogario 1989). Therefore, we assume that the role of *O. moubata* in the maintenance of the diseases is probably circumscribed to domestic pigs.

These results, although preliminary, suggests that the epidemiological cycles of ASFV in West Africa and Madagascar are different from those described in other regions of Africa. In West Africa, soft ticks might play an occasional role in maintaining the disease, but the virus is basically spread by direct contact with pigs. Further studies should be performed in order to confirm the suspected absence of virus among wild pig populations in both countries. In any case, if a sylvatic cycle exists, it is likely to be circumscribed to a tick-domestic pig cycle. The role and importance of this cycle in the epidemiology of the disease should also be investigated using analytical tools such as risk mapping, risk assessment or transmission compartment models.

ACKNOWLEDGEMENTS

We would like to express our gratitude to the Veterinary Services of Madagascar and Maison du Petit Elevage for supporting this work. We would also like to thank Prof. Antonio Encinas Grandes from the University of Salamanca for his availability and cooperation regarding the salival gland ELISA test. Finally, we are very grateful to Emmanuel Albina and personnel from ASF reference laboratory in Montpellier and Barbara Wieland (IAH/RVC) for their support in the laboratory analysis.

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DEVELOPMENT OF AN AVIAN INFLUENZA RISK MODEL FOR KWAZULU-NATAL, SOUTH AFRICA

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ABSTRACT

The KwaZulu-Natal Department of Health has identified Avian Influenza as a risk to the people of its' province and as such wanted to increase monitoring and surveillance. With limited resources available the monitoring needed to be directed to those areas which have a higher risk of having avian influenza introduced. A team of experts from veterinary, epidemiology, virology, ornithology and geographic information systems were tasked with developing a spatially explicit model which would identify areas of relatively high risk of the introduction of Avian influenza in order to direct more surveillance and monitoring to these areas. A model was developed using multi criteria evaluation within a geographic information system. The number of general practitioners in relatively higher risk areas undertaking surveillance as part of the "Viral Watch" programme have accordingly been increased in order to enhance the likelihood of early detecting of this disease.

INTRODUCTION

An influenza pandemic in 1918/19 was one of the most deadly pandemics of communicable diseases to have affected the world; this pandemic was directly responsible for over 20 million deaths worldwide, of which over 300 000 deaths occurred within South Africa (Smith, 2006). This resulted in the total paralysis of the health care system, which struggled to manage patients. It also resulted in a total inability to dispose of corpses with associated health risks. With the advent of new subtypes of highly pathogenic bird flu, otherwise known as avian influenza (AI), health experts anticipate the occurrence of major pandemics in the near future (National Department of Health, 2006). If the H5N1 strain of the virus mutates to become a threat to human populations, mortality as seen in the last century will be far greater due to a larger population, greater degree of urbanization and much greater and more mobile population which includes rapid international travel (Bruinzee et al. 2006)

It is against this background that the World Health Organisation and its' Member States have been encouraged to prepare contingency plans to best prevent and control future pandemics. The main challenge to health authorities is to develop adequate tools which may be used to predict where future pandemics will occur so as to best allocate scarce surveillance, monitoring and response resources.

The KwaZulu-Natal Department of Health together with a team, comprising

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veterinarians, epidemiologists, ornithologists, ecologists and virologists have utilized geographic information systems to develop an AI Risk Model for KwaZulu-Natal (KZN) which identifies areas where contamination of H5N1 is most likely to be introduced. The output of this model has been used to identify areas where human health practitioners should increase their surveillance and monitoring, but may also be used by animal health practitioners for similar purposes.

METHODOLOGY

A multi criteria evaluation (MCE) approach was used to develop the AI risk model. In decision theory, multi-criteria evaluation is the process in which several criteria are evaluated in order to meet a specific objective. Taking several criteria into account in an evaluation can be achieved through weighted linear combination. Important variables that would contribute towards the risk of the introduction of AI into KZN were identified and weighted relative to each other using expert opinion in the veterinary, wildlife, virology and epidemiology fields (Table 1). The spatial model was developed so as to be easily manipulated to accommodate more data or changes in variables as more information regarding the introduction of this disease becomes available.

Table 1: Factors considered which influence the risk of introduction of AI, source of data, and description of data manipulation undertaken to produce factor-specific maps of risk. All the data sets were limited to KZN and reclassified into 255 categories in order to normalise the data for inclusion into a multi criteria evaluation.

Dataset / Factor	Source	Description
Transport corridors (Roads & railways)	KZN Department of Transport (National and provincial roads); SA Explorer (railways)	A distance operator was applied to all transport corridors based on the assumption that the further away from a main transport line the less likely the chance of coming into contain with illegally transported poultry products. Each transport type was then multiplied by a factor to indicate its relative impact; the following weights were used: National Roads x 10, Provincial roads x 2 and Rail x 5. All types of transport with their relative factors were added together to obtain a composite dataset.
Entry points (official customs entry points into KZN)	KZN Department of Health	Official customs entry points into KZN were assumed to be the most likely points of import of poultry products contaminated with the H5N1 virus. These were also the most likely points where contaminated people from Asia would enter the country. A distance operator was applied to entry points based on the assumption that the further away from entry points the less likely the chance of coming into contact with illegally imported poultry products.

Poultry farms (poultry farms registered with the KZN Department of Agriculture)	Allerton Veterinary Services	Poultry farms represent higher risk source areas for the introduction f poultry diseases. A distance operator was applied to poultry farms as it was the assumption that the further away from poultry farms the less likely the chance of coming into contact infected poultry products.
Bird Rehabilitation Centres	KZN Wildlife (updated by Ben Hoffman, Predatory Bird Centre)	Bird rehabilitation centres represent areas to which sick birds are brought, and where humans and birds come into close contact, thus increasing the risk of transmission. A distance operator was applied to all bird rehabilitation centres based on the assumption that the further away from rehabilitation centres the less likely the chance of coming into contact infected birds.
Open water (pans, vleis, rivers, wetlands, lakes and farm dams)	National Land Cover Database	Water bodies, wetlands and vleis are areas where migratory birds congregate. A distance operator was applied to the water dataset based on the assumption that the further away from open water the less likely the chance of coming into contact with infected birds.
Precipitation (Mean Annual Rainfall)	South African Atlas of Agrohydrology and Climatology	Increased humidity increases the longevity of the pathogen in the environment.
Temperature (Mean Annual Temperature)	South African Atlas of Agrohydrology and Climatology	Lower temperatures increase the longevity of the pathogen in the environment.
Enumerator area type (small holdings, farms, tribal settlements, Traditional and informal dwellings) Poultry proxy)	2001 Census conducted by Statistics SA	The types of enumerator area used indicated the likelihood of poultry being held.
Income (Poultry proxy).	2001 Census conducted by Statistics SA	Households earning less than R800 per month were assumed to have a greater chance of living close proximity to poultry, and to regularly consume poultry products
Distribution and density of wild birds as potential avian influenza vectors	Avian Demography Unit, University of Cape Town (Bruinzee *et al*. 2006)	The distribution of migratory birds with the highest risk as internationally recorded of carrying Avian Influenza (mainly water fowl)

Figure number	Factor / Variable
Figure 1	Customs entry points
Figure 2	Wildlife rehabilitation centres
Figure 3	Poultry farms
Figure 4	Type of land use and income
Figure 5	Transport routes
Figure 6	Water bodies
Figure 7	Temperature and precipitation
Figure 8	Wild bird prevalence of carrying AI

Below are the spatially explicit variables utilised to create the avian influenza risk model.

Figure 1

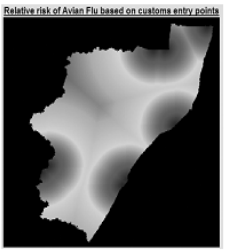


Figure 2

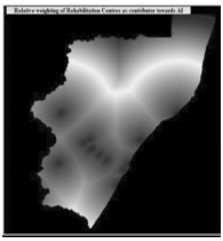


Figure 3

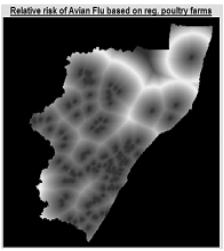


Figure 4

Figure 5

Figure 6

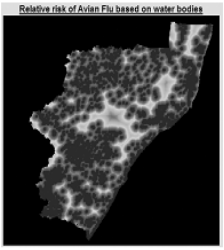


Figure 7

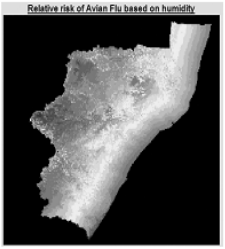


Figure 8

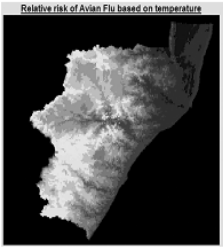
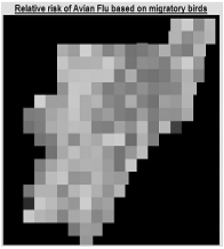


Figure 9



Relative weighting of variables was undertaken by experts using a pairwise comparison approach (Table 2). The WEIGHT Module in the Geographic Information System software IDRISI (ver. 14 (Kilimanjaro Edition)) (Clarke Labs, 2003) was used to develop a set of relative weights for the group of AI risk factors in a multi-criteria evaluation (Table 3). The weights were developed by providing a series of pairwise comparisons of the relative importance of factors in respect of risk of introduction of AI. The procedure by which the weights are produced follows the logic developed by T. Saaty under the Analytical Hierarchy Process (Clark Labs, 2003).

The factors and their resulting weights were used as input for the Multi Criteria Evaluation (MCE) module for weighted linear combination of the spatial data layers representing each risk factor (Figure 10).

Table 2: Pairwise comparison scores for risk factors [Scores: 9 - Relative to the column variable, the row variable is extremely more important; 6 - Relative to the column variable, the row variable is strongly more important; 4 - Relative to the column variable, the row variable is moderately more important; 1 - Relative to the column variable, the row variable is equally important; 1/4 - Relative to the column variable, the row variable is moderately less important; 1/6 - Relative to the column variable, the row variable is strongly less important; 1/9 - Relative to the column variable, the row variable is extremely less important].

	Entry Points	**Transport corridors**	**Poultry Farms**	**Income**	**Open Water**	**Tempera-ture**	**Precipi-tation**	**Rehab Centres**	**Migrating Wild birds**
Entry Points	1								
Transport corridors	1/3	1							
Poultry Farms	1/3	2	1						
Income	1/3	3	1/2	1					
Open Water	1/3	1/3	1/3	1/3	1				
Temperature	1/9	1/9	1/9	1/9	1/9	1			
Preci-pitation	1/9	1/9	1/9	1/9	1/9	1/2	1		
Rehabilitation Centres	1/3	3	1	1/2	4	9	9	1	
Migrating Wild birds	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1

Figure 10: Avian Influenza Risk Macro Model built in IDRISI Kilimanjaro
This model was built in order to prepare the variables for inclusion into the Multi Criteria Evaluation module

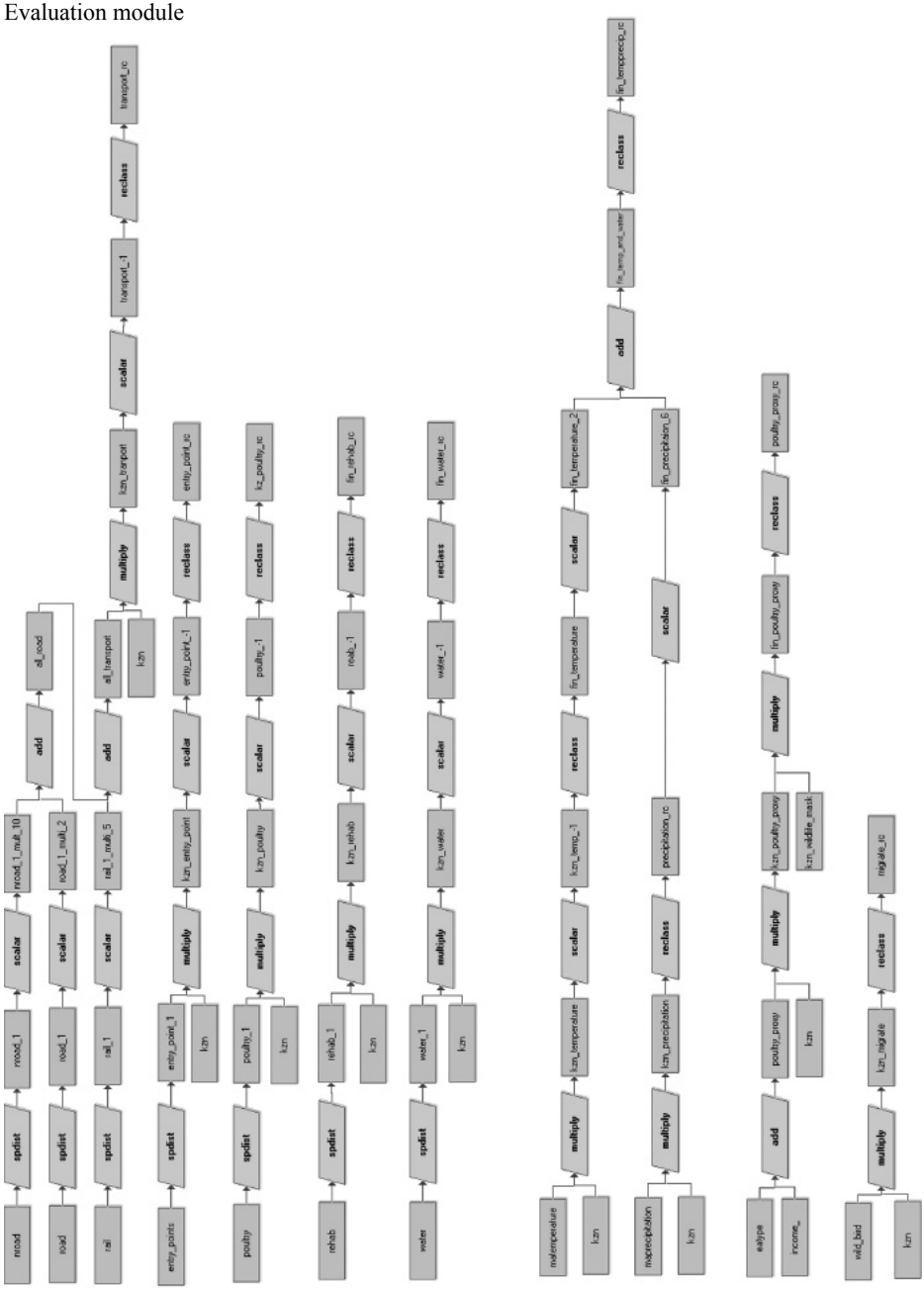


Table 3: Eigenvector weights of risk factors based on pairwise comparison scores; Consistency Ratio = 0.08, which is acceptable [Note: Eigenvector is a vector which, when acted on by a particular linear transformation, produces a scalar multiple of the original vector]

Figure number	Factor	Weight	Rank
Figure 1	Customs entry points	0.2658	1
Figure 2	Wildlife rehabilitation centres	0.2155	2
Figure 3	Poultry farms	0.1634	3
Figure 4	Type of land use and income	0.1469	4
Figure 5	Transport routes	0.1008	5
Figure 6	Water bodies	0.0672	6
Figure 7	Temperature and precipitation	0.0210	7
Figure 8	Wild bird prevalence of carrying AI	0.0193	8

Figure 11: Relative risk of the introduction of Avian Influenza in KwaZulu-Natal, South Africa.

RESULTS

The output of the analysis is a map of relative risk of introduction of AI in KZN (Figure 11). The Durban (Ethekwini Metro) and Richard's Bay (Mhlathuze Municipality) areas have the highest risk of AI introduction whilst the drier and more isolated central and north western areas have the lowest risk.

CONCLUSIONS

The modeling process based on weighted spatially explicit risk factors has identified the highest risk areas for the introduction of AI. It was recommend that scarce monitoring and surveillance resources be directed to the Durban and Richard's Bay areas in order to maximise the likelihood of early detection of an AI outbreak; early detection will in turn not only benefit human health but may also be of use in the spheres of agriculture and conservation. The approach used in this study has potential for surveillance planning and risk management for other human and animal diseases.

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Figure 1

A CENSUS SURVEY OF WESTERN CAPE OSTRICH FARMS TO IDENTIFY RISK FACTORS ASSOCIATED WITH SEROPOSITIVITY TO H5 AVIAN INFLUENZA VIRUS

P.N. Thompson[1], M. Sinclair[2] & B. Ganzevoort[3]

SUMMARY

In a 2005 serological survey, 16.3% of ostrich farms in the Western Cape Province of South Africa were found to be seropositive to H5 avian influenza (AI) virus. A census survey was subsequently performed using a questionnaire on those ostrich farms registered for export that still existed at the end of 2005 (367 farms, of which 82 were seropositive), in order to identify risk factors associated with farm-level seropositivity. For each farm, information was collected on the ostrich population, movements of birds, management practices, and frequency of contact between ostriches and various wild bird species. Logistic regression models were developed for the whole province and also for the two largest ostrich farming regions, "Klein Karoo" and "Southern Cape". Seroprevalence differed between areas, being highest in the Klein Karoo (31.6%). In all three models, increased risk was associated with increasing numbers of ostriches (excluding chicks) on the farm. Increased risk of seropositivity was associated with reduced frequency of cleaning of feed troughs (<1x/week vs. >1x/week), both overall (odds ratio (OR) = 4.49, $P = 0.007$) and in the Southern Cape (OR = 53.6, $P = 0.005$), and with failure to clean and disinfect transport vehicles, both overall (OR = 2.28, $P = 0.03$) and in the Klein Karoo (OR = 2.62, $P = 0.04$). Increased risk of seropositivity was also associated with increasing frequency of contact of ostriches with certain wild bird species: overall with white storks (*Ciconia ciconia*), in the Southern Cape with gulls (*Larus* spp.), and in the Klein Karoo with Egyptian geese (*Alopochen aegyptiaca*).

INTRODUCTION

Avian influenza (AI) is a growing public health concern. It has been calculated that the impact of AI on the poultry industry has increased 100-fold with 23 million birds affected in 1959 to 1998 and more than 200 million affected from 1999 to 2004 (Capua 2006). The disease is caused by influenza A viruses of the Family *Orthomyxoviridae.* Influenza A viruses are enveloped single-stranded RNA viruses. Subtypes are defined by the antigenicity of two major surface proteins on the envelope, namely haemagglutinin (H) and neuraminidase (N). Sixteen H (H1-H16) and nine N (N1-N9) subtypes have

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been recognised, translating into 144 possible combinations, with each virus containing one H and one N antigen in any combination. AI viruses are further divided in two pathotypes based on their ability to cause disease in chickens. Highly pathogenic avian influenza (HPAI) spreads rapidly, may cause serious disease and result in high mortality rates (up to 100% within 48 hours). Low pathogenic avian influenza (LPAI) can cause mild disease that might go undetected or show no symptoms at all in some species of birds (OIE 2006). HPAI viruses possess multiple basic amino acids (arginine and lysine) at the cleavage site of their haemagglutinin glycoprotein precursor (Wood *et al*. 1993). HPAI has only been associated with H5 and H7 subtypes and these are the only subtypes notifiable to the World Organisation for Animal Health (OIE) (Senne *et al*. 2005).

Ostrich farming in South Africa is heavily reliant on export of leather and fresh ostrich meat products to trade partners in the USA, Japan and Europe respectively. In July 2004 the H5N2 subtype of HPAI virus was isolated from ostriches (*Struthio camelus*) in the Eastern Cape Province of South Africa. Following this diagnosis a national avian influenza survey was conducted until May 2005. During this survey 124 out of 761 ostrich farms in the Western Cape Province tested serologically positive to H5 AI. In response to these findings 15 945 cloacal swabs were tested by polymerase chain reaction (PCR) to detect the agent. All tests yielded negative results (Sinclair *et al*. 2006).However, exports were banned from August 2004 until September 2005. In July 2006 H5N2 HPAI was again detected in ostriches, this time in the Albertinia district of the Western Cape Province. Once more exports were banned from July 2006 until the 1st of November 2006, when conditional exports (only from serologically negative farms) were allowed.

The world market share of the South African ostrich industry is approximately 60%. The main production systems and processing facilities are located within a limited semi-arid region (Klein Karoo) within the Western Cape Province, representing 70-80% of the South African industry (Olivier 2005). A prolonged ban of meat exports may lead to a loss of at least R50 million per month during the peak season. The ostrich industry employs 20000 people directly. A ban on the export of ostrich meat would therefore place the industry under stress and job losses would be inevitable.

The purpose of this questionnaire survey was to identify farm-level risk factors for seropositivity to H5 AI, with a view to mitigating these risk factors and thereby helping to ensure sustainable exports.

MATERIALS AND METHODS

A census survey was performed. The study population consisted of all export registered ostrich farms that were present in the Western Cape Province of South Africa during the March - May 2005 AI surveillance period and that were still registered at the end of 2005 (435 farms). The survey was designed as an interview based questionnaire survey and each property owner was personally interviewed by an animal health technician, who recorded the answers to the questions onto a data capture sheet.

The questionnaire included information on the contact details of the owner, the farm name, registration number and geographical location. Table 1 lists all the categorical potential risk factors recorded; the only continuous variable was the number of birds present on the farm (excluding chicks). The questions referred to conditions prevailing on the farms 12-18 months previously, at the time of the H5N2 isolation in the Eastern Cape and prior to the serological survey.

The results of the 2005 AI serological survey were used to classify the farms as either seropositive (one or more seropositive ostriches detected on the farm) or seronegative (Sinclair *et al*. 2006). Sufficient randomly selected samples had been collected from each epidemiological unit on each farm in order to detect a within-group seroprevalence of 10%. The sera had been tested using the haemagglutination inhibition (HI) test according to standard procedures (OIE 2005), using antigen from the H5N2 AI virus isolated from ostriches in the Eastern Cape in 2004. A HI titre exceeding 1/16 was regarded as positive.

Data analysis

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The independent variables were tested for univariable associations with the outcome variable (H5 seropositivity) using the Fisher exact test for categorical variables and simple logistic regression for continuous variables. All independent variables with $P < 0.20$ in the univariable analysis, and with < 15% missing values were included in the initial multiple logistic regression models.

Firstly a model was developed for all areas combined. In this model, "Klein Karoo" and "Karoo" were combined into a single region and "Agulhas" and "West" were combined ("Western Cape"). This was done on the basis of environmental and management similarities between these regions and in order to reduce the number of regions with very few or no seropositive farms. The model was developed by backward elimination until all independent variables were significant in the model with $P_{\text{lrtest}} < 0.05$. All other independent variables, including those not initially included, were then individually retested by addition back into the model, and retained if $P_{\text{lrtest}} < 0.05$. Biologically meaningful one-way interactions amongst remaining independent variables were then tested by addition into the model and retained if $P_{\text{lrtest}} < 0.05$.

In addition to the combined model, separate multiple logistic regression models were developed in the same way for each of the two largest ostrich farming regions, "Klein Karoo" and "Southern Cape". The fit of each logistic regression model was assessed using the Hosmer-Lemeshow goodness-of-fit test. All analyses were done using STATA version 8.2 (Stata Corporation, College Station, TX, USA).

RESULTS

The questionnaire was conducted on 84.4% (367/435) of the export ostrich farms in the study population. The owners of the remaining 68 farms could not be interviewed since they had either sold the farm, discontinued ostrich farming or were unavailable for the interview. Of these 367 farms, 82 (22.3%) were classified as seropositive (Sinclair *et al*. 2006), and this varied between regions (Table 1). The number of farms for which responses to each question were obtained is shown in Table 1.

In the univariable analyses, the number of birds present was positively associated with seropositivity ($P < 0.001$). There were also univariable associations between a number of categorical risk factors and seropositivity (Table 1). These included the use of large water troughs, the types of feed troughs used and their method and frequency of cleaning, the frequency of visits by Newcastle disease (NCD) vaccinators, the use of lucerne pastures as grazing, the disinfection and cleaning of vehicles, the movement of ostriches onto the farm during the autumn, and the frequency of contact of ostriches with certain wild bird species. These variables, except for *vehicle cleaned and disinfected*, which had a high proportion of missing values, were initially included into the multiple logistic regression models.

Table 1. Univariable association of categorical risk factors with farm-level AI H5 seropositivity in ostriches on 367 Western Cape ostrich farms

Variable	Level	Number of farms	Proportion seropositive (%)	P[1]
Region	Agulhas	30	6.7	<0.001
	Karoo	20	20.0	
	Klein Karoo	171	31.6	
	Southern Cape	124	17.7	
	West	22	0.0	
Type of birds present during 2004	Slaughter birds only	64	20.3	0.63
	Breeders only	15	13.3	
	Both	276	23.9	
Chicks present during 2004	no	97	26.8	0.26
	yes	262	21.0	
Small water troughs	no	287	21.3	0.28
	yes	77	27.3	

Medium water troughs	no	41	26.8	0.55
	yes	324	21.9	
Large water troughs	no	298	21.1	0.19
	yes	66	28.8	
Frequency of cleaning water troughs	>1x/week	55	23.6	0.94
	~1x/week	153	21.6	
	<1x/week	156	22.4	
Method of cleaning water troughs	Empty and refill	108	22.2	0.20
	Scrub	167	15.6	
Type of feed troughs	Open feed troughs	270	19.3	0.05
	Self-feeders	31	29.0	
	Both	62	32.3	
Frequency of cleaning feed troughs	>1x/week	87	8.0	<0.001
	~1x/week	102	21.6	
	<1x/week	146	30.8	
Method of cleaning feed troughs	Move only	101	32.7	0.04
	Empty and refill	65	18.5	
	Empty, refill and move	165	18.2	
	None	9	11.1	
Frequency of visits by NCD vaccinators	1x/year or less	67	14.9	0.05
	2x/year	124	18.5	
	4x/year or more	171	28.1	
Lucerne pastures used as grazing for ostriches	no	58	12.1	0.04
	yes	308	24.4	
Backyard poultry present	no	176	23.3	0.71
	yes	188	21.3	

Rented vehicle used	no	188	20.7	0.41
	yes	123	25.2	
Vehicle cleaned and disinfected	no	78	30.8	0.09
	yes	219	20.5	
Open water sources present	no	24	16.7	0.62
	yes	341	22.6	
Ostriches have access to open water sources	no	209	23.9	0.38
	yes	156	19.9	
Open water sources used as water points	no	250	24.0	0.23
	yes	115	18.3	
Movement of ostriches onto the farm:				
Summer	no	82	19.5	0.44
	yes	200	24.0	
Autumn	no	124	12.9	0.001
	yes	158	30.4	
Winter	no	189	20.6	0.29
	yes	93	26.9	
Spring	no	127	26.0	0.25
	yes	155	20.0	
Wild birds seen amongst ostriches:				
Egyptian Goose (*Alopochen aegyptiaca*)	Never	84	17.9	0.49
	Seldom	120	22.5	
	Frequently	162	24.7	
African Sacred Ibis (*Threskiornis aethiopicus*)	Never	160	20.0	0.002
	Seldom	131	16.8	
	Frequently	73	38.4	
Hadeda Ibis (*Bostrychia hagedash*)	Never	64	18.8	0.11
	Seldom	167	27.5	
	Frequently	135	17.8	

Cattle Egret (*Bubulcus ibis*)	Never	158	20.9	0.75
	Seldom	140	22.9	
	Frequently	68	25.0	
Pigeons (Columbidae)	Never	52	11.5	0.07
	Seldom	93	20.4	
	Frequently	221	25.8	
Finches and sparrows (Ploceidae & Passeridae)	Never	21	9.5	0.31
	Seldom	59	20.3	
	Frequently	286	23.8	
Gulls (*Larus* spp.)	Never	324	21.9	0.95
	Seldom	30	23.3	
	Frequently	10	20.0	
White Stork (*Ciconia ciconia*)	Never	219	21.0	0.16
	Seldom	130	22.3	
	Frequently	17	41.2	
Helmeted Guineafowl (*Numida meleagris*)	Never	72	30.6	0.04
	Seldom	118	25.4	
	Frequently	176	17.0	

[1] *P*-value for Fisher's exact test

The final logistic regression model for all areas combined is shown in Table 2. The risk of seropositivity increased with increasing numbers of ostriches on the farm, with reduced frequency of cleaning of feed troughs, with failure to clean and disinfect vehicles and with frequent sightings of White Storks amongst ostriches. The variable *vehicle cleaned and disinfected* was initially excluded from the model due to 19% missing values. However, when tested at the end for inclusion in the model, it was significant. Although its inclusion resulted in a reduction from 323 to 263 farms being included in the model, it did not materially change the coefficients for the other predictors; therefore it was retained in the final model.

Table 2: Final logistic regression model for farm-level AI H5 seropositivity in ostriches – all areas combined (263 farms)*

Variable	Level	*β*	Odds ratio	95% CI (OR)	*P*
Region	Klein Karoo and Karoo	0	1	–	–
	Southern Cape	-0.95	0.39	0.17, 0.90	0.03
	Western Cape	-2.25	0.11	0.02, 0.49	0.004
Number of birds on farm (excluding chicks)	Continuous	0.001	–	–	<0.001
Frequency of cleaning feed troughs	>1x/week	0	1	–	–
	~1x/week	1.39	4.02	1.33, 12.19	0.014
	<1x/week	1.50	4.49	1.52, 13.30	0.007
Vehicle cleaned and disinfected	Yes	0	1	–	–
	No	0.82	2.28	1.09, 4.77	0.03
Wild birds seen amongst ostriches:					
White Storks	Never	0	1	–	–
	Seldom	0.73	2.06	1.00, 4.27	0.05
	Frequently	2.02	7.51	1.71, 32.93	0.007

* Hosmer-Lemeshow goodness-of-fit test $\chi^2 = 4.36$ (8 d.f.), $P = 0.82$

For the Klein Karoo region, the final logistic regression model (Table 3) showed that increased risk of seropositivity was associated with larger numbers of ostriches on the farm, with failure to clean and disinfect vehicles and with frequent observation of Egyptian Geese amongst the ostriches. Similar to the first model, the variable *vehicle cleaned and disinfected* was initially excluded due to 30% missing values. However, when tested at the end for inclusion in the model, it was significant. Although its inclusion resulted in a reduction from 163 to 115 farms being included in the model, it did not materially change the coefficients for the other predictors; therefore it was retained in the final model.

Table 3: Final logistic regression model for farm-level AI H5 seropositivity in ostriches – Klein Karoo (115 farms)*

Variable	Level	*β*	Odds ratio	95% CL (OR)	*P*
Number of birds on farm (excluding chicks)	Continuous	0.001	–	–	0.002
Vehicle cleaned and disinfected	Yes	0	1	–	–
	No	0.96	2.62	1.06, 6.54	0.04
Wild birds seen amongst ostriches:					
Egyptian Geese	Never	0	1	–	–
	Seldom	-0.05	0.95	0.27, 3.30	0.94
	Frequently	1.21	3.36	1.08, 10.47	0.04

* Hosmer-Lemeshow goodness-of-fit test $\chi^2 = 8.42$ (8 d.f.), $P = 0.39$

For the Southern Cape region, the final logistic regression model (Table 4) showed that increased risk of seropositivity was associated with larger numbers of ostriches on the farm, with reduced frequency of cleaning of feed troughs and with frequent observation of gulls amongst the ostriches. The presence of chicks was associated with a reduced risk of seropositivity. No biologically meaningful interactions were found to be statistically significant in any of the models.

Table 4: Final logistic regression model for farm-level AI H5 seropositivity in ostriches – Southern Cape (112 farms)*

Variable	Level	*β*	Odds ratio	95% CL (OR)	*P*
Number of birds on farm (excluding chicks)	Continuous	0.001	–	–	0.01
Chicks present	no	0	1	–	–
	yes	-3.50	0.03	0.002, 0.43	0.01
Frequency of cleaning feed troughs	>1x/week	0	1	–	–
	~1x/week	3.40	30.07	2.38, 379.4	0.009
	<1x/week	3.98	53.60	3.32, 864.4	0.005

Wild birds seen amongst ostriches:					
	Never	0	1	–	–
Gulls	Seldom	1.89	6.61	1.39, 31.49	0.02
	Frequently	3.03	20.63	0.93, 458.6	0.06

* Hosmer-Lemeshow goodness-of-fit test $\chi^2 = 5.88$ (8 d.f.), $P = 0.66$

DISCUSSION

In this study we have shown an association between certain risk factors and farm-level seropositivity to H5 AI virus in ostriches. However, the HI test used for the determination of the serological status of the farms, although validated for chickens, has not yet been validated for ostriches. Therefore, although the tests were performed according to OIE guidelines, the sensitivity and specificity of the test are not known. In addition, serological testing had been done in order to detect a minimum within-farm seroprevalence of 10%, so farms with a lower prevalence of seropositive birds were more likely to have been missed, thus reducing the sensitivity of the farm-level test. However, it can be assumed that any misclassification of farm serological status would have been nondifferential, and that any bias in the estimated odds ratios would thus be towards the null (Copeland *et al*. 1977). This study may therefore have lacked sufficient power to identify certain risk factors for H5 seropositivity. The availability of validated tests for H5 and H7 AI subtypes in ostriches will greatly facilitate investigation of the epidemiology of notifiable AI viruses in this species.

Despite adjustment for the other predictor variables, ostrich farms in the Klein Karoo and Karoo region showed the highest risk for seropositivity, followed by the Southern Cape, while the Western Cape region showed the lowest risk. It is possible that this may be due to geographic spread of the virus from the Eastern Cape Province westwards, but may also reflect differences in other, unmeasured risk factors between the regions. The number of ostriches on the farm was consistently positively associated with the risk of seropositivity. This increased risk may partially be explained by a higher population density, resulting in more efficient viral transmission, and associated stress resulting in increased susceptibility to infection. The frequency of visits by Newcastle disease vaccinators was significantly associated with seropositivity on a univariable level, an association that disappeared in the multivariable analysis. This was probably due to confounding by size of farm, since the frequency of visits by vaccinators is largely a function of the number of birds on the farm.

The reason for the strong negative association seen in the Southern Cape region between the presence of ostrich chicks on the farm and the risk of farm seropositivity, is not known, but may be due to confounding with other, unmeasured management factors. None of the chicks tested during the 2005 survey were seropositive, indicating either that the chicks were hatched after the active infection period, or that chicks (due to different management practises) were not exposed to the risk factors.

The traditional ostrich-farming areas in the Western Cape Province report almost

yearly outbreaks of LPAI in ostriches, which have been attributed to introduction by wild birds and certain climatic patterns (Abolnik *et al*. 2006). During the winter months lower temperatures and wetter conditions are more favourable for the spread of the disease. Stress associated with transport may increase viral shedding (should the newly introduced ostriches be infected) and/or weaken the birds' resistance to infection from the environment. When this occurs in favourable climatic conditions, the disease is more likely to spread. This is consistent with the finding in the univariable analysis that risk of seropositivity was higher when ostriches were moved onto the farm during autumn. Our study also found that failure to clean and disinfect the transport vehicles further contributed to the risk of seropositivity.

Previous studies have showed that wild birds can act as reservoir for viral infections in ostriches (Pfitzer *et al*. 2000 and Alexander 2000). In 1998 the presence of a H6 AI serotype was demonstrated in an Egyptian Goose near Oudtshoorn shortly before an outbreak involving H6N8 in ostriches (Pfitzer *et al*. 2000). In 2004 a H5N2 LPAI virus was isolated from an Egyptian Goose in the Oudtshoorn district and was found to be highly similar to the H5N2 HPAI virus isolated from ostriches in the Eastern Cape later that year (Olivier 2006). Both instances suggest that the outbreaks in ostriches may have originated in wild waterfowl. Abolnik *et al*. (2006) showed that the AI viruses isolated from wild ducks in South Africa were most likely of Eurasian ancestry, and suggested that waders carry viruses from their breeding grounds and stopover sites, co-inhabited by Eurasian migrants, to South Africa each year. Viruses shed into local wetlands via the faeces may then be ingested by sympatric indigenous waterfowl, which then become infected and act as reservoir hosts that move extensively throughout the country. Certain wild bird species, for example sacred ibis, storks, gulls and Egyptian geese, are encouraged to visit ostrich camps by open water sources and feeding points. Their numbers often exceed the ostrich numbers in a camp especially where feed is provided *ad lib*. They congregate around the feeding troughs and watering points and contaminate them with their faeces. Should the wild birds be infected with an AI virus, failure to regularly clean feed troughs will increase the probability of the contamination reaching an infective level. In this study weekly, or less frequent, cleaning of feed troughs, compared to more frequent cleaning, had a strong positive association with the risk of farm-level seropositivity, particularly in the Southern Cape region. At the univariable level, emptying and refilling the feed troughs, compared to just moving them, reduced the risk of farm seropositivity. These findings, together with the association between the presence of certain wild bird species and the risk of seropositivity, supports the theory that infection of ostriches may occur via faecal contamination of feed by wild birds.

The role of water sources is less clear in this study, with no significant associations being found between types of water troughs, method or frequency of cleaning of water troughs, or the presence or utilisation of open water sources. However, a large number of responses to the question regarding the method of cleaning of water troughs had to be discarded. Initially, "permanent chlorination" was offered as an additional response to this question, but a markedly greater prevalence of seropositivity was seen amongst farmers giving this response. Further investigation revealed that chlorination was extremely rarely done prior to the 2005 serological survey, and that institution of

this practice was probably done by farmers who perceived their farms to be at risk, or even in response to positive serological tests on their own or neighbouring farms. Management of water troughs should therefore not be excluded as a possible risk factor, since AI viral transmission in waterfowl populations is thought to occur by a faecal-oral route via contaminated water, and the virus can survive for extended periods of time in water (Brown *et al*. 2007).

The species of wild birds that appeared to increase the risk of farm-level seropositivity varied by region. The assessment of individual species abundance was difficult to standardise and was subjective, since it depended on the farmers' observation and identification skills. Nevertheless, the various species included in the questionnaire were fairly easily distinguishable, and for several species the tendency for increasing abundance to be positively associated with farm seropositivity made biological sense. The crude association between the use of lucerne pastures as grazing for ostriches and the risk of farm seropositivity was probably a case of confounding due to a positive association between lucerne pastures and the abundance of certain wild bird species, namely Egyptian goose, African sacred ibis and white stork.

CONCLUSION

Increased risk of farm-level seropositivity to H5 AI in Western Cape ostrich farms during 2005 was associated with increased numbers of ostriches on the farm, with infrequent cleaning of feed troughs, with failure to clean and disinfect transport vehicles, and with increased frequency of contact with certain wild bird species on the farm. Proper management of feeding (and possibly also watering) troughs in order to reduce faecal contamination by wild birds, as well as biosecurity measures to reduce mechanical transmission between premises, should reduce the risk of seropositivity. The validation of the HI test in ostriches is urgently required in order to accurately monitor the occurrence of AI amongst ostriches and to further elucidate the epidemiology of AI in this species.

