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Monitoring experimental Alcelaphine Herpesvirus-1 infection in cattle by nucleic-acid hybridization and PCR

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ABSTRACT

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The DNA probe SW15 derived from the laboratory-attenuated Alcelaphine Herpesvirus-1 (AHV-1) strain WC11 as well as from the polymerase chain-reaction test (Hsu, Shih, Castro & Zee 1990), was used to detect viral DNA of malignant catarrhal fever (MCF) in six experimentally infected cattle. Heparinized blood samples were collected and tested at least three times a week over a period of up to 142 d. Results of hybridization and PCR tests were compared with the results of clinical examinations, and on various occasions with those of viral isolation and serum-neutralization assays as well as with those of pathology.

Three animals developed clinical signs and lesions typical of MCF, while the other three animals remained clinically healthy. All cattle seroconverted, and viral nucleic acid was detected by DNA hybridization and PCR at various intervals during the observation period. Virus isolation was successful in two of the clinical cases and all cattle seroconverted. Storage of blood samples at 4 °C for up to 10 d did not influence the hybridization and DNA-amplification results.

Keywords: Alcelaphine, Herpesvirus-I, cattle, nucleic-acid hybridization, PCR, malignant catarrhal fever, MCF

INTRODUCTION

Malignant catarrhal fever (MCF) is a sporadic, but almost invariably fatal, disease of cattle and many other species of wild ruminants such as deer and buffalo. It is characterized in cattle by high fever, mucopurulent nasal and ocular discharges with corneal opacity, generalized lymphadenopathy and, in most cases, severe inflammation and degenerative lesions in the mucosa of the upper respiratory tract and alimentary tract (Barnard, Van der Lugt & Mushi 1994; Plowright 1964; Reid & Buxton 1984; Metzler & Burry 1990). Malignant catarrhal fever occurs in one of two forms, namely the wildebeest-derived form caused by Alcelaphine Herpesvirus type 1 (AHV-1) (Plowright, Ferris & Scott 1960), and the sheep-associated form which is now known to result from infection with Ovine Herpesvirus type 2 (OHV-2) (Schuller & Silber 1990; International Committee on Taxonomy of Viruses: Herpesvirus study group 1992).

A presumptive diagnosis of MCF can be made on the herd history, clinical signs and pathology. Virus isolation is not practical, owing to the instability of the cell-associated AHV-1 and the fact that OHV-2 does not replicate in cell culture. Serology is of limited value owing to a lack of specificity. Only a small percentage of infected animals show seroconversion late in the course of the disease, and possible serological

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cross-reaction between herpesviruses and the generally low antibody titres makes serological interpretation difficult (Rossiter, Mushi & Plowright 1977). DNA probes were developed from various AHV-1 strains (Shih, Irving, Zee & Pritchett 1988; Seal, Klieforth & Heuschele 1990; Bridgen, Munro & Reid 1992; Michel 1993) to achieve a reliable and rapid diagnosis, to detect latently infected animals, and to investigate the epidemiology and pathogenesis of the disease. Methods involving the polymerase chain reaction have also been developed (Hsu, Shih, Castro & Zee 1990; Katz, Seal & Ridpath 1991; Tham & Young 1994).

In the present study, a non-radioactively labelled DNA probe, SW15 (Michel 1993) and the PCR method (Hsu *et al.* 1990) were evaluated and compared as diagnostic aids in cattle experimentally infected with AHV-1.

MATERIAL AND METHODS

Viruses, virus propagation and viral-DNA extraction

Genomic DNA of AHV-1 strains was prepared according to the method described by Michel (1993). The Rinderpest strain Kabete-O was propagated on Vero cells. Bovine foetal muscle cells were used to cultivate a field isolate of Bovine Viral Diarrhoea/Mucosal Disease (BVD/MD) virus. Following proteinase-K digestion and precipitation of proteins with saturated sodium chloride, viral nucleic acids were precipitated with three volumes of absolute ethanol. Foot and Mouth Disease (FMD) viral RNA was kindly provided by Dr N.T. van der Walt, Foot and Mouth Disease Laboratory, Onderstepoort.

