Detection of AHV-1 DNA in lung sections from blue wildebeest (*Connochaetes taurinus*) calves by in situ hybridization

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ABSTRACT

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In situ hybridization was performed on formalin-fixed, paraffin-embedded lung sections from eight blue wildebeest *(Connochaetes taurinus)* calves to investigate the role of this organ in the excretion of alcelaphine herpes virus-1 (AHV-1). A digoxigenin labelled *SmaI* fragment of AHV-1 was used as a DNA probe. Viral DNA was detected in the lungs of seven calves in which infection with AHV-1 had previously been confirmed, indicating the significance of the lower respiratory tract in viral replication and excretion. The results also confirmed the sensitivity of the in situ hybridization technique in detecting low amounts of viral DNA in infected cells in routinely processed tissues.

Keywords: Alcelaphine herpes virus-1, blue wildebeest, *Connochaetes taurinus*, in situ hybridization, lung, non-radioactive labelling

INTRODUCTION

Malignant catarrhal fever (MCF) is caused by the wildebeest-associated alcelaphine herpes virus -1 (AHV-1) (Plowright 1964) and based on circumstantial evidence also by the sheep-associated ovine herpes virus-2, which was classified more recently (Roizman, Carmichael & Deinhardt 1992; International Committee on Taxonomy of Viruses 1992).

The mode of transmission between reservoir host and cattle is still unknown (Plowright 1986). Infectivity of AHV-1 in wildebeest blood is strictly cell-associated and depends mainly on the retention of cell viability which makes transmission by blood-sucking arthropods rather unlikely (Plowright 1963, 1965). Wildebeest calves as well as adult wildebeest have, however, been found to excrete infectious alcelaphine herpes virus-1 in nasal secretions (Rweyemamu, Karstad, Mushi, Otema, Jessett, Rowe, Drevemo & Grootenhuis 1974; Mushi, Rossiter, Karstad & Jessett 1980; Barnard, Bengis, Griessel & De Vos 1989). Mushi and co-workers (1980) further showed by filtration of the nasal secretions that the infectivity in the nasal secretions is mainly extracellular. These results suggest transmission of AHV-1 among wildebeest and from wildebeest to cattle, preferably occurring in nasal and/or ocular secretions.

In situ hybridization is an important tool in the elucidation of the pathogenesis of infectious diseases because of its ability to detect nucleic acids in the cellular environment (Haase, Brahic, Stowring & Blum 1984; Warford 1988). In this study, in situ hybridization was used to demonstrate the localization of AHV-1 nucleic acid in the lungs of naturally infected, free-living blue wildebeest calves.

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MATERIALS AND METHODS

Cell cultures

Vero cell cultures were infected with the laboratory-attenuated AHV-1 strain WC 11. As soon as cytopathic effect was visible the cells were harvested and one drop of cell suspension was dried and fixed on a pretreated slide by immersion in ethanol/acetic acid (1:3) for 20 min. Uninfected Vero cell cultures served as negative controls.

Organ specimens

Between 1988 and 1993, eight blue wildebeest (*Connochaetes taurinus*) calves between approximately 1 and 4 months of age were immobilized in the Kruger National Park with etorphine hydrochloride (M99) and xylazine hydrochloride (Rompun). After the calves had been transferred to the laboratory, nasal mucus and ocular fluid were collected from calves 1–5 (Table 1; calf nos 2–4 and 8–9 in Barnard *et al.* 1989), while heparinized blood samples for slot-blot hybridization and virus isolation were collected from calves 6–8 (Table 1; calf nos 8–10 in Michel 1993). All calves were subsequently euthanased by the administration of an intravenous overdose of sodium pentobarbitone and a full post mortem examination performed on each carcase. Specimens from various organs including the lung were collected in 10 % buffered formalin for histology. Lung specimens from a bovine, negative for MCF, served as a negative control. Organ specimens were routinely processed and stained with haematoxylin and eosin (HE) for histopathological examination.

Infection in seven of these calves was previously confirmed by virus isolation (Barnard *et al.* 1989) and/or by slot-blot hybridization of DNA from blood leucocytes, nasal mucus and ocular fluid (Michel 1993).

Blood samples from three calves had previously been tested by filter hybridization and from one, also by virus isolation, and they delivered positive results (Michel 1993).

