Gammaherpesvirus carrier status of black wildebeest (*Connochaetes gnou*) in South Africa

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INTRODUCTION

Malignant catarrhal fever (MCF) is an economically important disease primarily of domestic cattle with a high case fatality rate. It is caused by either alcelaphine herpesvirus type 1 (AlHV-1) or ovine herpesvirus type 2 (OvHV-2). The major reservoir host of AlHV-1 is the blue wildebeest (*Connochaetes taurinus*), but it is generally accepted that the black wildebeest (*Connochaetes gnou*) is also a reservoir host. Malignant catarrhal fever in sika deer caused by CpHV-2 has been described as a chronic infection characterised by loss of body mass for 3–4 weeks prior to euthanasia10,11. The other virus causes classical MCF in white-tailed deer but the reservoir species has not been identified and the virus is therefore tentatively called malignant catarrhal fever virus-white-tailed deer (MCFV-WTD)12.

Lapses in control due to MCF may reach 7% annually in those areas in Africa where wildebeest and cattle share grazing7. Although infection and disease in cattle is usually fatal, surviving or carrier animals have been identified13. Traditionally, blue wildebeest have been regarded as the most important carrier of AlHV-1. However, black wildebeest have emerged as playing an important role in causing MCF in recent years. A comparison of black wildebeest-associated outbreaks in South Africa between the periods 1981–1983 and 1988–1990 revealed that the number of cases of MCF where only black wildebeest were involved has increased 7-fold14. This increase can be ascribed to the increase in the number of farms on which black wildebeest are kept.

Spread of the virus among blue wildebeest calves is likely to occur through close contact among calves during the 1st few weeks of life15. However, there is evidence that a proportion of wildebeest foetuses become infected *in utero*16. The infection therefore establishes itself in all animals in a population of blue wildebeest within the 1st months of life. The exact mode of transmission has not been elucidated but is most likely to be by aerosols. The virus appears to be excreted especially via ocular and nasal secretions from wildebeest calves17 and lambs18. It is only the cell-free virus secreted mainly by the calves or lambs that is infective to susceptible species19.

Histopathology remains the most definitive diagnostic procedure in dead animals, especially when there are legal or insurance considerations. Additional diagnostic tests used for sero-surveillance are based on the detection of antibodies against AlHV-1. Serological tests such as the serum-virus neutralisation and com-

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ABSTRACT

Malignant catarrhal fever (MCF) is an economically important disease primarily of domestic cattle with a high case fatality rate. It is caused by either alcelaphine herpesvirus type 1 (AlHV-1) or ovine herpesvirus type 2 (OvHV-2). The major reservoir host of AlHV-1 is the blue wildebeest (*Connochaetes taurinus*), but it is generally accepted that the black wildebeest (*Connochaetes gnou*) is also a reservoir host. No viral studies in the black wildebeest have been reported and the carrier status of black wildebeest has not been documented. Specimens were collected from several game farms and conservation areas in central South Africa representing the geographical area historically linked to the natural habitat of the black wildebeest. Specimens were obtained from 304 black wildebeest of different ages and sex, as well as 51 black wildebeest foetuses at different stages of gestation. Virus was isolated from a black wildebeest calf. Morphological features and antigenic characteristics suggested it to be a gammaherpesvirus closely related to AlHV-1. All serum samples tested positive with a competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) for group-specific malignant catarrhal fever virus antibody. A SYBR Green real-time PCR assay was developed for the detection of gammaherpesviral DNA. Only 15.8% of the animals tested positive with the real-time PCR assay whereas 90% of the foetuses tested positive. This finding suggests that, unlike OvHV-2 infection in lambs in which the infection takes place after weaning, the virus in black wildebeest is mainly transmitted *in utero* or soon after birth. The results suggest that black wildebeest are latent carriers of a gammaherpesvirus similar or closely related to AlHV-1 present in blue wildebeest and that it is likely that all black wildebeest are persistently infected.

Key words: alcelaphine herpesvirus type 1, black wildebeest, *Connochaetes gnou*, gammaherpesvirus, malignant catarrhal fever.