Probe preparation

An approximately 2-kb-SmaI fragment of the WC11 genome which had been cloned into the vector pUC18 (Michel 1993) was gel-purified twice on 1% agarose gels as described in Sambrook, Fritsch & Maniatis (1989). Probe DNA was labelled with horseradish peroxidase and the ECL-labelling-and-detection kit was used according to the manufacturer's instruction (Amersham). Briefly, SW15 DNA (10 ng/µℓ) was boiled for 5 min and immediately chilled on ice for 5 min. Equivalent volumes of DNA-labelling reagent and, after mixing, of glutaraldehyde solution, were added. The labelling mixture was incubated at 37°C for 10 min and stored on ice until used.

Clinical procedures

Daily, rectal temperatures were recorded and the animals were examined for clinical signs of disease such as lymph-node enlargement, depression, photophobia, lacrimation, scleral congestion, corneal opacity, nasal exudate, crusted muzzle, hyperaemia of mucous membranes, necrosis of buccal papillae, salivation and diarrhoea.

The onset of the clinical stage was defined by an increase in the body temperature above 40 °C. Case 1 was euthanased by an overdose of pentobarbitone sodium 7 d after onset of the clinical stage [day 49 post infection (p.i.)], case 2 after 5 d (day 29 p.i.) and case 3 after 4 d (day 45 p.i.). Cases 4–6 failed to develop clinical signs and were reinfected with the virus on day 87 p.i. at a dose of 1,29 x 109 PFU. They were given prednisolone (Prednivet, Truka-Panvet) (1mg/kg/d) intramuscularly and dexamethasone (Dexafort, Intervet SA) (1mg/kg/d) intravenously from day 132 p.i. until day 139 p.i. in an attempt to effect viral recrudescence and/or to induce clinical disease. Cases 4–6 were euthanased on day 142 (p.i.).

Samples and buffy-coat preparation

Heparinized blood (4 x 10 mℓ) for buffy-coat (BC) cell preparations was initially collected three times a week and after the onset of fever, daily, from each infected animal. The control cow was sampled three times a week over a period of 90 d. Following centrifugation for 20 min in a bench-top centrifuge at 1500 rpm, buffy coats were aspirated and washed twice with 4,2 mℓ of dd H_2O to which NaCl in a final concentration of 0,8% was added. The washed BC pellets were digested overnight at 37 °C in 2 mℓ of lysis buffer (10 mM Tris pH 7,5, 400 mM NaCl, 2 mM EDTA) containing 0,6% SDS and 62,5 µg of proteinase K. Proteins were precipitated with 0,5 mℓ of a saturated NaCl solution followed by low-speed centrifugation. DNA was precipitated from the supernatant with at least two volumes of ethanol and resuspended in TE-buffer.

Slot-blot hybridization

Nucleic-acid samples from purified viruses, tissue culture or blood samples were blotted on Hybond N+ membrane and fixed by ultraviolet light cross-linking (3 min). Following 1 h of prehybridization, the membrane was incubated overnight in ECL-hybridization solution containing 10 ng/mℓ of labelled SW15 probe. Post-hybridization washes were carried out under high-stringency conditions (0,1% SDS, 0,1 x SSC) at 62°C. Final detection of hybridization signals revealed AHV-1-positive samples after exposure of the membrane to ECL Hyperfilm for 30 min.

DNA amplification by PCR

Five microlitre from each BC-DNA sample was added to 45 µℓ of amplification reaction containing 10 mM Tris-HCl (pH 9,0), 50 mM KCl, 1,5 mM MgCl2, 1% Triton X-100, 1 mM dNTPs, 20 pmol of each of the two primers A and B (Hsu *et al.* 1990) and 1,5 U of Taq-DNA polymerase (Promega). The reactions were overlaid with 40 µℓ of mineral oil. Positive and negative control DNAs were included in each amplification experiment. After an initial denaturation step at 97°C for 3 min the Taq-DNA polymerase was

added as soon as the reaction mixture cooled down to 62°C. Then the temperature of the reaction mixture was raised to 72°C for 1 min. After 33 cycles of 93°C for 40 s, 62°C for 30 s and 72°C for 1 min, a final extension step of 5 min at 72 °C completed the amplification.