ACKNOWLEDGEMENTS

The authors would like to thank the Animal Health Technicians of the Western Cape Department of Agriculture for assisting with the interviews and Jacques Kotze for his assistance with the data capturing and GIS work.

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MYCOBACTERIUM BOVIS AND MULTI-SPECIES DISEASE TRANSMISSION: ASSESSING THE RISK

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SUMMARY

Mycobacterium bovis is the cause of tuberculosis (TB) in a wide range of mammalian species. Infection is endemic in cattle and farmed deer in most countries, and endemic in wild animal populations in North America, Europe, sub-Saharan Africa, and New Zealand. In domestic livestock the epidemiology of TB and disease control measures are well established, but are poor and rudimentary in wild animal populations. In domestic livestock TB is a disease of economic and human health concern, and most TB research in animals has focused on domestic animals. The significance of M. bovis in and for wild animals has not been established. Not all infected wild animal populations are a significant reservoir of infection and the presence of infection in a wild animal population is not of itself evidence that they will be. To determine the potential of wild animal species to be a reservoir of infection certain key questions must be addressed: the nature of the disease in individual animals, routes and levels of excretion, including their potential as prey, and the geographic distribution of infected populations. The role of wild animals in the epidemiology and control of TB in domestic animals will be illustrated with six well research examples.

INTRODUCTION

Mycobacterium bovis can cause tuberculosis (TB) in a wide range of mammalian species. TB is present in domestic cattle and deer in many countries. Infection is also present in wild animal ecosystems, in North America, continental Europe, sub-Saharan Africa, and New Zealand. In domestic livestock TB is a disease of economic and human health concern, hence most TB research in animals has focused on domestic animals. Whereas the epidemiology of TB and disease control measures is well established in domestic livestock, they are poor and rudimentary for wild animals. The significance of M. bovis in and for wild animal has not been established.

Tuberculosis in a wild animal population may not only have an impact within the affected species, but may also spillover into populations of other wild species and into domestic livestock (cattle and deer) where it may have a direct economic impact. Tuberculous wild animals may also be a risk to public health and the underlying factors affecting the risk to humans are basically the same as those for the risk to domestic livestock (Biet et al., 2005). Although this paper will focus on the risk to domestic

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animals from TB in wild animal, the principals described can equally be applied to transmission within a wild animal ecosystem. Below are described the questions that need to be addressed when assessing the role of an animal or population of animals within an economic or ecological system.

This paper will address the assessment of risk, using examples of Tb in wild animals as they have affected Tb control in livestock, and a detailed description of Tb in badgers.

ASSESSMENT OF RISK

There are a number of key issues when determining the significance of tuberculous wild animals and their status as a reservoir for domestic animals. These are: the nature of the infection in individual animals, the dynamic of infection in the population, the broad geographic distribution of infection in the species, local distribution of the wildlife species, and the interaction of the wild animal host with domestic animals. There are a range of epidemiological patterns of infection among different wild animal populations, and the mere presence of infection in a wild animal population is not, of itself, evidence that the species is a significant reservoir. In a multiple host system, the nature of the disease in each host species and the host populations must be determined, and it must be established that interactions that which could lead to disease transmission occur between the species.

Infected wild animal population are classed as either a maintenance or spillover host depending on the dynamics of the infection (Morris and Pfeiffer, 1995; Table 1). In a maintenance host infection persists by intraspecies transmission alone and a maintenance host can be the source of infection for livestock. In a spillover host infection will not persist indefinitely unless there is continued re-infection from another species. Although infection may not persist in a spillover host, the time required for infection to die out when the external source infection ceases will depend on the efficacy of intraspecies transmission. A spillover host can be a reservoir of infection for domestic livestock. The status of a wild animal population, whether a maintenance or spillover host, has significance when determining when control of the disease in a species is necessary to prevent transmission, especially that to domestic animals.

Table 1: Animal species found infected with Mycobacterium bovis in New Zealand and their potential as a source of infection for domestic livestock.

Species	**Principal site(s) of infection**	**Host status**	**Potential as source for domestic live-stock**
Brushtail possum	Respiratory tract	Maintenance	High
Deer - both domestic and feral	Respiratory tract	Maintenance	High
Cattle	Respiratory tract	Maintenance	High

Ferret	Gastro-intestinal tract	Spillover / Maintenance	Low to moderate, high in some specific areas
Pigs (feral)	Gastro-intestinal tract	Spillover	Low
Sheep	Respiratory tract	Spillover	Very low
Goat	Respiratory tract	Spillover	Very low
Rabbit	Single case	Spillover	Very low
Hare	Single case	Spillover	Very low
Stoat	Gastro-intestinal tract	Spillover	Very low
Weasel	NDa	Spillover	Very low
Hedgehog	Respiratory tract	Spillover	Very low
Cat	Skin and gastro-intestinal tract	Spillover	Very low
Dog	Gastro-intestinal tract	Spillover	Very low

[a] ND – not described

References: Coleman and Cooke, 2001; Morris and Pfeiffer, 1995; Gay et al., 2000

FACTORS AFFECTING TRANSMISSION

Both individual animal and population factors influence transmission within and between animal populations. The nature of the infection in individual animals factors that influence transmission include:

1. The route(s) of infection;
2. The anatomical location of infection and lesions, and the structure of lesions;
3. The routes and levels of excretion;
4. The routes of infection for the domestic livestock at risk; and,
5. An understanding of the minimum infective dose by each route.

These factors provide a picture of the dynamics of infection within the individual, how long an infected individual may remain within the population, the duration of their infectivity, the common modes of transmission, the possible risk from environmental contamination, how infection may be transmitted to livestock and the risk of transmission.

Foremost among the population factors that influence transmission is the “force of infection” (Caley and Hone 2002), or the risk of transmission, and is dependent on the prevalence of infection, average infectivity, and the need to fulfil conditions necessary for transmission. For each route of infection the conditions will differ; of particular importance is the distance, both in time and space, between the source of infection and susceptible animals. Factors relating to time and space include the duration of interactions, the distance between interacting infected and susceptible hosts, the effect

of time on the viability of excreted bacilli, and the effect of time on the physical location of the bacilli in the environment. For effective transmission M. bovis must survive in a place and form, in sufficient numbers and for long enough for the susceptible host to encounter an infective dose. With excreted bacilli, the period of survival of M. bovis outside of the host is an important determinant of the duration of risk.

In different wild animal species, M. bovis infections have different manifestations. In the Eurasian badger (Meles meles), disease spreads slowly with a high point prevalence of infection, few animals with high levels of excretion, and a low disease induced mortality (Little et al 1982). In contrast, infected possum populations usually have a low point prevalence of infection, a short period of clinical disease, a short period of very high infectivity and a high diseased induced mortality.

The state of the natural environment can influence the epidemiology of TB in wild animal populations. An altered environment may affect the individuals within the population and population dynamics. If the changes also increase the interaction between wild and domestic animals, these may facilitate transmission. Changes resulting from population management or disturbance of natural social structures may not only cause an exacerbation of disease in wild animal populations but may also be used to control the disease. Increased disease prevalence in white-tailed deer (Michigan, USA), were associated with greatly increased population density. In contrast, a decrease in TB prevalence in fallow deer (Phoenix Park, Dublin) where co-grazing of deer and cattle had been practiced, was achieved by the removal of cattle.

When investigating a wild animal species as a potential source of M. bovis for domestic livestock, it is essential to consider each of the key questions as it relates to each species. There may be a temptation to jump to conclusions, biasing assessments based on only one of the elements. Each species has to be assessed individually and the decision on its role determined after considering all the issues.

THE INFLUENCE OF WILDLIFE INFECTION ON CONTROL PROGRAMS IN DOMESTIC LIVESTOCK

In many environments the significance of wild animal Tb is its impact on the epidemiological of infection in domestic cattle populations. Hence wild animal tuberculosis as it affects TB control in domestic livestock has been well research. The scope of wild animal involvement in the epidemiology of bovine tuberculosis can be illustrated by reference to some well researched cases. For each of the following wild animal species the key characteristics of their role in the epidemiology will be described: the feral pig (Suis scrofa) and feral Asian water buffalo (Bubalus bubalis) in Australia, white-tailed deer (Odocoileus virginianus) in USA, and the brushtail possum (Trichosurus vulpecula) and the involvement of multiple species, including the special case of the ferret (Mustela furo), in New Zealand.

Feral pigs in Australia

Tuberculosis in the feral pig population in northern Australia is an example of a wild

animal population where the prevalence of infection was high but they did not play a role in the epidemiology of TB in domestic cattle because there was no significant excretion of M. bovis from the pigs.

Feral pigs in northern Australia are an exotic species derived from liberated or escaped domestic animals. In the 1970s TB was endemic in the large population in northern Australia with the prevalence in some areas >40% (Corner et al., 1981). The pigs were infected by scavenging carcases of infected cattle and water buffalo. However, few had generalised disease and in most the lesions were small (1 - 2 mm) well encapsulated foci that contained few bacilli. Mostly infection was confined to the mandibular lymph nodes and indicative of oral infection. Even though they shared the same environment as cattle, feral pigs were considered to be of no epidemiological significance in the transmission to cattle based on lesion distribution in the majority of pigs and the lack of excretion of M. bovis. The feral pig was a spillover and end stage host, not a source of infection for cattle, and no control of the feral pigs was undertaken. That decision was validated when 20 years later and after TB was eradicated from the cattle and water buffalo populations, a survey showed almost no TB in the feral pig population (McInerney et al., 1995). Even though infection could be transmitted from pig to pig by cannibalism, it was found that when the disease was controlled in the cattle and water buffalo, infection disappeared from the feral pigs.

Feral Asian water buffalo in Australia

Although the TB prevalence in feral buffalo was very high, these animals were also not considered a significant reservoir of M. bovis for cattle as buffalo and cattle did not interact. The Asian water buffalo was introduced into northern Australia in the early 1800s, and the population was estimated as >300,000 in the 1970s (Cousins et al., 1998). Buffalo are a gregarious, social species, forming dense groups especially when the plains are flooded during monsoon season. The pathology and epidemiology of TB in the buffalo was similar to that in cattle, and was respiratory in nature. In the 1970s over much of the buffalo's range, TB was endemic and in places the prevalence was >25%. Although the grazing range of buffalo and cattle overlapped extensively the two species did not interact and there was little opportunity for transmission. Infection cycling independently within the two species. It was concluded that because of the lack of interspecies contact, buffalo were not a significant source of infection for cattle. In Australia, TB in the cattle population was eradicated by means of test and slaughter and, as the buffalo were not indigenous, had little economic potential, and caused significant damage in ecologically sensitive wetlands, depopulation was justified as a means to control the disease.

Brushtail possums and multiple species involvement in New Zealand

Where there are mixed populations of different wild animal species, each infected with M. bovis, it can be difficult to determine which of the species are significant. In New Zealand the TB control scheme in cattle had made significant by the late 1970s to the point where eradication was considered achievable (O'Neil and Pharo, 1995). The recognition of wild animal involvement in the infection of cattle and domestic deer led

to a re-evaluation of the eradication scheme. In recent years great progress has been made in the control of the disease, with the number of infected cattle and deer herds decreasing over the last decade (Animal Health Board, 2004).

The role of wild animals in the epidemiology of TB in domestic animals was difficult to evaluate because of the number of species involved (Table 1). In order for control to proceed, detailed research was undertaken to identify the role of each infected wild animal species, and was based on the following criteria:

1. The pathology of infection in each species and their potential to excrete M. bovis that could lead to infection in other species by either direct contact, aerosol transmission or ingestion;
2. Interaction between the wild and domestic animals, particularly interaction conducive to transmission by one of the available routes.
3. The geographic distribution of the infected wild animals.

Using this approach, the brushtail possum was identified as the single most significant wild animal source of infection. Although the prevalence is generally very low, 1% - 2%, it may exceed 50% in rare situations (Coleman et al., 1994). TB in possums is a fulminating disease, with the clinical phase lasting only 2 - 6 months. Possums die from pulmonary TB, have extensive lung caseation and necrosis (Jackson et al., 1995), and excrete a highly infectious aerosol (Corner et al., 2002). Terminally-ill possums become disorientated, develop a staggering gait, and lose their fear of cattle (Julian 1981). Cattle and deer are attracted to the dying possum by its abnormal behaviour, and investigate by licking and biting, thus being exposed to infectious aerosols (Paterson and Morris, 1995; Sauter and Morris, 1995). Additional evidence supporting the role of possums was that the same strain types of M. bovis occurred in sympatric populations of cattle and possums, and that culling of infected possum populations decreased in the risk of breakdown in cattle herds (Caley et al., 1999).

Infection in other wild animal species did not have the characteristics necessary for them to be a significant threat to domestic livestock. However, the epidemiology and significance of TB in the ferret population remained unclear (see below).

Ferrets in New Zealand

Whereas the role of the possum in the epidemiology of the TB in livestock has been established, the role of the ferret remains unclear. M. bovis infection in ferrets is most frequently associated with the mesenteric lymph nodes, with a high proportion of animals showing no gross lesions and respiratory lesions are rare (Lugton et al., 1997a). Infected ferrets may excrete M. bovis in faeces. Tuberculous ferrets are found widely in New Zealand and the prevalence may exceed 90% (Lugton et al., 1997b). Based on the diet and lesion distribution in ferrets it is clear the principal route of infection is ingestion, and the most prevalent infected prey are possums (Caley et al., 2001). The ferret is primarily a spillover host but may be a maintenance host where high population densities exist, and these are uncommon in New Zealand (Caley and Hone, 2004).

Of critical concern is whether ferrets can act as a reservoir of M. bovis for livestock. Intuitively, one would suspect that they could, given the high prevalence in some

populations. It is argued that culling of ferrets is justified on the basis of the high prevalence, but the argument fails to take heed of the necessity for all the elements in the transmission pathway to be present; that is, a source of the organism, a susceptible host and the appropriate environment for transmission to occur. What are in doubt are the first and third elements: excretion from ferrets in a way that could lead to infection in cattle, and no appropriate interactions enabling cattle to encounter an infectious dose. M. bovis in faeces is not generally be available to cattle, and interactions between cattle and ferrets conducive to aerosol transmission are unlikely (Sauter and Morris, 1995). Although there may be well-founded ecological, political and public relations justifications for culling ferrets, culling to control TB in cattle can not be justified on epidemiological grounds.

White-tailed deer in Michigan (USA)

The first tuberculous wild white-tailed deer was detected in Michigan in 1975 and it was believed a chance spillover from cattle (Schmitt et al., 1997). A second deer was detected in 1994 when the state had acquired a TB-free status for cattle. A survey of hunter-killed deer in 1995 found 12 infected animals, and subsequent studies have shown that although the overall prevalence of TB in deer was low, there was a focus of high prevalence in the north of the state (O'Brien et al., 2002).

Tuberculous lesions in white-tailed deer are associated with the upper and lower respiratory tract and the lymph nodes of the head, especially the medial retropharyngeal lymph nodes (O'Brien et al., 2001). Deer excrete from lung lesions resulting in aerosol transmission between animals, but excretion from superficial draining lymph node lesions, oral transmission may also occur.

The high prevalence of M. bovis infection in deer was associated with the changed management of the wild deer population (Schmitt et al., 2002). White-tailed deer are part of the natural wild fauna of Michigan, and the population is managed for hunting. A change in the management of the wild herds lead to a dramatic increase in numbers, from 0.7 to 1.7 million in 40 years (O'Brien et al., 2002), and a change in the behaviour of the deer. The population in 2002 was above the natural capacity of the environment and had been achieved by supplementary feeding during the winter (a practice now banned) (O'Brien et al., 2002). Such feeding causes deer to congregate around feed dumps, and the high densities undoubtedly facilitated aerosol transmission. Oral transmission may also be facilitated through contamination of the food piles.

The re-emergence of TB in cattle in Michigan was believed to have been due to the reservoir of infection in the white-tailed deer. If deer were the source of infection for cattle, either directly or indirectly, the mechanism of transmission will be difficult to determine because transmission events were rare. The TB problem in Michigan deer is focused in a 5-county core area (O'Brien et al., 2002). The cattle population of the state is 1.9 million, with 11,000 cattle in 548 herds and 100,000 deer in the 5-county core area (Schmitt, personal communication, 2005). Over the 8 years up until 2004, there were 32 outbreaks in cattle in the state, including some not directly attributable to deer. In 26 of these outbreaks, only one or two infected cattle were involved (Judge, 2005). Studies

of cattle and deer behaviour on pasture show no direct interactions occur (DeLiberto et al., 2005). Wild animal surveys have found M. bovis infection in 16 other species (Schmitt et al., 2002) but none showed pathology that implicated them as a source of infection for cattle (Bruning-Fann et al., 2001). Therefore transmission process between deer and cattle if it occurs at all, is unclear. The low prevalence of TB in cattle could be due to either aerosol transmission from deer but interactions are rare, to transmission by ingestion, or by another uncommon and less obvious route. Alternatively, a third animal species may be involved.

TUBERCULOSIS IN BADGERS AND TRANSMISSION TO CATTLE

M. bovis infection is endemic in the wild badger population in Ireland and widespread in populations in the England and Wales (Eves, 1999; Nolan and Wilesmith, 1994). In both countries infection is also present in deer (free-ranging, semi-wild and semi-domestic deer) but in the UK is infrequent in other wild animals (Table 2; Delahay et al., 2002; Mathews et al., 2006).

The eradication of TB from domestic livestock in Ireland and Great Britain have been compromised by re-infection from infected badgers (Krebs et al., 1997; Gormley and Collins, 2000; Ó'Máirtín et al., 1998; Griffin et al., 2005). In both countries the badger is a protected species and of social and ecological importance. In Ireland, the prevalence of TB in badgers culled during the TB control program is >42% (Corner unpublished). That badgers are a major reservoir of infection in Ireland has been demonstrated by the significant drop in the incidence of TB in cattle following the systemic and sustained removal of infected badger populations (Ó'Máirtín et al., 1998; Griffin et al., 2005).

Tuberculosis in badgers

Tuberculosis in badgers is primarily a respiratory disease with transmission principally by infectious aerosols (Nolan and Wilesmith, 1994). After infection there is a slow progression to overt disease (Gallagher et al., 1998). Badgers seem able to limit the progress of infection and 50% - 80% of infected badgers have no visible lesions (NVL) (Clifton-Hadley et al., 1993; Corner unpublished). Badgers with M. bovis infection may survive for years (Little et al., 1982; Clifton-Hadley et al., 1993) and tuberculosis does not have any significant effect on the size or structure of badger populations (Wilkinson et al., 2000).

M. bovis is also transmitted by biting when wounds become contaminated with M. bovis in buccal cavity saliva. In high density badger populations in south-west England, bite wounds were seen as a significant route of transmission (Cheeseman et al., 1989; Macdonald et al., 2004). Lesions in the buccal cavity and pharynx have not been described, so it is assumed that the contamination originates from the lower respiratory tract.

Transmission to cattle

To understand and assess the risk of transmission of M. bovis to cattle from tuberculous

badgers, a number of factors must be considered: the susceptibility of cattle by each of the possible routes of infection, the minimum infectious dose for each route, and the probability (opportunity) of cattle to encounter an infectious dose by that route. Not only do badgers have to excrete M. bovis but the excreted organism must be available to the cattle, and the cattle must behave in such a way as to encounter the organism.

Table 2: M. bovis infection in some domestic and wild animal species in the United Kingdom.

Species found infected (Prevalence)				
Deer	Fallow deer	Roe deer	Sika deer	
Carnivores	Fox (1.2%)	Mink (0.5%)	Ferret (3.9%)	Domestic cat
Small mammals	Mole	Bank voles	Brown rat	

Species found not infected				
Carnivores	Stoat	Weasel	Polecat	Otter
Deer	Red Deer	Muntjac Deer		
	Domestic dog			
Medium mammals	Rabbit	Brown hare	Hedgehog	Grey Squirrel
Small	Common shrew	Pygmy shrew	Water shrew	Field voles
	Wood mouse	House mouse	Yellow-necked mouse	

References: Delahay et al., 2002; Mathews et al., 2006

Cattle are susceptible to infection with by a number of different routes but for each route their susceptibility differs. Under natural conditions, there are two routes by which M. bovis excreted by badgers could infect cattle, aerosol and ingestion. Cattle are very susceptible to respiratory infection, with the minimum infective dose ≤5 bacilli when delivered in an aerosol to the alveolar spaces (Chause 1913 cited by Francis, 1947; Neill et al., 1991). Only particles with an aerodynamic diameter of 1–10μ will after inhalation reach and be deposited in an alveolus.

The conditions necessary for successful aerosol transmission are exacting, and badgers and cattle must interact closely in both time and space. There is a very short period of risk due to the rapid bactericidal effects of desiccation, ozone, ultraviolet irradiation from sunlight, and dilution in, and the movement of air (Salem and Gardner, 1994; Cox, 1995; Gannon et al 2006). M. bovis is deposited onto soil or vegetation represent a low risk of causing respiratory infection. While infective droplet nuclei may

be resuspended after settling, dust particles generated from dried sputum containing M. tuberculosis were found not to be infectious because of their large size (>10 μ; O'Grady and Riley 1963).

In contrast, cattle are insensitive to oral challenge where the minimum infective oral dose is 104 - 107 bacilli (Francis, 1947; O'Reilly and Daborn, 1995; Palmer et al., 2004). M. bovis is very susceptible to the bactericidal effects of gastric secretions (Gaudier and Gernez-Rieux, 1962, cited by Smith and Moss, 1994) and M. bovis survives for only a short time in rumen fluid (B. Gannon pers com 2007). Only from a few of the most heavily infected badgers would excreta contain sufficient M. bovis to infect cattle by ingestion.

The infectiousness of tuberculous badgers can be divided into two periods. During the subclinical and early clinical stages of disease tuberculous badgers would behave normally; they may excrete infectious aerosols with increasing infectivity as the disease progresses (Gallagher, 1998). Aerosol transmission from subclinically infected badgers is unlikely on pasture because of the avoidance behaviour of healthy badgers (Benham, 1993; Sleeman and Mulcahy, 1993; Hutchings and Harris, 1999). During the terminal stage of disease badgers are highly infectious (Gallagher, 1998), they may change their behaviour, become active during daylight and lose their fear of cattle (Muirhead et al., 1974). As with possums, cattle are attracted to will investigate wild animals behaving abnormally. Terminally-ill badgers, excreting highly infectious aerosols, thus pose a risk to cattle interacting with them.

Badgers are known to enter cattle houses, and in this situation there is the opportunity for close contact with cattle. The potential for spread during long periods of contact is exacerbated by the spatial distribution of feed (in troughs or on the floor) which limits where badgers are likely to forage.

The evidence from the distribution of lesions is that oral infection of cattle is not significant. In addition, cattle are averse to ingesting food contaminated with badger urine or faeces (Benham and Broom, 1991). Further, excreted bacilli, particularly in urine, would rapidly become unavailable when absorbed and diluted in soil, or distributed over vegetation (MAFF, 1979) and the bacilli are not available for cattle to ingest at the rate required to deliver an infective dose (Gallagher, 1998). There is negligible risk to cattle from the slow intake of a low numbers of bacilli over a period of days or weeks. The paucity of reports of TB in wild rabbits or domestic sheep, two species very susceptible to experimental infection with M. bovis (Wilson and Miles, 1975; Cordes et al., 1981), strongly supports the view that pasture contamination is of minimal significance.

CONCLUSIONS

Identifying the role of each species in the epidemiology of M. bovis infection in a multi-species ecological system poses many problems. Much can be learned about the process from the research involving wild animal species where they are associated with the control programmes in domestic livestock. It is prudent to rely on scientific data when defining the role of the wild animal species and its significance as a reservoir

of infection, because if wrongly assigned it may be hard to subsequently counter the negative perception.

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DESCRIPTION OF A MEDIUM TERM NATIONAL STRATEGY TOWARD ERADICATION OF TUBERCULOSIS IN CATTLE IN IRELAND

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ABSTRACT

A compulsory National Bovine Tuberculosis (TB) eradication program has been operating in the Republic of Ireland since 1959. Substantial progress was achieved in the early decades, but since the mid '70s there has been no improvement despite the continuing application of an intensive national tuberculin testing program. Geographical information systems techniques have been used to identify areas of the county where tuberculosis is consistently identified at high incidence levels. Each year, circa 70% of all standard "skin test" reactors are drawn from roughly 30% of the area of agricultural land. These areas and the techniques used to delineate them are described. A strategy based on reducing the local densities of badgers, weighted toward more intensive removals in the areas of the country defined as "chronic" is described. In the short term this will result in lowering the risk of cattle herds becoming infected with TB from TB infected badgers in the local environment. The frequency of significant cattle:badger interactions will be reduced as a consequence of the local reduction in the density of both cattle and badgers. Badger culling is carefully planned and rigorously monitored and only take place as a sequel to an epidemiological investigation carried out by State Veterinarians who must follow a standardised protocol. The medium term strategy targets a 25-30% reduction in the national badger population. This strategy will be re-visited when the results of planned vaccine trials have been evaluated.

INTRODUCTION

A voluntary test and slaughter scheme commenced in the Irish Republic in 1954 when tuberculosis levels in cattle were at very high levels with 80% of herds and 17% of animals infected. The test used was the Single Intradermal Comparative Tuberculin Test (SICTT). The scheme became compulsory in 1959, involved the whole country by 1962 and has been in existence since. The Irish cattle population numbered 4.5

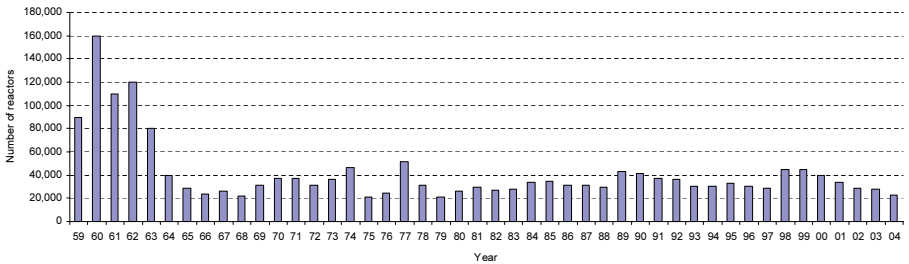

Figure 1: Test positives identified 1959–2004 in Ireland

million animals in 250,000 herds in the late 50's, and initially in excess of 100,000 test positive animals were removed annually (Watchorn, 1965). By 1965, test positive animals being removed had fallen to 40,000 animals annually, representing an animal prevalence of circa 0.5% (More, *et al*., 2006). The rapid progress witnessed in the early years of the eradication programme was not maintained and the testing programme has consistently identified between 20,000 and 40,000 test positives each year since 1965 (Figure 1). The national cattle herd is now 7 million bovines and these are farmed in 125,000 herds with an animal prevalence of 0.3%.

The European Union (EU) trading directive governing this area is Directive 64/432/EEC and the Irish scheme conforms to this directive. The SICTT skin test is applied to every herd as a surveillance test each year, and animals that react positively to the test are removed and slaughtered and the herds retested at 60-day intervals from when the test positives were removed until they have passed 2 clear herd tests. In addition to the annual surveillance test for all herds, animals cannot be slaughtered unless they have passed a SICTT within the previous 365 days. When infected herds are identified, neighbouring herds are tested at a frequency greater than the annual test while infection remains locally. Ancillary testing is carried out on infected herds using the Interferon-gamma blood test.

While such a comprehensive testing regime would be expected to successfully eradicate tuberculosis from the national cattle herd, as was the experience in many of our EU neighbours, this has not happened in Ireland nor in the United Kingdom of Great Britain and Northern Ireland. The reason for this is the presence of tuberculosis in a wildlife species, *Meles meles*, the Eurasian badger. Tuberculosis in this species is endemic in Ireland. Where badgers have been culled in areas where seriously infected cattle herds also exist, upwards of 40% of the badgers are culture positive for *M. bovis* (L. Corner, personal communication).

The first infected badger was detected in Ireland in 1974 (Noonan *et al*., 1975), and this led to a number of formal studies that attempted to identify a link between tuberculosis in badgers and tuberculosis in cattle in the same local areas. The first of these studies, called the East Offaly Study, was carried out between 1989 and 1994 and demonstrated that a marked reduction in levels of tuberculosis in cattle was observed when the local badger population was maintained at low levels (Dolan, *et al*., 1995). This study was confined to one geographic area, which was not representative of the land types found throughout Ireland, hence, the results from the study while compelling were not conclusive. A follow-up study, the Four Area Project (FOP), was carried out at 4 sites between 1998 and 2002 and reductions in tuberculosis in cattle were again observed following the removal of badgers (Griffin, *et al.,* 2005). These studies have shown that reducing the density of badgers over a wide area (the removal sites in each of the four areas averaged circa 250 km^2) to perhaps 20% of their original density and maintaining these lower densities over a number of years resulted in reduced levels of tuberculosis in cattle.

While it is acknowledged that eliminating badgers in Ireland would likely result in a more successful cattle tuberculosis eradication scheme, such a policy would be unacceptable at a number of levels. At the societal level, the destruction of one of our

important native large species of mammal would be completely unthinkable. The EU is a signatory of the 1989 Convention on the Conservation of European Wildlife and Natural Habitats (Berne Convention), and the Irish government ratified this treaty in 1982. Under Irish Law (The Wildlife Act, 1976), the badger is a protected species including protection of the underground burrows (setts) where badgers live and raise their young.

COMPONENTS OF THE NATIONAL STRATEGY

Social partnership

In Ireland since 1987, a key component in the shaping of government policy has been a process termed "Social Partnership". Since 1987, there have been 6 agreements between the Government of the day and groupings that are referred to collectively as the "social partners". The social partnership is built on 4 pillars (Trade Unions, Employers and Business representatives, Farming representatives and Community and Voluntary groupings) who with government agree on a programme that incorporates a wide range of measures such as taxation matters, wage rises and elements of social/economic policy. The process has led to a prolonged period of economic stability and to levels of national prosperity that were not attained previously. During the years 1991 to 2002, economic growth in the Irish economy averaged 7.3% per year--- albeit starting from a low base compared to then EU averages. These levels of growth matched the expansion in the economies of Singapore and China and spawned the labels "the Celtic and Asian Tigers" in the popular press.

Table 1: The relevant extract from the PPF section on animal disease eradication (page 68) states:

15. All parties involved agree to the adoption of necessary measures with the objective of reducing current levels of, and ultimately eradication, of Brucellosis, TB and other significant animal disease from the national herd. The primary constraint on containing and eradicating TB is the existence of a significant reservoir of infection amongst the wildlife population. To reduce the incidence of TB by 50%, and to make significant progress towards the eradication of brucellosis, within the next four years, the following measures will be taken:

- Commit specified staff resources in each District Veterinary Office (DVO) to carry out investigative work into the cause of breakdowns; and
- In addition to current arrangements relating to wildlife, take a pro-active approach in each DVO area, using 75 dedicated Departmental and Farm Relief Service personnel, to the removal of all sources of infection in the 20% of the country which yields some 50% of the current TB reactors; the distribution of these resources will be finalised in

The current interim eradication strategy is based on agreements reached in the partnership process of 2000 (Programme for Prosperity and Fairness (PPF), 2000). The mechanism whereby the matters agreed by the government and the social partners

become incorporated into policy measures, involves further negotiation between the government departments/agencies responsible for the policy areas and the individual groups from within the social partnership structure most closely involved in the area. In this case the Department of Agriculture and Food (DAF) is responsible for conducting the statutory bovine tuberculosis eradication programme and the Parks and Wildlife division of the Department of Environment, Heritage and Local Government are responsible for implementing the Wildlife Act, 1976, under which badgers are a protected species. The social partners with an interest in the area are the representatives of the farming bodies and the representatives of the conservation organisations such as Irish Wildlife Trust and Badgerwatch Ireland. The objective of the interim strategy is that it conforms with the spirit of what was agreed by the negotiators of the primary PPF document (Table 1).

Conservation safeguards

Because the ultimate eradication of tuberculosis in cattle is contingent on reducing the levels of tuberculosis in the national badger population, a research programme is being undertaken to quantify the protective effects of vaccinating badgers with bacillus Calmette-Guerin (BCG) vaccine. Experiments to date (L. Corner, pers. communication) have demonstrated that BCG administered orally to badgers results in protective effects against experimental infection with *M. bovis*. A large-scale field trial is planned, beginning in late 2006, where the protective effect of BCG vaccination of badgers in the wild will be evaluated. Final decisions regarding selecting components of the national strategy that will help lead to the ultimate eradication of tuberculosis must await the completion of this trial.

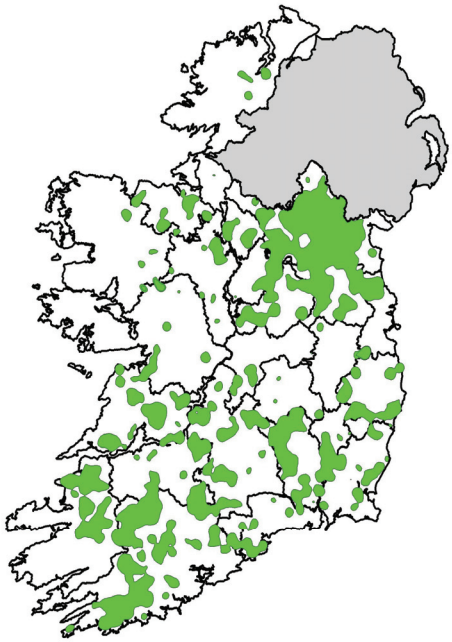

Figure 2: "Chronic" TB areas in Ireland

Assuming vaccination of badgers will be part of any final strategy, and as the business of farming must continue under the terms of the PPF agreement, a medium term eradication strategy was formulated. Conservation of a healthy badger population nationally is a key objective of the medium term strategy, so at the heart of the strategy is a commitment on the part of DAF to guarantee that capturing of badgers will be confined, cumulatively, to not more than 30% of the agricultural

land of the country over the lifetime of the strategy which began on 1st. January, 2004. At the end of 2006, capturing is ongoing on 14%* of agricultural land (*lands captured are calculated as within 0.5 km radius of any sett approved for capture).

The national capturing effort is not evenly applied, and is more intensive in those areas where tuberculosis in cattle herds is persistent (Figure 2). Using kernelling (spatial smoothing) techniques, it is possible to delineate areas comprising roughly 30% of the agricultural land which during 1998/1999/2000 yielded roughly 70% of all standard interpretation skin test positives via the SICTT (72 hour bovine increase by more than 4mm greater than 72 hour avian increase). In the areas marked green on the map, capture will not take place over more than 60% of the agricultural land, whereas elsewhere, capturing will be capped at 20% of agricultural land. Overall, this guarantee will ensure that capturing will never exceed an area greater than 30% of the agricultural land in Ireland, or conversely badger habitats in 70% of the agricultural land will be safeguarded.

Epidemiology of tuberculosis in Ireland in 2006

Over 40 years of annual SICTT testing, involving upward of 9 million tests each year on a cattle population of 7 million along with the speedy removal of all test positives and many lower grade skin-test increases (20% of animals removed as reactors are non-standard) has altered how tuberculosis in manifested in Irish cattle herds (Martin *et al*, 2001). Clinical respiratory disease is not seen and carcases with generalised tuberculosis at slaughter are identified in fewer than 10 instances per annum from 1.5 million animals slaughtered (DAF unpublished data). Tuberculosis is now a sub-clinical disease in bovines in Ireland, and due to the annual "herd surveillance" testing programme, that removed infected cattle in the early stages of the disease process. A clearer understanding of tuberculosis in Irish cattle herds is possible if one uses the concept of episodes (O'Keeffe *et al*., 1998). An episode is the interval between a herd being placed on movement control and the lifting of movement controls. In the Irish context this spans an interval of between 150 to 200 days in the majority of herds, during which time three SICCT herd tests are applied (O'Keeffe *et al.,* 1996). Under EU rules, a herd must pass two clear herd tests at 60-day intervals following removal of test positives. A simple classification system based on episodes is outlined in Table 2 (O'Keeffe *et al*., 1998).

Table 2: Episode classification criteria and frequency 1989-1997

	No. standard positives	No positives with gross lesions	Frequency
Group 1	2 or more	1 or more	24.5%
Group 2	1	1 or more	17.0%
Group 3	0	1 or more	2.7%
Group 4	2 or more	0	8.4%
Group 5	1	0	24.2%
Group 6	0	0	23.2%

These data represent information on 90,554 episodes, in 60,337 individual herds. In 2006, there are roughly 124,000 herds that include cattle. Of the breakdown episodes examined during the interval 1989-1997, 23.2% (Group 6) did not have even a single test positive animal identified during three herd tests over the 150-200 day interval. The majority of these herds were not affected with tuberculosis, but were cases where movement controls were applied as a precautionary measure. A further 41.2% of herds (Groups 2 and 5) had only one standard test positive identified over the three tests, and while the majority of these were infected animals, the degree to which tuberculosis infection is present in other animals in the herd is open to conjecture.

One of the elements in the PPF commitments (Table 1) was that breakdowns of tuberculosis would be investigated and a cause for the outbreak sought. Therefore all Group 1 and a majority of Group 4 type episodes are now investigated by DAF veterinarians using a standard methodology (O'Keeffe, 1999). The primary objective of this investigation is to establish if an introduced animal is the likely source of the breakdown, and if not, to establish if badgers where present in the local environment of the herd. The other common sources of infection (i.e. residual carry over of within herd infection and contemporaneously infected neighbouring herds) are also evaluated where identified.

Limited capturing of badgers

If an introduced animal(s) is ruled out as causing the breakdown, and if signs of badger activity are found on lands of, or on lands adjacent to the index herd, a survey of the local area is organised by DAF staff to a radius of 1 km out from the affected farm. Where setts (badger burrows) are located, the locations are recorded on a GIS database along with the sett characteristics. In Ireland the majority of tuberculosis breakdowns are clustered geographically (O'Keeffe, 1994). Before capturing at any setts can take place, candidate setts must first be approved for capture by a staff person from the DAF Wildlife Unit who independently verifies that the sett is within 2 km of a tuberculosis-affected farm. Badgers are captured using a stopped-body restraint, and humanely euthanased using a 0.22 calibre bullet. Trained contractors, monitored and supervised by DAF staff, carry out capturing.