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A single-tube nested PCR was developed by Dungu et al.23 for the detection of OvHV-2 in clinically affected animals as well as carrier sheep. This assay can be used to distinguish between sheep-associated MCF and wildebeest-associated MCE. Traul et al.24 developed a real-time PCR assay using the hybridisation probe format for the detection of AIVH-1. It targets open reading frame 3 (ORF 3) that encodes for a tegument protein. The authors concluded, however, that the conventional nested PCR (which targets ORF 50), also developed by them, was more sensitive (~1 log) than the real-time PCR.

The aims of this study were (i) to isolate an MCF-causing virus from a black wildebeest calf in tissue culture; (ii) to test serum specimens collected from black wildebeest for the presence of antibodies reactive to an MCF-causing virus, and (iii) to develop a real-time PCR assay for detection of MCF-causing gammaherpesvirus DNA carried by black wildebeest.

MATERIALS AND METHODS

Study area and samples collected

Black wildebeest specimens were collected on several game farms and conservation areas in central South Africa (26°S to 31°S and 24°E to 30°E). Specimens (EDTA blood, serum and tissue samples) were collected randomly from live (n = 89) and culled (n = 215) black wildebeest with no intention to compare age or sex groups (Table 1). Specimens (corneas and spleen) from 51 black wildebeest foetuses at different stages of gestation were also collected. The culling operation formed part of the Free State Nature Conservation’s annual population control of different species, in conjunction with live capture. Animals were culled during 2005 in the Tussen-die-Riviore, Gariep, Maria Moroka and Sandveld nature reserves. The animals were shot at night using long-range rifles fitted with silencers and were immediately bled. Tissue samples were taken when the carcasses were skinned and eviscerated. Cornea and spleen samples from the 51 foetuses were also collected during the culling operations. Specimens were collected from live

animals caught by a professional capture team, Thaba Manzi Wildlife Services, on 3 farms situated near Potchefstroom in the North West Province (Schoondal, Grootkoppe and Copperfield).

In addition, blood samples were collected from a young buffalo (Syncerus caffer) that was housed with older black wildebeest calves for a 6-month period in the holding pens of Thaba Manzi Wildlife Services. Buffalo are known to be susceptible to MCF in captivity and under stressful conditions (I Espie, National Zoological Gardens, Pretoria, pers. comm., 2005).

Viral isolation and identification

A 10 mL heparinised blood sample was collected from a 1-week-old black wildebeest calf (WBS) that was born in the holding pens at Thaba Manzi Wildlife Services. This sample was immediately taken to the Virology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for viral isolation. It was co-cultivated with secondary black wildebeest foetal kidney (BWFK) cells previously established from a black wildebeest foetus (2004). Following the development of typical cytopathic effects in the cell monolayer, the supernatant fluid and cell debris was examined for the presence of viral particles by electron microscopy at the Electron Microscopy Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, for confirmation as a herpesvirus. Wildebeest foetal kidney cells showing cytopathic effects were fixed onto a multi-well microscope slide and reacted in an indirect fluorescent antibody test with known positive polyclonal bovine serum and sheep anti-bovine FITC-conjugated IgG antibody (The Binding Site Ltd, Birmingham, UK). The positive bovine serum was identified with a competitive inhibition ELISA (VMRD Inc., USA).

Serology

Of the 299 black wildebeest serum samples collected, 269 were tested for the presence of MCFV antibody using a competitive inhibition ELISA kit (VMRD Inc., USA). The preparation of the serum samples and the test procedure were done according to the manufacturer’s instructions. The positive and negative controls used were supplied as standard controls with the kit. The positive control was run in duplicate and the negative control in triplicate as specified in the manufacturer’s instructions. The plate was read with an ultra microplate reader, Bio-Tek Instruments6, Model EL808 and the results prepared by the software programme KC Junior6. If the test sample caused more than 25 % inhibition, it was noted as positive. The following equation was used to determine the percentage inhibition from the raw data:

\[
\% \text{Inhibition} = 100 - \left[ \frac{\text{Sample O.D. (Ave.)}}{100} \times \text{Mean neg. control O.D.} \right]
\]

Real-time PCR assay

A SYBR Green real-time PCR assay was developed and evaluated for the specific detection of AIVH-1.