Pretreatment of microscopic slides

Precleaned microscopic slides were washed in "Extran" (Merck) overnight and rinsed in running tap water for 2 h. After they had been rinsed with double-distilled water, the slides were dipped in 2% 3-Aminopropyltriethoxy-silane (Sigma) for 5 s. The slides were fixed twice in acetone and rinsed twice in double-distilled water. Finally, the slides were allowed to air dry.

Pretreatment of cover slips

Cover slips were cleaned in 2% acetic acid for 30 min, rinsed twice in double-distilled water and once in ethanol. Dry cover slips were dipped in "Sigmacote" (Sigma) and dried at 120°C for 2 h.

Probe preparation

An approximately 2 kb SmaI fragment of the WC 11 genome that had been cloned into vector pUC18 (Michel 1993) was gel purified twice on 1% agarose gels as described by Sambrook, Fritsch & Maniatis (1989). The DNA probe was sonicated for 4 min (Branson Sonifier 250) and labelled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim). The probe concentration was determined by titration.

In situ hybridization

Four micrometre sections were cut from formalin-fixed, paraffin-embedded lung specimens, placed on pretreated slides and dried for at least 3 h at 40°C. Subsequently the sections were fixed in Karnovsky's fixative (Moench, Gendelman, Clements, Narayan & Griffin 1985) and air dried. Prior to hybridization the sections were dewaxed by washing them three times in xylene for 7 min at room temperature. Slides with cell suspensions were not dewaxed. All slides were rehydrated by sequential immersion in absolute ethanol for 10 min, in 70%, 50% and 30% ethanol for 5 min, each followed by a rinse in PBS with 5 mM $MgCl_2$. Denaturation occurred in 0,2 N HCL for 20 min followed by two washes in 2x SSC/5 mM EDTA at 50°C for 30 min. In order to make the nucleic acids accessible, the sections were incubated in 100 µg/mℓ pronase (slides with cell suspensions in 0,1 µg/mℓ) at 37°C for 30 min. The enzyme activity was stopped in 0,2% glycin for 10 min and the specimens were fixed in 4% paraformaldehyde for 25 min. After they had been rinsed in PBS/5 mM $MgCl_2$ the slides were dehydrated in graded alcohol and air dried.

For hybridization, each specimen was covered with 20 µℓ of the following hybridization solution: 4x SSC, 45% formamide, 5x Denhardt's solution (0,02% ficoll, 0,02% bovine serum albumin, 0,02% polyvinylpyrrolidone), 10% dextrane sulphate, 0,75 ng/µℓ of DIG-labelled probe. For controls each batch of in situ hybridizations included at least one lung section from the control cattle, which was hybridized with DIG-labelled probe, and one lung section from blue wildebeest which was treated with hybridization solution without labelled probe. Specimens were covered with siliconized cover slips and sealed with the rubber cement "Fixogum" (Marabu, Germany), denatured at 96°C for 5 min and chilled on ice for 4 min before overnight incubation at 42°C.

Posthybridization washes and detection

After removal of the cover slips the specimens were washed twice in 4x SSC/45% formamide at 42°C for 15 min, twice in 2x SSC for 5 min and twice in 0,2 x SSC at 50°C for 15 min. After a blocking step in 3% bovine serum albumin, the slides were incubated in

anti-DIG fab fragments (diluted 1:7 500) and colour detection was performed according to manufacturer's instructions (Boerhinger Mannheim). Some of the sections were stained with haematoxylin and eosin after in situ hybridization had been completed. Staining intensities were, however, reduced compared with routine HE staining.

Assessment of the in situ hybridization signals

Entire hybridized sections were viewed with a Zeiss light microscope (x 250–1 000). Only sections with a noise-to-signal ratio comparable with that of the negative controls that were hybridized simultaneously with the test sections, were used in the evaluation.

RESULTS

Histopathology

A mild or moderate interstitial pneumonia was present in all calves. Alveolar walls were infiltrated by lymphocytes admixed with lesser numbers of neutrophils and macrophages. All calves had variable peribronchial, peribronchiolar and perivascular accumulations of lymphoid cells. These changes in one calf were accompanied by a severe segmental lymphocytic arteritis.

In situ hybridization

Sonification of the DNA probe resulted in an average probe length of 400–800 bp that achieved more efficient hybridization than the full-length probe of 2 kb (data not shown). In situ hybridization with the digoxigenin-labelled DNA probe was first carried out on Vero cell cultures infected with WC 11 and uninfected controls (Fig. 1 and 2). Single, infected cells were clearly distinguished from uninfected cells by their varying degrees of purple to brown staining.