A further element of the medium term strategy is to limit any capturing during the months of January and February each year to areas that were captured previously. This measure was prompted by animal welfare concerns due to the risk of capturing lactating females that in turn would lead to the possibility of orphaned offspring. Returning to areas previously captured ensures a lower risk of capturing any badger, and an even lower risk of capturing a lactating female.

CONCLUSION

The strategy outlined is a pragmatic response, based on sound science, to a complex problem. The national badger population is a valued resource and the limitations applied to the proportion of lands where capturing will be permitted guarantee the survival of the species. The hope of developing an oral delivery system for BCG vaccination that will

reduce the impact of tuberculosis in badgers is a realistic one. Confining any capturing of badgers to areas where herds have first been identified with severe tuberculosis that the source was not introduced infected cattle, is a further safeguard against unnecessary removal of badgers. Removing heavily infected badgers from localities where cattle breakdowns have been identified can only but benefit the surviving test negative cattle as well as the badgers in the wider area surrounding the removal zones. Doing nothing when confronted by a known problem is not an option in the Irish situation. Evolving a strategy among a diverse range of stakeholders which is a sub-optimal solution for some, but which is accepted by all as a fair compromise, is triumph for common sense and a tribute to the generosity of all involved.

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THE DEVELOPMENT OF THE BCG VACCINE FOR USE IN BADGERS IN IRELAND

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ABSTRACT

Mycobacterium bovis infection is endemic in badger populations in Ireland and studies have clearly demonstrated that tuberculosis in the badger constitutes a significant reservoir of infection for cattle. Limited badger culling is carried out in areas with a high risk of transmission to cattle but this is considered as an interim strategy pending the development of a vaccine strategy.

The aim of the badger BCG vaccine research programme is to establish if BCG vaccine is protective against tuberculosis in badgers and to provide scientific support for the incorporation of vaccination into the national tuberculosis control and eradication programme. The vaccine programme follows a logical sequence of studies using both wild badgers and captive badgers housed in a purpose built facility that simulates the badgers' natural environment. To date, the studies have examined pathogenesis of infection and the development and evaluation of *in vitro* diagnostic assays measuring immunological responses to vaccination and to *M. bovis* challenge. The BCG vaccine has been shown to induce protection in badgers when delivered by any one of three different routes – subcutaneous injection, mucosal (combination of intranasal aerosol and conjunctival instillation) and oral. The protective response limits the distribution and severity of tuberculosis in experimentally challenged badgers. A field trial has been designed to demonstrate that BCG is protective in wild badgers under conditions of natural transmission and also to estimate vaccine efficacy. The outline of this field trial is described.

INTRODUCTION

In Ireland, the badger is recognised as the principal wildlife reservoir of *Mycobacterium bovis* (*M. bovis*) infection (Eves 1999) and contributes to the spread and persistence of tuberculosis in associated cattle populations (Gormley and Collins 2000). Its important role has been demonstrated by the significant drop in prevalence of tuberculosis in cattle following the large scale removal of badger populations in both the East Offaly and Four Area Badger Projects .

As part of the interim strategy to control bovine tuberculosis, the Department of Agriculture and Food remove badgers where, as a result of an epidemiological

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investigation, they have been identified as the probable source of a tuberculosis outbreak in cattle. However, there are limitations to the use of culling in the long term and the elimination of an important native mammal species is unacceptable and undesirable (O'Keeffe 2006). There is a growing consensus that while continued annual tuberculin testing of herds will serve to maintain tuberculosis in cattle at a low level, the problem of *M. bovis* infection in badgers will not be solved without a vaccine (Gormley and Collins 2000).

In 2001 the badger BCG vaccine research programme was established in Ireland to study the protection against tuberculosis induced by BCG vaccine in badgers. It was envisaged that vaccination will replace or complement culling as a means of controlling tuberculosis in badger populations. A captive badger facility, called the BROC (Badger Research Observation Complex), was constructed to carry out experimental infection and vaccination-challenge studies. Additional studies on wild badgers in their natural environment were also conducted.

PEN STUDIES WITH CAPTIVE BADGERS

Experimental infection studies

The first captive badger study conducted at the BROC was an experimental infection study. An infection model was required to provide measurable parameters through which vaccine protection could be examined. The objective of this study (BROC 1) was to identify a dose of *M. bovis* which, when delivered by the endobronchial route to badgers, generated a disease profile that mimicked natural infection. Low (<10 CFU), medium (10^2 CFU) and high (3×10^3 CFU) doses were used and the highest dose proved optimal for challenge studies . The badgers were very susceptible to infection by this procedure, even with a dose of <10 CFU.

Having established the utility of the endobronchial route and an effective challenge dose, a second study was designed to use the optimal infective dose (derived from BROC 1) and follow the progress of disease over time. The aim of BROC 2 was to study the progression of infection following endobronchial challenge and determine the optimum time for post mortem examination. Following infection, the disease progressed slowly with a uniform result across the badgers studied. The optimal time to examine disease was found to be 12-18 weeks post infection.

Vaccination-challenge studies

Having established the experimental infection procedure, it was necessary to determine if BCG was protective in badgers and to examine different routes of vaccination. Eight parameters were used to measure the outcome of the vaccination-challenge studies: gross pathology ((i) number of sites with lesions and (ii) gross lesion severity scores), histopathology ((iii) number of sites with lesions and (iv) number of extrathoracic sites) and bacteriology ((v) number of sites of infection, (vi) number of extrathoracic sites, (vii) bacterial burden in the lungs and (viii) bacterial burden in the thoracic lymph nodes).

The first vaccination-challenge study demonstrated that badgers vaccinated with BCG by the subcutaneous and mucosal (intranasal and intraconjunctival) routes generated protection when challenged by the endobronchial infection procedure. Vaccination by either route with ~10^6 CFU BCG Pasteur led to a significant difference in the gross lesion severity score of disease between vaccinates and non-vaccinated control animals.

If BCG vaccination of wildlife is to be a practical option in the field, an oral bait will be the most efficient means of large scale delivery. A lipid formulation that protects the live BCG from gastric secretions has been developed . Having demonstrated that BCG generated protection in badgers, and that a mucosal route was highly effective, we wanted to test the levels of protection generated by the BCG vaccine when delivered by the oral route. The vaccination-challenge study demonstrated that badgers vaccinated by oral routes with the lipid-encapsulated BCG were protected against challenge with *M. bovis*. A significant difference in the gross lesion severity score was found between vaccinates and non-vaccinated control animals and the other parameters followed a similar trend.

Having demonstrated that oral delivered BCG could induce protection the aim of the next experiment was to establish the duration of protection from a single oral dose of BCG. Badgers were vaccinated by oral routes with a lipid-encapsulated BCG and challenged up to 12 months post-vaccination. Although bacteriological and histopathological results are still pending, there was significant protection still evident at 12 months post-vaccination.

In line with a 1994 recommendation by the WHO/FAO, all initial BCG vaccine studies in badgers were conducted using BCG Pasteur. Currently, the only commercially available strain in Europe is BCG Danish. Therefore, the last study compared the protective effect of oral BCG Danish and oral BCG Pasteur. This study was completed earlier this year and all the results will become available later in the year. The gross lesion severity score have been indicative of protection in earlier studies and in this study vaccinates and non-vaccinated control animals showed a significant difference.

Immunodiagnostic assays for the detection of *M. bovis* in live badgers

Immunological assays for accurate diagnosis of *M. bovis* infection in live badgers are essential for disease surveillance in the badger population and to monitor any wildlife vaccination trial. As part of the captive badger studies a range of in vitro diagnostic assays, based on serology and cell mediated immunity (CMI), have been developed and assessed in collaboration with the Veterinary Laboratory Agency (VLA) in the UK. Serological tests for detection of *M. bovis* in live badgers include the Brock test, MAPIA (multi antigen print immunoassay), the Pritest and the Rapid test. The rapid test is the first 'animal-side' diagnostic test for tuberculosis in badgers providing results within 10 minutes . CMI based assays that have been developed for use in the badger include a lymphocyte transformation assay, a whole blood interferon-γ (IFNγ) ELISA and an ELISPOT also for badger INFγ. The immunoassays developed during the captive badger studies were validated in wild naturally infected badgers. Blood and serum were collected from 215 wild badgers that were subjected to a detailed post mortem with bacterial culture of tissues to accurately determine their true tuberculosis status.

The performance of the diagnostic immunoassays is currently being determined against this "gold standard".

BCG VACCINE FIELD TRIAL

Captive badger studies are an effective way of examining aspects of the immune response to vaccine and the ability of vaccine to generate protection. The BCG vaccine has been shown to be protective in captive badgers when they are challenged with a high dose of *M. bovis*. However, before vaccination can be used in the national bovine tuberculosis eradication programme it must be established that BCG can induce protection in a wild badger population subjected to natural challenge doses. In addition, to develop suitable strategies for vaccine application, vaccine efficacy (VE), that is, the number of new cases of tuberculosis prevented by vaccination, needs to be estimated. Of a number of trial designs available the most robust is to vaccinate a fixed proportion e.g. 50%, of a population. This study design follows the example of the demonstration of protection and the estimation of vaccine efficacy used in New Zealand for a field trial in wild brushtail possums.

The primary objective of the trial will be to validate the results of captive badger studies and demonstrate that BCG is protective in wild badgers under conditions of natural transmission and to estimate vaccine efficacy. Protection and vaccine efficacy will be estimated by comparing the prevalence of *M. bovis* infection in the two treatment groups. A secondary objective is to examine the effect of post-infection vaccination. The effect of this in badgers, if present, will be measured through differences in survival time and from the nature of the disease in infected-vaccinated versus infected-non-vaccinates. The case definition for protection and vaccine efficacy will be the bacteriological isolation of *M. bovis* from either clinical samples or from post mortem samples. Estimates of infection incidence will be estimated from changes in immunological parameters.

The field trial will, of necessity, use oral delivery of vaccine as that is the most probable and cost effective method for broad scale application of vaccine. The trial will provide a practical base for understanding the logistics of oral delivery to wild badger populations. The trial will be conducted over 4 years with vaccinations occurring during the first 3 years and badgers will be re-vaccinated annually. During the trial the badger population of 300 – 400 badgers with ~30% prevalence, will be captured 3 times a year using a capture-tag-release regime. At each capture blood samples will be collected for immunodiagnostic assays. Both cell-mediated and humoral immune responses will be used to determine the badger's infection status and to detect a change in infection status, that is, to detect the presence of pre-existing infection when badgers are recruited to the study and the appearance of new infection on recapture. At the end of the vaccine period the trial site will be depopulated and all the badgers will be subjected to a detailed post mortem examination with collection of tissues for bacteriology and histopathology.

The outcome of the field trial will be used to develop strategies for the use of the vaccine in the field and to inform policy. It will also provide support for the registration

of the vaccine for use in badgers and will inform debate on the use of live vaccines. At present, several strategies for vaccine use can be considered but the choices need to be refined before implementation. These decisions can best be made with the assistance of modelling. Prevalence studies, studies on tuberculosis pathogenesis in badgers, badger reproduction strategies, and the diet of badgers and studies of bait-uptake will be conducted in parallel with the field trial and will provide valuable information for modelling where, when and how to vaccinate badgers. The infection transmission rates between badgers, and between badgers and cattle, need to be estimated to allow for accurate predictive modelling. These rates will be estimated from field studies and in controlled studies with captive badgers and cattle. Captive badger studies examining vaccine safety and efficacy issues will continue as these will be required for vaccine registration. If BCG vaccine is effective in controlling or eradicating the disease in badgers then the likelihood of eradicating this zoonotic disease from the cattle population will be greatly increased.

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STUDY OF THE EPIDEMIOLOGY OF BOVINE TUBERCULOSIS (BTB) IN SOUTH AFRICAN LIVESTOCK AND WILDLIFE BY MOLECULAR TYPING OF *MYCOBACTERIUM BOVIS*- PRELIMINARY RESULTS

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ABSTRACT

For better understanding of the transmission of bovine tuberculosis, reliable methods are required to determine the spread of individual *Mycobacterium bovis* strains. Four different molecular typing techniques [*IS* 6110, Polymorphic-G-C Rich Sequence

(PGRS), Variable Number of Tanderm Repeat (VNTR) and Spoligotyping] were applied to characterize *M. bovis* strains causing bovine tuberculosis in South African livestock and wildlife. A total of 90 isolates were analyzed, 30 from cattle, 46 from African buffalo (*Syncerus caffer*, Kruger National Park) and 14 from species other than buffalo and cattle (10/14 from the Kruger National Park). *IS* 6110 revealed 5 different banding patterns isolates with copy numbers ranging from 2-4 in 30 cattle isolates from 12 different farms in 4 Provinces i.e., Mpumalanga (13), Limpopo (2), North West (7) and Kwazulu Natal (8). Isolates harboring 2 copy numbers were predominant and identical (16/30). PGRS proved to be a better typing tool when compared to *IS* 6110 as 5 different banding patterns were produced from only 14 of the 30 isolates from 5 different farms, suggesting that each farm was infected by a different *M. bovis* strains. IS6110 revealed 5 different banding patterns in 26 Buffalo isolates. Two distinct VNTR genotypes were revealed by the Frothingham primers in another 20 buffalo isolates when VNTR typing was applied. These genotypes also differed in their spoligotypes (spoligotype 54 and 21), and there was some minor variation in a single VNTR loci within Spoligotype 21. From the results it is unlikely that the two genotypes have evolved from each other. All the species other than buffalo were of spoligotype 54, suggesting that although two molecular types are present in buffalo from KNP, only one type is spilling over into animals other than buffalo.

INTRODUCTION

Bovine tuberculosis, caused by *Mycobacterium bovis* has a complicated epidemiology involving interactions between cattle, and between cattle and infected wild life (Menzies and Neill, 2000, Johnston *et. al.,* 2005). In outbreaks, factors such as the movement of cattle, the spread of the disease from neighboring premises and the persistence of the infection on farms are of particular importance (Pollock and Neill, 2002) together with the role of infected wildlife, most notably the African buffalo (Syncerus *caffer*) in South Africa (Bengis, 2000). Since the 'National tuberculosis

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control and eradication scheme was introduced in 1969 (S.A), bovine tuberculosis could be successfully controlled in commercial cattle herds. In the Kruger National Park, African buffalo serve as the main reservoir of the disease since the initial diagnosis in 1990 (Bengis *et. al.,* 1996). The disease has now reached epidemic proportions in the Southern regions of the park and has effectively spread among buffalo herds in a northern direction leaving only a small pool of uninfected buffalo herds in the northern half of the park. *M. bovis* has spilled over into lions, cheetah, chacma baboon, greater kudu, leopard, spotted genet and hyena, and warthog (De Vos *et. al.,* 2001, Keet *et. al.,* 1996, Michel *et. al.,* 2000). The establishment of the Great Limpopo Transfrontier Park (GLTP), which link the KNP with the Limpopo National Park in Mozambique might increase the possibility of the disease eventually spreading in the GLTP and out of the park to neighboring livestock and also pose a threat to humans (Tuberculosis in wildlife and in the wildlife/livestock/humans interface: problems and solutions. Maputo and Kruger National Park, 4-7 October, 2004).

For control purposes, it is important to identify strains of *M. bovis* and trace their routes of transmission and subsequently identify the sources of the infection and clarify how the disease was spread (Anon, 2004). Genotyping (DNA fingerprinting) methods such as Restriction Enzyme Analysis (REA) and Restriction Fragment Length polymorphism (RFLP) using insertion sequence IS 6110 and the polymorphic GC-rich sequence (PGRS) as probes have provided useful epidemiological information (Collins, 1998, Michel and Hlokwe *et. al.,* 2006, unpublished data) even though they are laborious and slow techniques.

PCR based spoligotyping has been widely used to genotype *M. bovis* isolates (Haddad *et. al.,* 2004) and was found to be highly reproducible, rapid and reproduces reliable phylogenetic data in the United Kingdom (Smith *et. al.,* 2003), however the current spoligotyping assays lacks discrimination for *M. bovis* isolate in other countries like Northern Ireland (Skuce *et. al.,* 2002) and Australia, Canada, the Republic of Ireland and Iran (Cousins and others 1998). A novel class of genetic markers collectively known as 'variable number of tanderm repeats (VNTR) markers, was identified during the genome sequencing project of the *M. tuberculosis complex* bacteria. Several of these markers vary in copy number between the sequenced genomes for reference strains of *M. tuberculosis complex* bacteria and within test panels of clinical isolates of the *M. tuberculosis complex* bacteria including *M. bovis*. The VNTR markers are amplified by the PCR using specific flanking primers. The sizes (number of base pairs) of the unit that is repeated in tanderm at each locus is known from the sequencing of the reference strains, so that the number of repeats present at each locus for any typed isolates can be inferred to the length of the PCR product. The VNTR genotype is then expressed as a linear string of numbers recording the number of tanderm repeat at each locus assayed (Frothingham *et. al.,* 1998). Roring *et. al.,* (2004) described the NVTR type markers as highly discriminative for typing *M. bovis* isolates, especially those with low IS 6110 copy numbers.

The present study is aimed at evaluating different typing techniques to characterize and analyze *M. bovis* strains causing bovine tuberculosis in South African lifestock and wild life for disease control purpose.

MATERIALS AND METHOD

Animal species and tissue samples

Table 1.1: Shows the types and number of animal species, number of tissue samples cultured and location of the animals

Type of species	No. of animals	No. of tissue samples	District/Province
Buffalo	39	46	KNP, MP
Cattle	20	30	12 Farms: MP, LP, NW, KZN
Lion	6	6	4: KNP; 1: Hoedspruit; 1: unknown
Kudu	1	1	KNP
Impala	1	1	Malelane
Meercat	1	1	Unknown
Leopard	2	2	KNP
Warthog	1	1	KNP
Bushbuck	1	1	KNP
Hyena	1	1	KNP

Isolation and identification of *M. bovis*

Routine diagnostic tissue samples (collected between end of 2003 to 2006) from cattle, buffalo, Lion, Kudu, Impala, Meercat, Leopard, Warthog, Bushbuck and Hyena submitted in the Tuberculosis laboratory (Onderstepoort Veterinary Institute) were used for *M. bovis* isolation following the method described by Bengis *et.al.,* 1996. The resulting *M. bovis* isolates (identified by Ziehl Neelsen and PCR procedures) were subcultured on Lowenstein-Jensen media slopes supplemented with pyruvate and incubated at 37 ºC until sufficient growth was observed.

Chromosomal DNA extraction

The extraction of DNA from *M. bovis* isolates was done by using the Puregene DNA isolation kit following the manufacturer's instructions with minor modifications. Overnight glycine-treated cells were heat killed at 94°C for 10 minutes and allowed to cool down. 500µl of MSG extraction buffer (50g/L Monosodium Glutamic Acid; 6.06g/L Tris; 9.3g/L EDTA) were added to the culture and cells were loosen by using an innoculating loop. 300µl of lysis solution were added following centrifugation, and 5µl RNAse A solution (4mgml) was added to the sample and incubated at 37°C for 30 minutes. 150 µl of protein precipitation solution were added and the sample was centrifuged at maximum speed for 2 minutes, followed by precipitation of the aqueous

phase with isopropanol. The resulting pellet was dissolved in 40µl of TE buffer (10mM Tris-HCL; 1mM EDTA).

Restriction Fragment Length Polymorphism- IS 6110 and PGRS typing

DNA restriction digest, Southern blotting, and hybridization

Chromosomal DNA was digested with P*vu* II (IS6110 typing) and Alu I (PGRS typing) restriction enzymes in a 37°C water bath overnight. The resulting fragments were electrophoressed on 0.8% and 1.2% agarose gels for IS6110 and PGRS typing respectively. Southern blotting was done by using the Hybond N^+ membrane in 20X standard saline citrate (175.3g/L NaCL; 88.2g/L Trisodium Citrate hyhrous) overnight (Skuce *et. al.,* 1994 and Cousins *et. al.,* 1998b). The IS6110 and PGRS probes were synthesized and labeled by using the DIG probe synthesis kit, and hybridization of the probes was done by using the DIG-Easy Hybridization granules following the manufacturer's instruction. For detection, DIG wash and block buffer set was used and CSPD was a chemiluminescence substrate for alkaline phosphatase. P*vu*II and Alu I restriction enzymes, CSPD and all the kits used in this study were purchased from Roche diagnostics. The Hybond N^+ membrane was from AEC-Amersham. Analysis of the resulting IS 6110 and PGRS banding patterns was done manually.

Spoligotyping

Spoligotyping (PCR- based) of 20 buffalo isolates and 14 isolates from species other that buffalo and cattle were performed according to the method of Kamerbreek *et. al.,* (1997), at the Veterinary Laboratory Agencies, Weybrigdge, United Kingdom.

VNTR typing

VNTR typing (PCR-based) of 20 buffalo isolates and 14 isolates from species other that buffalo and cattle was performed according to the method of Frothingham *et. al.,* (1998), at the Veterinary Laboratory Agencies, Weybrigdge, United Kingdom. The frothingham primer (Applied Biosystems) sets targeting six loci (A, B, C, D, E, F) were used in a final reaction volume of 25µl including 2µl of *M. bovis* cell lysate or DNA. PCR products were analyzed by using both ABA 377 Sequencer (all twenty isolates) and by gel electrophoresis (only 2 isolates)

RESULTS AND DISCUSSION

DNA finger printing of bacterial pathogens like *M. tuberculosis* and *M. bovis* has been widely used to determine the routes of transmission and monitor control programmes. In this study, four different typing techniques (IS 6110, PGRS, Spoligotyping, VNTR typing) were applied to characterize and analyze *M. bovis* isolates from South African cattle and wildlife for epidemiological purposes. A total of ninety isolates from cattle (30), buffalo (46), 14 isolates from different species other than cattle and buffalo, which included lion (6), Kudu (1), impala (1), meercat (1), leopard (2), warthog (1),

bushbuck (1) and hyena (1). The districts of origin of the animal species are indicated in table 1.1.

IS *6110* typing

Cattle isolates (Table 1.2)

Thirty *M. bovis* isolates were subjected to Southern blot analysis using DIG labeled IS *6110* as a probe. Five different patterns were found, and the number of IS *6110* copies ranged from two to four. Isolates containing 2 copies were predominant (16 of 30) and the banding patterns of their DNA fingerprints were identical, even though the cattle were from different farms. DNA fingerprints of 2 isolates from epidemiologically related farms were amongst those similar fingerprints. In this case, infection by a common source was suggested and was later confirmed by PGRS typing in the previous study by Michel and Hlokwe *et. al*., 2006 (unpublished data). Isolates from different tissue samples originating from two cattle on same farm were found to be identical and had the highest copy number of all isolates. This similarity of the banding patterns indicates a systemic infection with a single *M. bovis* clone.

Buffalo isolates (Table 1.3)

Twenty six *M. bovis* isolates from buffaloes in the KNP were subjected to IS *6110* typing. Five different patterns were obtained and the IS *6110* copies ranged from 4-6. None of the DNA fingerprints from buffalo isolates were similar to those in cattle in this study, and therefore no link of transmission of bovine tuberculosis between the animals under study.

PGRS typing (Table 1.2)

Fourteen *M. bovis* isolates originating from 8 cattle on 6 different farms were subjected to a different restriction enzyme. For this purpose, the DNA from these isolates was digested with a restriction enzyme AluI, and PGRS probe was used. PGRS typing was performed to further differentiate *M. bovis* isolates from different farms with identical IS *6110* pattern. Five different DNA fingerprints were produced. Isolates from one animal produced identical pattern as it was the case in IS *6110* typing, and so were isolates from same or epidemiologically related farms.

Table 1.2 Shows Mycobacterium bovis isolates from cattle analyzed by IS 6110 and PGRS typing

TB No	Farm	District	Province	IS *6110* Pattern	IS *6110* Copy No	PGRS pattern
3924B	1	Middleburg	Mpumalanga	IS C1	2	PG C1
3931A	2	Naboomspruit	Limpopo	IS C1	2	PG C2
3931B	2	Naboomspruit	Limpopo	IS C1	2	PG C2
3980E	3	Middleburg	Mpumalanga	IS C1	2	PG C1
4518	4	Malelane	Mpumalanga	IS C2	3	PG C3
4520	4	Malelane	Mpumalanga	IS C2	3	PG C3
4524	4	Malelane	Mpumalanga	IS C2	3	PG C3
4572A	5	Bulhoek	NorthWest	IS C1	2	PG C4
4572B	5	Bulhoek	NorthWest	IS C1	2	PG C4
4572C	5	Bulhoek	NorthWest	IS C1	2	PG C4
4572D	5	Bulhoek	NorthWest	IS C1	2	PG C4
4572E	5	Bulhoek	NorthWest	IS C1	2	PG C4
4572F	5	Bulhoek	NorthWest	IS C1	2	PG C4
4592A	6	Middleburg	Mpumalanga	IS C1	2	PG C5
4613	7	Middleburg	Mpumalanga	IS C1	2	*
4615	7	Middleburg	Mpumalanga	IS C1	2	*
4617	7	Middleburg	Mpumalanga	IS C1	2	*
4620	7	Middleburg	Mpumalanga	IS C1	2	*
4759A	8	Bergville	Kwazulu Natal	IS C3	4	*
4759B	8	Bergville	Kwazulu Natal	IS C3	4	*
4760A	8	Bergville	Kwazulu Natal	IS C3	4	*
4760B	8	Bergville	Kwazulu Natal	IS C3	4	*
4760C	8	Bergville	Kwazulu Natal	IS C3	4	*
4760D	8	Bergville	Kwazulu Natal	IS C 3	4	*
4764C	9	Dipolaseng	Mpumalanga	IS C4	3	*
4765B	10	Vryberg	NorthWest	IS C5	4	*
4770B	11	Ogies	Mpumalanga	IS C4	3	*
4792D	8	Bergville	Kwazulu Natal	IS C3	4	*
4792E	8	Bergville	Kwazulu Natal	IS C3	4	*
4844	12	Nkomazi	Mpumalanga	IS C2	2	*

Table 1.3 Shows M. bovis isolates from Buffalo analyzed by IS 6110

TB No	Location	IS 6110 Pattern	IS 6110 Copy No
4577F	Skukuza	IS B1	5
4577D	Skukuza	IS B1	5
4581F	Skukuza	IS B1	5
4645H	Skukuza	IS B2	5
4649A	Skukuza	IS B1	5
4649D	Skukuza	IS B1	5
4684E	Skukuza	IS B1	5
4687B	Skukuza	IS B1	5
4801D	Skukuza	IS B1	5
4801E	Skukuza	IS B1	5
4858F	Skukuza	IS B1	5
4860F	Skukuza	IS B1	5
4862B	Skukuza	IS B1	5
4931C	Skukuza	IS B1	5
4932A	Skukuza	IS B1	5
4934C	Skukuza	IS B1	5
4804A	Skukuza	IS B3	4
4804B	Skukuza	IS B3	4
4804F	Skukuza	IS B3	4
4806A	Skukuza	IS B4	6
4812C	Skukuza	IS B2	5
4812J	Skukuza	IS B1	5
4816F	Skukuza	IS B1	5
4817B	Skukuza	IS B1	5
4818C	Skukuza	IS B5	6
4818D	Skukuza	IS B5	6

Spoligotyping and VNTR typing (Table 1.4)

The genetic profiles of 20 *M. bovis* isolates originating from buffalo and 14 isolates from different animal species other than buffalo and cattle were analyzed by Spoligotyping and VNTR typing. 0f 14 isolates from other species, 10 were from species from the Kruger National Park, and so were all buffalo isolates. VNTR typing determines the number of tanderm repeats in the chromosomes of *Mycobacterium tuberculosis* complex bacteria. Frothingham's primers sets (A-F) were used to test 6

loci on the chromosomes of *M. bovis* isolates. PCR products were analyzed by using both ABA 377 Sequencer (all twenty isolates) and by gel electrophoresis (Frothingham *et. al.,* 1998). The *M. tuberculosis complex* bacteria (including *M. bovis*), contains a DNA polymorphism within the direct repeat (DR) locus. The region comprises of a series of DR sequence of DNA, interspersed with no-repetitive sequences (Spacers). 43 non-repetitive sequences have been identified for use in spoligotyping. The presence or absence of specific spacers allows typing of isolates (Kamerbreek *et al.,* 1997).Two distinct genotypes were obtained in buffalo isolates, i.e., 6 4 3 4*4 3.1 (spoligotype 54, V1) and 6 5 5 4*3 3.1(spoligotype 21, V2) (with 10 isolates each). However, there was some minor variation in a single VNTR locus for two isolates with VNTR type V2. The findings of these study show that it is unlikely that V1 and V2 strains might have evolved from each other as they differ in 2 VNTR loci and also their spoligotypes. 8 of 10 isolates from species other than buffalo from the KNP were found to have identical VNTR profile (6 4 3 4^{*} 4 3.1). The VNTR profiles of the other 2 isolates differed to the rest of the isolates only on 1 and 2 VNTR loci, suggesting that the strains might have evolved from the one strain. The VNTR profile of a lion isolate from a game lodge in Hoedspruit shared a common profile with isolates form KNP, thus indicating an epidemiological link. VNTR profile of a meercat isolate (unidentified location) was found to be completely different from all other profiles and therefore suggesting infection by a different *M. bovis* strain type. All *M bovis* isolates from 14 species other than buffalo were of one spoligotype (spol 54). The results of this preliminary study show that although there seems to be two distinct *M. bovis* strain types in buffalo from KNP, only one strain (Spol 54) is spilled over and causes bovine tuberculosis in other animal species.

Table 1.4: Shows M. bovis isolates from buffaloes and other wild species analyzed by VNTR and spoligotyping

TB No.	Species	District	VNTR Type	VNTR Profile A B C D E F	Spoligotype
4649A	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
3894B	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
4766A	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
3890C	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
3096	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
3892D	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
2649B	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
4587H	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
3891A	Buffalo	KNP	V2	6 4 5 4* 3 3.1	Spol 21
3880B	Buffalo	KNP	V2	6 5 5 4* 1 3.1	Spol 21

3BN	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
3898I	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
4573D	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
3117	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
3119	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
4508E	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
3891A	Buffalo	KNP	V2	6 5 5 4* 3 3.1	Spol 21
2649A	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
3898F	Buffalo	KNP	V1	6 4 3 4* 4 3.1	Spol 54
2649D	Buffalo	KNP	V2	6 4 3 4* 3 3.1	Spol 21

CONCLUSION

From preliminary results of the study, it is concluded that:

- PGRS is a better genetic maker for strain differentiation in cattle isolates with few IS *6110* copies.
- IS *6110* typing is a better tool to differentiates buffalo isolates better with more that two IS 6110 copies.
- All isolates from same animal analyzed produced identical DNA fingerprints.
- No concurrent infection was observed as all isolates from same herds had same DNA fingerprinting patterns.
- Buffalo and cattle strains were different (when analyzed by IS *6110*), and therefore there is no indication of transmission from buffalo to cattle, or visa versa.
- IS *6110* and PGRS typing results were reproducible- same results were obtained from isolates that were previously typed.
- Two main buffalo *M. bovis strain* types exists in the Kruger National Park, but only one of them actually spill over to lions, leopards, warthogs, kudu, bushbuck, and hyena.

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OVERVIEW OF THE HEALTH IMPACT AND EPIDEMIOLOGICAL RISK ASSESSMENT OF SYRPHID FLY LARVAE WITH REFERENCE TO THE 2006 OUTBREAK

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ABSTRACT

A maggot recovered from the Plankenbrug River in December 2005, proved to be an immature stage of the drone fly, Eristalis tenax. E. tenax is a member of the so-called 'filth flies'. Filth flies are synanthropic insects since they found in close association with humans, human dwellings or animals kept for human use. These flies are strongly attracted to animal manure, human faeces and heavily contaminated and decaying organic waste.

An outbreak of rat-tailed maggots was reported after widespread power failures caused severe environmental pollution in the form of sewage overflows. In addition to this unexpected and sudden source of massive pollution, there is widespread pollution entering the environment from failed municipal sanitation systems that are slowly adding huge loads of sewage contamination to the environment.

Filth flies and their maggots breeding on or in contact with fecal matter make them important mechanical vectors of disease-causing organisms. Some of the pathogens carried by these insects are enteropathogenic Escherichia coli, Salmonella spp., Shigella spp. Cryptosporidium parvum and other water- and foodborne pathogens. E. tenax has also been conclusively proven to transmit Mycobacterium avium subspecies paratuberculosis on cattle farms. Mechanical transfer of C. parvum oocysts and Giardia lamblia cysts by maggots of filth flies can occur by surface contact, excretion (defecation), regurgitation or dislodgement.

Direct ingestion of water or food contaminated by maggots can cause human and animal myiasis. The usual symptom is diarrhoea but the actual syndrome depends on which part of the body is invaded. There is evidence that body orifices such as the rectum can be invaded by these maggots without passing through the intestinal tract. The occurrence of myiasis has been recorded in developing countries more frequently than in developed countries with properly maintained sewerage systems. Myiasis constitutes only a moderate health risk, but the presence of maggots in or around water represents a warning sign of contamination.

The syrphid flies are strongly attracted to human food and animal feed. Their maggots are strongly attracted to sewage and manure and carry important pathogens. Their ability to act as vectors for transmission of these pathogens constitute an often overlooked "bridge" between contaminated sewage or decaying matter and water or foodstuffs that had been in direct contact with the maggots.

There is a need for more awareness and more research on these flies and their potential to carry disease.

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INTRODUCTION

During a routine water sampling session of the Plankenbrug River on 5-12-2005, a small planktonic maggot was observed in one of the sample bottles. On closer inspection, it proved to be a maggot that has not been seen in any of the river samples over the previous 8 years. The maggot was tentatively identified as the immature form of the drone fly (also called hover fly) Eristalis tenax or less likely the closely related, but much more rare, Eristalinus tenax. The two are very closely related species that are virtually indistinguishable during their larval stages. They have similar life cycles and feeding habits. (Note: E. tenax in this paper refers only to Eristalis tenax.)

Subsequently, especially during the first few months of 2006, South Africa experienced a hitherto unprecedented occurrence of rat-tailed maggots associated mainly with the water reticulation systems in cities and towns. The maggots were reported in numerous locations in the metropolitan areas of Cape Town as well as many other towns and clustered communities across South Africa. These maggots were almost always reported by concerned home dwellers as associated with water obtained from formal piped water supplies. The maggots were reported in water that had just been drawn from taps, and in baths, showers and sinks inside homes in urban areas. A few home dwellers also reported discovering the maggots in their gardens. Those maggots reported to occur in urban gardens were mostly associated with the distribution of compost obtained from municipal sewage sludge operations.

BACKGROUND INFORMATION

E. tenax is a member of the so-called 'filth flies'. Filth flies are synanthropic insects since they found in close association with humans, human dwellings or animals kept for human use (Graczyk T.K., Knight R. & Tamang L. 2005). These flies are strongly attracted to animal manure, human faeces and heavily contaminated and decaying organic waste (Graczyk T.K., Knight R., Gilman R.H.& Cranfield M.R., 2001). The United States Food and Drug Administration regulatory frameworks recognize 21 species of filth flies as "disease-causing flies" based on strict scientific criteria (Olsen A.R., 1998). E. tenax is among the 21 species so designated (Olsen A.R., 1998). The presence of these species near human habitation or in any location where they can constitute a danger requires preventative and corrective actions as appropriate under sanitary standard operating procedures, good manufacturing practices or pest control programmers (Olsen A.R., 1998).

The larval stages of these flies (maggots) are found in liquid habitats heavily polluted with manure or excreta. They are encountered in manure pits, excreta lagoons, run-off ditches around animal feedlots, and in rivers heavily contaminated with untreated sewage. They feed on liquid manure, excreta or decaying waste or the bacteria that grow on these substrates. The syrphid flies are classified as a pollution-tolerant species. The larva does not have legs or pincers and move by means of a flicking action of the body. It cannot "crawl" up vertical surfaces, especially smooth ones any higher that it can flick its body.

When the larvae are ready to pupate, they often migrate a short distance to a slightly drier environment. The larval skin dries up, hardens and begins to change color. After 3-5 days the adult fly will emerge to mate and lay eggs as on (or closely associated with) manure or sewage to start another life cycle. This breeding cycle is slowed in winter or in cold spells, but not completely absent.

In January 2006 the Western Cape suffered a prolonged series of power failures across the province, closely followed by similar outages in other parts of the country. The design of urban sewerage systems require that at the time of construction sumps should be installed that are capable of catching overflow sewage for a prescribed minimum time (usually 4 hours) in case of power failures. In most urban areas these sumps have not been enlarged to keep pace with expansion of the built-up areas they serve. Some sumps now overflow in as short a time as 30 minutes. During the extended power failures, thousands of tonnes of sewage overflowed and contaminated large areas. The smell of raw sewage provided a powerful attractant to lure syrphid flies into urban areas where they do not normally occur in great numbers. The sewage overflows also provided a breeding ground for the flies.

In addition to the sudden disaster of sewage spills associated with power failures, the rivers around the urban areas of South Africa are becoming steadily more polluted with untreated or poorly treated sewage as municipal infrastructures buckle under poor management and inadequate funding. It is therefore not surprising to discover rat-tailed maggots in the Plankenbrug River, one of South Africa's most polluted rivers. The presence of rat-tailed maggots in a river is seen as a sign that such a river is heavily polluted with untreated excreta.

TRANSMISSION OF INFECTIOUS DISEASES BY SYRPHID FLIES

Filth flies and their maggots breeding on or in contact with fecal matter make them important mechanical vectors of human disease-causing organisms (Graczyk T.K., Knight R., Gilman R.H.& Cranfield M.R., 2001). According to the USA Food and Drug Administration guidelines some of the pathogens carried by these insects are enteropathogenic Escherichia coli, Cryptosporidium parvum, Salmonella spp., Shigella spp. and other water- and foodborne pathogens (Olsen A.R., 1998).

Filth flies and their maggots that come into contact with fecal matter contaminated with C. parvum oocysts can harbour these oocysts both externally and internally and will mechanically deposit them on other surfaces (Graczyk T.K., Fayer R., Cranfield M.R., Mhangami-Ruwende B., et al., 1999). Therefore, synanthropic flies can serve as mechanical vectors for C. parvum oocysts and under poor sanitary conditions could be involved in the transmission of human and animal cryptosporidiosis. C. parvum infection is particularly prevalent in pre-weaned cattle (Graczyk T.K., Knight R. & Tamang L. 2005). According to studies done on the oocysts recovered from such maggots, the oocysts are still viable after transmission through the maggots' digestive tract (Graczyk T.K., Knight R. & Tamang L. 2005; Graczyk T.K., Fayer R., Cranfield M.R., Mhangami-Ruwende B., et al., 1999). This study also revealed that the oocysts

shed by infected calves were carried by the flies for at least three weeks.