Generation of an internal DNA probe by PCR

The target DNA for the second amplification was a 608-bp internal sequence of the amplification product described by *Hsu et al.* (1990). The primers in the reaction mixture described above were replaced by the primers MC1 and MC2, and the dNTP mixture consisted of 17,5 µM DIG-11-dUTP (Boehringer Mannheim), 32,5 µM dTTP and 50 µM dGTP, dCTP and dATP each. The labelling efficiency of the DIG-labelled probe was determined by titration and direct detection.

Detection of amplification products

A 25 µℓ aliquot of each sample was blotted on a Hybond Nylon membrane and hybridized with 30 ng/m of the DIG-labelled, internal DNA probe. Following post-hybridization washes at conditions of high stringency (0,1 x SSC, 68°C), samples containing the specific amplification product could be identified by chemiluminescent detection (Boehringer Mannheim).

Virus-isolation and serum-neutralization test

Heparinized blood samples for virus isolation and serum samples for the neutralization test were collected from cases 1–3 on the first day of onset of the clinical phase (days 42, 24 and 41 p.i., respectively), from case 4 on day 78 p.i., and from cases 5 and 6 on day 142 p.i. For virus isolation, buffy-coat cells were cocultivated with primary cultures of lamb foetal kidney cells (generation < 10) and passaged at least four times. The neutralization test was performed on primary cultures of calf foetal thyroid (CFTh) cells with WC11 as antigen.

Pathology

A necropsy was performed on each animal and tissue specimens for light microscopy were collected in 10 % neutral buffered formalin. The eyes from each animal were fixed in Zenker's solution. Tissue blocks were processed routinely, embedded in paraffin wax, and sections were stained with haematoxylin and eosin (HE).

RESULTS

Animals and animal inoculation

Six male cattle (one bull and five oxen; cases 1–6) of different breeds, from an isolated breeding herd of the Onderstepoort Veterinary Institute (OVI), were used. The herd had had no contact with sheep or wildebeest. The animals were infected with a virulent AHV-1 isolate ("Skukuza") at a dose of 3,15 x 108 plaque-forming units (PFU). All cattle were kept in a paddock during the experiment, except case 1 which was initially kept in an isolation stable and was moved to the paddock on day 41 p.i. One non-infected control cow was kept separately and sampled twice a week.

Clinical responses of cattle to AHV-1

Three cattle (cases 1–3) developed clinical signs of MCF. The incubation period varied between 23 and 41 d (average 35 d). Nasal and eye discharges were initially serous and mild, and later became mucopurulent and purulent. The eyes showed progressive centripetal opacity. The oral mucosa were hyperaemic and oedematous with increased salivation. Oral congestion accompanied superficial necrosis of buccal papillae. In case 1 moderate submandibular oedema and an exudative dermatitis on the prepuce and interdigital space was seen. Terminal diarrhoea was observed in case 1. Signs in cases 4–6 were limited to mild inflammation of the upper respiratory tract.

Slot-blot hybridization and PCR

Control DNAs

The SW15 probe did not hybridize with nucleic acids from BVD/MD, FMD or Rinderpest viruses (data not shown). As previously shown, the SW15 probe did not hybridize with DNA from the bovine herpesviruses 1, 2 and 4, the plasmid vector pUC 18 or uninfected cattle (Michel 1993). The sensitivity of the SW15 probe determined in a dilution series of homologous SW15 and WC11 DNA was 0,1 pg and 10 pg, respectively (data not shown).

Generation of an internal DNA probe by PCR

In order to generate a DNA probe for the detection of the amplification product of primers A and B, an internal pair of primers was designed, based on the nucleic-acid sequence of the cloned fragment of Hsu *et al.* (1990). Primer MC1 is located at position 1730 to 1755:

5' GATAGCACTCATGTCTGTGAATGTGG 3'

and primer MC2 is located at position 2312 to 2337:

5' GTGTAGTTATTGCTTGTGCTCCCGTA 3'

of the non-coding DNA strand.

