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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)*

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ABSTRACT

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Two WC 11 specific DNA fragments, 3 kb and 2 kb in size, respectively, were cloned and evaluated as probes for their use in diagnostic and epidemiological investigations of malignant catarrhal fever (MCF). Field specimens including blood, ocular fluid, nasal mucus and urine of blue wildebeest *(Connochaetes taurinus)* calves in the Kruger National Park, South Africa, were tested and found positive for excretion of MCF-virus by slot blot hybridization. In 2 cases MCF-virus DNA was detected in the urine of the calves. No hybridization was observed with DNA from other bovine herpesviruses.

INTRODUCTION

An apparently healthy black wildebeest (*Connochaetes gnu*) was identified as a reservoir of malignant catarrhal fever (MCF) by Mettam, as early as 1923, when he inoculated blood samples from it into cattle. The causative virus was first isolated by Plowright (1960). Another 30 years passed before molecular studies allowed the characterization of this gammaherpes-virus (Bridgen, Herring, Inglis & Reid 1989; Seal, Klieforth, Welch & Heuschele 1989; Bridgen 1991). Isolates derived from wildebeest were designated Alcelaphine Herpesvirus 1 (AHV-1), whereas isolates derived from hartebeest were termed Alcelaphine Herpesvirus 2 (AHV-2) (Roizman, Carmichael & Deinhardt 1992). Related herpesviruses have been isolated from animals with MCF at the Oklahoma City Zoo (Castro, Daley, Zimmer, Whitenack & Jensen 1982) and recently also from a roan antelope in a wildlife park in Scotland (Reid & Bridgen 1991). Neutralizing antibodies against AHV-1 have been found in several other wild ruminants in Africa and the USA (Plowright 1982). Epidemiological evidence implicates domestic sheep as a carrier of another, not yet characterized MCF causing herpesvirus (Schuller, Cerny-Reiterer & Silber 1990), especially in temperate zones (Goetze & Liess 1929, 1930; Reid, Buxton, Pow & Finlayson 1986), but also in Africa (De Kock & Neitz 1950). Both forms of the disease are generally diagnosed by the complex of clinicopathological features, which include sudden, persistent pyrexia, ocular and nasal discharge, generalized enlargement of lymph nodes, necrosis of mucosae, skin and eye lesions as well as diarrhoea and in some cases dermatitis (Plowright 1964, 1986). Traditional virological techniques such as serological methods and

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virus isolation are applicable only to a certain extent for the diagnosis of MCF. Low titres, cell association and especially the instability of the virus in clinical specimens are the main causes of a limited success rate of virus recovery in tissue culture. Cross reactions with other bovine herpesviruses further complicate the diagnosis of MCF. In the past, several approaches have been taken to investigate the possible routes of virus transmission in wildebeest-associated outbreaks. They implicate transmission by oral and nasal aerosols (Barnard, Bengis, Griessel & De Vos 1989a; Mushi & Rurangirwa 1981; Plowright 1965; Rweyemamu, Karstad, Mushi, Otema, Jessett, Rowe, Drevemo & Grootebhuis 1974), intrauterine infection (Barnard 1990; Plowright, Ferris & Scott 1960) as well as possible involvement of an intermediate host (Barnard, Van de Pypekamp & Griessel 1989b). However, it proved difficult to reproduce and verify these results due to the abovementioned technical limitations. The diagnostic use of a nucleic acid probe specific for the MCF virus therefore offers the possibility to overcome some of these obstacles as shown by other researchers (Katz, Seal & Ridpath 1991; Seal, Klieforth & Heuschele 1990; Shih, Irving, Zee & Pritchett 1988) and at the same time may provide a reliable tool for studying the epidemiology and pathogenesis of MCF. This paper describes the preparation of 2 WC 11-specific DNA probes and their application in the study of malignant catarrhal fever epidemiology.

MATERIALS AND METHODS

Virus strains and cell cultures

The cell culture adapted virus strain WC 11 was propagated on Vero and MDBK cells. The MCF isolate Skukuza, a field isolate derived from a blue wildebeest in the Kruger National Park (unpublished data), was cultured on fetal lamb kidney (FLK) cells. All cells were maintained in Eagle's minimal essential medium supplemented with 10 % irradiated bovine serum, benzyl penicillin (400 U/mℓ) and streptomycin (200 µg/mℓ) at 37 °C.