Mechanical transfer of C. parvum oocysts and Giardia lamblia cysts by maggots of filth flies can occur by excretion (defecation), regurgitation or dislodgement (Graczyk T.K., Knight R. & Tamang L. 2005; Graczyk T.K., Grimes B., Knight R., Da Silva A.J. et al., 2003). The potential for mechanical transmission of disease-causing organisms is judged to be high (Graczyk T.K., Knight R. & Tamang L. 2005). The author has personally observed the maggots of E. tenax excreting into water in her laboratory. These maggots were retrieved from highly polluted water containing E. coli at a concentration of >1 million per 100 ml water.

Cryptosporidiosis can be initiated in healthy individuals by ingesting as few as 30 oocysts, but it is believed that in people with impaired immune systems a single oocyst can initiate infection (Graczyk T.K., Knight R., Gilman R.H.& Cranfield M.R., 2001). Cryptosporidiosis is an especially important disease for immunocompromised individuals, be they human or animal. For instance, a high number of advance HIV/AIDS cases treated at Tygerberg Hospital for chronic, non-inflammatory diarrhoea, tests positive for coccidian parasites which include C. parvum (personal communication - Dr Emille Reid, Adult Infectious Diseases Unit, Tygerberg Hospital).

E. tenax has also been conclusively proven to transmit Mycobacterium avium subspecies paratuberculosis on cattle farms (Fischer O.A., Matlova L., Dvorska P., Bartos M. et al., 2005). M. avium is a serious disease of cattle and species of M. avium can also cause serious disease in humans, especially those who are malnourished, suffering from chronic diseases or those with impaired immune systems. These studies also showed that M. avium paratuberculosis could survive in the intestinal tract and internal organs of E. tenax ((Fischer O.A., Matlova L., Dvorska P., Bartos M. et al., 2005).

Direct ingestion of water or food contaminated by maggots can cause human myiasis (also sometimes referred to as pseudomyiasis) (Garcia-Zapata M.T., de Sousa Jr E.S., Fernandes F.F. & Santos S.F. 2005; Mumcuoglu I., Akarsu G.A., Balaban N. & Keles I., 2005). Myiasis is the infestation of live humans or vertebrate animals by these maggots. The usual symptom is diarrhoea but the actual syndrome depends on which part of the body is invaded. There is evidence that body orifices such as the rectum can be invaded by these maggots without passing through the intestinal tract (Whish-Wilson P.B., 2000). The occurrence of myiasis has been recorded in developing countries more frequently than in developed countries with properly maintained sewerage systems. Myiasis constitutes only a moderate health risk, but the presence of maggots in or around water represents an often overlooked "bridge" between contaminated sewage or decaying matter and the volume of such water in contact with the maggots.

NEED FOR SCIENTIFIC RESEARCH

Prof Thaddeus Graczyk of Johns Hopkins University, who is the undisputed world authority on transmission of disease via filth fly vectors feels that the mechanical transmission of pathogens and the epidemiological involvement of synanthropic flies

have not received adequate scientific attention (Graczyk T.K., Knight R. & Tamang L. 2005). Further research is necessary to elucidate the mechanisms involved in retaining the infectivity of pathogens transported by all life stages of filth flies, the efficiency of transport via the exoskeleton versus the gastrointestinal tract of the insects, and the temporal and spatial dispersal of pathogens from contaminated sites (Graczyk T.K., Knight R. & Tamang L. 2005).

Given the clear relationship between the presence of E. tenax maggots and human and animal pathogens, the unhelpful approach by various authorities to the outbreak in 2006 and the refusal to admit that the maggots could pose health risks were not conducive to the development of strategies to prevent a recurrence.

This brief overview is by no means complete. The aim was to present sufficient evidence to show that the maggots of these flies are involved in the transmission of serious diseases of humans and animals. The worst affected sections of the human population are those who live under unsanitary conditions as well as those who have debilitating health conditions. In South Africa that comprises a significant portion of the population.

ACKNOWLEDGEMENTS

Mrs M Slabbert, senior laboratory technologist, Dept Medical Microbiology, Tygerberg Hospital who isolated the maggot from the Plankenbrug water sample.

Mr. L Brown of the Dept of Conservation Ecology and Entomology of the Faculty of Agriculture of the University of Stellenbosch who confirmed the tentative identification of E. tenax from a photograph.

Dr E Reid, Senior Specialist Infectious Diseases, Faculty of Health Sciences, University of Stellenbosch and Tygerberg Hospital for discussion of the manuscript and providing information on cryptosporidiosis in HIV patients.

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BARTONELLA INCIDENCE AND DIVERSITY IN ENDEMIC SOUTH AFRICAN MURID RODENTS OCCURRING COMMENSALLY WITH HUMANS

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SUMMARY

Bartonella is an emerging pathogen that displays high levels of bacterial and host species diversity, with 16 Bartonella species currently being recognised. Bacterial species diversity is known to be high in field-caught South African rodents, but has not been assessed in rodents occurring commensally with humans. Consequently, routinely snap-trapped rodents from rural households in Limpopo Province, South Africa were screened for the presence of this bacterial genus using PCR. All samples identified in this manner as being positive for Bartonella presence were subjected to further analyses aimed at determining the identity of the rodent host species as well as the identity of the Bartonella species present in the liver of each animal. The former was achieved by genetic characterization of the cytochrome-B gene of the rodent mitochondrial genome, whilst the latter was reliant on sequence analysis of the bacterial citrate synthase gene. Results indicate the presence of numerous recognised, as well as novel Bartonella species, some of which appear to readily cross rodent-species barriers, whilst others appear confined to particular rodent genera. Although many Bartonella lineages appeared to be restricted to South Africa, links between strains from the Limpopo Province and those from the United Kingdom and the Far East were recovered, the most significant of which was the identification of a strain that has > 98 % sequence identity with a strain cultured from an Indonesian rat, and that clusters within the B. elizabethae lineage. The recent discovery of the otherwise Asian endemic, Oriental house rat, Rattus tanezumi in South Africa, provides a plausible explanation for the local occurrence of this Rattus-associated Bartonella lineage that has been linked to cases of human endocarditis elsewhere, whilst the known presence of other invasive Rattus species in South Africa points to a probable role of members of this genus in transmitting exotic Bartonella strains to endemic rodent species.

INTRODUCTION

Species of the fastidious, gram-negative bacterial genus *Bartonella* have a natural cycle that typically comprises infection of the erythrocytes of a vertebrate reservoir host with transmission between hosts being *via* a haematophagous arthropod vector (Breitschwerdt & Kordick, 2000). At least 16 species are currently recognised for this

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emerging, opportunistic pathogen, which occur across a wide range of host species, including humans, with almost half of these having zoonotic potential. *Bartonella* species that are known to be the causative agents of human diseases such as Trench fever, Carrion's disease, endocarditis and cat scratch disease, include *B. quintana, B. bacilliformis, B. elizabethae, B. henselae* and *B. clarridgeiae* (Breitschwerdt & Kordick, 2000). Variable clinical presentation is often associated with *Bartonella* infections, particularly in immune-compromised individuals, and in South Africa *B. henselae* is known to be present in HIV-positive patients presenting with bacillary angiomatosis (Frean et al 2002).

Bartonella has a worldwide distribution and has been identified in numerous wild and domestic mammalian species, as well as in a wide range of haematophagous invertebrates (Breitschwerdt & Kordick, 2000 and references therein). The occurrence of *Bartonella* in field-caught South African rodents was recently confirmed (Pretorius et al 2004), but has not been assessed in rodents occurring commensally with humans. The known zoonotic potential of many of the formally recognised *Bartonella* species, together with the high HIV infection rates in South Africa make it imperative to investigate *Bartonella* in rodents that occur in close contact with humans, particularly in light of their recognised potential to facilitate *Bartonella* transmission. The aim of this study was therefore to assess *Bartonella* incidence and diversity in genetically identified murid rodent species caught in rural, community households in the Limpopo Province, South Africa by means of a PCR-based sequencing approach which confirms bacterial presence and permits bacterial species identification on the basis of the nucleotide sequences generated. However, as prior results from this study site indicated that the two endemic genera most commonly trapped within rural community households were *Aethomys* and *Mastomys* (von Maltitz et al. 2006), unequivocal species identification was reliant on genetic characterisation, as both genera contain cryptic, sympatric species that are not readily distinguished on the basis of either external or cranial morphology (Chimimba et al. 1999; Venturi et al. 2004).

MATERIALS AND METHODS

Samples used in this study

Murid rodents were trapped in four villages in the Limpopo Province of South Africa, namely Bloublommetjieskloof, Mapate, Nkomo-B and GaPhaahla. Snap traps were placed in ten community households of each village and inspected daily. Sampling locality and date together with standard museum data (Deblase & Martin 2001) were recorded for each rodent trapped in this manner, prior to removal of the liver which was stored in absolute ethanol.

Genetic Characterisation Of Host And Bacterial Genes

Total genomic DNA was extracted from liver samples using the High Pure PCR template preparation kit (Roche), according to supplier specifications. In order to accurately identify the host species, the entire cytochrome-B (*cyt-b*) gene of the murid

mitochondrial DNA (mtDNA) genome, which is approximately 1.2 kbp in length, was amplified and sequenced as previously described by Bastos and co-workers (2005). A 513 bp fragment of the citrate synthase (*gltA*) gene of the *Bartonella* bacterial genome was targeted with primers designed specifically for this study, with positive PCR results confirming bacterial presence. Each positive bacterial gene amplicon was purified directly from the tube using the High Pure PCR product purification kit (Roche), according to manufacturer specifications, and used as template for cycle sequencing with Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer), at an annealing temperature of 50°C.

Host species and bacterial species identification

Species identification of the rodent host was determined by phylogenetic analysis of the complete 1140 bp fragment of the *cyt-b* mtDNA, as described previously (Bastos et al. 2005). Nucleotide sequences generated from all *Bartonella* positive PCRs were aligned with all available homologous sequences on the Genbank database (www.ncbi.nlm.nih.gov). A gene phylogeny was subsequently inferred using distance and parsimony methods with nodal support being assessed by bootstrap resampling. The MEGA3 program (Kumar et al.2004) was used to generate distance trees, whilst PAUP* (Swofford 2003) was used for the parsimony analyses. The best-fit model selected under the Akaike Information Criterion (AIC) in ModelTest version 3.06 (Posada & Crandall, 1998), was used to guide model selection for the distance analyses in MEGA.

RESULTS

Phylogenetic analysis of the complete *cyt-b* gene of the host mtDNA revealed the presence of eight endemic murid rodent species recovered from snap traps in Limpopo households. These eight species belong to six genera (*Aethomys*, *Mastomys*, *Tatera, Saccostomys*, *Steatomus*, *Lemniscomys*), that fall within three distinct subfamilies, namely the Murinae, Dendromurinae and Gerbillinae. *Bartonella* was detected in 14 of the 25 murid rodents that were screened, corresponding to a prevalence of 56 %.

Nucleotide sequencing of a ~500bp region of the citrate synthase (*gltA*) gene was used to infer a bacterial gene phylogeny, which together with a sequence similarity value of < 96%, advocated by La Scola et al. (2003) for the discerning *Bartonella* species from partial *gltA* nucleotide sequences, was used to delineate species. Seven distinct bacterial clades were identified in this manner from the *Bartonella*-positive Limpopo Province samples that were sequenced (Fig. 1). Three of these appear to be genus-specific (labeled '*Tatera*-specific', '*Mastomys*-specific 1' and '*Mastomys*-specific 2' in Fig. 1), whilst members of the remaining four clades contain bacteria recovered from diverse, unrelated murid rodent genera. Of these bacterial clades demonstrating low species fidelity, one corresponds to the formally recognized species, *Bartonella elizabethae*, another appears specific to South African rodents and coincides with a bacterial lineage described by Pretorius and co-workers (2004), whilst the remaining two represent novel species.

Within the three species-specific clades, the *Tatera*-specific *Bartonella* sequences (ARC81-82) group together with field-caught conspecifics sampled in the Free State

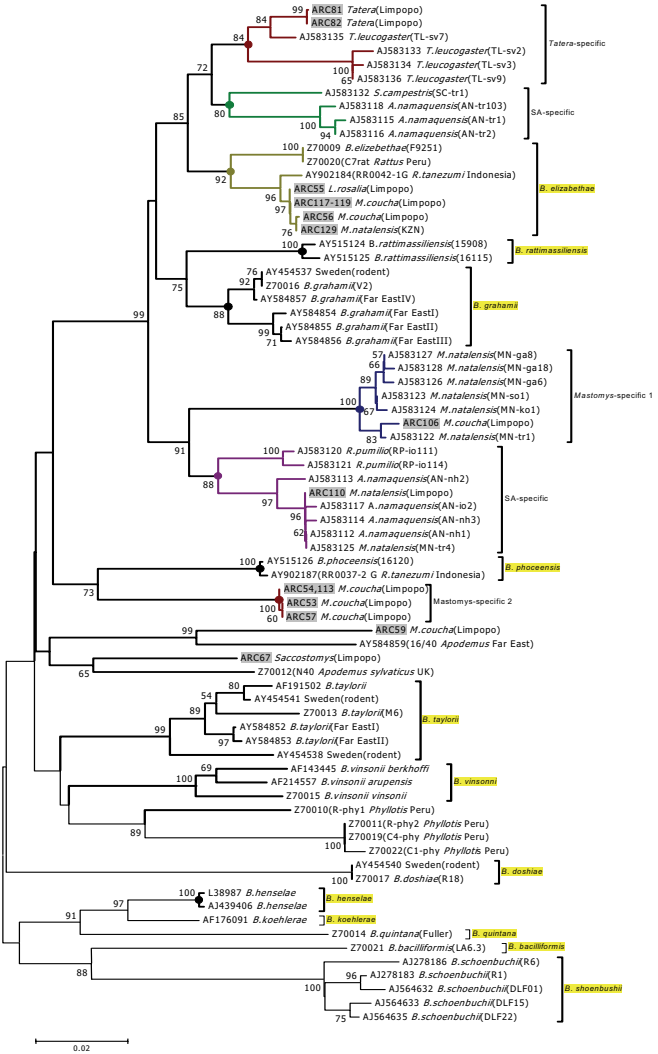

Fig. 1 Neighbor-joining tree based on partial gltA gene sequences of Bartonella type-specimens and those generated from endemic rodent samples from the Limpopo Province, South Africa.

province, South Africa (Pretorius et al. 2004), whilst the *Mastomys*-specific 1 *Bartonella* sequence (ARC106), identified from a *Mastomys coucha* specimen, groups together with samples designated *Mastomys natalensis*, from the Free State Province. The *Mastomys*-specific 2 lineage is representative of a new and previously undescribed *Bartonella* species.

DISCUSSION

A complete understanding of the epidemiology of any disease not only requires that the disease agent be studied, but also that the biology and behaviour of the hosts and vectors should also be known. The latter rests entirely on accurate species identification of both the host and the vector species, and formed a crucial part of this study in which cryptic host species predominated at the study site. This required accurate identification by genetic characterisation of the *cyt-b* gene of the rodent host, and revealed the presence of eight endemic species.

Bartonella incidence in endemic murid rodents sampled in the Limpopo Province was found to be 56 %, which is comparable to that identified in field-caught rodents from the Free State Province (Pretorius et al. 2004). It also falls within the prevalence ranges detected in other parts of the world. For example, woodland rodents from the UK were shown to have an overall *Bartonella* incidence of 64 % (Birtles et al. 2001), whilst the prevalence in different rodent species in China ranged from 19 % to 71 % (Ying et al. 2002).

Phylogenetic analysis of the *gltA* sequences from the 14 *Bartonella*-positive rodent samples characterized in this study, revealed high, and localized levels of bacterial species diversity, with seven distinct *Bartonella* species being identified. Three of these clustered within field-caught rodent clades identified previously from the Free State Province in South Africa (Pretorius et al. 2004), one within the *B. elizabethae* lineage, and the remaining three were distinct and considered on the basis of genetic distance and the phylogeny to be representative of novel *Bartonella* species.

The six Limpopo sequences (ARC55-56, ARC117-119 and ARC129) that fell within the *B. elizabethae* clade were recovered from three endemic murid rodent species, namely, *Lemiscomys rosalia*, *Mastomys coucha* and *M. natalensis,* and were most closely related to a *Bartonella* strain cultured from a *Rattus tanezumi* specimen, from Indonesia (RR0042-1G). In light of the recent discovery of the Asian exotic rat, the Oriental house rat, *Rattus tanezumi*, in South Africa (Bastos et al. 2005), this finding is not surprising. It is however highly significant as it represents the first record in South Africa of a bacterial species with recognized links to human endocarditis, in endemic rodents occurring commensally with humans. Its presence together with evidence of common ancestry between *Bartonella* from endemic rodent species and *Bartonella* from the UK and the Far East, points to a broader probable role of *R. tanezumi* and other invasive *Rattus* species in transmitting *Bartonella* to endemic rodent species.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Chris Chimimba (MRI-UP) for review of this manuscript. I'm also grateful to Frikkie Kirsten and Emil von Maltitz (both from the ARC-PPRI) for providing the rodent samples used in this study. This study was funded by an NRF Focus Area Grant awarded to ADSB. Any opinions, findings and conclusions or recommendations expressed in this material are those of the author and therefore the NRF does not accept any liability in regard thereto.

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A QUALITATIVE ASSESSMENT OF THE RISK OF FOOT AND MOUTH DISEASE OUTBREAKS OUTSIDE THE WESTERN BOUNDARY OF KRUGER NATIONAL PARK

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SUMMARY

Since the year 2000 at least five declared outbreaks of foot and mouth disease (FMD) have occurred in the area adjacent to the Kruger National Park (KNP) despite the implementation of control measures. Although only one of these outbreaks threatened the free zone, it is necessary to develop decision tools in order to help in the implementation of efficient disease control strategies. To address this issue, a qualitative risk assessment was performed to evaluate the risk of FMD outbreaks of KNP origin occurring across the park's Western Boundary. The OIE assessment method was used based on data collected during a three month period between February and May 2007 and expert opinion. Although the data were not sufficient to allow a quantitative risk assessment to be performed, the investigation served as a useful initial approach prior to undertaking a quantitative risk assessment. Risk was assessed using the following parameters: prevalence of infection in buffalo in the KNP, permeability of the fence along its Western Boundary, the potential for contacts between livestock and buffalo, the level of immunity in cattle herds and the efficiency of surveillance measures. The magnitude of the consequences is derived from the probability of transmission and spread. The method and results are presented to serve as a basis for further discussion and for the development of a quantitative risk assessment model.

INTRODUCTION

The development of transfrontier conservation areas (TFCAs) represents substantial challenges for animal disease control. The increase of habitats for wildlife provides larger geographic areas for the reproduction and dispersal of wild species and their pathogens, therefore multiplying the chances of animal health interactions at the wildlife/livestock interface (Bengis 2005). South Africa is unique regarding the management of control of foot and mouth disease virus (FMDV) where the disease is endemic only in the Kruger National Park (KNP), with all three SAT viruses (SAT 1, 2 & 3) efficiently

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maintained in the resident African buffalo (*Syncerus caffer*) populations. Animal health authorities of South Africa have been efficient in containing the disease within the borders of the KNP by the application of different control and preventive measures. These measures consisted of the erection of a 400km perimeter fence along the Western Boundary of KNP, adjacent area zonation with the application of serial vaccination programmes around the KNP in the immediate buffer zone, intensive surveillance measures by means of regular inspections in the buffer and surveillance zones, and movement control of cloven hoofed animals and their products between zones. Due to the success in containing the disease, the country was granted zoned FMD-free status without vaccination by the *Office International des Epizooties* (OIE) in 1996. Indeed, prior to 2000, the last outbreak of the disease in domestic stock in the FMD free zone had occurred in 1957 and the last outbreak in the buffer zone occurred in 1983. However, between 2000 and 2007, at least 5 outbreaks with confirmed origins from KNP wildlife have occurred along the Western Boundaries of the KNP, some reaching the outskirts of the free zone and threatening agricultural exports on one occasion. Despite having had one outbreak beyond the buffer zone in 2000, South Africa has managed to regain its FMD free zoned status. All these outbreaks were successfully contained after varying periods of time. However, the reasons for the increased incidence of FMD outbreaks need to be analysed. To improve our understanding of the dynamics of the disease and its control systems, a comprehensive qualitative risk assessment of the occurrence of FMD outbreaks outside the KNP was performed.

MATERIALS & METHODS

The method used to conduct this qualitative risk assessment is based on the OIE *Terrestrial Animal Health Code* normally applied to manage the risks associated with imports of live animals and animal products (Murray 2004). The theoretical bases for any risk assessment, whether qualitative or quantitative, are the same. Once the hazard has been identified, in this case, an FMD outbreak, the risk to be assessed is a function a) of the probability that infection will occur and b) of the magnitude of the consequences of such an occurrence. The probability of occurrence of the outbreak is in turn the product of the release assessment of FMDV from the KNP to adjacent provinces and the probability of exposure of cattle herds to the virus. The appraisal of the magnitude of the consequences must take into account both the probability of dissemination of the pathogen (transmission plus spread) and the economic impact of the disease. For a qualitative assessment different authors propose each of these events to be characterised by a number of parameters and each parameter to be analysed on the basis of all available information (Murray 2004, Zepeda 1998). In addition, the probability of occurrence of each event is assessed for classification by means of the following descriptive scale:

– Negligible, when the probability of occurrence of the event is sufficiently low to be ignored, or if the event is possible only in exceptional circumstances
– Low, when the occurrence of an event is a possibility in some cases

– Moderate, when the occurrence of the event is a possibility
– High, when the occurrence of the event is clearly a possibility.

These parameters are subsequently combined with each other according to a matrix (Murray 2004, Zepeda 1998) to give an overall idea of risk.

In this study, the probability of occurrence of a hazard (FMDV infection and the consequences of an epizootic) is equal to the probability of escape of the virus (from the KNP to adjacent areas in South Africa) combined with the probability of the exposure of domestic animals susceptible to the pathogen. As FMDV cannot be transmitted to humans, the consequences of an epizootic are solely socio-economic.

RESULTS

1. Hazard identification

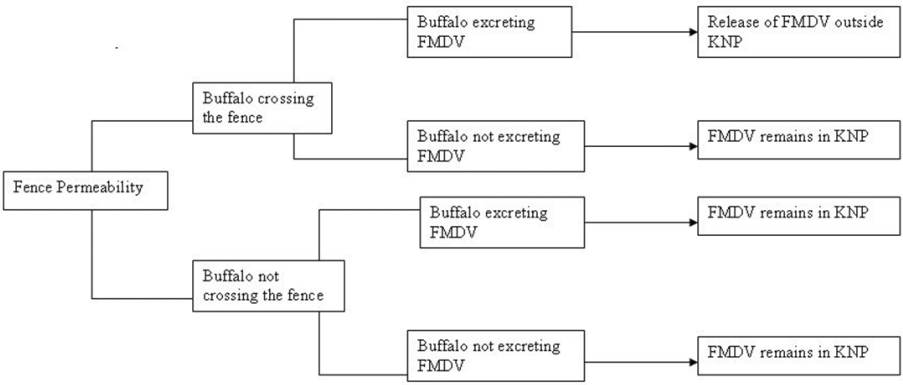
In the present analysis we focused on outbreaks of FMDV occurring along the borders of the KNP produced by buffalo strains (SAT 1, SAT 2 and SAT 3). Although other species of wildlife such as impala and kudu, may be involved in the epidemiology of FMDV in the KNP, we have considered the African buffalo as being the ultimate long-term source of the virus for cattle and the main potential role player in the transmission of FMDV to cattle in South Africa (Vosloo, Bastos, Sahle, Sangaré & Dwarka 2005).

2. Release assessment

The three parameters were examined in order to determine the probability of exit of the virus beyond the Western Boundary of the KNP:

- o permeability of the fence
- o the prevalence of infection in the buffalo population
- o the population density and distribution of buffalo in the KNP

Figure 1: Possible pathway of release of the FMD virus outside the boundaries of the KNP

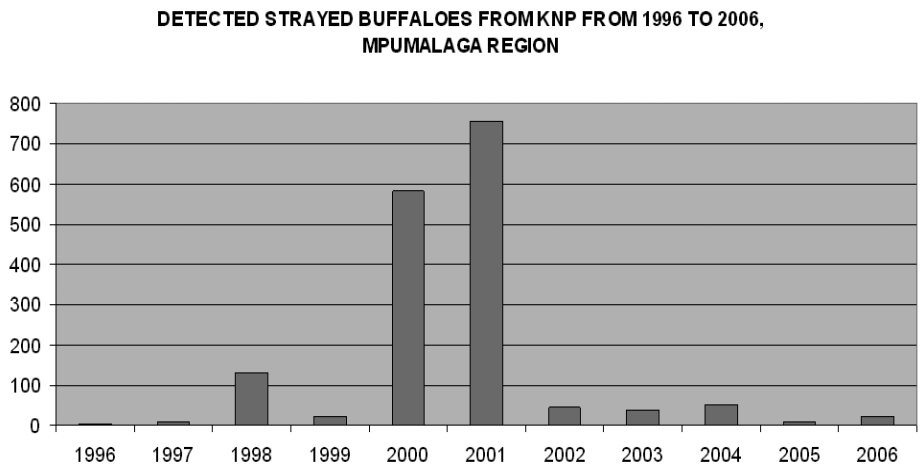


2.1. Fence permeability

The initial KNP disease control fence, erected between 1958 and 1961 to prevent outbreaks of FMD, has been largely successful in limiting wildlife movements for almost a half a century. This fence was upgraded and electrified between 1997 and 1999. Nevertheless, during the last years, its efficiency in deterring large mammals from escaping the KNP has substantially decreased due to a combination of different multiple factors such as:

- A dramatic increase in the elephant population in the KNP resulting in subsequent elephant related fence breaks (up to 45 fence breaks in one year only in Mpumalaga province).
- Increased human/fence line activity: Illegal immigrants from Mozambique (estimated at least 3000/year) are believed to damage the fence at different points and fence components such as batteries and solar panels are regularly stolen. These activities negatively affect the structural integrity of the fence and reduce the deterrent effect of the fence against large mammals.
- Climatic factors: Climatic extremes with major floods have substantially damaged the fence from time to time with the last event in 2000 / 2001. In addition, sections of the fence may also be seasonally damaged at major drainage crossing points and river flows after heavy rains.

Figure 2: Histogram showing detected strayed buffalos from KNP in Mpumalanga region.



All of these factors have a different impact on fence permeability resulting in a substantial number of stray buffalo escaping from the KNP premises (Figure 1). Therefore, the overall probability of KNP buffalo crossing the fence can be considered moderate.

2.2. Prevalence of infection in the buffalo population

Most young buffalo become infected between 3 and 8 months of age, when maternal antibodies wane. In the acute stages of infection, young buffalo excrete FMDV in roughly the same quantities and by the same routes as cattle and are potentially highly infectious (Gainaru, Thomson, Bengis, Esterhuysen, Bruce & Pini 1986). Between May and November, large quantities of virus circulate among buffalo herds and this is the "childhood" epidemic period when they are most likely to be a source of infection for other species (Bastos, Boshoff, Keet, Bengis & Thomson 2000, Thomson 2002). Fourteen days after initial infection, the viruses can no longer be recovered from the tissues, secretions or excretions, with the exception of pharyngeal cells, in about 60% of cases in the so-called ``carrier animals'' (Anderson, Foggin, Atkinson, Sorensen, Madekurozva & Nqindi 1993). Condy, Hedger, Hamblin & Barnett (1985) demonstrated that FMDV can be maintained in buffalo populations for at least 24 years or longer as long as susceptible cohorts of animals become available on a regular basis. Carriers transmit the infection poorly to cohorts and to other susceptible species (Thomson, Vosloo & Bastos 2003). Nevertheless, although not proven, there is the hypothesis that in certain cases, stress could act as a cause for carrier animals (cattle) to start excreting virus and cause outbreaks (Kitching 2002). This aspect hasn't been sufficiently studied and therefore with the current knowledge available, we can only consider that the probability of finding a potential FMD shedder buffalo in the KNP (a young animal actively infected) as low.

2.3. Population density and distribution of buffalo population in the KNP

The population of buffalo in the KNP has been growing since the last population crash in the early 90's. The current population of buffalo (between 33,000 and 35,000 head) is the highest figure in the last 14 years (R. Bengis, personal communication). Buffalo congregate around sites of available surface water during the dry season between May and November when water is scarce (Keet, Hunter, Bengis, Bastos & Thomson 1996). These high densities of animals, linked with an important circulation of virus between August and November, constitute a high risk virus source around water points and in river beds. These are hot spots for transmission between wildlife species and may compound risk if nearby fences are permeable. Therefore in the dry season, the risk of a susceptible buffalo being a source of infection might increase from low to moderate.

2.4. Assessment of release of FMD

Using the matrix proposed by Zepeda (1998), the authors conclude that the probability of FMDV release outside the KNP is a function of the combination of risks related to the fence permeability (moderate) and the probability of young buffalo excreting the virus which is low in the rainy season and low to moderate in the dry season. Therefore, the risk of FMDV release outside the KNP is rated as low to moderate depending on the season.

3. Probability of exposure

The parameters considered in determining the probability of exposure were as follows:

- Probability of air-borne virus transmission
- The potential for transmission from buffalo to susceptible cattle in the buffer zone with vaccination
- The probability of virus spread within the buffer zone with vaccination
- The probability of virus transmission among cattle within the buffer zone without vaccination
- The probability of virus spread outside the buffer zone without vaccination
- The probability of transmission into the free zone

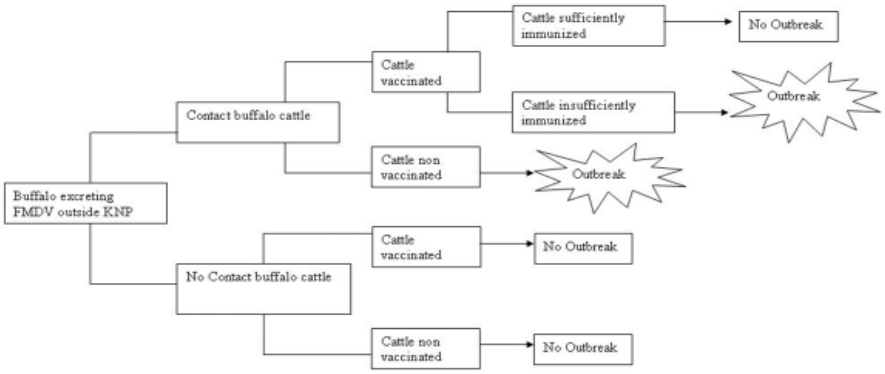
3.1. Probability of air-borne virus transmission

Circumstantial evidence accumulated over many years has indicated that long distance air-borne transmission is highly unlikely in southern Africa. In a quantitative risk assessment in Zimbabwe, the likelihood of this event was considered remote (Sutmoller, Thomson, Hargreaves, Foggin & Anderson 2000). This suggests that potential transmission of FMDV from buffalo to cattle occurs basically by direct contact.

3.2. Probability of contact between buffalo and cattle

Considering the high number of cattle around the KNP, and the gregarious instinct of buffalo, the probability of contact if a small group of buffalo escape from KNP is at least moderate. In fact, a buffalo escaping the KNP will naturally join a cattle herd if it becomes available, searching for the protection of con-specifics particularly in a foreign environment and even more if the buffalo group is small (one or few individuals – especially heifers and young bulls). Older bulls are less likely to socialise with cattle. This risk is higher in areas of communal farming, where densities of cattle are higher and there are fewer fences and will also depend on the amount of time that buffalo

Figure 3: Possible pathway of transmission of FMDV from buffalo to cattle and subsequent spread



remain outside the KNP premises, which on some occasions can remain undetected for several weeks.

3.3. Probability of transmission between cattle and buffalo

Bastos, Bertschinger, Cordel, van Vuuren, Keet, Bengis, Grobler, & Thomson (1999) suggested that sexual transmission of FMDV between male buffalo and cattle can occur. According to different authors, there is evidence that transmission from infected buffalo to cattle occurs fundamentally during acute stages of the disease (Thomson, Vosloo & Bastos 2003, Vosloo, Bastos, Sangare, Hargreaves & Thomson 2002). The occurrence of carriers in transmitting virus has only rarely been found (Dawe, Flanaghan, Madekurozwa, Sorensen, Anderson, Foggin, Ferris & Knowles 1994, Dawe, Sorensen, Ferris, Barnett, Armstrong & Knowles 1994, Kitching 2002, Vosloo, Bastos, Kirkbride, Esterhuysen, Janse van Rensburg, Bengis, Keet & Thomson, 1996).

Therefore, the probability of transmission of FMDV when an escaped buffalo comes into contact with cattle outside the KNP in the vaccination zone is low.

3.4. Probability of spread within the buffer zone with vaccination

In the buffer zone with vaccination, cattle of all ages are vaccinated twice a year. Vaccinated animals are F-branded and do not leave the zone except when destined for slaughter. However, animals are not individually identified in every herd and this can lead to a low level of risk of cattle from the vaccination zone moving into the buffer zone without vaccination. Moreover in some areas, despite inspection and compulsory vaccination of cattle, vaccination coverage can be low for instance due to lack of motivation of veterinary technicians or local communities to visit the diptanks. Therefore, in some communal areas vaccination coverage of cattle herds can be below 60% if the diptank system collapses. The current risk of cattle herds being insufficiently immunised is considered to be low to moderate depending on the area, being higher in areas of communal farming and specially those where the involvement of communities in dip-tank surveillance is compromised. Moreover, the level of protection of the vaccine depends on the degree of similarity between the circulating strains of virus and attenuated strains used in the vaccine (Bastos, Haydon, Sangare, Boshoff, Edrich & Thomson 2003, Vosloo, Bastos, Sahle, Sangaré & Dwarka 2005). Until 2006, animals were vaccinated with trivalent (SAT-1, SAT-2 and SAT-3) vaccine consisting of buffalo isolates originating from KNP. However since 2006, the trivalent vaccine is purchased from other countries and the origin of the strains is likely to be distant from those circulating in KNP. Therefore, the level of protection of the current vaccine against FMDV challenges from the KNP buffalo population in cattle is probably lower, since efficiency of vaccines is directly linked with antigenic relations between the strains used for its production and the strains circulating in the field.

Conditions are more conducive for spread of infection in communal areas due to mixing of animals from different farmers at dip tanks and communal grazing areas. Moreover, individual farming units and animals are often not identifiable (Bruckner, Vosloo, Du Plessis, Kloeck, Connoway, Ekron, Weaver, Dickason, Schreuder, Marais

& Mogajane 2002). Therefore the probability of spread of the FMDV among cattle populations in the buffer zone with vaccination can be considered moderate.

3.5. The probability of transmission to the free zone

For an animal to be moved from the buffer zone with vaccination into the buffer zone without vaccination, it needs to be non vaccinated (non F branded) and quarantined for at least 21 days. During that period the animals are physically inspected and serologically tested. There is a possibility for some animals from the buffer zone with vaccination to occasionally cross illegally into the non vaccinated area. The probability of infected animals going undetected by physical inspection outside the buffer zone with vaccination is moderate, particularly if the clinical signs of the disease are mild. However, the probability of an infected animal going undetected by serology is negligible (the test has a sensitivity of 95% and specificity of 98%). Due to the implementation of these control measures, the probability of transmission from the buffer zone with vaccination into the buffer zone without vaccination is low**.**

For an animal to move from the buffer zone without vaccination into the free zone, it has to go through the same quarantine and control process. Therefore, the risk of an infected animal from the buffer zone reaching the free zone is considered negligible.

3.6. Exposure assessment

The probability of contact between escaped buffalo and cattle outside the park and the probability of transmission from excreting buffalo to cattle have both been rated as moderate and low, respectively. The probability of cattle in the buffer zone with vaccination developing the disease is rated as moderate in communal areas depending on the level of immunity among herds. Nevertheless, the probability of transmission and spread into the buffer zone without vaccination is rated as low. The probability of spread and transmission above the buffer zone without vaccination is rated as negligible. Therefore the exposure assessment can be qualified as moderate in the buffer zone with vaccination while it is considered low in the buffer zone without vaccination and negligible onwards.

4. Magnitude of consequences

The consequences of an outbreak of FMD outside KNP are solely economic, since the virus cannot be transmitted to humans and the public health consequences are null. Economic consequences can be direct, arising from the direct effects of outbreaks on production, but mainly on the costs of disease control (culling, vaccination) and restrictions on trade (movement control). Indirect impact comes from the costs of surveillance after the outbreak, fence management and maintenance, regular surveillance and monitoring activities at dip tanks and trade losses (impaired movement, embargoes, sanctions). The level of consequences will depend on the area where the outbreaks occur (Table 1). In the buffer zone, most consequences are biological and include calf mortalities, loss of milk production by lactating cows, loss of weight in beef cattle, secondary infection of hooves and teats. The impact of FMD control measures on the

livelihoods of local communities such as strict movement control, insufficient trading opportunities or lack of availability of resources (grazing, water) is certainly more important than the impact of a single outbreak and should also be considered. However, since trading opportunities in the vaccination zone are limited, the impact of economic consequences at a national level, should an outbreak occur in the vaccination zone, can be considered as low. In the case of an outbreak spreading out of the buffer zone with vaccination, the economic impact becomes more important. The magnitude of consequences of an outbreak reaching the free zone are high (millions of Rands) since South Africa would loose its zoned FMD-free status for international trade in agricultural products, until the outbreak is contained and the FMD-free status re-established (at least during 3 months after the last clinical case).

5. General assessment

In the current situation, the overall situation of risk will differ depending on the season of the year and the FMD zone, where the consequences of an outbreak will be different. However, in all cases the result of the risk estimation regarding the occurrence of an outbreak and its consequences is moderate (see Table 1).

Table 1: Overall estimation of risk according to different scenarios.

Zone	Season	Combined probability of outbreak	Consequences	Risk estimation
Vaccination	Dry Rainy	Moderate Low	Low	Moderate Low
Non vaccination	Dry Rainy	Low	Moderate	Moderate
Free	Dry Rainy	Negligible	High	Moderate

DISCUSSION

The OIE risk analysis framework implemented in this study is usually a tool to manage disease risks associated with the imports of live animals or animal products from an exporting country (Murray 2004). The application of this method in the context of South Africa is innovative in that it measures the risk of hazard occurrence between different zones in the same territory.