After amplification had been completed, a prominent DNA fragment was detected by agarose-gel electrophoresis which migrated according to its predicted size of 608 bp (Fig.1). It hybridized with the amplification product produced by the primers A and B and the XbaI restriction fragments (Hsu *et al.* 1990) (data not shown).

Buffy-coat DNA samples

Heparinized blood samples were collected from six experimentally infected cattle and one control animal before and during the period of clinical disease, and DNA was extracted from the buffy coats. The earliest time of virus detection in white-blood-cell-DNA was determined by conventional DNA-DNA hybridization with the SW15 probe and the PCR method in combination with hybridization (Fig. 2). Most of the buffy-coat DNA samples were tested with both assays, while all samples were tested by hybridization with the SW15 probe. The results of the PCR and hybridization assays are summarized in Table 1. The earliest time of AHV-1 DNA detection by both methods was 2 d p.i. in case 1, 3 d p.i. in case 3, 6 d p.i. in case 4 and 8 d p.i. in case 6. PCR delivered the earliest positive results in cases 2 and 5 after 5 d p.i. and 8 d p.i., respectively, compared to 8 d p.i. and 31 d p.i. by conventional probe hybridization, respectively. The number of positive results per animal was significantly higher when tested by PCR than it was by hybridization alone, although intermittent positive and negative results from cases 4–6 were obtained by both assays. With only two exceptions, all samples from cases 1–3 were positive by PCR, while the hybridization results were consistently positive only in case 1. DNA samples collected from the control cow were found to be negative by both tests throughout the observation period.

To evaluate the effect of time on the detection of virus in the blood samples, one of the four 10 mℓ blood samples from each animal was digested and DNA was extracted directly after collection. A second sample was digested 2–3 d later, a third 4–5 d and a fourth 9–10 d later. Neither hybridization nor PCR results of the four blood samples from each animal differed when correlated with the time elapsed from sample collection to sample processing.

The corticosteroid treatment which was carried out over a period of 8 d in an attempt to enhance viremia or induce clinical MCF had no effect on either aspect.

Serology and virus isolation

The results of the serum-neutralization test and the virus isolation are summarized in Table 2. Significant antibody titres were found in cases 1–3 at the time of onset of fever, while detectable antibody titres in cases 4–6 were observed only 142 d p.i. Virus isolation was successful in cases 1 and 3.

Pathology

Although varying in degree, gross lesions compatible with MCF were present in cases 1–3. Lesions in the other animals comprised mild swelling of the lymph nodes of the head in cases 4 and 5, a segmental catarrhal enteritis in cases 4–6, and mild oedema of the lungs in cases 5 and 6. Typical microscopical changes of MCF, characterized by lymphoid hyperplasia and vascular and epithelial lesions associated with the infiltration of predominantly lymphoid cells (Barnard *et al.* 1994), were present in cases 1–3. No histological changes indicative of MCF were evident in cases 4–6. In these cases, lesions in the small and large intestines were characterized by the infiltration of moderate numbers of plasma cells and lymphocytes in the lamina propria, and also by fusion and loss of villi, and congestion. In several areas of the large intestine in case 4, crypts which were occasionally dilated and filled with inflammatory cells, herniated into underlying submucosal lymphoid follicles. The affected lymph nodes in cases 4 and 5 showed mild lymphoid hyperplasia. A mild subacute interstitial pneumonia was present in cases 5 and 6.