Virus purification

Infected cell cultures were incubated on roller bottles for 1–3 weeks up to the lytic phase. Virus was pelleted from the cell-free culture fluid and centrifuged in a 15–60 % sucrose gradient at 22 000 rpm for 4 h. All light-scattering bands with a sucrose density between 40 % and 51 % were pooled and the virus was pelleted and resuspended in TE-buffer (0,01 M Tris, 0,001 M EDTA pH 7,6).

DNA extraction and restriction enzyme analysis

Purified virions were lysed by the addition of 0,1 % SDS and digested with 50 µg/mℓ proteinase K (Boehringer Mannheim) and 50 mM EDTA. The digest was extracted once with phenol (Tris-buffered), twice with phenol/chloroform (1:1) and twice with chloroform/isoamylalcohol (24:1), followed by an overnight precipitation with 2 volumes of absolute ethanol at –20 °C. The DNA pellet was resuspended in TE-buffer. Approximately 1,5 µg of MCF-virus DNA was digested with 7,5 u of Sma I restriction enzyme according to the manufacturer's instructions (Boehringer Mannheim). The digest was separated on a 0,8 % agarose gel in Tris-acetate buffer, containing 0,5 µg/mℓ ethidium bromide (Maniatis, Fritsch & Sambrook 1982).

Cloning of WC 11-specific fragments

WC 11 DNA was partially digested with Sma I endonuclease and cloned into the Sma I restriction site of pUC 18 (Maniatis *et al.* 1982). After transformation of *E. coli* JM 105 (Chung, Niemela & Miller 1989) white colonies were selected on IPTG/X-gal and ampicillin containing LB plates and screened for inserts by a modified minipreparation method (Saunders & Burke 1990). Two recombinant clones containing WC 11 DNA fragments of 2 kb and 3 kb, respectively, were selected and prepared on a large scale. After digestion with Sma I endonuclease the DNA fragments were twice gel-purified followed by a phenol/chloroform extraction.

Non-radioactive probe labelling

The cloned and gel-purified restriction endonuclease fragments of the WC 11 were subsequently designated SW 2 and SW 15. The nucleotide analog digoxigenin 11-dUTP (DIG) was incorporated into SW 2 and SW 15 by the random primed labelling technique (Feinberg & Vogelstein 1983) according to the manufacturer's instructions (Boehringer Mannheim). The labelling efficiency was determined by detecting the freshly DIG-labelled probe titrated on a nylon membrane. Its colour intensity was compared to that of a labelled control DNA supplied by the manufacturer.

Field specimens

Ocular fluid (collected from the inside of the lower eyelid), nasal mucus (collected by nasal swab) and blood samples were collected from 10 3-months-old blue wildebeest *(Connochaetes taurinus)* calves in the Kruger National Park. Urine samples were collected from 3 of the 10 calves. Forty heparinized blood samples as well as 12 nasal swabbings and 12 ocular fluid samples were collected from 1 of the cattle herds of the Animal and Dairy Science Research Institute, Irene, situated in a region free of sheep and wildebeest. No clinical cases of MCF have ever occurred in this centre.

Preparation of field specimens for virus isolation and DNA extraction

Blood samples

Leucocytes were prepared from the buffy coats (Barnard *et al.* 1989a) of 10 mℓ of heparinized blood. After osmotic lysis of the erythrocytes by mixing with 6 volumes of sterile distilled water and 2 volumes of 3,6 % sodium chloride the leucocytes were pelleted by low speed centrifugation in a bench centrifuge. One half of a leucocyte sample, 100 µℓ of each ocular fluid sample (of an initial volume of about 400 µℓ) and the complete nasal mucus samples (recovered from a swab) underwent proteinase K-digestion in the presence of 0,5 % SDS at 37 °C overnight. After phenol extraction and ethanol precipitation the DNA pellets were resuspended in 50–250 µℓ TE-buffer and stored at –20 °C. The rest of each sample was co-cultivated with FLK cells in 25 mℓ plastic flasks in the presence of fungizone (20 mg/ℓ). Control samples were processed for DNA-DNA hybridization alone.

Urine samples were centrifuged at 3 000 rpm for 10 min to separate sediment and supernatant. The supernatant was removed and concentrated by centrifugation at 25 000 rpm for 75 min. This supernatant was discarded and the pellet treated with 100–500 µℓ of 2 M NaCl/0,5 M NaOH, vortexed briefly, and allowed to stand for 15 min (Chou & Merigan 1983). An equal volume of 3 M NaAc (pH 5,5) was added to restore neutral pH.