Although most of the information provided in this study is not new, it provides a synthesis of the different outbreaks that had occurred during the last few years and a good basis for discussion between different role players as a part of the risk communication process. Our analysis shows that the probability of an FMD outbreak occurring outside of KNP is moderate due to a diversity of factors. In the first line of intervention, the

pathway steps where the risks are higher and there is some margin of improvement are:

- The increased permeability of the fence
- The lower level of immunity among some herds in communal areas

Permeability of the fence is likely to be reduced by increasing surveillance and the pace of fence repairs along the fence perimeter. Management strategies for the control of elephant populations are under consideration in the KNP and the erection of a new fence system more solid but non electrified is being implemented in the north of the Park (D. Keet, personal communication).

In addition efforts in the buffer zone to maintain or improve the dip tank system and the implementation of vaccines containing the correct strains should allow an increase in the level of herd immunity and a subsequent mitigation of the risk of outbreak occurrence from moderate to low. Until those measures are improved, the risk of new outbreaks is at least moderate, particularly during extreme dry seasons and South Africa should take note of this. The experience acquired with previous outbreaks has shown that once the disease spreads among domestic cattle outside the KNP, active surveillance and emergency disease control actions have managed to identify and contain the disease before it reached the free zone, preserving the zoned disease-free status. On the other hand, these control measures clearly inhibit rural and social development of thousands of people living in the buffer zone and probably need re-assessment.

The qualitative risk assessment method presented here has clear limitations, especially regarding the choice and subjectivity of categories for the different parameters of risk chosen. However, it represents a first approach for a more comprehensive quantitative assessment which requires more time and available data. Equally, it provides a very useful tool to discuss different scenarios and disease management situations and to identify gaps of knowledge that can be used to manage other animal health challenges regarding at the wildlife/livestock interface in the context of TFCA's.

ACKNOWLEDGEMENTS

We are very grateful to Drs. Willie Ungerer and Grietjie De Klerk from the National Veterinary Services, and Erika Kirkbride from EDD-OVI for providing reports and information on recent outbreaks. We would like to acknowledge Dr. Edwin Dyason for providing information on Limpopo Province, Johan Walters and Vuyo Magadla for their help in the assessment of control measures and Dr. Dewald Keet, for his invaluable information regarding the northern part of the fence.

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A METHOD FOR ESTIMATING THE DISTRIBUTION OF SPECIES SUSCEPTIBLE TO FOOT-AND-MOUTH DISEASE IN THE KRUGER NATIONAL PARK REGION

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SUMMARY

Landscape distribution of susceptible species is needed to model the spatial spread of infectious diseases. Our objective is to develop 2 individual species animal count maps – for African buffalo and domestic cattle susceptible to foot-and-mouth disease (FMD) in the Kruger National Park Region (KNPR) of the Republic of South Africa (RSA) – using retrospective census and land use data. The KNPR includes the Kruger National Park, Limpopo and Mpumalanga provinces. We applied dasymetric mapping methods to disaggregate animal population census data to a finer resolution using ancillary data. In this study, ancillary data used to define species distributions included stocking rate or carrying capacity and land suitability parameters. A geographical information system (GIS) was used to identify suitable land and vegetation locations (x,y coordinates) for each species. Covariate-specific animal counts were modelled using a Poisson distribution. The resulting animal count data was mapped and will be used to model FMD spread within the KNPR.

INTRODUCTION

Information concerning the landscape distribution of susceptible species is necessary to model the spatial spread of infectious diseases. The Kruger National Park Region (KNPR) is vital for surveillance of infectious diseases because the continuous interaction between human, wildlife and livestock creates potential for their transmission. For the purpose of this study the KNPR is defined to include the Kruger National Park (KNP), Limpopo and Mpumalanga provinces in the Republic of South Africa (RSA) as shown

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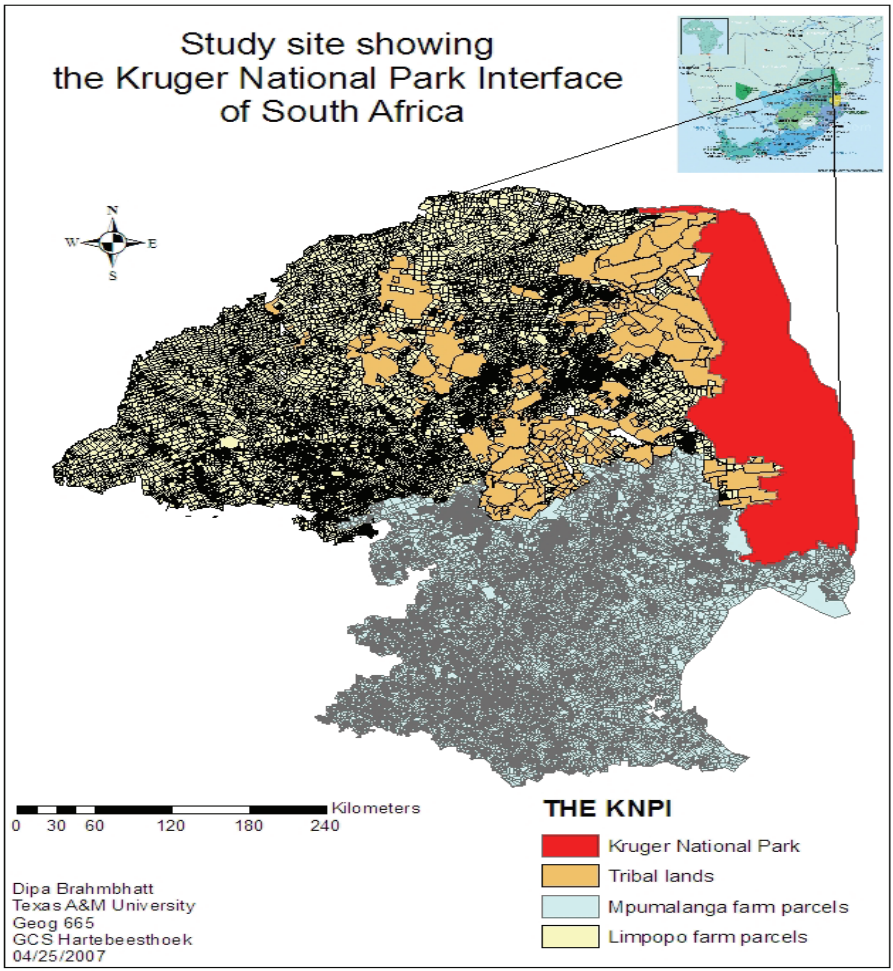

Figure 1: The Kruger National Park Region showing the Kruger National Park, Limpopo and Mpumalanga Provinces of RSA included in this study.

in figure 1. The KNP is separated from the provinces by a fence on its western boundary which is approximately 400 km in length. One of the infectious diseases of greatest concern in the KNPR is foot-and-mouth disease (FMD) which is a highly contagious, but rarely fatal viral disease affecting cloven hoofed wildlife and domestic livestock.

A number of wildlife species in Africa have been shown to be affected by FMD such as the African buffalo (*Synercus caffer)*and (*Aepyceros melampus*) in the KNP , and wildebeest (*Connochaetes taurinus*) in the Serengeti . Infected African buffalo are considered a source of FMD for susceptible livestock close to the KNP and impala in the KNP . Impala can act as intermediaries for the transmission of FMD virus from buffalo

to cattle which could be due to their ability to jump perimeter fences . Phylogenetic analyses of outbreaks in cattle and impala caused by SAT 1 strains between 1971-1981 in the RSA indicated that several of the outbreaks had spread from impala to cattle . In addition, epidemiological investigations into the outbreaks in cattle in 2000, caused by SAT 1 and the SAT 2 outbreak in 2001 were traced back to buffalo that escaped from KNP. The exact mechanisms and spatial factors responsible for disease transmission to domestic animals remain unclear.

One of the limitations of modelling the distribution of susceptible species on a landscape is that the available census data is typically at an aggregate level. In the RSA this is available in the form of choropleth maps at district levels per province. Some of the limitations of these maps include smoothing the variations of local domestic cattle distributions, representing artificial transitions in domestic cattle counts at district boundaries and suggesting that cattle are distributed homogenously in the district even though some areas have no livestock .

The objective of this study is to develop methods to estimate the distribution of FMD susceptible species (domestic cattle and African buffalo) in the KNPR and further use this data to model the spread of FMD in the KNPR of the RSA.

MATERIALS AND METHODS

The dasymetric method was modified (S. Rollo, L. Highfield & M. Ward, unpublished data) by randomly allocating points on selected areas followed by disaggregating data using a Poisson distribution and varying stocking rates.

Retrospective Ancillary Data

The distribution of animal species can be approximated using vector based dasymetric mapping methods defined as using additional information to disaggregate coarser resolution population data to finer resolution . This method was modified by including a Poisson distribution (S. Rollo, L. Highfield & M. Ward, unpublished data). The additional information necessary to disaggregate census data was stocking rate (livestock) for commercial and communal farmers/ carrying capacity (wildlife), river network and vegetation layers. The stocking rate is defined as the number of animals per square kilometre and was calculated using 2004 census data acquired from National Veterinary Services (NVS). Commercial farmers differ from communal farmers because they have defined boundaries around their land, exclusive rights for the properties and produce competitively for export markets in the RSA. On the other hand, most communal farmers are primarily subsistence farmers, have undefined boundaries and generally have open access rights to defined grazing areas . The spatial commercial farm parcels, communal lands and river network for Limpopo and Mpumalanga provinces in 2000 were acquired from the NVS. The wildlife census was acquired from South African National Parks (SANPARKS). This consisted of the 2001 African buffalo mega herbivore census data for the entire KNP.

The 2004 South African National Biodiversity Institute (SANBI) vegetation map for RSA, Swaziland and Lesotho was acquired from the Department of Environmental Af-

fairs and Tourism (DEAT). This vegetation map consists of 441 vegetation types which are classified into 9 major biomes representing large natural areas defined by vegetation type and climate . The 9 biomes are named as follows Albany thicket, desert, forest, grassland, Nama-Karoo, savanna, succulent Karoo and wetlands. All Geographic Information Systems (GIS) data was available as vector files with a scale of 1:50000.

Data Management in ArcGIS

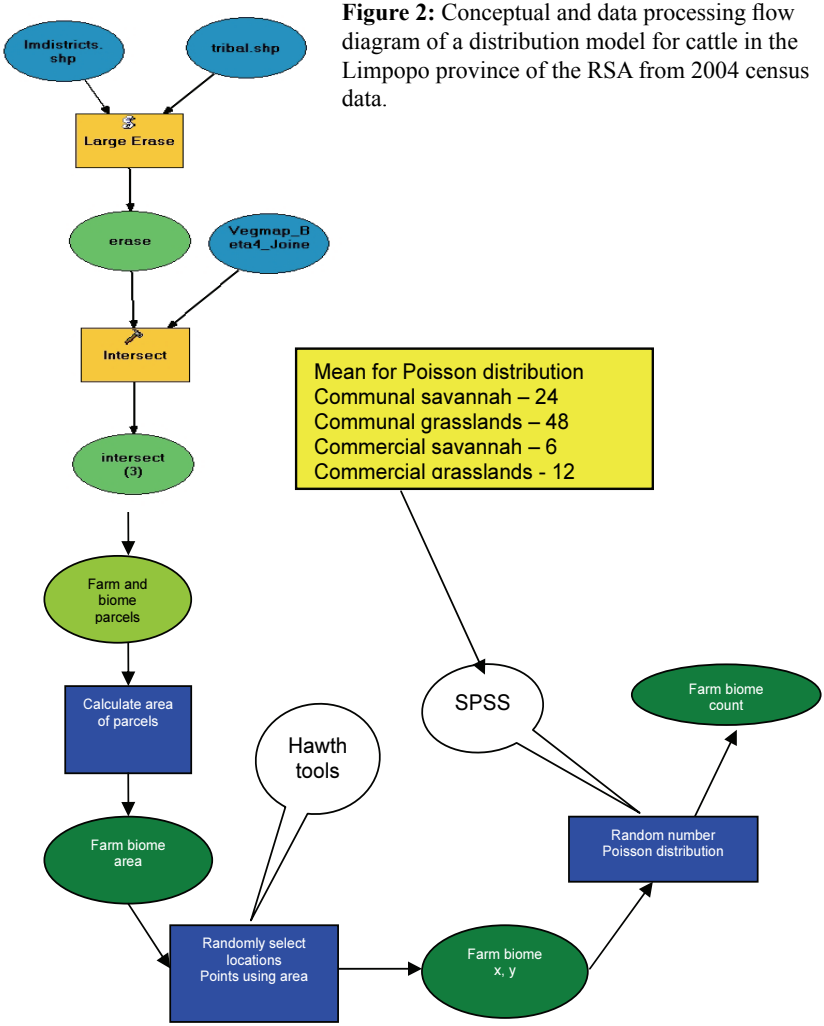

Figure 2: Conceptual and data processing flow diagram of a distribution model for cattle in the Limpopo province of the RSA from 2004 census data.

All the tasks for managing the data were done in ArcGIS . The land parcels for Limpopo and Mpumalanga provinces were projected the same as the vegetation layers. There were 28,183 farm parcels in Limpopo province. From these, 376 parcels which had no farm names were excluded. Some commercial land parcel boundaries were overlapping with communal lands and were erased from the communal lands. This resulted in two separate farm specific layers; one for commercial parcels and another for communal lands as shown in the top part of figure 2 using X-pro tools extension in ArcGIS. The stocking rates for commercial and communal lands extracted from the process above were calculated in Excel using 2004 census data.

The 2001 mega herbivore file for African buffalo was projected to UTM Zone 36 South (WGS 84). The data consisted of bull buffalo (single bulls and herds of bulls) and buffalo herd (total which includes calves) on latitude and longitude point locations in the KNP.

Geoprocessing

The commercial parcels were intersected with the vegetation layer in ArcGIS. Intersection results in a polygon with intersecting boundary and area between the commercial parcels and vegetation layer. The 47 different vegetation types in Limpopo province were reclassified into 4 major biomes (savannah, grasslands, forests and wetlands) using the South African National Biodiversity Institute classification scheme . The areas of each of these biomes which intersected with commercial parcels were calculated. Geographic locations were randomly selected using hawth tools extension in ArcGIS. These points were exported to SPSS where the Poisson distribution was used to model counts of cattle at each location. The mean for the Poisson distribution was determined using stocking rate information calculated for commercial and communal lands.

Grasslands have a high biodiversity and is important for dairy, beef and wool production in South Africa hence we assumed that it has a higher stocking rate than savannah biomes. The above procedure was repeated for communal farm parcels. The conceptual and data processing flow diagram is shown figure 2.

The procedure was based on the assumption that there were no cattle distributed in forests and wetlands because they are unsuitable biomes. Grasslands were considered the most suitable biome for grazing and the stocking rate was doubled for this biome. It was also assumed that the Poisson distribution was adequate to describe the distribution of cattle in the Limpopo province.

In the winter season (April to September) the African buffalo have been shown to not go further than 1 kilometre from water sources . This information was used to create seasonal maps for bull buffalo and buffalo herds in winter (April to September) and summer (October to March). The bull buffalo and buffalo herds were overlapped with the river network for the KNPR. The distance from each point to the rivers was calculated by joining the buffalo data files with the spatial attributes of the river. The maximum distance from the buffalo to the river shown in table 1 was used as a snap threshold for distributing census data to the river network in the KNP. The census points were snapped to rivers using hawth tools extension in ArcGIS. A snap threshold of 10 and 8 kilometres was used for bull buffalo and buffalo herd respectively.

Table 1: Descriptive statistics for the distance between buffalo locations and the closest river for bull buffalo and herds of buffalo.

DISTANCE to nearest RIVER (kilometres)	BULL BUFFALO	BUFFALO HERD
Mean	1.82	2.01
Median	1.03	1.66
Standard Deviation	1.91	1.54
Minimum value	0.07	0.24
Maximum Value	10.00	7.54

In the African buffalo distribution maps the assumptions are that there is no known migratory pattern data or population growth pattern model available for African buffalo, African buffalo shifts are seasonal where in the winter they do not go further than 1 kilometre from water sources, and the impact of the transfrontier conservation areas and re-distribution of wildlife species is not accounted for in the model.

PRELIMINARY RESULTS AND DISCUSSION

The area of commercial parcels was 73593.56 square kilometres and had 430 906 cattle (2004 census) giving a stocking rate of approximately 6 cattle per square kilometre. The area of communal lands was 27021.70 square kilometres and had 637 182 cattle (2004 census) giving a stocking rate of approximately 24 cattle per square kilometre. The stocking rate for savannah was approximated to 6 for commercial parcels and 24 for communal lands. We assumed that grasslands would have double the stocking rate of savannah. The maps are not presented here because they may affect subsequent publication in peer review journals. The resulting maps can be used to identify geographic locations with highest counts for cattle and African buffalo. This is important because locations with high counts of livestock may have higher economic losses during FMD outbreaks. The resulting maps can also be used in transmission models to evaluate spatial and temporal extent of the outbreak.

ACKNOWLEDGEMENTS

This work could not be possible without assistance from Dr. Ungerer, Dr. de Klerk, Dr. Lang, Dr. Bengis and Dr. Dyason with the National Veterinary Services (NVS); Izak Smith with South African National Parks and Dr. Deon Marias with Department of Environmental Affairs and Tourism (DEAT) for providing with retrospective GIS data and expert opinion when needed. This work was supported by the McMillian Foundation and International Education Fee of the Texas A&M University.

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AN OUTBREAK OF DERMATOSPARAXIS IN A COMMERCIAL DRAKENSBERGER CATTLE HERD IN SOUTH AFRICA

Holm, D. E.[1], Van Wilpe, E., Harper, C.

SUMMARY

Dermatosparaxis, also known as cutaneous asthenia or Ehlers-Danlos syndrome, is a heritable (autosomal recessive) collagen dysplasia causing hyper extensibility and fragility of the skin of various species. In cattle this mutation was studied in the Belgian Blue breed, and described as a 3 bp change followed by a 17 bp deletion in the gene coding for the enzyme Procollagen 1 N-Protease (pNPI).

This outbreak in a commercial Drakensberger herd in the Northern Free State followed the introduction of a new bull in 2000 that developed severe skin lesions. Some of his calves were similarly affected and one of his affected sons was kept as breeding bull. Two affected calves were referred to the Production Animal Clinic (Onderstepoort Veterinary Academic Hospital) in October 2005. Detailed clinical examination revealed only skin abnormalities. During a farm visit, 18 cases with skin lesions were seen in the herd of 146 animals (12.3%). No mortalities occurred amongst affected calves. The lesions seen were either large lacerations with sharp skin edges that could be torn easily in acute cases, slow healing defects with contracted edges in sub acute cases or large, but thin scars in chronic cases. Lesions were mostly seen on the lateral extremities of the thorax and abdomen, and on bony protrusions elsewhere. Another abnormality was a thin, lax skin, that could easily be stretched or torn, and that seemed to be poorly attached to the sub cutis. Animals with lesions were all born during 2001, 2002, 2004 or 2005.

Transmission electron microscopy (TEM) examination of skin biopsies revealed haphazard arrangement of collagen fibrils within collagen bundles with the fibrils not being as tightly packed as normal and some having a curved appearance. The fibrils showed size variation and slight irregular outlines were evident on cross-section, consistent with mild Dermatosparaxis.

DNA samples of some animals in the herd were analysed using fluorescently labelled primers to amplify the region of the pNPI gene that contained the mutation described in Belgian Blue cattle, but this mutation could not be demonstrated in any of them.

It is concluded from this case study that a milder, delayed form of dermatosparaxis with a different gene mutation to that described in Belgian Blue cattle existed in Drakensberger cattle in South Africa, which possibly also explains the milder ultrastructural abnormalities. Further investigation is required.

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INTRODUCTION

Dermatosparaxis ("tearing of skin"), also known as cutaneous asthenia or Ehlers-Danlos syndrome, is a heritable collagen dysplasia causing hyper extensibility and fragility of the skin. In some species, joint laxity and blood vessel abnormalities have also been described (Colige et al, 1999). The condition has been known in humans since the 17th century, and was first described as a collagen abnormality by Ehlers (Denmark, 1901) and Danlos (France, 1908) (Van Leuven, 1987). This autosomal recessive defect has been reported in humans, dogs, mink, cattle, sheep, cats and horses (Van Leuven, 1987), but apart from humans only in sheep (white Dorper) in South Africa (Van Halderen et al, 1988). Cattle breeds known to be affected include Belgian Blue, Holstein, Charolais, Hereford, Simmental and crossbred cattle (Scott, 1988 and Steffen, 1993).

Clinical signs and severity of the condition vary between species (Van Leuven, 1987), and several different clinical forms (with differing ages of onset) of the disease have been described in humans (Nuytinck et al, 2000). In Dorper sheep the onset is at 3 weeks to 2 months of age, and severe skin lesions develop that necessitate euthanasia (Van Halderen et al, 1988), although different clinical forms have been described in other breeds of sheep (Van Leuven, 1987). In horses a milder and delayed clinical form exists, that occurs only at 6 to 12 months of age, and smaller skin lesions (2 - 8cm diameter) develop mostly over the thorax and back (Scott, 1988).

In Belgian Blue (and Holstein) cattle onset is soon after birth, and it is reported that in some instances the skin of a foetus can be removed completely during manipulation of dystocia. In the Belgian and Holstein breeds the condition is reported to be lethal, and typical clinical signs include initial subcutaneous oedema of the limbs, eyelids and dewlap and large skin defects in the same areas that are prone to infection (Hanset et al, 1974). These lesions heal slowly with thin, papyraceous scars (Scott, 1988). Hanset et al (1974) further report that early diagnosis can be made by veterinarians due to the ease of penetration of the skin by needles when drugs are administered. In the Hereford breed, onset at approximately 2 months of age, severe skin fragility leading to large lesions on the head and body and also joint laxity and thickened skin has been described (O'Hara et al, 1970). Apart from the above clinical abnormalities, abnormal sound of the voice and poor vision has been reported in calves (Van Leuven 1987).

Ultrastructural changes in the Hereford have been described as randomly distributed, loosely packed collagen fibrils within collagen fibres, leading to fibres with diffuse bizarre, randomly linked and linear ("hieroglyphic") appearance on cross-section (in contrast with the normal round appearance) (O'Hara et al, 1970).

Due to the use of Artificial Insemination and inbreeding in Belgian Blue cattle, the defective gene, after its first appearance, spread quickly and widely within this breed (Lapiere et al, 1971). This spread however, also led to early recognition of the autosomal recessive heritability of this condition (Hanset et al, 1974). The molecular cause of the condition was shown to be a defect in the procollagen protease enzyme, leading to abnormal orientation of collagen fibrils within collagen fibres, and resultant weak fibres (Lapiere et al, 1971). The genetic mutation has been studied in detail in humans and

also in Belgian Blue cattle, and described in the latter as a three base pair (bp) change followed by a 17 bp deletion in the beginning of the coding sequence of the gene coding for the enzyme Procollagen 1 N-Protease (pNPI) (Colige et al, 1999). This mutation is similar to the type VII C form of Ehlers Danlos syndrome in humans, and it changes the reading frame of the message, resulting in the synthesis of a truncated protein. It has been recognised that heterogeneity may occur with Ehlers-Danlos Syndrome in humans, due to the fact that other mutations to those previously described, are causally involved in the disorder (Nuytinck et al, 2000).

MATERIALS AND METHODS

Two affected Drakensberger (indigenous South African beef breed) calves, a bullock of about 6 month old, and a heifer of about 7 months old, were admitted to the Production Animal Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH) (Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, South Africa) in October 2005. Following their admission, full clinical work-up including haematology and serum chemistry was performed.

The two calves were sedated and prepared for skin biopsies on the second day. Skin samples were taken using a biopsy punch from the centre and periphery of the lesions, as well as from apparently normal skin, after regional infiltration of lignocaine dorso-cranial to the biopsy sites. Samples were sent for histopathological and electron microscopical examination.

Lesions were treated conservatively by applying antiseptics, fly repellants and aqueous cream daily. Over the two week period that the calves were in the hospital, the lesions showed very slow healing progress, although the difference was more noticeable in the lesions seen on the male calf.

Discharge of the two calves was followed by a farm visit where a thorough history was taken, all animals in the herd were examined for skin abnormalities, weighed (using a weigh tape), body condition scored and whole blood samples were taken of all animals in the herd for later genotyping. Relationship to other cattle in the herd was recorded in cases where it was known to the herdsmen.

Haematology and serum chemistry were performed by the clinical pathology laboratory of the OVAH using standard techniques.

Electron microscopy was performed by the Electron Microscopy Unit of the Faculty of Veterinary Science, University of Pretoria after initial fixation of samples in buffered formalin, using standard techniques.

Genotyping was performed by the Veterinary Genetics Laboratory, Faculty of Veterinary Science, University of Pretoria, using standard PCR techniques and using primers for amplification of the region of the pNPI gene known to be affected in Belgian Blue cattle.

OUTBREAK HISTORY

The affected herd of commercial Drakensberger cattle, is kept on a small farm

adjacent to the town Virginia, in the Northern Free State province of South Africa. The farm is situated between the town and a large inactive gold mine. Management on the farm is limited, no data of calving date, mass etc are recorded and there is a year-round breeding season with a multi-sire system. Cattle were identified by branding the year of birth only. During the last half of 2000, the farmer bought a 3-year old Drakensberger bull from a stud herd for breeding purposes. In July 2001, this bull started developing skin lesions, after being in a fight with another bull. The lesions seen on this bull were described by the veterinarian as large oedematous lesions on the skin of the neck, thorax and preputium, that drained some serous fluid on lancing, and that eventually became necrotic, sloughed and then healed slowly. These lesions responded poorly to treatment, and new lesions developed from time to time. After several attempts at treating this bull, he was culled during 2002.

Calves born to this bull started developing lesions at about 4 months of age according to the owner. These lesions were similar to those seen in the bull although the appeared mostly on the lateral extremities of the thorax and abdomen, and were also non-responsive to treatment. Skin biopsies were taken during October 2003 of an affected calf. A diagnosis of eosinophilic dermatitis (suspected insect bite hypersensitivity) was made. One of the male calves of the original bull, born in 2001, was kept on the farm as a breeding bull. This bull also developed some skin lesions, although milder than those seen in the original bull. This latter bull's offspring also seemed to develop skin lesions when they reached about 4 months of age according to the owner.

Several attempts at treating these lesions were unsuccessful, but no mortalities occurred amongst the affected calves according to the owner. The owner further reports that after the slow healing process of the lesions, the animals seemed to develop only occasional and much milder lesions once they have reached about two years of age. The farmer and veterinarian were under the impression that the incidence of the disease was slowly increasing during 2005, and a heritable skin condition was suspected at this stage. Because of this, the second bull was culled, and the help of the Production Animal Clinic of the OVAH was called in during October 2005. Since the middle of 2006 no new cases have been reported in this herd.

CLINICAL SIGNS

Detailed clinical examination revealed only skin abnormalities. All other organ systems were normal, and all vital signs were within normal limits. Body condition was 2 and 2.5 (5-point scale) for the two calves respectively.

Skin abnormalities in the two calves were large ulcerated lesions on the lateral extremities (abdomen and bony protrusions of the hips) that seemed to be of different clinical stages. On the bull calf, the lesion was very large (50cm x 30cm), raw, with fresh skin edges and covered with thin scabs (see figure 1). According to the owner,

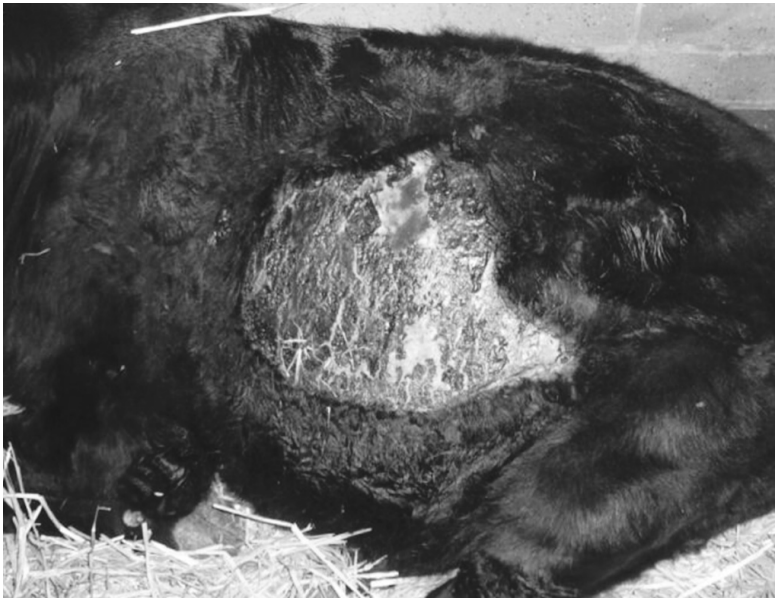

Figure 1: Large lesion seen on the lateral extremity of the male calve's abdomen.

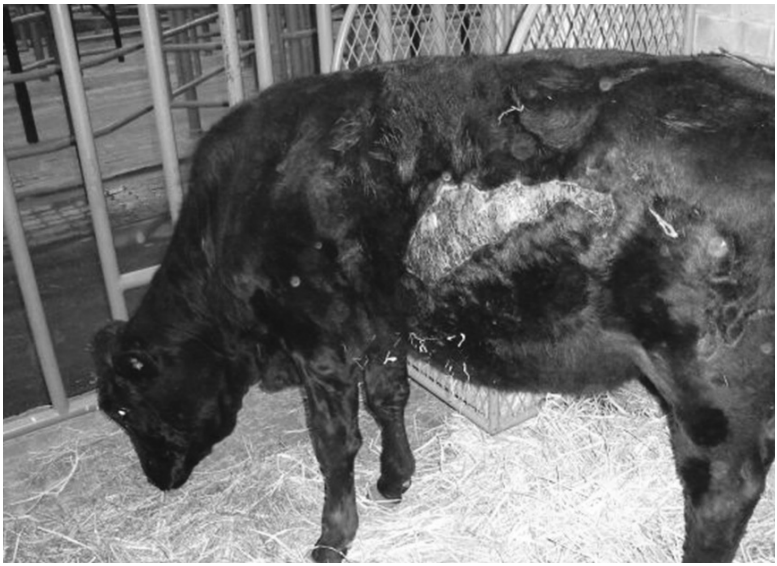

Figure 2: More chronic lesion seen on the side of the 7 month old heifer.

these lesions were approximately 2 weeks old at the time of admission. On the heifer calf, the lesions seemed to be older: they were smaller than in the first calf, with contracted edges that were poorly defined compared to the lesion of the first calf. However, these lesions were also raw and covered with a similar thin scab (see figure 2). According to the owner, the lesions on the heifer were approximately 4 weeks old at the time of admission. Apart from the lesions present, the skins of these animals also seemed thin and could be stretched fairly easily. Stretching of the skin revealed a sensation that the skin consisted of two layers that could be separated without difficulty.

Haematology revealed a mature neutrophilia in the heifer (4.82 x 10^9 neutrophils/litre, normal range: 0.6 – 4.0 x 10^9 neutrophils/litre). Both calves had a low Mean Cellular Volume (MCV), the values were 36.8 and 33.1 *f*litres respectively (normal range: 40 – 60 *f*l). Serum chemistry showed high Creatinine Kinase (CK) levels in both calves (186 and 221 units/litre respectively, normal range: 12 – 146 units/litre), low urea levels in both calves (1.1 and 2.7 mmol/litre respectively, normal range: 3.6 – 10.7 mmol/litre) and both calves had a low Albumin/Globulin ratio (0.79 and 0.76 respectively, normal range: 0.9 – 1.4).

EPIDEMIOLOGY

There were a total of 146 cattle in the herd. Thirteen animals (8.9%) presented with skin lesions of differing severity and clinical stages. Some animals developed fresh skin lesions during our investigation due to trauma induced by the handling facilities. Thin and lax skin was found in 24 (16.4%) animals.

The prevalence of skin lesions amongst female animals was 8.1% (9/111) and amongst male animals was 11.4% (4/35) ($P = 0.77$). The Odds Ratio (OR) for male animals compared to female animals, was 1.45.

Animals with skin lesions at the time of the farm visit were all born during 2001, 2002, 2004 or 2005. There were no lesions amongst animals born before 2001 and during 2003, and skin lesions were most prevalent amongst animals born during 2001

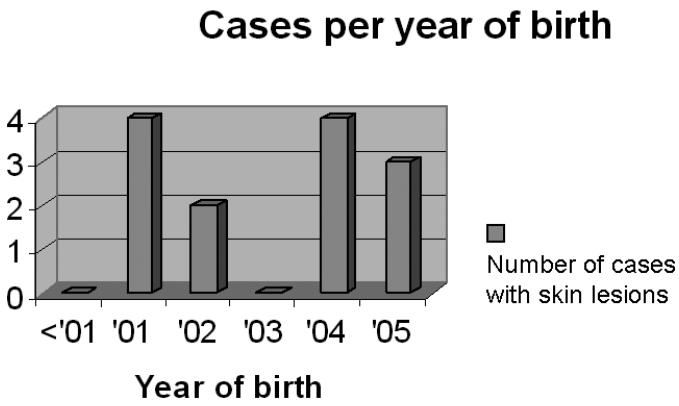

Figure 3: The age distribution of animals with typical skin lesions

(figure 3). Thin, stretchable skin occurred in animals of all ages, but was most prevalent in animals born during 2005.

The lightest animal with lesions weighed 140kg, and there were 8 other calves lighter than 140kg without lesions. There was a male calf with lesions born during 2005 that weighed 210kg, and one born during 2004 that weighed 290kg, representing good growth for this herd. The mean mass of affected animals was 298kg (95% C.I. = 218 – 378kg) and of those not affected by skin lesions was 357kg (95% C.I. = 332 – 381kg), $P = 0.17$. Mean Body Condition Score was 2.78 and 2.86 for affected and unaffected animals respectively ($P = 0.51$).

Due to the lack of records neither familial relationships between animals in the herd, nor temporal distribution of the occurrence of lesions could be established to confirm the farmer's observation that calves only started to develop skin lesions once they have reached approximately 3 or 4 months of age.

ELECTRON MICROSCOPY

Skin specimens, initially fixed in buffered formalin for light microscopy, were subsequently processed for transmission and scanning electron microscopy by standard techniques.

Transmission electron microscopy (TEM) of the reticular dermis revealed the

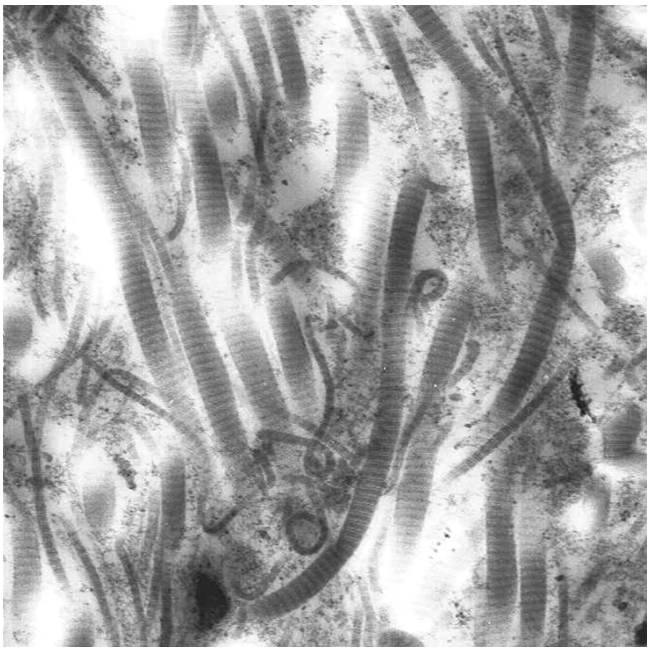

Figure 4: Haphazard organization and size variation of collagen fibrils within a collagen fibre.

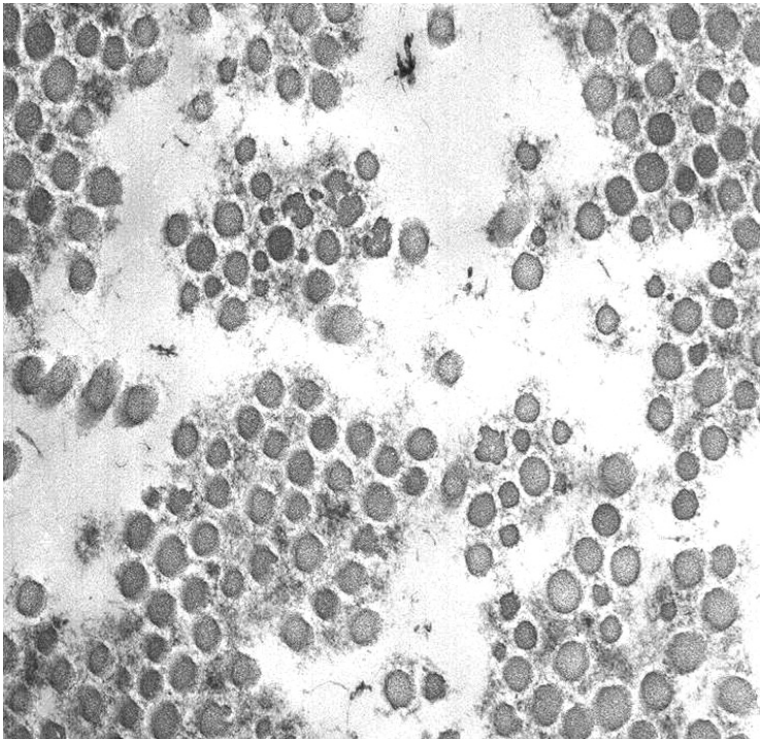

Figure 5: Cross section through a collagen fibre showing the irregular outline of single fibrils. Note the difference in fibril diameter.

presence of collagen fibres containing abnormal fibrils distributed among fibres with a normal architecture. The abnormality was demonstrated in the haphazard arrangement of collagen fibrils within the collagen fibres (figure 4). Some of the fibrils also displayed a curved shape in longitudinal profile and were not as tightly packed within the fibre. The fibrils also showed a variation in size (figures 4 and 5) and slight irregular outlines of individual fibrils were evident in cross sections (figure 4). Active fibroblasts with dilated endoplasmic reticulum, containing finely granular material, was present. The striated periodicity of the fibrils was normal. Scanning electron microscopy reflected the TEM findings in demonstrating the loose, convoluted appearance of the fibrils within the fibres and the size difference between individual collagen fibrils.

GENOTYPING

The standard PCR protocol, routinely used for genotyping cattle at the Onderstepoort Veterinary Genetics Laboratory and including only the primer pair described for dermatosparactic cattle produced only the 101bp fragment in all animals tested, including the affected calves, suspected carriers and suspected non-carrier animals. The mutation

known to occur in Belgian Blue cattle (84bp fragment) could not be demonstrated in any of the samples tested.