TABLE 1 Results of virus isolation, slot-blot hybridization and in situ hybridization on eight blue wildebeest calves

Calf no.	Virus isolation[a]	Slot-blot hybridization[b]	In situ hybridization
1	+	ND	–
2	+	ND	+
3	+	ND	+
4	+	ND	+
5	+	ND	+
6	C	+	+
7	+	+	+
8	C	+	+

ND = Not done
C = Specimen contaminated
– = Negative result
\+ = Positive result
[a] As described by Barnard *et al.* 1989
[b] As described by Michel 1993

The results of the in situ hybridization on lung sections of eight blue wildebeest calves are summarized in Table 1. The noise-to-signal ratio observed was generally very low (Fig. 1–3). The results showed an overall low incidence of detectable infection with the number of AHV-1 DNA-containing cells varying from five to 40 per section. Consistently positive results

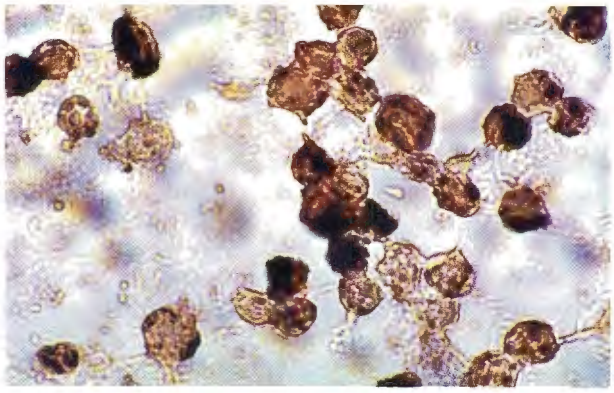

FIG. 1 AHV-1-infected Vero cell culture hybridized with DIG-labelled probe

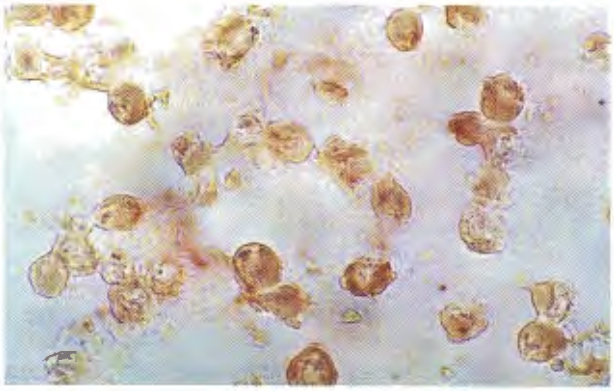

FIG. 2 Uninfected Vero cell culture control hybridized with DIG-labelled probe

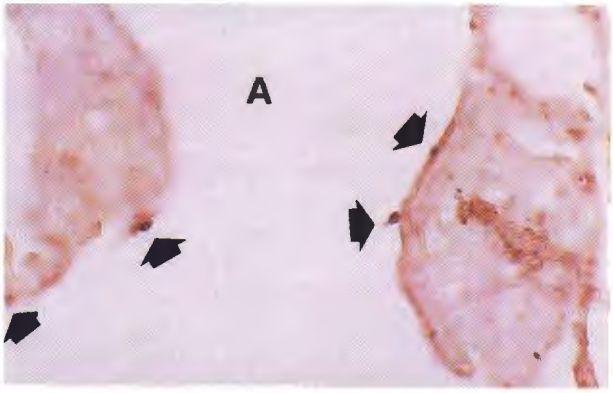

FIG. 3 Lung sections of a blue wildebeest calf: AHV-1-fected alveolar lining cells (arrows) after hybridization with DIG-labelled probe A: Alveola

in consecutive sections were found in the lungs of seven of the eight blue wildebeest calves.

The substrate-labelled individual cells gave a purple staining, but with a lack of nuclear and cytoplasmic detail. Positive cells were constituent cells of the alveoli, sometimes lining the alveolar septa (Fig. 3) of mononuclear cells. Occasional labelled cells were demonstrated in the bronchiolar epithelial cell of three calves found positive on in situ hybridization.