Southern blot and slot blot hybridization

Southern blots were carried out as described by Southern (1975) on nitrocellulose membranes (Boehringer Mannheim). For dot blot hybridizations 1 µℓ aliquots of heat-denatured DNA were spotted on a nylon membrane and baked at 80 °C for 1 h prior to hybridization. For slot blot hybridizations DNA-aliquots of 50 µℓ were denatured with 25 µℓ 1M NaOH, additionally boiled for 5 min and immediately chilled on ice for 5 min, according to Diaz-Mitoma, Preiksaitis, Leung & Tyrell (1987). An equal volume of 2 M NH4Ac (pH 7) was added to each sample directly before application to a 0,45 µm nylon membrane via alternate slots of a 24-slot manifold apparatus. Each filter contained 100 ng of WC 11 as well as 150 ng of bovine control DNA. The filter membranes were prehybridized and hybridized according to the manufacturer's instructions.

Virus isolation

When the cell cultures co-cultivated with the different field specimens showed signs of degeneration, they were rinsed with Eagle's medium and supplemented with uninfected FLK cells. On a few occasions the cultures were passaged with or without the addition of new FLK cells.

RESULTS

Characterization of the SW 2/SW 15 combined DNA probe

Initial characterization of the probe confirmed hybridization of undigested DIG-labelled WC 11 DNA with SW 2 and SW 15. Then both WC 11 specific DNA fragments were DIG-labelled and hybridized to a Southern blotted Sma I digest of WC 11 DNA. The probes hybridized specifically to the 3,0 kb fragment of WC 11 (SW 2) and to the 2,0 kb fragment (SW 15). Furthermore, both probes proved to hybridize specifically to various amounts of WC 11 DNA and MCF field isolate Skukuza, but not to the same amount of uninfected bovine DNA, pUC 18 DNA or DNA extracted from IBR, BHV 2 or BHV 4 (Fig. 1). From the same dot blot their sensitivity was determined to be at least 1 ng of WC 11 DNA. In the next step the specificity of the probes was examined for field samples. DNA, extracted from the leucocytes of 40 blood samples, 12 ocular fluid samples and 12 nasal mucus samples collected from a dairy herd free of recorded cases of MCF, was tested for AHV-1 by slot blot hybridization. As shown in Fig. 2 no hybridization signal was observed with the negative control samples.

Slot blot hybridization for the detection of MCF virus DNA in field specimens

DNA was extracted directly from ocular fluid, leucocyte and nasal mucus samples of 10 blue wildebeest *(Connochaetes taurinus)* as well as from their different generations of cell culture passages. It was

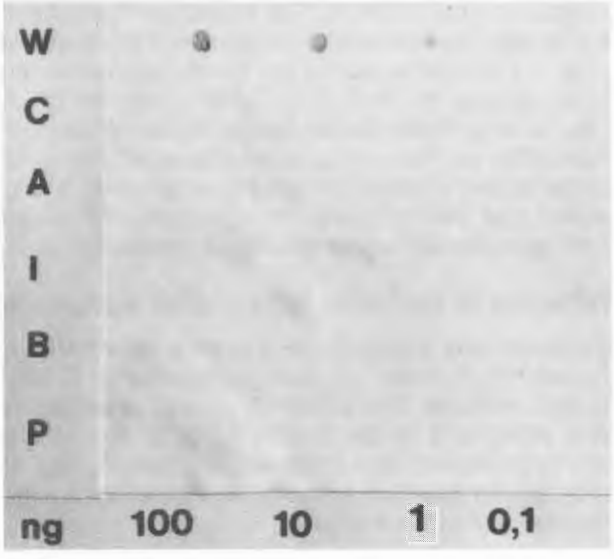

FIG. 1 Dot blot hybridization of an equimolar mixture of the digoxigenin labelled SW 2 and SW 15 probes with seriaten-fold dilutions of W: WC 11 DNA, C: DNA extracted from fetal lamb kidney cells, A: BHV 2 DNA, I: BHV 1 DNA and B: BHV 4 DNA, P: pUC 18 DNA