DISCUSSION

Dermatosparaxis was the most likely disease that fitted the history, epidemiology and clinical signs in this outbreak. Differential diagnoses for localised anasarca in cattle include increased hydrostatic pressure (heart failure, vascular obstruction), hypoproteinemia (nutrition, intestinal-, liver- or kidney disease), vascular damage (infectious diseases such as besnoitiosis and lumpy skin disease, hypovitaminosis A) and uroperitoneum (mostly male animals) (Radostits et al, 2000). These could be ruled out with reasonable confidence by the epidemiology and clinical chemistry in this outbreak. Other hereditary skin diseases of cattle with roughly similar clinical signs include only familial acantholysis that has as yet only been described in Angus cattle in New Zealand (Scott, D.W., 1988), and that could be ruled out by electron microscopic examination.

There was a fairly low prevalence of skin lesions in the herd (<10%) at the time of examination, taking into consideration that this included all (chronic and acute) cases. This, together with the distribution of year of birth of the affected cattle that fitted the history of the times that the two suspected carrier bulls could have conceived calves, was suggestive of an autosomal recessive genetic mutation as a possible cause of the abnormalities seen. According to the farmer these were not the only bulls present in the herd at the time, and when that it considered one would expect a reasonably high prevalence of carrier females present in the herd.

There was no evidence to suggest that the presence of skin lesions affected growth or production, and the difference in mean mass of affected and healthy animals was confounded by the fact that the healthy part of the herd was older than the affected animals. This was most likely due to the fact that the herd produces weaner calves for the feedlot industry, and most of the affected animals would have been sold at around 7 months of age. Despite the above, the difference in mean body mass of affected and healthy cattle was not significant, and similarly there was no evidence to suggest that body condition was affected by the presence of skin lesions. If the normal pre-weaning growth rate of beef calves is considered, it seems most likely that the 8 animals that weighed less than 140kg at the time of examination were younger than 4-5 months of age, although exact birth dates were not known. The fact that no lesions were found in these 8 calves supported the observation made by the farmer that calves were only affected once they reached about 4 months of age. This is in disagreement with previous descriptions of the clinical onset of dermatosparaxis in cattle, but similar to reports in horses (Van Leuven, 1987).

Although the odds of a male animal being affected were 1.45 times more than that for a female animal, it has to be kept in mind that most male animals in the herd are sold as weaners at the age of +/- 8 months, making the male component of the herd much younger than the females, and this gender predilection can thus be explained by the age distribution within the herd. Previous reports of dermatosparaxis in cattle have also

found no correlation with gender (Hanset et al, 1974).

The only significant clinical signs were the skin abnormalities. The mild and chronic inflammatory response (neutrophilia and decreased albumin:globulin ratio) seen was most likely due to secondary infection of the large skin lesions. The other serum chemistry abnormalities were most likely caused by transport (increased CK - muscle injury) and starvation (decreased urea - rumen metabolism), as serum samples were taken from the hospitalised calves on the same day as transport over +/- 300km. The clinical signs recorded in this outbreak were less severe than those previously described in cattle, and the absence of mortality due to the disease was also in disagreement with previous findings (Hanset et al, 1974 and O'Hara et al, 1970). In the case of the Drakenberger cattle, lesions were limited to the lateral extremities of the thorax, abdomen and bony protrusions of the pelvis. This, as well as the later onset of clinical signs represents evidence that the condition in Drakensberger cattle is similar to, but not exactly the same as that seen in Belgian Blue, Holstein, Hereford and other breeds of cattle, and resembles more the disease described in horses (Van Leuven, 1987 and Scott, 1988).

The abnormalities found in the electron microscopic examination of the dermis of the two hospitalised calves was consistent with dermatosparaxis. Longitudinally sectioned collagen fibrils normally associate closely, with their packing into smooth surfaced bundles being near parallel. Normal fibrils in transverse sections usually appear circular in outline (Eyden et al, 2001). The abnormal fibrils found in the skin lesions of the two calves exhibit a wide diameter range and irregular contours in cross-sectional profile (figure 5). These findings indicate a mild form of dermatosparaxis, as not all the collagen fibres in the two calves exhibited abnormal fibrils. In addition, when compared to the two calves, the ultrastructural findings in ovine (Bavinton et al, 1985) and feline (Holbrook et al, 1980) dermatosparaxis showed a greater percentage of distorted collagen fibrils and most of the transverse fibril profiles displayed flanged contours. Similarly, the abnormalities seen in the Drakensberger cattle seems to be less marked than those in the Hereford, where so-called "hieroglyphic" appearance of collagen fibres was described in TEM examination (O'Hara et al, 1970). This is further evidence that the form of dermatosparaxis seen in Drakensberger cattle is milder than that described in other breeds.

The mutation of the pNPI gene described in Belgian Blue cattle (Colige et al, 1999) could not be demonstrated in this case in any of the affected cattle that were tested. This suggests that the mutation in the Drakensberger breed is probably not the same as in the Belgian Blue, and once again supports evidence that the mutation in Drakensberger cattle is probably unique and needs further investigation. In humans, it has been suggested that heterogeneity may occur in Ehlers-Danlos Syndrome (Nuytinck et al, 2000), and evidence from this study suggests that the same may be true in cattle. However, the difference in the clinical signs between affected Drakensberger cattle and other breeds may rather suggest a unique form of dermatosparaxis.

CONCLUSION

This outbreak investigation provides strong evidence that a milder, delayed form of dermatosparaxis with a different gene mutation to that described in other cattle breeds existed in Drakensberger cattle in South Africa. Further investigation is required.

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NEW INSIGHTS INTO THE EPIDEMIOLOGY OF CALF MORTALITIES DUE TO VANADIUM EXPOSURE

Gummow, B.[1], Holm, D.[1]

SUMMARY

The pathogenesis and epidemiology of vanadium poisoning is complex and multilayered involving determinants at an environmental and animal level as well as at a microscopic cellular level and even a chemical level. The presence and interaction of these determinants produces the course of disease that is ultimately manifested and noticed clinically. One of the functions of an epidemiologist is to figure out what determinants are playing a role in the course of the disease so that preventive measures can be implemented. This paper looks at the question why do calves being exposed to vanadium pollutants become ill at doses that have no apparent effect on adult cattle? This question has puzzled researchers and clinicians for many years. A sentinel study was carried out on the property of a vanadium mine between 1999 and 2005 using Brahman cross cattle divided into two exposure groups (High and Low) comprising a core of 10 and 20 cattle respectively plus offspring. During the period 1999-2004 cattle in the High exposure group were exposed to environmental doses of vanadium ranging from 0.05-23.96 mg V/d/kg body weight (mean=2.6 mg/d/kg) and the Low exposure group to doses of vanadium ranging from 0.01-12.72 mg/kg/d (mean=1.2 mg/kg/d). A total of 17 out of 39 calves died in the High exposure group and 12 out of 56 calves died in the low exposure group between 1999 and 2004. Kaplan-Meir survival curves are given for both groups and a no adverse effect level (NOAEL) for vanadium exposure is discussed. A significant difference was seen between survival rates in the High exposure group and the Low exposure group. Radiographic evidence showed that calves with clinical signs ingest large quantities of soil, which is probably related to their behaviour during periods when they experience GIT disturbances. The behavioural ingestion of soil in environmentally contaminated areas results in increased risks to calves and could explain why calves become ill while their adult counterparts remain healthy. This paper reports on these findings as well as new clinical findings of buccal cavity and vulva erosions and discussed some of the determinants that could explain these.

INTRODUCTION

Why is Vanadium important?

Vanadium (V) is classified as a transition element on the periodic table with the atomic number 23. It is widely distributed in nature and the average level of vanadium

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in the earth's crust is normally 100-150 ppm (Faulkner Hudson, 1964; Richie, 1985; Waters, 1977). The prevalence of vanadium exceeds that of well-known metals such as copper and lead (Nriagu, 1998), and equals that of zinc and tin (Byerrium et al., 1974; Windholz, 1983; Grayson, 1983). Vanadium compounds exist in over 50 different mineral ores at concentrations of between 10-4100 ppm and in association with fossil fuels, particularly coal (at concentrations of between 19-126 ppm in ash) and crude oil (at concentrations of between 3-257 ppm) (Nriagu, 1998). About one third of the vanadium resources are located in Africa and North America, and about 24% are found in Europe and <4% in both Asia and South America (Nriagu, 1998). About 83% of vanadium recently produced from mines comes from vanadiferous magnetite (Fe_2O_3) in South Africa, China and Russia (Hilliard, 1992). The remaining 17 % of worldwide vanadium production from primary sources is recovered from the oil industry.

South Africa is the worlds leading producer of vanadium and accounts for 50% of the current global output. Other producing countries include Russia, China and the USA. Western Australia has large deposits of magnetite ores containing vanadium and the tar sands of Alberta, Canada, represent a huge reservoir of vanadium (Nriagu, 1998). These remain largely untapped sources of vanadium.

The vanadium oxides are most often used by industry, primarily in the manufacturing of steel, where it is used as ferrovanadium or as a steel additive (Reilly, 1991; Toxicological profile for Vanadium, 1992). Vanadium is thus used in producing rust-resistant, spring, and high-speed tool steels. It is also used in the production of components for aircraft engines and weapon systems, making it a strategic mineral for armament manufacturers. In addition, because the metal has good structural strength and a low-fission neutron cross-section, it is useful in nuclear applications. Vanadium foil is used as a bonding agent in cladding titanium steel. Vanadium pentoxide is used in ceramics and is a catalyst. It is also used as a mordant in dying and printing fabrics and in the manufacture of aniline black (CRC handbook of chemistry and physics, 1977). Small amounts of vanadium are used in making rubber, plastics, and certain other chemicals (Reilly, 1991; Toxicological profile for Vanadium, 1992).

Vanadium poisoning has long been an occupational health risk within industry (Faulkner Hudson, 1964). In South Africa, mining companies and farmers are often found in close proximity to one another and it is no surprise that sporadic incidents of vanadium poisoning in cattle have been reported in South Africa over many years (Unpublished archival records of the Onderstepoort Veterinary Institute (OVI), Private Bag X05, Onderstepoort, 0110, 1961/1962, 1975/1976; Gummow et al., 1994; McCrindle et al., 2001). Outbreaks of vanadium poisoning in cattle are however, not confined to South Africa and a number of reports from other parts of the world (mainly Europe) have been published since the 1960's (ter Heege et. al., 1964, Frank et al., 1992; Frank, et al., 1996). Little work has however been done on the impact of chronic exposure to vanadium pollutants in ruminants or on the public health implications of that exposure (Toxicological profile for Vanadium, 1992). It was therefore decided in 1999 to set up a sentinel cattle herd within the bounds of a vanadium mine to study the effects of chronic vanadium exposure in more detail.

The use of animals as environmental sentinels

The primary goal of an animal sentinel system is to identify harmful chemicals or chemical mixtures in the environment before they might otherwise be detected through human epidemiology studies or toxicology studies in laboratory animals. Once identified, exposures to them could be minimised until methods can be devised to determine specific aetiological agents or until suitable prophylactic measures can be established. Animal sentinel systems therefore have potential value as early warning systems for new hazards, as indicators of potential human exposure to complex mixtures or in complex environments, and as monitors of the effectiveness of remedial measures or other environmental management actions (Hornshaw et al., 1983). A primary objective of this study was to investigate whether a long-term animal sentinel system for the vanadium mining industry could act as a monitoring system for pollution problems and thus address the concerns of surrounding farmers. Various potential biomarkers and production and health parameters were monitored in the cattle over the five-year period.

MATERIALS AND METHODS

Animal model system and justification of model

The animal model system comprised 30 Bos indicus beef breeding females with a 20% heifer replacement rate farmed extensively in a 200 ha area adjacent to a vanadium mine. Bos indicus beef cattle were used because they require less intensive management than dairy cattle, are more resistant to tick borne diseases, can be farmed extensively and thus have the most exposure to contaminated grazing. Bos indicus cattle were also more representative of the surrounding farmers cattle, which were farmed extensively. Cattle have been the species most involved in vanadium pollution problems in South Africa.

The area adjacent to the vanadium mine was used because it was not required for mining at this stage and it made economic sense to find an alternative use for the land. In addition, the area was known to have high background concentrations of vanadium in the surface soil and was adjacent to land farmed by farmers that had complained in the past that vanadium mining was having an influence on their animals' welfare.

Experimental design

A cohort study was carried out using 30 Bos indicus beef cattle of the same approximate mass. They were purchased in 1999 as heifers and randomly divided into two groups; A High Exposure (HE) Group of 10 and a Low Exposure (LE) Group of 20 cattle. The HE Group was farmed in an area immediately adjacent to the mine where high background concentrations of vanadium were thought to occur. The LE Group was farmed approximately 2-3 km from the first group in an area thought to have much lower background vanadium concentrations (D. Stein, Environmental officer, and L. Ford, Chemical engineer, personal communication, 1999). Both groups were farmed as an extensive beef cattle enterprise. The herd size was limited by the amount of

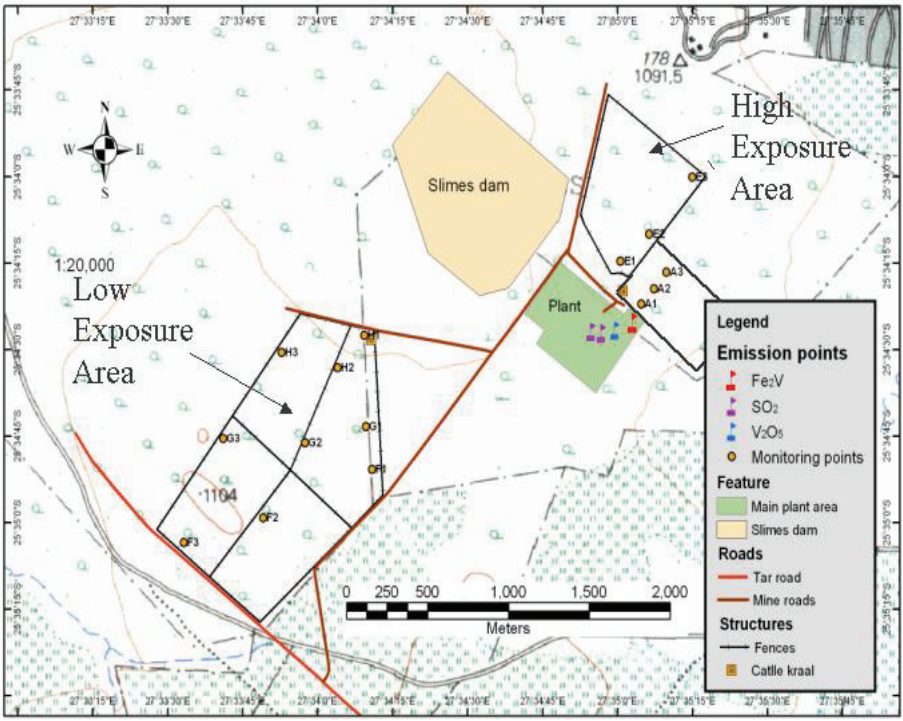

Figure 1: Map of the area where the trial took place showing the primary sources of vanadium release in relation to the LE and HE camps and the points where samples were routinely collected in those camps.

available grazing and a stocking density of approximately 1 animal per 5 ha was used as a guideline.

Observations and analytical procedures

The farm was visited by at least one of the veterinary co-workers once every 3 months, to monitor the health status of the herd, collect samples and bring records up to date.

A record system was kept by the Department of Production Animal Studies at the Faculty of Veterinary Science, University of Pretoria for analyses purposes.

A weather monitoring station was placed on the mine's property to measure wind direction, wind speed, ambient temperature, humidity and rainfall.

Nine deposit samplers per camp were placed at strategic points along three transections within each camp to capture airborne particulate matter. Each transect began near the processing plant and extended out to the boundary of the camps (Fig. 1). Soil and grass samples were collected once every 3-4 months at the same points as the nine deposit samples per camp. A variety of grass species were sampled at each sampling point in each transect. At the same time, water samples were taken from the drinking

troughs. The soil, grass and water samples were analysed by the Institute of Soil, Climate and Water, Pretoria, South Africa, for concentrations of heavy metals and in particular vanadium, using standard internationally accepted methods and quality control procedures (USEPA Standard Methods, 1986; Handbook of standard soil testing methods for advisory purposes, 1990).

At the same time the soil and grass samples were taken during the year, various potential biomarkers and production and health parameters were monitored in the cattle. These included bone, hair, milk, urine and faeces for vanadium determination and blood for haematology and chemical pathology.

RESULTS AND DISCUSSION

The animal health parameters that were being monitored during the five year period of the work serve as consequences of exposure. They included monitoring body weights (Fig. 2) as an indicator of possible effects on weight gain, calving percentages and pregnancy rates as indicators of cow fertility and general cattle health status.

Figure 2: Average weight of two groups of South African adult Brahman-cross sentinel cattle exposed to vanadium between 1999 and 2004.

Throughout the five years of the study, adult cattle showed few clinical signs or evidence of adverse effect. Production in terms of weight (Fig. 2) and calving percentages (70-80% for HE group; 50-75% for LE group) were within expected parameters for a herd of this nature. Clinical pathology parameters (BUN, AST, GGT) gave no indication of liver or kidney malfunction. Apart from conditions routinely seen in herds of this nature, the only abnormal clinical finding was occasional evidence of unexplained ulcers on the lips and nostrils with related nasal discharge and salivation in the high exposure herd. These lesions were also seen in calves, where they were usually a lot more severe (Fig. 3 and 4). There was no evidence of an increased rate of abortion or still births as

Figure 3: Lesions on the lips of South African sentinel calves exposed to chemicals emitted by the vanadium mine (1999-2004).

reported in previous outbreaks (Gummow et al. 1994).

During 1999 the first batch of calves were born and began showing clinical signs. One calf died and two were euthanased for necropsy out of ten calves born in the HE camp. The signs were different when compared to a previous outbreak of chronic vanadium poisoning in calves that occurred in South Africa in 1991 (Gummow et al. 1994) and it was uncertain if they had died of vanadium poisoning. Signs not seen in the 1991 outbreak were ulcers on the muzzle, nostrils (Fig. 3) and in a few cases vulva, as well as dyspnoea as a result of upper respiratory tract congestion and swelling and a profuse muco-purrulent nasal discharge (Fig. 4). It was thought the lesions might be viral in origin but electron microscopy done later in the study failed to reveal any viral particles in the lesions. Post mortems were non-specific and it was still uncertain that the cause of death was vanadium as these signs had not been described before. Since adult cattle showed no clinical signs or adverse effects and the cause of the signs was uncertain it was decided to continue with the project.

Other signs that became apparent were signs of facial paralysis as reported by Frank et al. (1990) and visio-spatial problems as seen in humans (Barth et al., 2002), but not blindness. These nervous signs were not noticed in the 1991 outbreak (Gummow et al., 1994). It is thought however that the neurological lesions are biochemical in nature and related to disruption of transmitters rather than physical lesions. Why certain nerves are

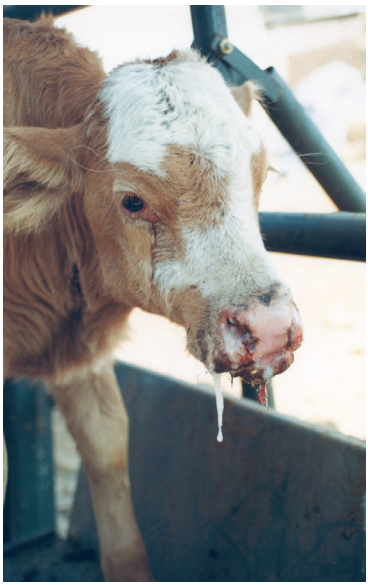

Figure 4: Signs of nasal discharge, salivation and dyspnoea seen in South African sentinel calves (1999-2004).

targeted remains a mystery.

As the project continued, a trend developed whereby a proportion of calves in the HE camps would become ill and frequently die, while those in the LE camps rarely showed signs. This is illustrated in the Kaplan-Meier survival analysis curves worked out for the two groups using all mortalities regardless of clinical signs (Fig. 5). The mortalities in the LE group of calves appeared, from necropsy, not to be directly related to vanadium but are never-the-less reflected in the survival curves. A total of 17 out of 39 calves died in the High exposure group and 12 out of 56 calves died in the low exposure group between 1999 and 2004. From the survival curves it can be seen that most deaths occur within the first 16 weeks after birth but mortalities can continue to occur over the next few years as surviving claves remain poor doers.

A question that remained unanswered throughout the years of work was why do suckling calves become sick and not adult cattle? Finding the answer to this question is the subject of continued work at the Faculty of Veterinary Science, University of Pretoria. Recent work, using radiographs of the rumens of affected calves has shown that calves ingest a lot more soil than was previously anticipated (Fig. 6) and that it remains in the rumen for a period of at least weeks if not months. Chemical analysis of the rumen gravel in affected calves has shown this to be high in vanadium. How much is soluble vanadium is still to be determined. However, this behaviour of eating soil may now explain the lesions on the lips and nostrils of these animals and why calves are more susceptible than adult cattle. It is thought that ingestion of some vanadium

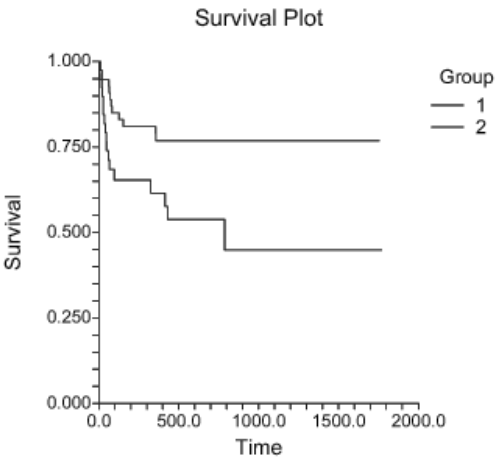

Figure 5: Kaplan-Meier survival analysis for sentinel calves in the HE group (1 – --bottom line) and the LE group (2 - …. top line), South Africa 1999-2004.

causes irritation of the GIT and that the natural response to this is for calves to eat some soil. In the case of polluted soils this has the potential to cause a cycle of irritation and ingestion resulting in large doses of vanadium or other pollutants being taken in. It also means that soil concentrations of vanadium and other metals, may play a much more important part of risk estimation for calves than was previously thought.

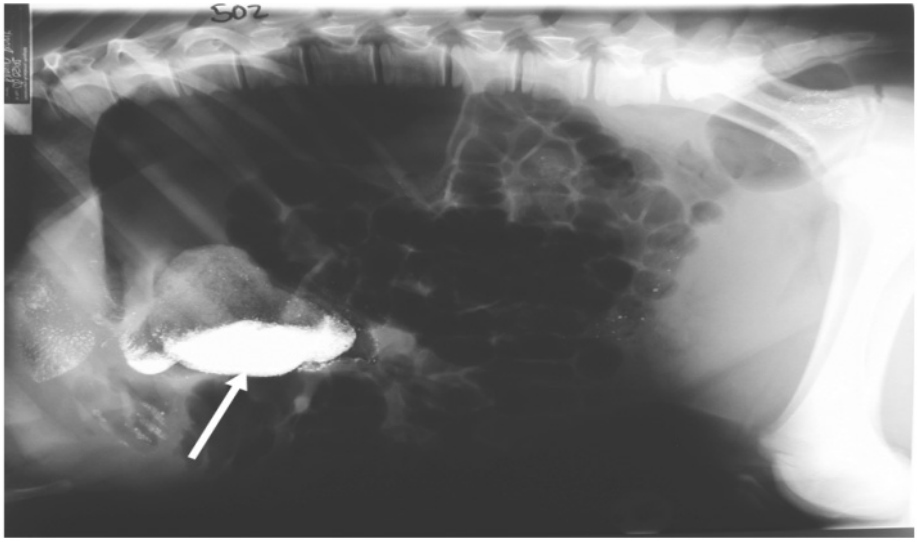

Figure 6: Radiograph of soil (arrow) containing metals found in the rumen of a 6 w old affected sentinel calf, South Africa, 2005.

By 2003 it appeared that when calves in the HE camp were born within 200 m of the vanadium processing unit (VPU), they developed signs and often died but if born further away survived. This theory was tested in 2004 when the calving area for the HE calves was moved beyond this 200 m radius and all calves born during that year survived. Confounding this was evidence that the pollution levels from the mine had also been decreasing over the years of the study.

The relevance of these findings is that a high percentage of calves in the HE areas invariably became sick and died or were euthanased, while calves in the LE areas did not. Hence the NOAEL must lie somewhere between the two exposure doses (Gummow et al., 2006a;). The exposure model created during the project, estimated a composite external dose over the five-year period of between 0.05 and 23.96 mg vanadium/kg body weight/day ($\bar{x}$=2.6) for the HE group and between 0.01 and 12.72 mg/kg/d ($\bar{x}$=1.2) for the LE group. There was only a 5% probability of values being <0.56 or >6.58 mg/kg/d for the HE group and <0.33 or >2.73 mg/kg/d for the LE group (Gummow et al. 2006a). The study therefore, showed that the composite NOAEL for cattle farmed in the vicinity of a vanadium processing unit, with its related chemical mixes, lies somewhere between 1.2 mg V/kg/d and 2.6 mg V/kg/d (Gummow et al., 2006a). This finding allows accurate risk estimation for areas with similar identified hazards (Gummow et al. 2006b).

CONCLUSIONS

The behavioural ingestion of soil in environmentally contaminated areas results in increased risks to calves and could explain why calves become ill while their adult counterparts remain healthy. It could also explain the ulcers seen on the nose and buccal cavity of exposed animals. At the concentrations of vanadium found in the HE camp there is a definite risk to calf health, with related production and economic impact to farmers. Viable extensive cattle farming cannot take place at these levels of exposure. At concentrations of vanadium found in the LE camp, there is minimal impact on cattle health, and extensive cattle farming should be sustainable at these levels of exposure. The NOAEL for intake of vanadium in cattle lies between 1.2 and 2.6 mg V/kg/d.

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THE VALUE OF REPRODUCTIVE TRACT SCORING AS A PREDICTOR OF FERTILITY AND PRODUCTION OUTCOMES IN BEEF HEIFERS

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SUMMARY

In this study 272 beef heifers were studied from just prior to their first breeding season (15 October 2003), through their second breeding season and until just after they had weaned their first calves in March 2005. The study consisted of two main parts: in the first part, the economic effects of an oestrus synchronisation protocol using prostaglandin were tested.

In the second part of this study, reproductive tract scoring (RTS) by rectal palpation was performed on the same group of heifers one day before the onset of their first breeding season. The effect of RTS on several reproduction and production outcomes was tested, and the association of RTS with the outcomes was compared to the associations of other input variables such as mass, age, body condition score (BCS) and Kleiber ratio using multiple or univariable linear or logistic regression.

RTS was associated with pregnancy rate to the 50 day AI season ($P < 0.01$), days to calving ($P < 0.01$), calf weaning mass ($P < 0.01$) and pregnancy rate to the subsequent breeding season ($P < 0.01$). These associations were mostly independent of associations with mass, age and BCS before the onset of the first breeding season. RTS was a better predictor of fertility than was Kleiber ratio, and similar in its prediction of calf weaning mass.

It was concluded from this study that RTS is a unique predictor of heifer fertility, compares well with (but is independent of) other traits used as a predictor of production outcomes and is likely to be a good predictor of life production of the cow.

INTRODUCTION

Age at puberty (AP) in heifers is conveniently defined as the age when a heifer displays visual signs of oestrus for the first time (Pineda, 2003). Although AP can be determined for individual animals by continuously observing for signs of oestrus, it is impractical in a large group of heifers (Anderson et al 1990). In the past, conformation, mass, body condition score and calculated indices such as the Kleiber ratio (Scholtz and Roux, 1988) have been used to select heifers for breeding in South Africa and elsewhere. Anderson et al. (1990) developed a standardised reproductive tract scoring (RTS) method to measure age at puberty of heifers directly. This method involves rectal palpation of the reproductive tracts and ovarian structures and is scored from 1 to 5, where heifers with scores 1 and 2 are not cycling, those with score 3 are on the verge

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of puberty, and those with scores 4 and 5 are cycling (see Table 1 below). Anderson et al (1990) recommends three possible applications of the RTS system: firstly as a screening test to determine the pubertal status of heifers before the breeding season, secondly as an indication of the nutritional requirements of heifers when sufficient time is allowed before the breeding season, or thirdly as a selection tool for AP. For the latter application it is important to do the examination at a strategic time, when approximately 50% of the heifers are cycling (Pence and Bredahl, 1998).

The RTS score as a method of selection, has been found to be correlated to AP, response to synchronisation and pregnancy rate to synchronised oestrus, and has an estimated heritability (h^2) of 0.32 (Anderson et al., 1990).

Rosenkrans and Hardin (2002) evaluated the accuracy and repeatability of the RTS system and found it to be a repeatable (within veterinarian and between veterinarians) method to estimate pubertal status. Kappa values (a measure of degree of agreement beyond chance) were 0.64 and 0.46 for agreement within veterinarian and between veterinarians respectively. These Kappa values represent moderate to substantial agreement beyond chance (Landis and Koch, 1977).

Table 1: Reproductive Tract Scoring system (Anderson, 1991)

		Ovaries			
Reprodutive Tract Score	**Uterine horns**	**Length (mm)**	**Height (mm)**	**Width (mm)**	**Ovarian structures**
1	Immature < 20mm diameter, no tone	15	10	8	No palpable structures
2	20 - 25mm diameter, no tone	18	12	10	8mm follicles
3	25 - 30mm diameter, slight tone	22	15	10	8 - 10mm follicles
4	30mm diameter, good tone	30	16	12	> 10mm follicles, Corpus Luteum possible
5	>30mm diameter, good tone, erect	>32	20	15	> 10mm follicles, Corpus Luteum present

The objective of this study was to compare RTS with mass, age, BCS and Kleiber ratio before breeding for predicting beef heifer performance under South African conditions.

MATERIALS AND METHODS

A prospective study was performed to determine the association of RTS and other predictors of heifer performance with production and reproduction outcomes.

A group of 272 Bovelder heifers, at Johannesburg Water's Northern Farm was selected for this trial in October 2003. The Bovelder is a synthetic South African beef breed. Northern Farm is located 30 km North of Johannesburg, South Africa (latitude 25°50'S) on the highveld of Gauteng (1380m above sea level). Annual rainfall averages 690mm per year, falling between September and May, and daily minimum and maximum temperatures (mean) in this region are 14.9°C and 27.0°C in January, and 2.7°C and 18.2°C in June (De Villiers, 2006). Processed water from the water purification works is used to irrigate kikuyu, clover and ryegrass pastures on this farm, and supplemental to these pastures, cattle are fed grass hay, silage and mixed ration diets at times when the pastures cannot fulfill the nutritional needs.

The heifers' ages at the start of the breeding season ranged from 364 to 486 (median 431) days. Two days before the onset of the insemination season (day -1), all heifers were weighed, body condition scored (BCS) (5-point scale) and reproductive tract scored (RTS) (Anderson et al., 1991). Mass ranged from 261 to 407 (mean 314.4) kg.

Frozen semen of 11 different bulls was allocated to heifers according to normal farm practice. Farm management and other staff were blinded to RTS category, and heifers were managed as one group. The insemination season started on 15 October 2003 (day 1). Heifers were inseminated once a day (9am) on observed oestrus by one experienced AI technician. Oestrus detection was done by visual observation and marking during each night, and a scratch-off sticker type oestrus detection aid for each heifer's first oestrus (*Estrus Alert,* CRI, PO Box 717, Howick, 3290, South Africa).

Days into the AI season and semen batch were recorded for all inseminations during the breeding season, as part of the normal farm record system. A veterinarian performed pregnancy diagnoses (PDs) by rectal palpation 90 days after the removal of bulls. Abortions, birth date, -mass and gender of calf, dystocia, calf mortality, cow mortality and weaning mass were subsequently recorded. Before the onset of the subsequent breeding season (1 November 2004), researchers recorded BCS again. Similar records were collected during the subsequent breeding season, and the trial was terminated on 1 April 2005. All calves were weaned on the same day (29 March 2005).

Days to pregnancy was defined as the number of days into the breeding season that it took for a heifer to become pregnant. Days to first insemination and days to calving were defined similarly, and the first day of the calving season was defined as the day when the first calf was born. When a heifer did not achieve the specified status by the end of the time period a maximum value was given to that heifer (eg. 50 in the case of days to first insemination), but these values were censored for the purpose of Cox regression.

All data were entered into a spreadsheet for analysis. Proportions were compared using the Fisher exact test for 2x2 tables, or the Chi-square test for 2xk tables, while means and medians were compared using ANOVA and Kruskal-Wallis one-way ANOVA on ranks respectively. The log-rank test was used for time-to-event data (such as days to

calving). Multivariable analyses included multiple regression for continuous outcomes and logistic regression for binary outcomes. RTS and BCS were treated as continuous variables, after checking that their univariable associations with each outcome was roughly linear. Kleiber ratio was not used in the multivariable analyses because it is calculated using mass and age as factors and will necessarily be closely associated with mass and age. Statistical analyses were done using NCSS 2004 (NCSS, Kaysville, UT, USA) and Epicalc 2000 (http://www.brixtonhealth.com/epicalc.html).

RESULTS

Associations between RTS and other pre-breeding indices

Using univariable linear regression, age, mass and BCS before the onset of the breeding season were associated with RTS ($P = 0.03$, $P < 0.01$ and $P < 0.01$ respectively). Table 2 is a summary of the multiple regression model for RTS, where age, mass and BCS are given as independent variables. It shows pre-breeding age as being the only variable with significant association with RTS ($P < 0.01$).

Table 2: Effects of pre-breeding age, mass and BCS on RTS (multiple regression)

Variable	Coef	SE	95% CI		P
Age	0.013	0.003	0.006	0.019	<0.01
Mass	0.002	0.003	-0.003	0.007	0.40
BCS	0.219	0.195	-0.163	0.601	0.26

Associations with days to first AI

Heifers with RTS 2 had a median days to first AI of 11, which was significantly higher than heifers in all other RTS categories ($P = 0.04$). Heifers with RTS 3 had a median days to first AI significantly lower than those with RTS 2, and significantly higher than those with RTS 5, but similar to those with RTS 4 (table 3). Table 4 gives a summary of the multiple regression model for days to first AI, with RTS, BCS and age before the breeding season as independent variables.

Table 3: Median days to first AI by RTS category

		Days to first AI	Pregnancy rate to AI	Days to calving	Weaning mass (calf)	Pregnancy rate to subsequent AI
RTS	n	median	%	median	mean (kg)	%
1	16	6^{ab}	31^{a}	53.5^{ab}	194^{ab}	63^{ab}
2	70	11^{b}	40^{a}	52^{a}	186^{b}	61^{b}
3	81	8^{ab}	53^{a}	28^{bc}	213^{ac}	72^{ac}
4	74	8^{a}	70^{b}	15^{c}	207^{ac}	85^{ac}
5	30	6^{a}	80^{b}	18^{c}	213^{ac}	90^{ac}

[abc] Superscripts that differ: P < 0.05

[n] Numbers at onset of trial

Table 4: Independent effects of pre-breeding RTS, mass, BCS and age on days to first AI

Variable	Coef	SE	95% CI		P
RTS	-1.051	0.444	-1.921	-0.182	0.02
Mass	0.027	0.019	-0.010	0.063	0.15
BCS	-4.428	1.404	-7.180	-1.675	<0.01
Age	-0.014	0.024	-0.061	0.034	0.57

Associations with Pregnancy Rates

Pregnancy rates for the 50 day AI season for the different RTS categories are summarised in table 3: heifers with RTS 1 to 3 had a significantly lower pregnancy rate than those with RTS 4 and 5.

Final pregnancy rates (including the period of bull breeding) were 56%, 76%, 81%, 92% and 93% for heifers with RTS of 1,2,3,4 and 5 respectively. Significance of these differences was similar to those differences seen in the pregnancy rates to the AI season.

Both RTS and Kleiber ratio showed univariable associations with pregnancy outcome ($P < 0.01$ and $P = 0.05$ respectively). When RTS was compared to mass, BCS and age before the breeding season with regards to their independent associations with pregnancy to the 50 day AI season (logistic regression), only RTS had an independent association that was statistically significant ($P < 0.01$) (see table 5).

Table 5: Effects of pre-breeding RTS, mass, BCS and age on pregnancy to AI

Predictor	Coef	SE	95% CI		P
RTS	0.572	0.129	0.319	0.825	<0.01
Mass	0.008	0.005	-0.003	0.018	0.15
BCS	0.211	0.385	-0.545	0.966	0.58
Age	-0.002	0.006	-0.016	0.011	0.74

Similarly, when RTS was compared to mass, BCS and age before the breeding season with regards to their associations with final pregnancy (including the period of bull breeding), once again only RTS had a significant independent association ($P < 0.01$).

Associations with days to calving

Medians of days to calving were 53.5, 52, 28, 15 and 18 for heifers with RTS 1,2,3,4 and 5 respectively. There was a significant difference in median days to calving between heifers with RTS 2, and those with RTS 3,4 or 5 ($P = 0.02$) (table 3).

Table 6 gives a summary of the multiple regression report of days to calving, with RTS, mass, BCS and age before the breeding season as independent variables. RTS has

the most significant (negative) independent association with days to calving indicating that an increase in RTS leads to an earlier calving date.

Table 6: Independent effects of pre-breeding RTS, mass, BCS and age on days to calving

Variable	Coef	SE	95% CI		P
RTS	-7.018	1.856	-10.680	-3.356	<0.01
Mass	0.156	0.082	-0.006	0.319	0.06
BCS	-11.794	6.102	-23.837	0.025	0.05
Age	-0.114	0.103	-0.318	0.010	0.27

Univariable linear regression showed that RTS was associated with days to calving ($P < 0.01$) while Kleiber ratio tended to correlate with days to calving ($P = 0.10$).

Associations with calf weaning mass

Calves of heifers with RTS 1 and 2 (n = 33) had a mean weaning mass of 186.7kg (90% CI 176.0 – 197.4 kg) while calves of heifers with RTS 3, 4 and 5 (n = 102) had a mean weaning mass of 210.1 kg (90% CI 203.8 – 216.4 kg) ($P < 0.01$). The only group of heifers with a single RTS that weaned calves with a mean weaning mass significantly lower than the mean weaning mass of calves from other RTS category heifers, were those with RTS 2 (185.8 kg, $P < 0.01$).