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(i) DNA extractions

DNA was extracted from a total of 774 black wildebeest samples (200 µL of whole blood or 2 g of tissue) representing 304 adult animals and 51 foetuses using the QiAamp® DNA Mini Kit (QiAGEN®, Southern Cross Biotechnologies) according to the manufacturer’s instructions. Extracted DNA was eluted in 100 µL elution buffer and stored at −20 °C. In addition, DNA was extracted from (i) the viral isolate obtained from WB5 and (ii) a blood sample of a bovine (Bovine 4408) that was confirmed to have died of blue wildebeest associated MCF (supplied by the Biotechnology Section of the Onderstepoort Veterinary Institute, Pretoria) to serve as positive control DNA. DNA extracted from an OvHV-2 positive sheep sample of Norwegian origin and double-distilled water served as negative controls.

(ii) Primer design and real-time PCR conditions

A SYBR Green real-time PCR assay was developed using the AlHV-1-specific nested primers C500–3 (5’–TCT GGC CCG TGC TGC AGC AAG ACT CTC AG–3’) and C900–4 (5’–TAT AGT AGA ATC CCG TCT GAG TGG TAG CTG–3’) as described by Li et al.7. These primers were derived from the sequence of AlHV-1 open reading frame (ORF) 50, a region of the AlHV-1 genome reported to be associated with virulence for rabbits8. The primers amplified a 274 bp region of the gene. The amplification mixture consisted of 2 µL LightCycler® FastStart DNA Master SYBR Green 1 (Roche® Applied Science), 3 mM MgCl2 (Roche® Applied Science), 10 µmol of each primer (Inqaba Biotech, SA) and 2.5 µL extracted DNA made up with sterile double distilled water to a final volume of 20 µL. Temperature cycling was performed in a LightCycler v2.0 (Roche®) and the real-time PCR results analysed by the Roche® LightCycler Software 4.0. An initial activation step at 95 °C for 10 minutes was followed by 45 cycles of denaturation at 95 °C with a 10 s hold; primer annealing at 65 °C with a 10 s hold; and product extension at 72 °C with a 20 s hold. Following amplification, a melting curve analysis was performed by heating the samples from 65 °C to 95 °C with a heating rate of 0.1 °C per s. Fluorescence values were continuously measured at 530 nm. For verification, the PCR products were subjected to gel electrophoresis on 2 % agarose gels stained with ethidium bromide to confirm the absence of nonspecific products or primer dimers. To confirm specificity, amplicons of the positive controls, WB5 and Bovine 4408, were subjected to direct sequence analysis.

(iii) Quantitative sensitivity

To determine the analytical sensitivity of the assay, a standard curve was generated using a 10-fold dilution of template amplified on the LightCycler™ real-time system. The real-time PCR products obtained from the positive controls (WB5 and Bovine 4408) were used. The PCR products were purified using the QIAquick PCR Purification Kit (QiAGEN, Southern Cross Biotechnologies), according to the manufacturer’s instructions, and their concentrations were determined by optical density measurements at 260 and 280 nm. Each dilution was assayed in triplicate using the real-time PCR conditions as described above. The standard curve was generated using the LightCycler™ software (Roche Diagnostics, Mannheim, Germany).

RESULTS

Cytopathic effects compatible with herpesvirus replication were visible 18 days after inoculation of cell cultures. The virus was identified by means of electron microscopy and an indirect fluorescent antibody (IFA) test. Viral particles were roughly 100 nm in diameter, enveloped and icosahedral in shape (Fig. 1).

Of the black wildebeest serum samples (n = 269) tested with the CI-ELISA, all yielded antibody reactive to the MCF group of viruses. The young buffalo bull (BB) tested negative with an inhibition lower than 25 %. Almost all of the samples, 97.3 % (n = 262), caused inhibition above 70 % with only 7 samples causing inhibition between 25 and 69 %.