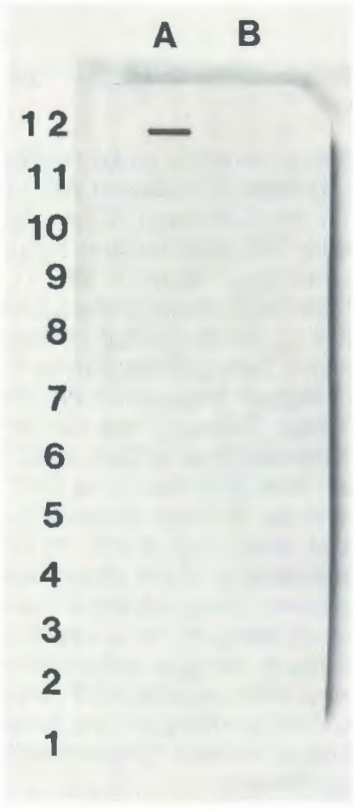

FIG. 2 Slot blot hybridizations of an equimolar mixture of the SW 2 and SW 15 probes with DNA extracted from the leucocytes of control blood samples A1–A10, control nasal mucus samples B1–B6 and control ocular fluid samples B7–B12. A11: 150 ng FLK DNA. A12: 100 ng WC 11 DNA

shown in slot blot hybridizations that 9 of the 10 blue wildebeest calves tested positive in at least 1 specimen type (Table 1). The intensity of the hybridization signals indicated a variation in the amount of AHV-1 DNA harboured in the leucocytes of the different calves (calf no. 6 in Table 1 and no. B4 in Fig. 3). In general, a stronger hybridization reaction was obtained from cell culture passaged than from unpassaged material of the same sample. It was found that the test was not influenced by fungal contaminations present in several samples.

Detection of MCF viral DNA in urine specimens

Sediment and supernatant of urine samples from 3 calves (no. 8, 9 and 10) were processed for hybridization with the SW 2/SW 15 probe. Supernatant and pellet of 2 of the 3 urine samples (no. 9 and 10) hybridized to the DNA probe (Table 1, Fig. 4) with supernatant of calf no. 9 exhibiting a very strong hybridization signal.

Virus isolation

Cell cultures inoculated with part of each specimen, as mentioned above, were passaged and constantly monitored for development of cytopathogenic effects

FIG. 3 Slot blot hybridization of an equimolar mixture of the SW 2 and SW 15 probes with DNA extracted from leucocytes (l) and ocular fluid (o) of blue wildebeest calves. A1: calf 1-l, A2 calf 4-l, A3 calf 8-l, A4 calf 7-l, A5 calf 9-l, A6 calf 2-l, A7 calf 10-l, A8 calf 2-o, A9 calf 8-o, A10: TE-buffer, A11: 150 ng fetal lamb kidney DNA, A12: 100 ng WC 11 DNA, B1 calf 4-o, B2 calf 1-o, B3 calf 9-o, B4 calf 6-l, B7 calf 5-l

for a period of 3 weeks. Due to fungal contamination cytopathic changes indicative of virus replication in cell cultures were inconclusive for all specimens except for calf no. 9. Single foci of rounded cells, typical for AHV-1, were observed.

DISCUSSION

Molecular hybridization provides a valuable alternative to conventional diagnosis of MCF, as it does not depend on infective virus particles, cell-free virus or antisera (Plowright 1986). Instead, this process allows the rapid and specific detection of viral DNA in even crude field specimens.

The specificity of the combined SW 2/SW 15 probe in field specimens was confirmed by a hybridization experiment including DNA from 40 control bovine

Table 1 Slot blot hybridization of specimens including leucocytes, ocular fluid nasal mucus and urine collected from blue wildebeest (Connochaetes taurinus) calves with the SW 2/SW 15 probe. Leucocyte and ocular fluid specimens were hybridized directly as well as after different numbers of passages on FLK cell cultures

Calf no.	Specimen type	Cell culture passage level 0	1	2	3	4
1	Leucocytes	+	+	++	+	*
	Ocular fluid	-	+	+/-	nd	-
	Nasal mucus	nd	*	*	*	*
2	Leucocytes	+	+/-	+	*	*
	Ocular fluid	-	+	+/-	*	*
	Nasal mucus	nd	-	-	-	*
3	Leucocytes	+	+/-	-	*	*
	Ocular fluid	-	++	+	*	*
	Nasal mucus	nd	+	-	*	*
4	Leucocytes	+	++	+	*	*
	Ocular fluid	-	++	+	+	+
	Nasal mucus	nd	+/-	*	*	*
5	Leucocytes	-	-	*	*	*
	Ocular fluid	-	+/-	+/-	*	*
	Nasal mucus	nd	-	+/-	+/-	*
6	Leucocytes	++	+++	*	*	*
	Ocular fluid	-	-	+	+	+
	Nasal mucus	nd	+	+	+	*
7	Leucocytes	+/-	+	+	+	*
	Ocular fluid	-	-	-	*	*
	Nasal mucus	nd	+	-	-	*
8	Leucocytes	+	+/-	+	*	*
	Ocular fluid	-	-	*	*	*
	Nasal mucus	nd	+	*	*	*
	Urine	-	nd	*	*	*
9	Leucocytes	+	+	+	*	*
	Ocular fluid	+/-	+	++	*	*
	Nasal mucus	nd	*	*	*	*
	Urine	+++	nd	*	*	*
10	Leucocytes	-	+	+	*	*
	Ocular fluid	-	+	+	*	*
	Nasal mucus	nd	*	*	*	*
	Urine	+	nd	*	*	*