DISCUSSION

The mean incubation period for MCF observed in this experiment was 35 d (24–42 d), which correlates with the known variability in incubation periods after experimental infection of 11–34 d, 20–36 d and 13 d (Plowright 1964; 1968; Selman, Wiseman, Wright & Murray 1978; Pierson, Hamdy, Dardiri, Ferris & Schloer 1979). Of six infected animals, three developed clinical MCF (cases 1–3) (Table 1). Buffy-coat specimens of case 1 were found to be positive by conventional probe hybridization and PCR/hybridization from day 2 p.i. until the animal was euthanased 7 d after the onset of clinical signs. These PCR results were similar to those obtained by Katz *et al.* (1991) in the detection of AHV-1 in bovine leucocyte specimens. The majority of samples collected from cases 2 and 3 were found to be positive by PCR, but negative by conventional probe hybridization. These findings demonstrate the PCR approach to be more sensitive than the DNA-hybridization assay alone. Three animals (cases 4–6) did not develop clinical signs of MCF but showed intermittent positive and negative results by both assays. The PCR detection was again found to reveal more virus-positive samples than DNA hybridization does (Table 1). Persistent infection with OHV-2 after recovery of clinical MCF was confirmed recently(Michel & Aspeling 1994). Like in other herpesvirus infections which are known for their ability to establish latent, life-long infections (Epstein & Achong 1979, Bagust 1986, Weller 1971, Armstrong, Orr, Ablashi, Pearson, Rabin, Luetzeler, Loeb & Valerion 1976), a certain percentage of cattle in a population might become latently infected with AHV-1 following contact with a reservoir host. Such apparently healthy animals might then remain clinically normal or develop acute disease, possibly depending on internal or external "trigger mechanisms". Stress has been suggested as being a "trigger mechanism" in inducing MCF outbreaks in deer in New Zealand (Audige 1992). How-

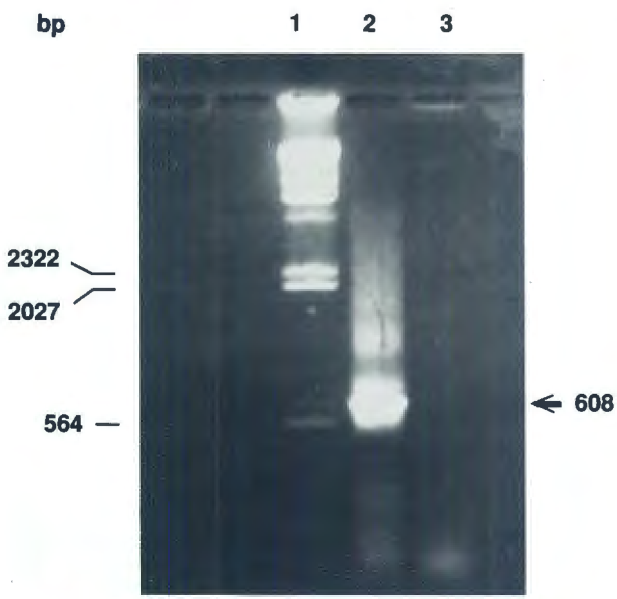

FIG. 1 Analysis of PCR-amplification reactions by agarose-gel electrophoresis

Lane 1: Lamba-DNA, HindIII digested
Lane 2: AHV-1-specific amplification product from a leucocyte lysate of a known positive case of bovine MCF with the use of the oligonucleotide primers MC1 and MC2
Lane 3: Control leucocyte lysate amplified with oligonucleotide primers MC1 and MC2

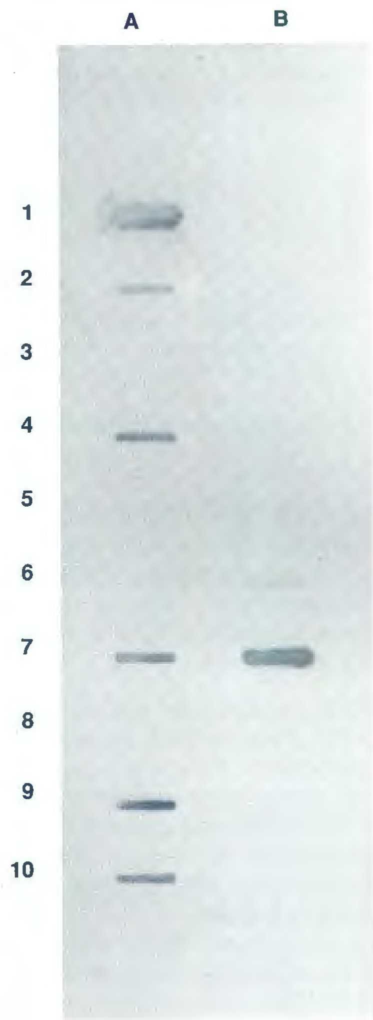

FIG. 2 Slot-blot hybridization of PCR-amplified leukocyte samples from cattle experimentally infected with AHV-1