Univariable linear regression showed a significant association between RTS and calf weaning mass ($P < 0.01$), and also between Kleiber ratio before the onset of breeding and weaning mass of the calf ($P = 0.05$). Table 7 is a summary of the multiple regression model for weaning mass of the calves, where pre-breeding RTS, mass, BCS and age were given as independent input variables. When days to calving was included as independent variable in the multiple regression model for calf weaning mass as shown in table 7, only age and days to calving were significantly associated with calf weaning mass ($P < 0.01$).

Table 7: Effects of pre-breeding RTS, mass, BCS and age on weaning mass of the calves (multiple regression).

Variable	Coef	SE	95% CI		P
RTS	6.296	2.728	0.898	11.693	0.02
Mass	-0.073	0.131	-0.331	0.185	0.58
BCS	5.528	8.637	-11.559	22.616	0.52
Age	0.250	0.146	-0.038	0.538	0.09

Associations with pregnancy rates after the subsequent breeding season

Heifers with original RTS of 2 that were still on the farm one year later had a significantly lower pregnancy rate to the subsequent AI season than those with RTS of 4 and 5 ($P = 0.03$) (table 3).

Univariable logistic regression showed a significant association between RTS (before the first breeding season) and pregnancy outcome after the second AI season ($P < 0.01$), while Kleiber ratio was not associated with pregnancy rate after the second AI season.

Receiver-operating characteristic (ROC) analyses of pregnancy outcomes

ROC analysis for RTS, yielded an area under the curve (AUC) of 0.65 for its prediction of pregnancy outcome after the 50 day AI season (Figure 1A), and an AUC of 0.66 for its prediction of pregnancy outcome after the subsequent AI season. For both BCS and Kleiber ratio, ROC analysis of pregnancy outcome after the 50 day AI season yielded an AUC of 0.50. Combining BCS and Kleiber ratio with RTS in a model of pregnancy to the 50 day AI season yielded an AUC of 0.67 (Figure 1B).

DISCUSSION

In this study RTS and BCS, which are arguably the more subjective measurements (compared to mass and age), showed independent association with production outcomes in more instances than the objective measurements (tables 4, 5, 6 and 7). The fact that RTS is a subjective measure did not negatively affect the strength of association of RTS with the outcomes. RTS showed a significant independent association with all the outcomes (Tables 4 - 7) while BCS showed significant independent association with two of the outcomes (days to first AI and days to calving). Age tended to have an independent association with weaning mass (table 7), while pre-breeding mass had a near-significant independent association with days to calving only (table 6). This demonstrates that RTS

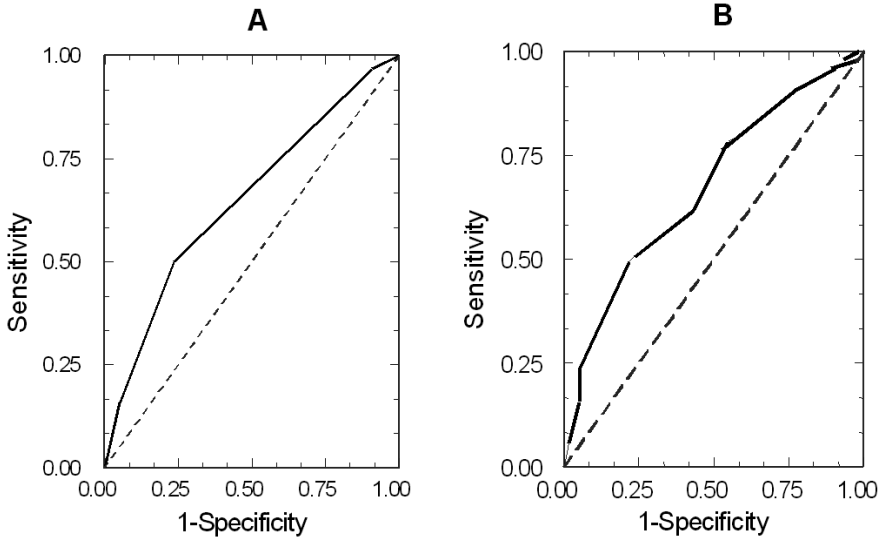

Figure 1: ROC curves for RTS (A) and RTS, BCS and Kleiber ratio (B) on pregnancy outcome after the 50 day AI season.

had a unique association (independent of mass, BCS and age) with production as well as reproduction outcomes. The fact that RTS became an insignificant independent factor of calf weaning mass when days to calving was added to the regression model shown in table 7, shows that the effect of RTS on calf weaning mass is through its association with days to calving rather than a direct association with calf weaning mass. This highlights the fact that by selecting for reproduction in a cow-calf operation, production outcomes will also benefit.

Comparing RTS with Kleiber ratio as predictors of heifer performance

Kleiber ratio is calculated as growth (ADG) per metabolic mass ($mass^{0.75}$), and because ADG is a factor of mass, age and birth mass, Kleiber ratio is therefore also a factor of mass, age and birth mass. The variation in birth mass is relatively small, and Kleiber ratio is mainly a function of mass and age. For this reason, Kleiber ratio could not be included in multiple regression models where mass and age before the breeding season were used as input variables, because it would be too highly correlated with them. To compare the suitability of RTS and Kleiber ratio in predicting production and reproduction outcomes, univariable regression was used. Simple logistic regression was used for pregnancy outcomes, and linear regression for numerical outcomes. RTS showed a consistently more significant association with reproduction outcomes than Kleiber ratio, and similarly significant association with calf weaning mass. This is evidence that RTS can be used as a primary selection tool for heifers before the onset of breeding without any detrimental effect on production.

If RTS had been used as a selection criterion in this group of heifers before breeding, and RTS 2 was used as the cut-off point (from Table 3), selection of the best 94% of heifers would have increased the pregnancy rate to the 50 day AI season from 56% to 58% ($P = 0.79$). Using RTS 3 as the cut-off point (selecting the best 68% of heifers) would have resulted in an increase in pregnancy rate to the 50 day AI season from 56% to 64% ($P = 0.10$). Although impractical because of the proportion of heifers that would have needed to be culled, using RTS 4 as cut-off would have resulted in an increase in pregnancy rate from 56% to 73% ($P < 0.01$). It seems that in this group of heifers it would have been most sensible to use RTS 3 as cut-off for selection. Of course, this will not always be the case, and it depends on the timing of RTS and the level of puberty reached by the group of heifers by that time. If the best 68% of heifers in this group were selected using Kleiber ratio, it would not have increased pregnancy rate to the 50 day AI season (56% vs. 57%, $P = 0.96$). The superiority of RTS as a selection tool for fertility outcome is well demonstrated by this.

RTS cannot be compared to BCS in the same way as above, because the cut-off points for RTS and BCS are different. Receiver-operating characteristic (ROC) analysis is a useful tool to compare the predictive value of RTS and other measures on pregnancy outcome, although the idea of RTS is not simply to predict pregnancy outcome, but rather as a selection tool for fertility. RTS has a higher area under the curve (AUC) value using ROC analysis (0.65) than BCS or Kleiber ratio, and the latter two do not add any predictability to RTS when used in a combined model (Figure 1). In fact, both

BCS and Kleiber ratio have AUC values of 0.5, indicating that they are not useful for predicting pregnancy outcome.

Long term benefits of using RTS as selection tool

Selecting for RTS leads to a reduction in days to calving (Table 3), which allows heifers more time to recover from the stress of calving and to become prepared for the next breeding season. First calf cows are known to be the group under most pressure to re-conceive in the subsequent breeding season, due to the fact that they are still growing and also nursing a calf, which puts tremendous pressure on their energy and protein metabolism, to the disadvantage of fertility (Chenoweth and Sanderson, 2001). RTS has been shown in this study to not only have effect on the immediate calving season, but also on the subsequent calving season (table 3). The proportion of heifers with RTS 4 and 5 that remained in the herd until their second breeding season was 80/104 (77%), while that proportion for heifers with RTS 1 to 3 was 90/167 (54%), demonstrating a significantly increased survival of heifers with higher RTS ($P < 0.01$).

Apart from this, amongst the heifers that were retained until their second breeding season, there was a strong association between RTS (before first breeding season) and pregnancy outcome of the second breeding season, most likely due to the effect of RTS on days to calving. The effect of days to calving on pregnancy rate of the subsequent breeding season is well known (Chenoweth and Sanderson, 2001)

Using the data presented in these results, it can be calculated that using RTS 3 as cut-off for selection would have tended to increase the pregnancy rate of the subsequent breeding season from 76% to 81% ($P = 0.37$). Further, if Kleiber ratio had been used to select the best 68% of heifers, it would have tended to decrease the pregnancy rate to the subsequent breeding season from 76% to 68% ($P = 0.19$).

It can be seen here that one has to take account not only of the direct benefit of using RTS as selection tool for heifers, but also the effect that selection using RTS will have on life production of the cows.

Due to its ease of measurement, good heritability and association with feed conversion ratio and therefore energetic efficiency (Nkrumah, 2004), Kleiber ratio has been used on many beef farms for many years as one of the (or the only) selection tools for replacement heifers. This evidence suggests that RTS is a valid way of selecting for heifer fertility before the heifers are bred for the first time, rather than to use performance indices after the breeding or calving season as culling measures. Evidence from this study suggests that selecting for RTS will not select against production measures such as Kleiber ratio, although the analyses are not sufficient to prove that for certain. It must be noted that the association between RTS and weaning mass of the calves is most likely as a result of the effect that RTS has on days to calving. This was confirmed in the results: when days to calving was added to the regression model, days to calving, and not RTS, was a significant independent variable of weaning mass as outcome. RTS is primarily an indicator of age at puberty, and therefore a predictor of fertility outcome, and should not be used as the only selection measure for heifers.

CONCLUSION

It is concluded that RTS before the onset of the breeding season is a predictor of heifer reproductive performance, even after adjustment for age, mass and BCS. It is a better predictor of fertility than other traits commonly used, compares well with other traits in predicting production outcomes, and is likely to be a predictor of life production of the cow.

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MOLECULAR EPIDEMIOLOGY OF AFRICAN HORSE SICKNESS VIRUS BASED ON THE NS3 GENE

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ABSTRACT

African horse sickness virus (AHSV) is the aetiological agent of African horse sickness (AHS), an Office International des Epizooties (OIE) listed disease. AHS is a non-contagious but serious disease of equids that can cause up to 95% mortality in horses and is endemic to sub-Saharan Africa.

Between 2004 and 2006, in excess of 140 AHS viruses were isolated from blood and organ samples submitted from South Africa, Namibia and Zimbabwe to the Faculty of Veterinary Science, University of Pretoria. All serotypes were represented, with a range of 3 to 60 isolates per serotype. The NS3 nucleotide sequences of these isolates were determined and the phylogeny of NS3 investigated.

This study confirms the presence of three distinct NS3 phylogenetic clades (α, β and γ). Some serotypes (6, 8 and 9 in α; 3 and 7 in β; 2 in γ) were restricted to a single clade, while other serotypes (1, 4 and 5) clustered into both the α and γ clade. The high sequence variation in NS3 between and within serotypes makes the identification of various AHSV quasispecies possible.

The use of genetic characterisation is illustrated in an outbreak of AHS within the AHS Surveillance zone of South Africa. The index case was a horse at Elsenburg Agricultural Research Farm which died on the 31st January 2004. The serotype involved was confirmed to be serotype 1. As all isolates from the area were also of the same serotype, a point introduction was assumed. NS3 sequencing of isolates from the outbreak, however, showed two distinct groups that separated into the α and γ clades, suggesting more than one introduction into the area.

The NS3 gene is the second most variable gene of the AHSV genome and is therefore well suited for sequencing. In addition, the small size of the gene (755 – 764 bp) and conserved terminal regions facilitates easy and quick sequencing. The significance of re-assortment of field with vaccine strain viruses cannot be established as NS3 sequence data of vaccine strains is unavailable. The establishment of a NS3 sequence database is important for characterising outbreaks of AHS and tracing the origin of epizootics. It will be an essential resource for elucidating the epidemiology of AHS.

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INTRODUCTION

African horse sickness virus (AHSV) is the aetiological agent of African horse sickness (AHS), an Office International des Epizooties (OIE) listed disease. AHS is a non-contagious but serious disease of equids that can cause up to 95% mortality in horses and is endemic to sub-Saharan Africa. AHSV is transmitted by midges (Culicoides spp.) and the extension of the insect's range due to climate change makes the international spread of AHS a very real possibility.

AHSV is a member of the genus *Orbivirus*, family *Reoviridae* of which bluetongue virus (BTV) is the type species. The virus consists of 10 segments of double stranded (ds)RNA that encode seven structural proteins (VP1 to VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3a). The genome is surrounded by an inner capsid, which forms the virus core and has two distinct layers of proteins. The inner layer or "subcore" is composed of VP3 and associated with it are three minor structural proteins (the core associated enzymes VP1, VP4 and VP6). The outer surface of the core particle is composed of VP7. Surrounding the inner capsid is the outer capsid composed of VP2 and VP5 proteins. VP2 contains the major serotype specific, neutralisation antigens and is in centrally involved in cell attachment and penetration (Stone-Marschat *et al.*, 1996, Burrage *et al.*, 1993).

The NS3 gene (s10) is the smallest segment of the AHSV genome, between 755 and 764 bp long. It contains two overlapping in-phase open reading frames that encode two nonstructural proteins, NS3 and NS3a (Van Staden & Huismans, 1991, Mertens *et al.*, 1984). NS3 is larger than NS3a as result of an additional 10 amino acids at the N-terminal end. NS3 is an integral membrane protein (Bansal *et al.*, 1998, Wu *et al.*, 1992, Hyatt *et al.*, 1991) and appears to be involved in the final stages of viral morphogenesis and release of virions (Wirblich *et al.*, 2006, Martin *et al.*, 1998, Stoltz *et al.*, 1996, Hyatt *et al.*, 1993). It is cytotoxic in insect cells (van Niekerk *et al.*, 2001a, van Staden *et al.*, 1995) and may play a role in the determination of virulence (O'Hara *et al.*, 1998).

NS3 is the second most variable AHSV protein after the outer capsid protein VP2, and can vary 37% across serotypes and 28% within serotypes (van Niekerk *et al.*, 2001b). AHSV NS3 is not as highly conserved as BTV NS3, which vary by only 7 - 10% (van Niekerk *et al.*, 2001b, Hwang *et al.*, 1992) and equine encephalosis virus (EEV), which vary by 17% (van Niekerk *et al.*, 2003). Conserved regions within AHSV NS3 have been reported to be the initiation codon for NS3a, a proline rich area between residues 22 to 34, a stretch of highly conserved amino acids from residues 46 to 90 and two hydrophobic regions (residues 116 to 137 and 154 to 170 respectively) predicted to form transmembrane helices (van Staden *et al.*, 1995).

The use of NS3 sequence data to characterise and trace AHS outbreaks was explored. Between 2004 and 2006, in excess of 140 AHS viruses were isolated from blood and organ samples submitted from South Africa, Namibia and Zimbabwe to the Faculty of Veterinary Science, University of Pretoria. The NS3 nucleotide sequences of these isolates were determined and the phylogeny of NS3 investigated. The data was used to explore the epidemiology of an AHS outbreak in the Western Cape in 2004.

MATERIALS AND METHODS

Virus isolation

Virus was isolated from heparin blood, blood clots and spleen or lung samples submitted to the Faculty of Veterinary Science, University of Pretoria.

Blood in heparin was centrifuged at 440 *g* for 10 – 15 min (Hettich zentrifugen, Universal 16). The white blood cells (buffycoat) were harvested, diluted with a phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (PBS+) and gentamycin (50 mg/l, Virbac) and dispensed into two sterile cryotubes (Nunc), which were frozen at -70°C.

Approximately 1.5 × 1.5 × 1.5 cm tissue was selected and ground to a fine paste using sterile acid-washed sand and a pestle and mortar. 10 ml of a PBS+ gentamycin (50 mg/l) mixture was added to the macerated tissue and the suspension transferred to a 20 ml sterile MacCartney bottle. The mixture was centrifuged at 440 *g* for 10 – 15 min (Hettich zentrifugen, Universal 16), the supernatant filtered through a 0.22 µm filter (Microsep) and then dispensed into sterile cryotubes (Nunc).

0.5 ml of processed blood or organ sample was inoculated into 25 cm^2 tissue culture flasks (Adcock Ingram Scientific) containing a confluent layer of BHK-21 cells (Howell *et al.*, 2002). Cell cultures were incubated at 37°C, observed and checked daily for any cytopathic effects (CPE). The growth medium was replaced every 2 - 3 days. Cultures showing no CPE after 10 -14 days were passaged by inoculating 0.5 ml of the culture onto a freshly prepared cell monolayer. As soon as 100% of the cell monolayer showed cytopathic changes or had detached from the surface due to degeneration, the cells and supernatant were harvested and tested for the presence of AHSV by a competitive ELISA (Hamblin *et al.*, 1990). Cultures showing no CPE after three passages were classified as negative for AHSV.

Plaque inhibition neutralisation test

The AHSV serotype was determined with a plaque inhibition neutralization test. 1.14×10^6 Vero cells (Howell *et al.*, 2002) were added to each well of a 6-well cell culture plate (Adcock Ingram Scientific), incubated at 37ºC in a humid atmosphere of 5% CO_2 in air until a confluent monolayer of cells was obtained. The growth medium was removed and a 1:50 dilution of the tissue culture sample was inoculated onto three wells, 1.5 ml per well. The remaining 3 wells were inoculated with 1.5 ml of a 1:100 dilution. After adsorption for 1 h at 37ºC in a humid atmosphere of 5% CO_2 in air, virus suspensions were removed and 3 ml of an agarose overlay was added. After the agarose had set, a fish spine bead containing a reference serotype specific AHSV serum was placed on the surface of the overlay, three beads per well. Plates are incubated at 37ºC in a humid atmosphere of 5% CO_2 in air for at least 5 days or until plaques were visible. Inhibition of the development of plaques around the bead confirmed the serotype of the virus.

RNA extractions

The tissue culture flasks were mixed and 1 000 µl of mixture transferred to a 1.5

ml eppendorf. Samples were spun at 3 000 rpm for 10 min in a 5417C centrifuge (Eppendorf) and the supernatant discarded.

RNA extractions from the cell pellet were performed with either TRIzol® (Invitrogen™) or the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For Trizol® extractions, 1 ml of Trizol® was added to each cell pellet. 900 µl isopropanol (Sigma) and 1 ul GlycoBlue™ (Ambion) was added to the aqueous phase and the mixture kept for at least 2 hours at -20°C. RNA was resuspended in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0, Ambion). For the RNeasy Mini kit, the extraction from animal cells protocol was followed. Cell pellets were lysed in 600 µl RLT buffer and the RNA eluted in 50 µl nuclease-free H_2O (Ambion). Samples were stored at -70°C. To denature the RNA, 1 µl 0.2 M methylmercury(II) hydroxide (Alfa Aesar) was added to every 5 µl of thawed RNA. The mixture was incubated for 10 min at room temperature before reduction with 1 µl 1 M 2-mercaptoethanol (Sigma).

Reverse transcription – polymerase chain reaction (RT-PCR)

A one-step RT-PCR was performed with the GeneAmp® Gold RNA PCR core kit (Applied Biosystems) according to the manufacturer's instructions. Two 25 µl reactions were performed per sample, containing 2 µl denatured RNA, 0.5 µl 20 nM MQ.AHS. NS3.1-22F (5'-gtt taa att atc cct tgt cat g) and 0.5 µl 20 nM MQ.AHS.NS3.749-769R (5'-gta agt cgt tat ccc ggc tc) terminal primers per reaction. Cycling conditions for the RT-PCR were 42°C for 12 min; 95°C for 10 min; 40 cycles of 94°C for 20 sec, 57°C for 30 sec and 72°C for 60 sec; 72°C for 7 min and a hold at 4°C on a GeneAmp® PCR System 9700 (Applied Biosystems).

Five µl PCR product mixed with 1 µl loading dye (Fermentas) was visualised by UV trans-illumination of a 1.5 % ethidium bromide stained agarose gel electrophoresed at 110 volts.

The two RT-PCR reactions per sample were combined and the mixture cleaned up using a QIAquick® PCR purification kit and a QiaVac 6S vacuum manifold (Qiagen) according to the manufacturer's instructions. Samples were eluted in 60 ul EB buffer (Qiagen).

Sequencing

A BigDye® Terminator v3.1 cycle sequencing kit was used for sequencing. At least two forward and two reverse ¼ reactions (4 – 7 µl DNA, total volume per reaction = 20 µl) were performed per sample according to the manufacturer's instructions. Sequence reactions were performed in 96-well plates using a GeneAmp® PCR System 9700. Cycling conditions were as described in the Applied Biosystems protocol.

After cycling, 50 µl 100% ethanol (Merck), 2 µl 125 mM EDTA (Ambion) and 2 µl 3M sodium acetate pH 5.5 (Ambion) was added to each sample and incubated for 15 min at room temperature. Samples were centrifuged at 4 230 rpm (2 500 *g*) for 30 min in a Sorvall® RC6 centrifuge (Kendro Laboratory Products). After the centrifuge had stopped, the plate was inverted immediately and spun at 1 000 rpm (acceleration 7) for 1 min to remove the supernatant. 70 µl 70% ethanol was added to each reaction and the plate centrifuged at 3 140 rpm (1 650 *g*) for 15 min. After the centrifuge had stopped, the

plate was inverted immediately, spun at 1 000 rpm (acceleration 7) for 1 min to remove the supernatant and air dried.

10 µl Hi-Di™ formamide (Applied Biosystems) was added to each sample and the mixture heated to 94°C for 2 min, followed by cooling at 4°C. Samples were analyzed with an ABI 3130xl Genetic Analyzer using POP-7 polymer and a 36 cm capillary (Applied Biosystems).

Phylogenetic analysis

Phred (Ewing *et al.*, 1998, Ewing & Green, 1998) and the Staden package (Staden *et al.*, 2000, Staden, 1996) were used for base calling and sequence assembly.

A sequence alignment was done with MAFFT (v5.8) (Katoh *et al.*, 2005, Katoh *et al.*, 2002).

A cladogram was generated with MrBayes (v3.1.1) (Ronquist & Huelsenbeck, 2003, Huelsenbeck & Ronquist, 2001) using a GTR+G substitution model (as determined with MrModeltest2 (Nylander, 2004)), three heated chains and 1×10^6 generations. The consensus tree was edited in MEGA (v4) (Tamura *et al.*, 2007, Kumar *et al.*, 1994).

Results

Over 140 AHS viruses were isolated from samples submitted to the Faculty of Veterinary Science, University of Pretoria between 2004 and 2006 (Figure 1).

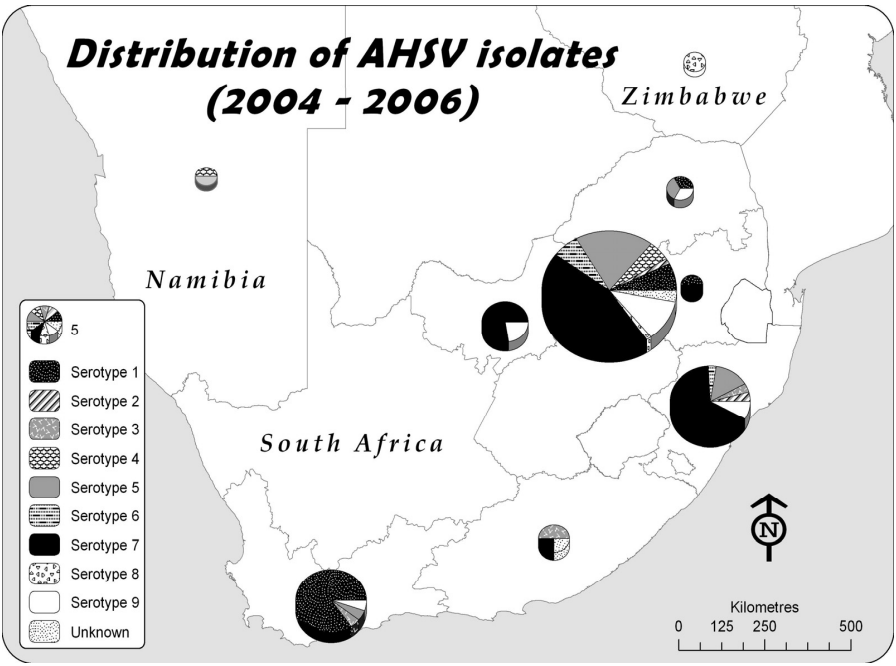

Figure 1: Classification of AHSV isolates collected between 2004 and 2006 by province, and serotype.

All serotypes were represented, with a range of 3 to 60 isolates per serotype. Most of the samples were submitted from Gauteng (53%), followed by Kwazulu-Natal (18%) and the Western Cape Province (14%). The majority of AHSV serotypes submitted from Gauteng were either serotype 7 (42%) or serotype 5 (22%). Outbreaks of serotype 1 occurred in the Western Cape Province in 2004 and serotype 7 in KwaZulu-Natal in 2005, which account for the majority of those serotypes submitted from those provinces.

This study confirms the presence of three distinct NS3 phylogenetic clades (α, β and γ), as previously reported (Figure 2)(van Niekerk *et al.*, 2003, Martin *et al.*, 1998, Sailleau *et al.*, 1997). Some serotypes (6, 8 and 9 in α; 3 and 7 in β; 2 in γ) were restricted to a single clade, while other serotypes (1, 4 and 5) clustered into both the α and γ clade. The high sequence variation in NS3 between and within serotypes makes the identification of various AHSV variants possible (Figure 3).

AHS outbreak, Western Cape, 2004

The use of NS3 characterisation is illustrated in an outbreak of AHS within the AHS Surveillance zone of South Africa. The index case was a horse at Elsenburg Agricultural Research Farm which died on the 31st January 2004. The outbreak resulted in the death of 17 horses, with the last death on the 28th March at Kalbaskraal. The serotype involved was confirmed to be serotype 1. As all isolates from the area were also of the same serotype, a point introduction was assumed. NS3 sequencing of isolates from the outbreak, however, shows two distinct groups (Figure 3) that separate into the α and γ clades (underlined isolates, Figure 2), suggesting more than one introduction into the area. The outbreak started on Elsenburg Agricultural Research Farm (Figure 4 and Table 1). No viral isolations were performed from the first two horses that died as annual rye grass toxicity was diagnosed and AHS was not suspected. Three horses died subsequently (22nd – 24th February) and one horse was clinically ill from AHS (26th February). AHSV serotype 1 was isolated from all samples submitted. NS3 sequencing showed that all these isolates belonged to the γ clade and no other clades were identified at Elsenburg.

A few days after the deaths at Elsenburg, horses died from AHS at Troughend, Oakhill and Daktari (26 – 28th February), about 15 km south of Elsenburg. NS3 sequencing identified these isolates as belonging to the α clade and AHS did not, therefore, spread from Elsenburg to these farms. The sequences (Figure 3) show clearly that the introduction of AHS to these farms was from a separate source to that of Elsenburg and that there were at least two separate introductions of AHSV to the Western Cape.

AHSV isolated from the remainder of the outbreak all belonged to the α clade. Isolates in this clade showed sequence variation in the NS3 gene and if these mutations were acquired during the outbreak, it would be useful for tracking the spread of the virus and determining a chain of infection. The data showed that, rather than mutations acquired during the course of the outbreak, there was a set of quasispecies present at the beginning of the outbreak. Quasispecies can be described as a population of viruses without a defined unique sequence structure, but rather as a weighted average of a large number of different individual sequences (Biebricher & Eigen, 2006, Domingo *et al.*, 1978).

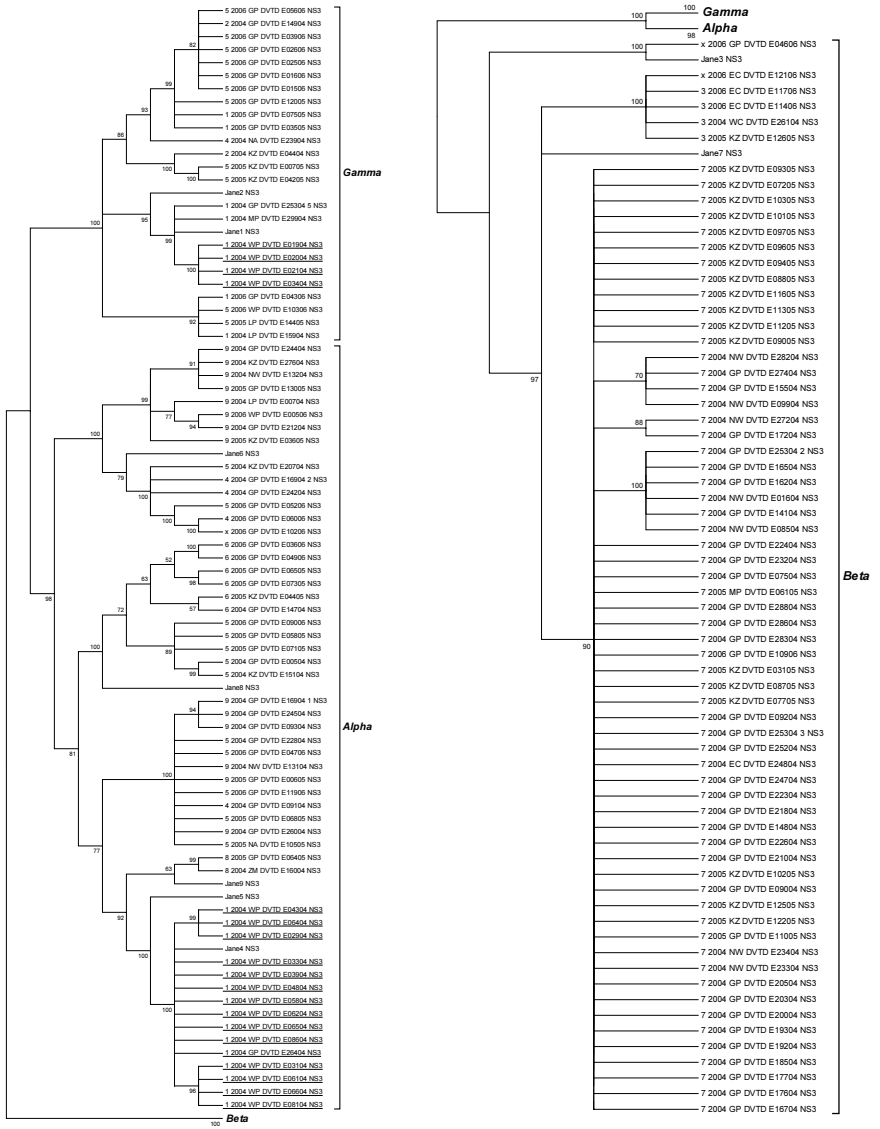

Figure 2: An unrooted cladogram of AHSV NS3 sequences, determined using Bayesian inference. Three distinct clades (α, β and γ), supported by high posterior probabilities (indicated on cladogram), are evident. Isolate names reflect serotype (x - unknown), year of isolation and province/country (EC - Eastern Cape, GP - Gauteng, KZ - KwaZulu-Natal, LP - Limpopo, MP - Mpumalanga, NA - Namibia, NW – North-West, WP – Western Cape and ZM – Zimbabwe) where isolated. Jane isolates refer to reference virulent field strains. Underlined isolates refer to samples collected during the AHS outbreak in the Western Cape Province in 2004.

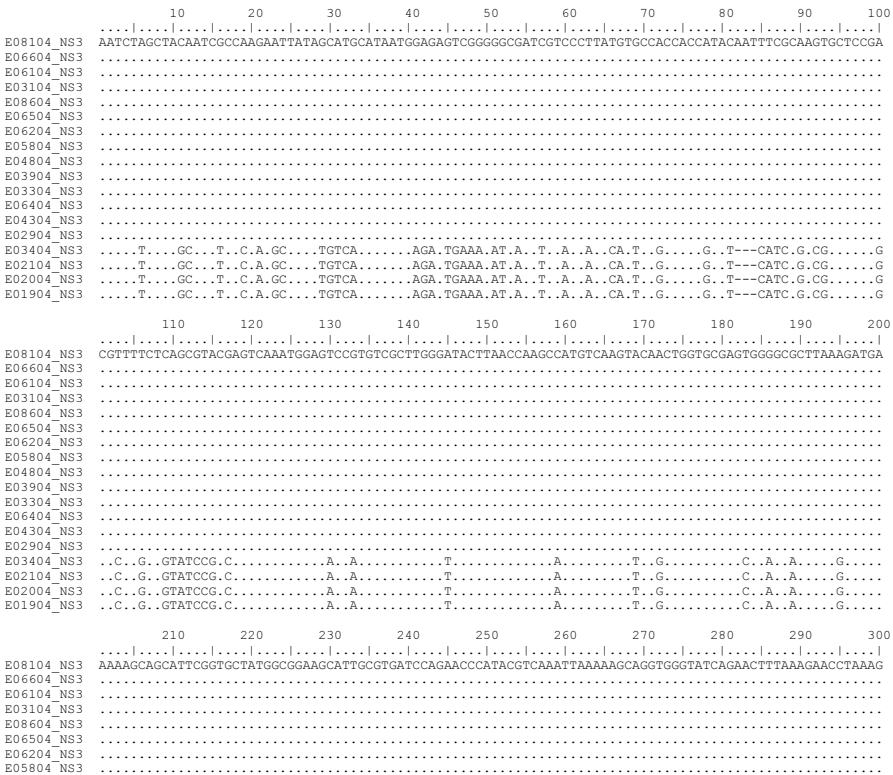
10 20 30 40 50 60 70 80 90 100
E08104_NS3 AATCTAGCTACAATCGCCAAGAATTATAGCATGCATAATGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCCGA
E06604_NS3
E06104_NS3
E03104_NS3
E08604_NS3
E06504_NS3
E06204_NS3
E05804_NS3
E04804_NS3
E03904_NS3
E03304_NS3
E06404_NS3
E04304_NS3
E02904_NS3
E03404_NS3 .....T....GC...T..C.A.GC....TGTCA.......AGA.TGAAA.AT.A..T..A..A..CA.T..G.....G..T---CATC.G.CG......G
E02104_NS3 .....T....GC...T..C.A.GC....TGTCA.......AGA.TGAAA.AT.A..T..A..A..CA.T..G.....G..T---CATC.G.CG......G
E02004_NS3 .....T....GC...T..C.A.GC....TGTCA.......AGA.TGAAA.AT.A..T..A..A..CA.T..G.....G..T---CATC.G.CG......G
E01904_NS3 .....T....GC...T..C.A.GC....TGTCA.......AGA.TGAAA.AT.A..T..A..A..CA.T..G.....G..T---CATC.G.CG......G
110 120 130 140 150 160 170 180 190 200
E08104_NS3 CGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGCCATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGA
E06604_NS3
E06104_NS3
E03104_NS3
E08604_NS3
E06504_NS3
E06204_NS3
E05804_NS3
E04804_NS3
E03904_NS3
E03304_NS3
E06404_NS3
E04304_NS3
E02904_NS3
E03404_NS3 ..C..G..GTATCCG.C.............A..A...........T.............A.........T..G..........C..A..A.....G.....
E02104_NS3 ..C..G..GTATCCG.C.............A..A...........T.............A.........T..G..........C..A..A.....G.....
E02004_NS3 ..C..G..GTATCCG.C.............A..A...........T.............A.........T..G..........C..A..A.....G.....
E01904_NS3 ..C..G..GTATCCG.C.............A..A...........T.............A.........T..G..........C..A..A.....G.....
210 220 230 240 250 260 270 280 290 300
E08104_NS3 AAAAGCAGCATTCGGTGCTATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTATCAGAACTTTAAAGAACCTAAAG
E06604_NS3
E06104_NS3
E03104_NS3
E08604_NS3
E06504_NS3
E06204_NS3
E05804_NS3
E04804_NS3
E03904_NS3
E03304_NS3
E06404_NS3
E04304_NS3
E02904_NS3
E03404_NS3 ...G.....G..T..A..GG....A..G..G...A.A.....G..G..G..CA.AA.......GCGA..A..A......CA....C....A.CA..G..A
E02104_NS3 ...G.....G..T..A..GG....A..G..G...A.A.....G..G..G..CA.AA.......GCGA..A..A......CA....C....A.CA..G..A
E02004_NS3 ...G.....G..T..A..GG....A..G..G...A.A.....G..G..G..CA.AA.......GCGA..A..A......CA....C....A.CA..G..A
E01904_NS3 ...G.....G..T..A..GG....A..G..G...A.A.....G..G..G..CA.AA.......GCGA..A..A......CA....C....A.CA..G..A
310 320 330 340 350 360 370 380 390 400
E08104_NS3 ATGGAGTTAGCAACAATGCGTCGAAAGAAATCGGCATTAAAAATAATGATCTTTATTAGTGGATGCGTAACGTTAGCTACATCGATGGTTGGGGGATTGA
E06604_NS3
E06104_NS3
E03104_NS3
E08604_NS3
E06504_NS3
E06204_NS3
E05804_NS3
E04804_NS3
E03904_NS3
E03304_NS3
E06404_NS3
E04304_NS3
E02904_NS3
E03404_NS3 G.T..A..GAGCGGG.....AA.G.......T.ATT..G.......T..G.....T.C.C.AA......TA.G.....T..TC.A.....A..TA..T
E02104_NS3 G.T..A..GAGCGGG.....AA.G.......T.ATT..G.......T..G.....T.C.C.AA......TA.G.....T..TC.A.....A..TA..T
E02004_NS3 G.T..A..GAGCGGG.....AA.G.......T.ATT..G.......T..G.....T.C.C.AA......TA.G.....T..TC.A.....A..TA..T
E01904_NS3 G.T..A..GAGCGGG.....AA.G.......T.ATT..G.......T..G.....T.C.C.AA......TA.G.....T..TC.A.....A..TA..T

Figure 3:. ....