The positive controls, WB5 and Bovine 4408, were used to evaluate the newly developed SYBR Green real-time PCR assay. A melting curve analysis of the PCR products was performed to verify that the fluorescent signal obtained in the real-time PCR originated from specific PCR products and not from artefacts like primer-dimers (Fig. 2). The average melting temperature (Tm) of the amplified control WB5 was 89.45 °C (±0.37 °C), and Bovine 4408 was 88.74 °C (±0.38 °C). The negative controls (OvHV-2 positive sheep sample and water control) had an average Tm of 85.03 °C (±0.56 °C). The latter is not unusual when SYBR Green is used and is caused by primer-dimer formation9. This phenomenon was confirmed by visualising the products on an agarose gel (data not shown). The positive results were in accordance with the presence of a 274 bp band on agarose gel (data not shown). The real-time PCR amplicons of the 2 control viruses, WB5 and Bovine 4408, were sequenced, and sequence analysis confirmed that WB5 was closely related to AlHV-1. A BLAST search showed 93.3 % similarity to the ORF 50 fragment of the published AlHV-1 genome4. The sequence of Bovine 4408 was 98.5 % similar to the ORF 50 fragment of the published AlHV-1 genome. The BLAST search showed no significant similarity to OvHV-2.

To determine the sensitivity of the AlHV-1 real-time PCR, a standard curve was generated using the LightCycler™ software. Results have shown the assay had an amplification efficiency of 1.935. An amplification efficiency of 2.00 is considered ideal, and corresponds to a doubling of copy number for every PCR cycle10. However, any double-stranded PCR artifact (in this case primer-dimers) contributes to signal intensity, which
results in the overestimation of the concentration of the target sequence. To solve this, a melting curve analysis was performed to distinguish between product and primer-dimers. For true quantification, the PCR must be optimised and artifact-free. Based on the melting curve analysis, the AlHV-1 assay could reliably detect dilutions with the lowest DNA concentration corresponding to 250 copies of the target DNA. The assay showed variable results at lower concentrations of target DNA per reaction.

In total, 774 wildebeest blood and tissue samples (representing 304 adult animals and 51 foetuses) were tested for the presence of gammaherpesvirus DNA using the SYBR green real-time PCR assay. Only 106 samples (13.7 %) tested positive for the presence of viral DNA. Of the 304 adult animals tested, 48 animals (15.8 %) yielded a positive real-time PCR test result from one or more of the samples collected from that animal (i.e. either EDTA blood, spleen and/or cornea) (Fig. 3). Of the foetuses tested, 46 (90.2 %) yielded a positive real-time PCR result from either the spleen or cornea sample, or both. Five foetuses tested negative for the presence of viral DNA (Fig. 4).

**DISCUSSION**

South Africa is the only natural habitat of the black wildebeest. To date the blue wildebeest has been regarded as the most important carrier of AlHV-1. Black wildebeest have been emerging as important carriers of MCFV in recent years. However, no viral studies in the black wildebeest have been reported and the nature of the virus carried by them was not known.

Virus is excreted by the natural host, the blue wildebeest, in a cell-free form for a brief period following infection and can be isolated from nasal swabs and blood leukocytes during this period\(^\text{17}\). Viral isolation from clinically affected animals can be achieved from cell suspensions of peripheral blood leukocytes, lymph nodes or other affected tissues\(^\text{15}\). In this study, virus was isolated from a 1-week-old black wildebeest calf (WB5) and it was confirmed to be a herpesvirus by electron microscopy. The size of approximately 100 nm, the icosahedral capsid and tegument were compatible with typical herpesvirus morphology. Based on the morphological, phenotypic, antigenic and genetic characteristics, it was found to be a gammaherpesvirus closely related to AlHV-1.

All 269 of the black wildebeest serum samples collected from live and culled black wildebeest from game farms and...
First documented confirmation of black wildebeest as carriers of a gammaherpesvirus antigenically similar to or closely related to AlHV-1. The black wildebeest sample represented the geographical area historically linked to their natural habitat and 100% of the tested population were positive.