0 – original sample
+ – positive
++, +++ – positive reaction above average
* – not available due to death of cell culture
- – negative
+/- – suspicious
nd – not done

blood samples and 24 control bovine nasal mucus and ocular fluid samples. The combined SW 2/SW 15 probe did not react with any of the control samples, nor could any cross-reaction with other bovine herpesviruses be observed (Fig. 1). This coincides with the finding (Seal, Klieforth & Heuschele 1987), that the Alcelaphine herpesviruses are genetically distinct from other bovine herpesviruses.

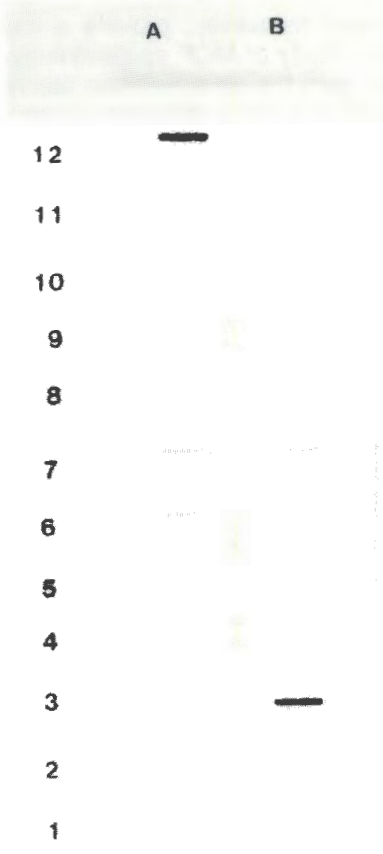

FIG. 4 Slot blot hybridization of DNA extracted from urine specimens of 3 blue wildebeest calves with an equimolar mixture of the SW 2 and SW 15 probes. A1–A5: control bovine urine pellet samples, A6: urine supernatant calf 10, A7: urine pellet calf 9, A8–A10: empty, A11: 150 ng fetal lamb kidney DNA, A12: 100 ng WC 11 DNA, B1: urine supernatant calf 8, B2 control bovine urine pellet, B3: urine supernatant calf 9, B4-5: control bovine urine pellets, B6: urine pellet calf 8 and B7 urine pellet calf 10

It also coincides with the findings from previous AHV-1 specific DNA probes constructed by other investigators (Seal *et al.* 1990; Shih *et al.* 1988). Although these previous AHV-1 specific probes were developed for molecular studies as well as for diagnostic purposes, the investigations described in this paper represent the first results for an epidemiological survey of MCF based on the hybridization technique. Ten blue wildebeest calves (*Connochaetes taurinus*), aged about 3 months, were screened for evidence of their role as carriers (Castro *et al.* 1982; Plowright *et al.* 1960) and excretors (Barnard *et al.* 1989a; Rweyemamu *et al.* 1974) of AHV-1. Various samples of each calf were hybridized with the SW 2/SW 15 probe and in all cases at least 1 sample of each calf was found positive. This suggests the percentage of MCF-carrying wildebeest calves to be considerably higher than described previously (Barnard *et al.* 1989a; Mushi, Karstad & Jesset 1980; Plowright *et al.* 1960). This might indicate that the DNA probes, in spite of their

rather limited sensitivity, provide a more suitable tool for the study of MCF epidemiology under field conditions than the virus isolation technique (Plowright *et al.* 1960). Virus isolation results were inconclusive mainly due to contamination and poor growth of cell cultures. The presence of AHV-1 DNA in the urine of 2 blue wildebeest calves is the first indication that AHV-1 can be excreted in the urine. Virus excretion via urine is, however, known for cytomegaloviruses (cmv) where detection of cmv DNA extracted from urine samples is accomplished with very high specificity (Chou & Merigan 1983). The intensity of the hybridization reaction indicates a significant amount of virus DNA present in the urine sample of calf no. 9. This might be an indication for thus far unrecognized routes of virus dissemination.

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