DISCUSSION

In the present study, lung sections of eight blue wildebeest calves were examined by in situ hybridization with the use of a digoxigenin-labelled DNA probe. We found AHV-1-specific nucleic acid sequences in seven out of the eight wildebeest lungs with numbers of infected cells varying from five to more than 40 per section. The specificity of the hybridization, especially in cases with low numbers of infected cells, was confirmed in consistent hybridization signals in two to three consecutive sections. Compared with similar work done in experimentally infected rabbits (Bridgen, Munro & Reid 1992) our non-radioactive in situ hybridization technique proved to be a rapid and reliable detection system for AHV-1 with a high sensitivity in blue wildebeest. The system failed to detect AHV-1 infection in only one of the eight calves which had previously been confirmed positive by virus isolation (Table 1).

This is the first report on the in situ detection of AHV-1 in blue wildebeest. Although it remains to be established whether the viral DNA in the bronchiolar and alveolar cells reflect a productive infection, these findings provide strong support for the earlier suggestion by Rweyemamu *et al.* (1974) that AHV-1 is excreted via the lower respiratory tract of blue wildebeest calves. Previously, cornea and upper respiratory tract, including the turbinates, were thought to be involved mainly in the replication of AHV-1 (Mushi, Rurangirwa &Karstad1981). However, the actual replication sites of this virus have not been established to date.

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REFERENCES

BARNARD, B.J.H., BENGIS, R.G., GRIESSEL, M.D. & DE VOS, V. 1989. Excretion of acelaphine herpesvirus-1 by captive and free-living wildebeest (*Connochaetes taurinus*). *Onderstepoort Journal of Veterinary Research*, 56:131–134.

BRIDGEN, A., MUNRO, R. & REID, H.W. 1992. The detection of alcelaphine herpesvirus-1 DNA by in-situ-hybridization of tissues from rabbits affected with malignant catarrhal fever. *Journal of Comparative Pathology*, 106:351–359.

HAASE, A., BRAHIC, M., STOWRING, L. & BLUM, H. 1984. Detection of viral nucleic acids by in-situ-hybridisation. *Methods in Virology.* 7:189–226.

INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES: HERPESVIRUS STUDY GROUP. 1992. The family Herpesviridae: an update. *Archives of Virology*,123:425–449.

MICHEL, A.L. 1993. Generation of a nucleic acid probe specific for the alcelaphine herpesvirus 1 and its use for the detection of malignant catarrhal fever virus DNA in blue wildebeest calves (*Connochaetes taurinus*). *Onderstepoort Journal of Veterinary Research*, 60:87–93.

MOENCH, T.R., GENDELMAN, H.E., CLEMENTS, J.E., NARAYAN, O. & GRIFFIN, D.E. 1985. Efficiency of in situ hybridization as a function of probe size and fixation technique. *Journal of Virological Methods*, 11:119–130.

MUSHI, E.Z., ROSSITER, P.B., KARSTAD, L. & JESSETT, D.M. 1980. The demonstration of cell-free malignant catarrhal fever herpesvirus in wildebeest nasal secretions. *Journal of Hygiene*, 85:175–179.

MUSHI, E.Z., RURANGIRWA, F.P. & KARSTAD, L. 1981. Shedding of malignant catarrhal fever virus by wildebeest calves. *Veterinary Microbiology*, 6:281–286.

PLOWRIGHT, W. 1963. *XVIIth World Veterinary Congress, Hanover, Proceedings*, I:519.

PLOWRIGHT, W. 1964. Studies on malignant catarrhal fever of cattle. Doctoral thesis. Faculty of Veterinary Science of the University of Pretoria.

PLOWRIGHT, W. 1965. Malignant catarrhal fever in East Africa. II. Observations on wildebeest calves at the laboratory and contact transmission of the infection to cattle. *Research in Veterinary Science*, 6:69–83.

PLOWRIGHT, W. 1986. Malignant catarrhal fever. *Revue Scientifique et Technique.* Office Internationale des Epizooties, 5: 897–918.

ROIZMAN, B., CARMICHAEL, L.E. & DEINHARDT, F. 1992. Herpesviridae: definition, provisional nomenclature and taxonomy. *Intervirology*, 16:201–217.

RWEYEMAMU, M.M., KARSTAD, L., MUSHI, E.Z., OTEMA, J.C., JESSETT, D.M., ROWE, L., DREVEMO, S. & GROOTENHUIS, J.G. 1974. Malignant catarrhal fever virus in nasal secretions of wildebeest: a probable mechanism for virus transmission. *Journal of Wildlife Diseases*, 10:478–487.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. New York: Cold Spring Harbour Laboratory Press.

WARFORD, A. 1988. In situ hybridization: a new tool in pathology. *Medical Laboratory Sciences*, 45:381–394.