A SYBR Green real-time PCR assay was developed and evaluated. It was shown to be specific for detection of AlHV-1 DNA; OvHV-2 DNA was not detected. Sequence analysis confirmed the results. The standard dilution series approach to generate a standard curve for quantitative real-time PCR gives a good estimate of the efficiency of the PCR assay. In this case, however, primer-dimers contributed to the signal intensity, which result in the overestimation of the concentration of the target sequence. To solve the problem, melting curve analysis was used to discriminate between product and primer-dimer. It is also important to keep in mind that clinical samples are complex and may contain inhibitory substances. The developed AlHV-1 SYBR Green assay proved to be sensitive enough to reliably detect 250 copies of the target DNA. It was not reproducible at lower DNA concentrations. The hybridisation probe format real-time PCR developed by Traul et al. for the detection of AlHV-1 was shown to reproducibly detect 10 copies of target DNA. The authors concluded, however, that the conventional nested PCR that was developed by them was more sensitive (−1 log) than the real-time PCR.

Only 106 of 774 (13.7%) black wildebeest samples (blood in EDTA, spleen, cornea and foetal samples) tested positive using this real-time PCR. This could be attributed to the lack of sensitivity of the assay. The 2 controls, namely the virus isolated from black wildebeest calf WB5 and the bovine that died of MCF, Bovine 4408, tested positive with every LightCycler run. The viral input DNA in both these was higher than those of field samples from carrier animals. DNA input could therefore be increased as one possible way of increasing the sensitivity of the assay. The choice of samples could also be re-evaluated as the virus may be more concentrated in other organs, for example in lymph nodes and the central nervous system. Working with only EDTA blood, spleen and cornea, 15.8% (48 of 304) of the animals tested positive for the presence of AlHV-1 DNA on 1 or more samples. Three per cent (9 of 301) of the blood samples tested positive; 9.3% (15 of 163) of the spleen samples and 13% (27 of 208) of the cornea samples.

The majority of animals that tested positive were culled during the latter part of the winter and early spring before the first seasonal rains. Nutritional stress may be a reason for the higher concentration of circulating virus at that time of year. Forty-eight animals had circulating virus detectable with real-time PCR. Thirteen of 38 (34.2%) animals culled in the Sandveld Nature Reserve during September 2005 were positive, mostly from spleen samples. The Gariep Nature Reserve had 12 of 73 (16.4%) real-time PCR positive animals that were culled during August 2005. The positive results were equally distributed between the spleen and cornea. Black wildebeest were culled in June 2005 on Maria Moroka Reserve and 11 of 59 animals (18.6%) were positive, mainly on cornea samples. Tussen-die-Riviere Nature Reserve yielded 6 of 43 (14.0%) positive animals and samples were taken during April, May and June 2005. The time of culling therefore seems to influence the results. The other reserves had between 0 and 14% positive animals but on 3 of these reserves (Schoondal, 7.4%, Grootkoppe, 12.5%; Copperfield, 0%), only live animals were caught and therefore only blood samples collected. Most of the animals that tested positive were adult animals but this component of the population was over-represented as culling operations focused on the adult animals for meat harvesting purposes. Foetuses testing positive on real-time PCR were evenly distributed between the reserves where animals were culled.

Ninety per cent of the foetuses tested positive with the PCR on one or both of the cornea and spleen samples. Only 5 of the 51 foetuses (9.8%) tested negative on...
both samples with real-time PCR. The results showed that unlike lambs, most of the black wildebeest calves are infected in utero. Of the 51 foetuses, 78.4 % (40 of 51) had positive spleen samples, 29.4 % (15 of 51) had positive cornea samples and of these, 19.6 % (9 of 46 positive foetuses) or 17.7 % (9 of 51 foetuses) were positive with both tissue types. The real-time PCR results confirmed that foetuses already become infected in utero.

In summary, this study represents the 1st documented confirmation of the gammaherpesvirus carrier status of black wildebeest in South Africa. Black wildebeest are latent carriers of a gammaherpesvirus similar or closely related to AlHV-1 present in blue wildebeest, and it is likely that all black wildebeest are persistently infected. Also, unlike OvHV-2 infection in lambs in which the infection takes place after weaning, the virus in black wildebeest is mainly transmitted in utero or soon after birth.

ACKNOWLEDGEMENTS

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