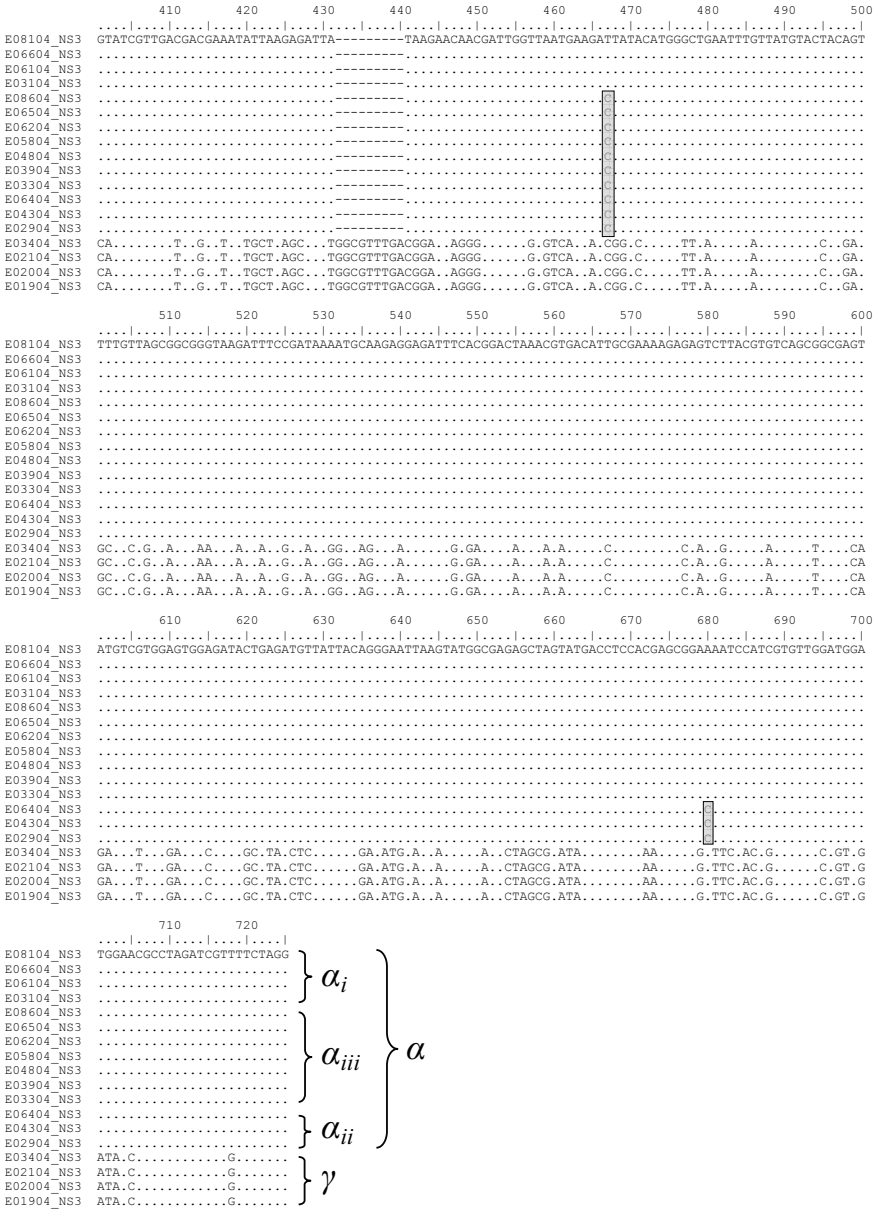

Figure 3: NS3 sequence alignment of AHSV isolates collected during the AHS outbreak in the Western Cape Province in 2004 (in interleaved format). Dots indicate homology with E08104. α and γ clades indicated, as well as sequence variation (grey boxes) within a clade (α_i, α_{ii} and α_{iii}).

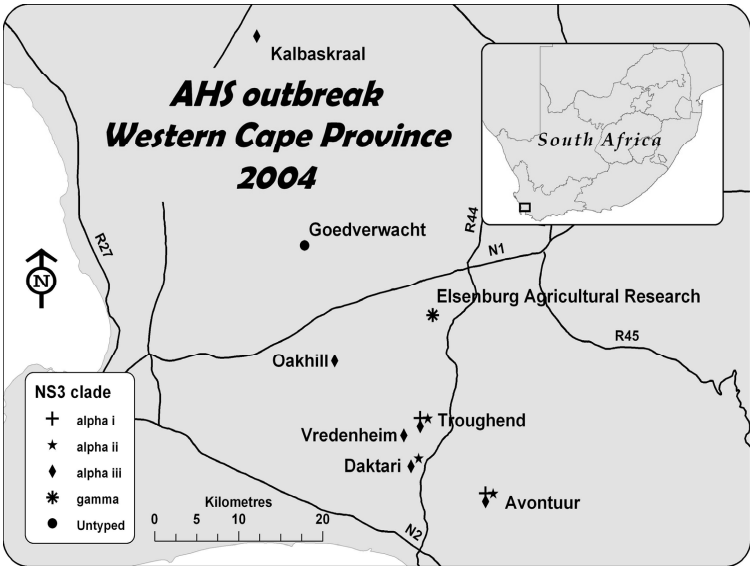

Figure 4: Phylogenetic analysis of AHSV serotype 1 suggests two separate introductions of the virus (α and γ clade) into the Western Cape Province in 2004. Quasispecies within the α clade indicated with subscript (α_i, α_{ii} and α_{iii}).

Date	Locality	Horse name	Case no.	NS3 clade
22 February 2004	Elsenburg Agricultural Research	Michelle	E02004	γ
22 February 2004	Elsenburg Agricultural Research	Laura	E01904	γ
24 February 2004	Elsenburg Agricultural Research	Mara	E02104	γ
26 February 2004^{+}	Elsenburg Agricultural Research	Mandy*	E03404	γ
26 February 2004	Troughend	SA saddle horse 1	E03104	α_i
27 February 2004	Oakhill	Murphy	E03304	α_{iii}
28 February 2004	Daktari	Murphy Brown	E02904	α_{ii}
03 March 2004^{+}	Troughend	Melody Fire*	E03904	α_{iii}
04 March 2004^{+}	Avontuur	Bow Street Belle (blood)	E04304	α_{ii}
09 March 2004	Avontuur	Bow Street Belle (organs)	E04804	α_{iii}
13 March 2004	Daktari	Calypso	E05804	α_{iii}
15 March 2004^{+}	Vredenheim	Bruin Perd (blood)	E06204	α_{iii}
16 March 2004^{+}	Avontuur	Special Edition (blood)	E06104	α_i
17 March 2004	Vredenheim	Bruin Perd (organs)	E06504	α_{iii}
17 March 2004	Troughend	SA saddle horse 2	E06404	α_{ii}
18 March 2004	Avontuur	Special Edition (organs)	E06604	α_i
24 March 2004	Avontuur	2003Stars and Stripes	E08104	α_i
29 March 2004	Kalbaskraal	Amber	E08604	α_{iii}

Table 1: Isolates from the AHS outbreak in the Western Cape Province in 2004 where the NS3 gene was sequenced and typed. Date indicates day horse died or when ante-mortal samples were collected ($^{+}$). * Horse clinically ill, did not die.

Quasispecies α_i, α_{ii} and α_{iii} were all present at Troughend, Oakhill and Daktari at the beginning of the α clade outbreak and within 3 days of each other. In addition, α_{ii} and α_{iii} isolates were obtained from the same horse (Bow Street Belle, Table 1), indicating AHSV quasispecies in one horse. Rather than a sequential acquisition of mutations during the outbreak, a genetically diverse population of viruses were present from the start of the outbreak and therefore no inference could be made about a chain of infection using the genetic data.

It could not be determined where the AHSV responsible for the Western Cape outbreaks originated from. The closest related 2004 serotype 1 γ clade viruses are E25304, E29904 and α clade E26404 (Figure 2). All these isolates were obtained after the outbreak in the Western Cape occurred and there were no serotype 1 viruses in the sequence database obtained before.

DISCUSSION

The NS3 gene is the second most variable gene of the AHSV genome after VP2 and is therefore well suited for use in characterising AHSV strains. In addition, the small size of the gene (755 – 764 bp) and conserved terminal regions facilitates easy and quick sequencing. It is not clear why there is such a large variation in the NS3 gene. NS3 is membrane-associated and therefore may be under immunological pressure, unlike other AHSV non-structural proteins that are intracellular and much more conserved than NS3 (M Quan, unpublished data).

In excess of 140 AHS viruses were isolated and sequenced from blood and organ samples submitted to our laboratory between 2004 and 2006. The virus collection is not representative of the AHS situation in the country during that period as samples submitted are biased towards horses in close proximity to the laboratory, i.e. Gauteng and the formal equine sector, due to the costs involved in veterinary examinations and diagnostic tests. Very few samples were submitted from the Eastern Cape, yet it has one of the highest unvaccinated horse populations in the country (Livestock figures 2004, Department of Agriculture).

The phylogenetic analysis confirmed the presence of three distinct NS3 phylogenetic clades (α, β and γ), as previously reported (van Niekerk *et al.*, 2003, Martin *et al.*, 1998, Sailleau *et al.*, 1997). In our analysis, serotype 8 clustered only in the α clade. The β clade was made up exclusively of serotype 3 and 7. In contrast, van Niekerk *et al.* (2003) found that serotype 8 clustered in both the α and β, while Martin *et al.* (1998) found that it clustered in the γ clade.

There was a difference in the amount of variation within serotype. Serotype 7 isolates showed very little variation in their NS3 sequence, even though samples were submitted from the North-West, Gauteng, KwaZulu-Natal and Eastern Cape Provinces. On the other hand, the NS3 sequences from serotype 5 isolates were very diverse, appearing in the α and γ clade and in different clusters within a clade. The difference in variation between serotypes may be a reflection of the disease characteristics at the time. An AHS epidemic occurred in KwaZulu-Natal in 2005, caused by serotype 7. In an epidemic

situation, a viral population of fairly low genetic diversity would be expected, due to a rapidly expanding population in a short time period and under little selective pressure. In an endemic situation, on the other hand, a viral population of high diversity should be expected, due to a long period of slow population growth under constant immunological pressure.

There was no correlation between NS3 sequence and serotype. It is interesting to note that other serotypes, such as serotype 4 (Jane4) share the exact same NS3 sequence to some of the serotype 1 isolates from the Western Cape (Figure 2). This may be as result of reassortment between different serotypes of AHSV infecting the same horse and sharing parts of their segmented genome.

The significance of reassortment of field with vaccine strain viruses cannot be established as NS3 sequence data of vaccine strains are unavailable. It is possible that the serotype 1 α or γ clade might represent a vaccine strain. Widespread vaccination is practiced in the area with 70% of horses vaccinated against AHSV (Sinclair, 2006). In addition, concern was expressed that the closest related 2004 serotype 1 γ clade viruses (E25304 and E29904) to the Western Cape isolates were as a result of vaccination, as both horses became ill 3 weeks after vaccination against AHSV (Figure 2). However, the NS3 sequence from E25304 and E29904 was exactly the same as that obtained from Jane1, a documented virulent field strain. Likewise, The Jane4 sequence is exactly the same as the serotype 1 α clade viruses from the Western Cape and Jane4 is also a virulent field strain. The phylogeny suggests that none of the Western Cape isolates represent vaccine strains.

The use of molecular epidemiology of the NS3 gene was illustrated with the AHS outbreak in the Western Cape in 2004, by showing that there was more than one introduction of AHSV to the area. In contrast to the serotype 1 γ clade viruses, which shared exactly the same NS3 sequence, the serotype 1 α clade virus isolates showed genetic variation, which indicated a population of AHS viruses with differing genome sequences (quasispecies), rather than representing an accumulation of mutations over time. It was therefore not possible to identify a chain of infection for the outbreak in the Western Cape.

The establishment of a NS3 sequence database is important for characterising outbreaks of AHS and tracing the origin of epizootics. The sequence data of the AHSV vaccine strains is a gap in the current NS3 sequence database. In addition, it would be ideal to sequence VP2, or a portion there of, and to combine that data in the phylogenetic analysis. The database will be an essential resource for elucidating the epidemiology of AHS, but needs to be backed up with a sensitive and accurate surveillance system and submission of samples for viral isolation and characterisation.

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COMPARATIVE UTILITY OF THREE ALCELAPHINE HERPESVIRUS-1 GENOME REGIONS FOR MOLECULAR EPIDEMIOLOGICAL STUDIES OF MALIGNANT CATARRHAL FEVER IN SOUTH AFRICA

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SUMMARY

Malignant catarrhal fever (MCF), locally referred to as 'snotsiekte', is generally a fatal disease of domestic cattle and has a world wide distribution. The disease can also be contracted by wild ruminants and pigs. The causative agents of MCF, which occurs naturally in domestic sheep and wildebeest, are two closely related viruses of the subfamily *Gammaherpesvirinae*, namely ovine herpesvirus-2 (OvHV-2) and alcelaphine herpesvirus-1 (A1HV-1), respectively. Disease control in South Africa is reliant on the segregation of these asymptomatic carriers and susceptible host species. The aim of this project was to identify a suitable, phylogenetically informative region in the genome which would assist in determining the relatedness of outbreak strains and permit assessment of field heterogeneity. Three gene regions of A1HV-1 field strains identified for PCR amplification and nucleotide sequencing were ORF75, and the genes encoding the R-transactivator protein and glycoprotein B (together with its 5' non-coding region). Phylogenetic analyses across all three gene regions revealed the presence of two distinct A1HV-1 clades in South Africa, with the relative gene variability being R-transactivator > ORF75 > glycoprotein B. Whilst viral groupings within the two major clades were consistent across all three gene regions, the overall better phylogenetic signal within ORF75 indicates that this gene region is best suited for distinguishing closely related viruses and is therefore of greater epidemiological value.

INTRODUCTION

Malignant catarrhal fever (MCF) is a sporadic, usually fatal disease of cattle, pigs, deer and certain other susceptible ruminants, such as Bison (Barnard *et al*., 1994; Hart *et al*., 2007) and exhibits a worldwide distribution. Outside Africa the disease is associated with contact between sheep and affected species (usually cattle but also deer as is the case in New Zealand) while within Africa both sheep and wildebeest are known carriers of the virus, transmitting it to susceptible domestic species through contact (Barnard *et al*., 1994). As neither carrier host shows clinical symptoms of the disease and as no known vaccine exists, control of the disease is reliant on separating cattle from sheep and wildebeest (Barnard *et al*., 1994). The disease is characterised in cattle by mucopurulent nasal and ocular discharges, keratoconjunctivitis with corneal opacity, and enlargement

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of the peripheral lymph nodes (Dunowska *et al*., 2001). The mucous discharge from the nasal and ocular cavities is where the colloquial name 'snotsiekte' for the disease originates, as this is what the hunters and Voortrekkers of the 18th Century observed when penetrating South Africa (Barnard *et al*., 1994).

In South Africa, MCF is caused by two closely related dsDNA viruses of the subfamily *Gammaherpesvirinae*, namely ovine herpesvirus-2 and alcelaphine herpesvirus-1 that persist sub-clinically in their natural host (Dunowska *et al*., 2001; Hart *et al*., 2007). The disease caused by AlHV-1 is commonly called the wildebeest-associated or African form of MCF and is restricted to Africa and zoological collections associated with wildebeest (Ensser *et al*., 1997; Li *et al*., 2003). Most adult wildebeest are naturally infected with A1HV-1 and transmission to cattle is said to occur under close contact situations, as in shared cattle and wildebeest grazing and watering areas. Transmission has, however, been known to occur across fences up to 100 metres away, which indicates an alternative means of transmission to that via air (Barnard *et al*., 1994). Interestingly, it has been shown that infection rates of cattle peak between January and May (after wildebeest calving season – December to February) and September to November (when the wildebeest calves are approximately11 months old). This may be an indication that mainly calves shed infectious virus although all wildebeest are regarded as carriers. Domestic sheep are worldwide reservoirs of MCF and sheep associated MCF is caused by ovine herpesvirus-2 (OvHV-2). Transmission from sheep in Africa is said to occur throughout the year with cattle to cattle transmission being uncommon (Barnard *et al*., 1994).

In the early 1900's there was an increase in cattle farming in South Africa and a decrease in the number of game animals with the result that few to no outbreaks of the disease were observed and therefore little to no attention was paid to MCF. Game farming has been on the rise since the 1970's as a result of the realisation of the economic gains of game farming and the awareness of wildlife conservation. With the increase in game farming an increase in the prevalence of MCF has been observed (Barnard *et al*., 1994). Although the occurrence of MCF in cattle is usually sporadic, outbreaks do occur with losses being substantial (Hart *et al*., 2007). Losses of as high as 34 % have been described in the northern part of South Africa (Barnard *et al.,* 1994)

Not much information is available on the molecular epidemiology of AlHV-1. We therefore endeavoured to assess the genetic variation of AlHV-1 in South Africa by identifying a suitable region of the genome and using this to determine variation in a number of field isolates. It was thought that such information could allow the identification of the source of the virus which could assist in the control of the disease.

MATERIALS AND METHODS

Samples used in this study

DNA extracted from samples that were PCR positive for either A1HV-1 or OvHV-2 (Bremer *et al*., 2005), were obtained from Onderstepoort Veterinary Institute (OVI) and are from suspected MCF cases within South Africa from 2000 to 2007 (Figure 1).

PCR amplification, purification and sequencing

The three regions of the genome that were identified as possible candidates for the genetic characterisation of AlHV-1 were: in ORF75 and in the genes encoding the R-transactivator protein and glycoprotein B. Polymerase chain reaction (PCR) was performed using a Perkin Elmer Gene Amp 2400 in a final volume of 25µL containing 20 pmol of each primer, in the presence of 1 unit of Taq DNA polymerase (Biotools, Madrid Spain). Primers used were MF combined with MR-AB1/2 (ORF75) (Bremer *et al*., 2005, Bastos and Sole, unpubl), PWF and PWR (R-transactivator) (Sole and Bastos, unpubl) and gpBups with gpBR2 (glycoprotein B) (Bremer, unpubl).

The amplified gene products were purified from the tube using the High Pure PCR Product Purification kit (Roche, Mannheim, Germany) according to manufacturer specifications. Sequencing reactions were performed at the primer-specific annealing temperature with version 3.1 of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin- Elmer, Foster City, USA)

Sequence analysis

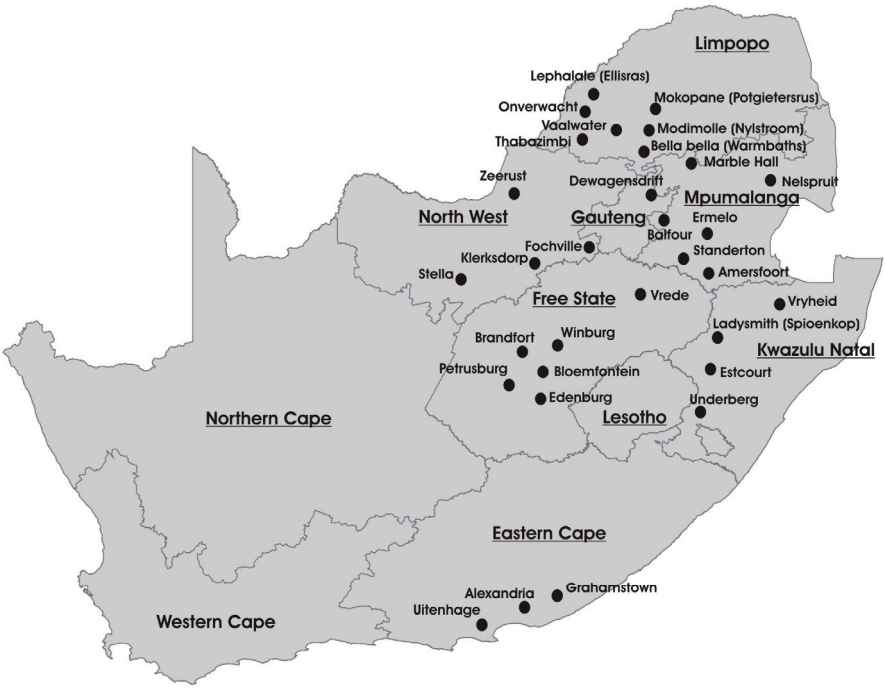
Sequences for all three gene regions were generated for nine clinical samples and aligned and edited in Vector NTI™ 10 (Invitrogen). The resulting three molecular sets of data were subjected to preliminary sequence analyses prior to phylogenetic analysis. The average nucleotide p-distances, number of parsimoniously informative sites, transition/tranversion ratio as well as overall base composition were calculated in MEGA 3.1 (Kumar *et al*., 2004) for each region.

Phylogenetic trees, for each gene region were reconstructed in MEGA 3.1 (Kumar *et al*., 2004) using the neighbor-joining method. Confidence levels for the nodes in the resulting p-distance trees were assessed by means of 1000 bootstrap replicates.

Table 1: Summary of the nine samples, from *Bos taurus*, for which sequences were generated for the three gene regions (ORF75, R-transactivator, glycoprotein B) compared in this study.

No.	Sample Ref No.	Sample Type	Locality	Year of outbreak
1	1147 *	EDTA	Onverwacht	2004
2	1219.1 *	Blood clot	Vaalwater	2004
3	1240 *	Blood clot	Ellisras	2004
4	1252 *	Blood clot	Standerton	2005
5	1505.2 *	Blood Clot	Thabazimbi	2005
6	1508 *	Brain	Potgietersrus	2005
7	1511 *	EDTA	Winburg	2005
8	1931 *	Blood clot	Uitenhage	2006
9	1939 *	Spleen	Alexandria	2007

Figure 1: Schematic representation of localities where suspected clinical samples were obtained.



RESULTS

Sequence statistics

The combined sequence length of all three regions was1489 base pairs (bp) made up as follows ORF75 = 448 bp, R-transactivator = 484 bp and glycoprotein B = 557 bp. Base frequencies and parsimoniously informative sites differed considerably between regions (Table 2). R-Transactivator had the most parsimoniously informative sites with ORF75 the lowest and glycoprotein B an intermediate number. The distribution of bases (a, c, g, t) across ORF75 appeared similar for all bases while R-Transactivator and glycoprotein B had fewer guanine bases than adenine, thymine and cytosine (Table 2). R-Transactivator and glycoprotein B exhibited an almost similar transition/transversion ratios whilst an almost three-fold higher bias in transitional changes were observed for ORF75.

Table 2: General sequence statistics calculated over the three regions used in this study.

	PI	V	C	R	a	c	g	t
ORF 75	10	31	417	4.0	23.4	24.1	28	24.5
R-Transactivator	39	59	425	1.4	28.4	30.2	19.2	22.2
glycoprotein B	27	32	525	1.6	29.8	26.1	19.7	24.3

^ PI – Parsimoniously informative sites, V – Variable sites, C- Conserved sites, R – transition/ transversion ratio a – adenine, c – cytosine, g – guanine, t – thymine

The overall average sequence divergences were 1.9 %, 2 % and 4.5 % for glycoprotein B, ORF 75 and R-transactivator, respectively. Sequence divergence between samples across all gene regions ranged from 0.2 % to 8.1 % (results not shown). These pairwise comparisons indicate that glycoprotein B is least variable whilst the R-Transacticator is the most variable, across the gene regions sequenced.

Phylogenetic analysis

Neighbor-joining trees were constructed for each gene region (Figure 2a, b, c). Branches with bootstrap support below 50 % were collapsed. Across all three gene regions two distinct lineages were consistently obtained (labelled I and II - Figure 2a, b, c), and contained the same samples. Strong support was noted for these lineages with ORF75 having 64 % and 99 % support, R-Transactivator 73% and 100 % support and glycoprotein B, 96 % and 100 %, support for lineages I and II, respectively. The samples did not group according to geographic area or year of outbreak but intermixed between the two (Figure 2).

Based on the fact that the glycoprotein B region only identified three gene variants (as seen in the phylogenetic tree (Figure 2c) and as pairwise differences between isolates was low, subsequent genetic characterisation focussed exclusively on the R-Tranactivator and OF75 regions. The resulting neighbour-joining phylogram is presented in Figure 3 a and b. The two major lineages (labelled I and II), recovered previously with the nine taxon datasets were again obtained with strong support (ORF75 had 67 % and 99 % bootstrap support and R-Transactivator had 96 % and 100 % bootstrap support, for each lineage respectively). The ORF75 region revealed the presence of 10 distinct sequence variants within South Africa, two of which clustered within lineage II and the remainder within lineage I. In contrast, 22 distinct South African sequence variants were obtained following R-Transactivator sequencing, 19 of which constituted lineage I, whilst the remaining three formed lineage II. Neither tree revealed grouping of isolates according to geographic origin or year of outbreak.

Figure 2:

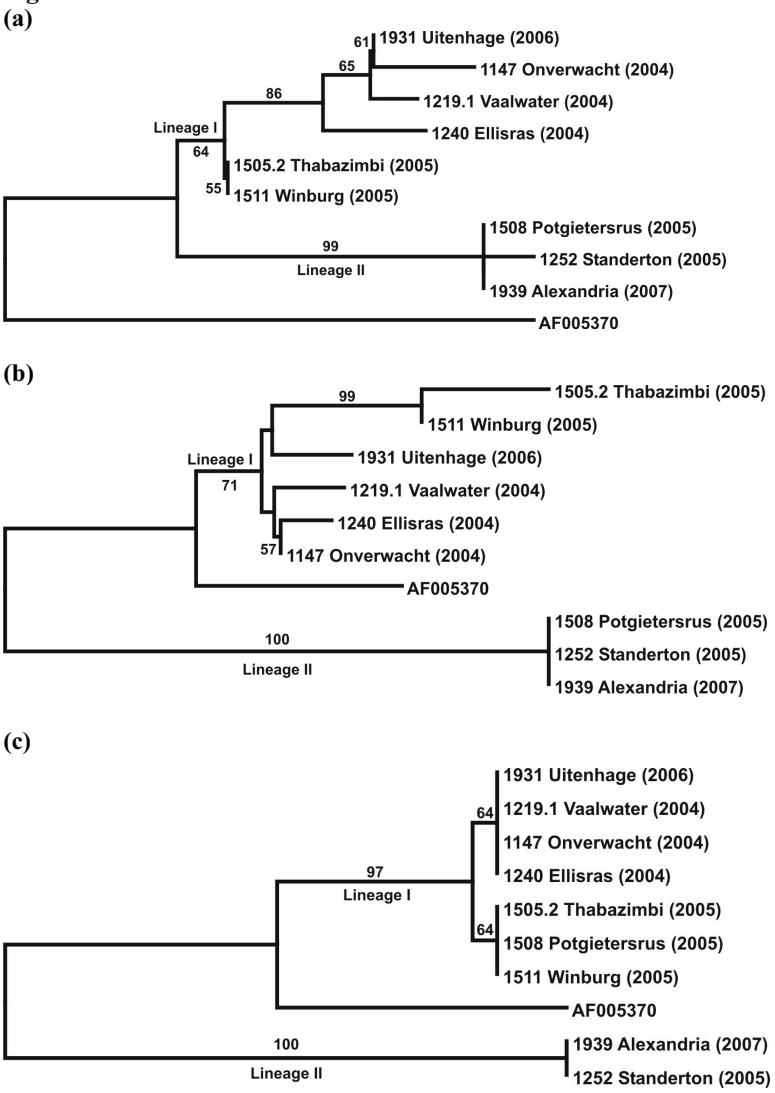
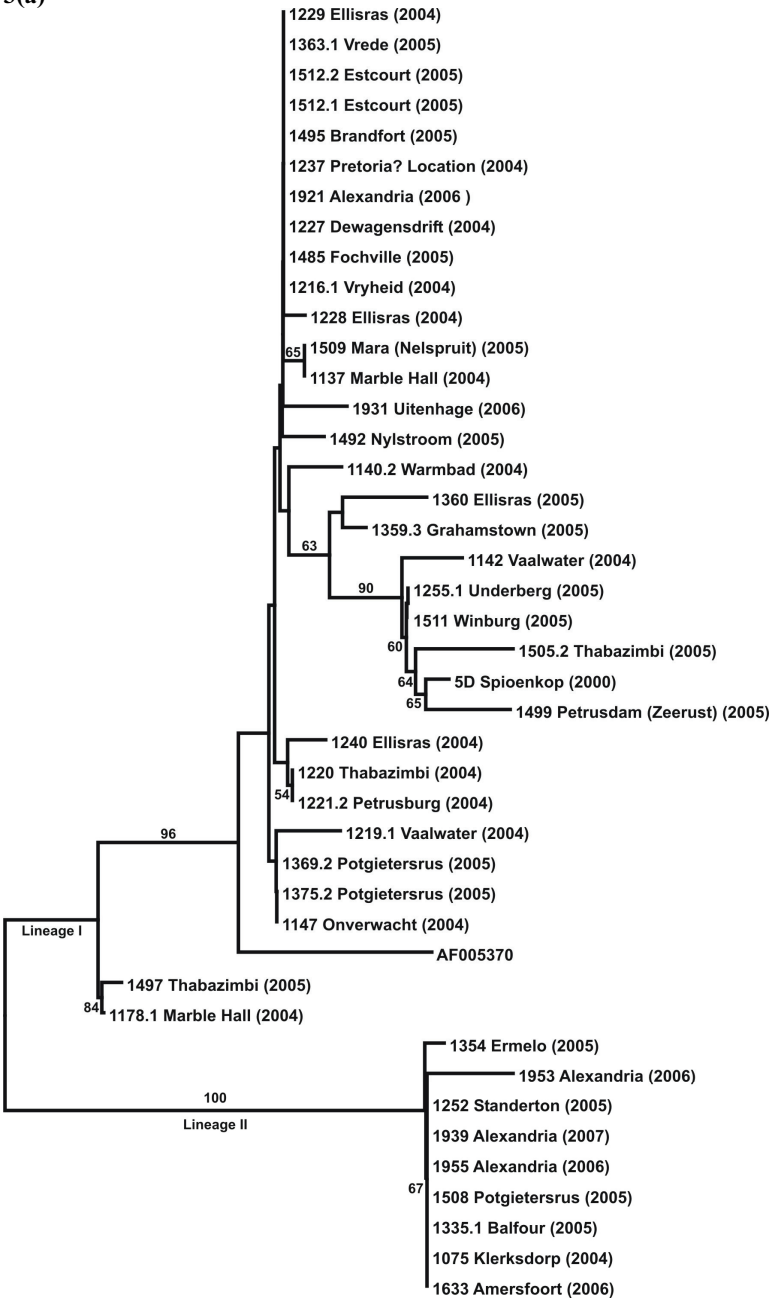


Figure 2a-c.: Neighbor-joining phylogram based on (a) ORF75, (b) R-Transactivator and (c) glycoprotein B gene regions characterised in this study. Bootstrap support (based on 1000 bootstrap replicates) and greater than 50 % is given next to each node. Numbers in brackets correspond to year of outbreak.

Figure 3:

3(a)


1229 Ellisras (2004)
1363.1 Vrede (2005)
1512.2 Estcourt (2005)
1512.1 Estcourt (2005)
1495 Brandfort (2005)
1237 Pretoria? Location (2004)
1921 Alexandria (2006)
1227 Dewagensdrift (2004)
1485 Fochville (2005)
1216.1 Vryheid (2004)
1228 Ellisras (2004)
65
1509 Mara (Nelspruit) (2005)
1137 Marble Hall (2004)
1931 Uitenhage (2006)
1492 Nylstroom (2005)
1140.2 Warmbad (2004)
1360 Ellisras (2005)
1359.3 Grahamstown (2005)
63
1142 Vaalwater (2004)
90
1255.1 Underberg (2005)
1511 Winburg (2005)
60
1505.2 Thabazimbi (2005)
64
5D Spioenkop (2000)
65
1499 Petrusdam (Zeerust) (2005)
1240 Ellisras (2004)
1220 Thabazimbi (2004)
54
1221.2 Petrusburg (2004)
96
1219.1 Vaalwater (2004)
1369.2 Potgietersrus (2005)
1375.2 Potgietersrus (2005)
1147 Onverwacht (2004)
Lineage I
AF005370
1497 Thabazimbi (2005)
84
1178.1 Marble Hall (2004)
1354 Ermelo (2005)
1953 Alexandria (2006)
100
1252 Standerton (2005)
Lineage II
1939 Alexandria (2007)
1955 Alexandria (2006)
67
1508 Potgietersrus (2005)
1335.1 Balfour (2005)
1075 Klerksdorp (2004)
1633 Amersfoort (2006)

(b)

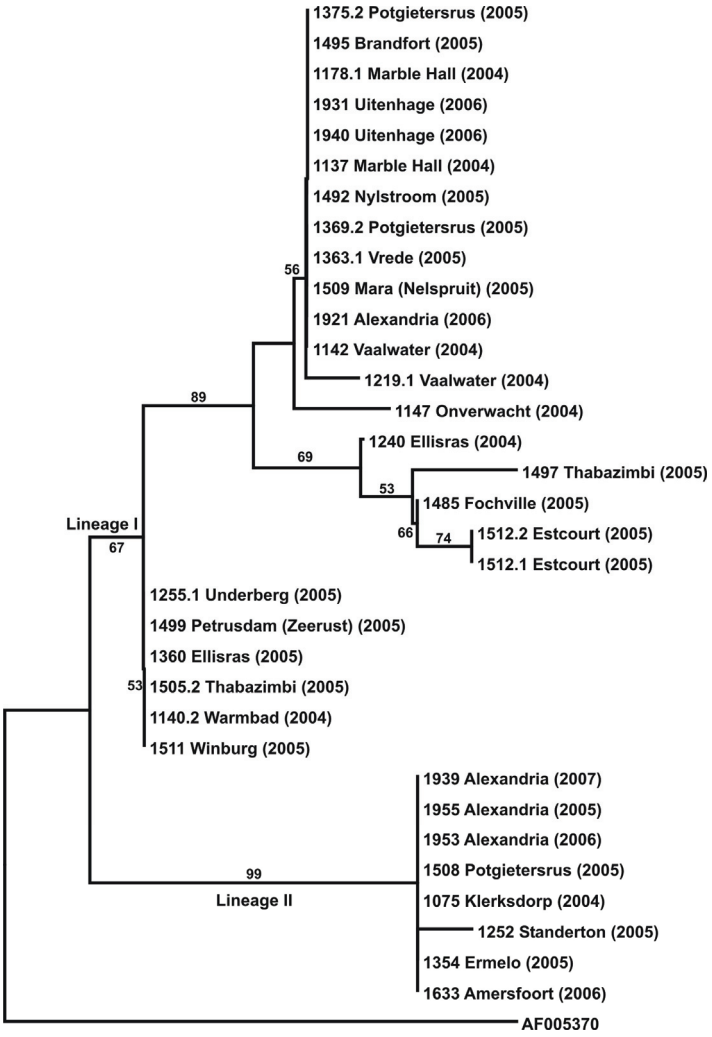


Figure 3a-b: Neighbor-joining phylograms based on (a) R-Transactivator and (b) ORF75 gene regions. Bootstrap support (based on 1000 bootstrap replicates) and greater than 50 % is given next to each node. Numbers in brackets correspond to year of outbreak.

DISCUSSION

The aim of this study was to identify a gene region within the A1HV-1 genome that is best suited to distinguishing between strains across a geographic gradient within South Africa, with the aim of determining the relatedness of AlHV-1 from different regions. Based on the pairwise differences the R-Transactivator region had the highest level of genetic variability, pointing to a likely potential for being the most suitable candidate gene. However, from the phylograms in figure 3 ORF75 shows greater resolution for lineage I while R-Transactivator indicates better resolution for lineage II. This conclusion can be drawn from the fact that there is better bootstrap support within lineage I and II as seen in figure 3a and 3b, respectively, which in turn leads us to the conclusion that both gene regions should be used when attempting to distinguish between outbreak strains.

In South Africa we have clearly identified two distinct lineages that are supported by all three gene regions targeted for the genetic characterisation of MCF. Lineage I contains isolates from the Limpopo Province, Free State, Gauteng, KwaZulu Natal and the Eastern Cape with lineage II containing outbreak isolates from Limpopo, Mpumalanga, North West and the Eastern Cape (Figure 1). Isolates in both lineages correspond to isolates obtained between between 2004 and 2007. Viruses recovered from a number of suspected clinical cases obtained from Uitenhage and Alexandria in the Eastern Cape during late 2006 and early 2007 are closely related to those recovered from the 2004/2005 cases in Limpopo Province, Mpumalanga and KwaZulu Natal. Based on this one can see quite clearly that outbreaks from different years are quite closely related, note the sister relationships in each of the respective trees between localities and year of outbreak i.e. isolate 1633 (Amersfoort - Mpumalanga) sampled from a suspected case in 2004 appears closely related to 1939 (Alexandria – Eastern Cape) sampled from a suspected case in 2007 in both ORF75 and R-transactivator (Figure 3a and 3b). This indicates that the same virus is infecting different animals from differing and geographically far apart (approximately 1000km) localities. Added to this our data shows an overall sequence variation of around 2 % (ORF75 = 1.4 % and R-transactivator = 3.3 % for overall sets of data) which strongly suggests that these sequences are derived from the same viral ancestor (Dunowska *et al*., 2001). As no insect vectors that transmit the virus have been identified so far (Barnard *et al*., 1990), it would appear that it is not the virus per se that is moving between these vast localities but man moving infected wildebeest into uninfected areas which in turn precipitate outbreaks. At this stage this is speculative and based solely on the phylogenetic information within the trees therefore further investigation into this would be of added value.

Conclusions drawn from this study are that the R-transactivator and ORF75 regions are the most phylogenetically informative and should be used in combination for the genetic characterisation of the MCF virus within South Africa. Secondly, farmers moving wildebeest and sheep, naturally infected with MCF virus, between farms and/or localities should be aware that by moving infected animals an MCF outbreak could occur leading to the loss of cattle which in turn may be a major economic loss.

ACKNOWLEDGMENTS

The Biotechnology Division is thanked for extracting DNA from clinical samples submitted to OVI for MCFV testing. Dr M Romito is thanked for reviewing the manuscript and the financial support of the Red Meat Board of South Africa and Department of Agriculture is gratefully acknowledged.

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POSTERS

HELICOBACTER AND BARTONELLA INCIDENCE IN INVASIVE RODENTS OF THE RATTUS SPECIES COMPLEX (RODENTIA: MURIDAE) IN SOUTH AFRICA

M. E. Mostert[1], [2], C. T. Chimimba[1],[2] & A. D. S. Bastos[1]

Members of the genus Rattus are generally invasive, occurring throughout the world in close association with humans. In South Africa, three species are known to occur, all of which are exotic and have a widespread distribution. These species, together with other members of the Rattus genus, are associated with economic losses resulting from the extensive damage that they cause to crops, stored grain, infrastructure and human property. Of greater concern is that most members of the genus are also known carriers of a range of zoonotic diseases that may be harmful to, and even cause death in humans as well as domestic animals, particularly in the developing world. These zoonotic diseases are generally not readily detected in rodents as they tend to be avirulent in the host reservoirs and only virulent in humans. Some of the rodent diseases of greatest concern in South African are bubonic plague, toxoplasmosis and leptospirosis, but these most likely only represent a fraction of the total disease diversity in the rodent host. In this study, the incidence of bacteria belonging to the genera Bartonella and Helicobacter present in members of the South African Rattus species complex was investigated by direct PCR amplification of the bacterial host genome. As both diseases have zoonotic potential, confirmation of their presence will have bearing on the formulation of appropriate rodent management strategies, particularly in those areas where human and rodent populations are highly concentrated.

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