1A = positive control (100 ng WC11 DNA); 1B = negative control (100 ng bovine DNA). Positive leucocyte samples 2A, 4A, 7A, 9A, 10A, 6B, 7B. Negative leucocyte samples 3A, 5A, 6A, 8A, 8B, 9B, 10B. 1B–5B: no samples applied

TABLE 1 Examination of blood-leucocyte samples of six cattle experimentally infected with AHV-1 with PCR and DNA probe hybridization

Case no.		Test occasions in days p.i. (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 142)
1	a	+ + + +++ +
	b	− + ++++ +++++ ++ + + +++ −
2	a	−++++ +++++++
	b	−− −−++− −−−++−−
3	a	− + + + −− + + ++++
	b	−− + +−− −−−−−−−−−− −++++
4	a	− −+−+ −−++ − + + + + − − − + − + + −++− − + −
	b	− −+−+− −−+−+−−++− −− −+ + −++− −−− ++−+−−−−−−−−++−++−−−−+−−−−−−−−−
5	a	− − + +− +−+ + + + − + + + − + + +−++− + − −+ + −−+
	b	−−−−−−−− −−−−−−−++++ − +− + ++++ +++ ++++−++−−−+−−−++−+−+−−+−−−−−−−−−
6	a	− − ++ + − + − + + + + −+−+− +−− + + + −
	b	−−−− +++ +++++−−−++− −−− + ++++ +−− +−−+−−−+−+++−−−−−−++−−−− −−−−−−

[a] = results obtained by PCR amplification
[b] = results obtained by DNA-DNA hybridization
\+ = positive hybridization/PCR result
– = negative hybridization/PCR result
p.i. = post infection

TABLE 2 Serology and virus isolation of 6 cattle experimentally infected with AHV–1

Case No.	Day of onset of clinical disease[a]		Day 77 p.i.[b]		Day 142 p.i	
	SNT[c]	VI[d]	SNT	VI	SNT	VI
1	1:> 256	+ (2p)[e]				
2	1:512	– (4p)				
3	1:256	+ (3p)				
4			1:32	– (6p)	1:256	– (5p)
5			–	– (6p)	1:64	– (5p)
6			–	– (6p)	1:64	– (6p)

[a] Onset of clinical disease of cases 1–3 were days 42, 24 and 4 p.i., respectively
[b] p.i = post infection
[c] SNT = serum-neutralization test, given as positive (+) or negative (–) A titre of 1:8 was considered as negative
[d] VI = virus isolation
[e] 2p, 3p, 4p, 5p and 6p denotes the number of passages done

ever, the role of immune suppression in the development of MCF is controversial (Heuschele, Nielden, Oosterhuis & Castro 1985; Milne & Reid 1990). In this experiment, the corticosteroid treatment failed to induce clinical MCF and had no effect on the detection of AHV-1 DNA.

PCR provides an alternative and more sensitive method for the early detection of AHV-1 in buffy-coat specimens. The data presented here indicates the recurrence of periods when no detectable amount of AHV-1 DNA was present in the blood leucocytes of infected cattle. Similar conditions were observed in studies involving persistent Epstein-Barr-virus infections in humans (Yao, Rickinson & Epstein 1985). In these cases the pharynx was found to serve as a site of latency where chronic low-grade replication of EBV provides reinfection of B cells. Sites of latency have also been demonstrated for a number of other herpesvirus infections (Williams, Bennett, Bradbury, Gaskell, Jones & Jordan 1992; Edington, Welch & Griffiths; 1994, Gutekunst, Pirtle, Miller & Stewart 1980). Whether the AHV-1, as a member of the subfamily, Gammaherpesvirinae, causes a persistent infection of peripheral blood leucocytes as a primary or secondary phenomenon, still needs to be investigated.

To prevent false negative results during such intervals, it is recommended that two blood samples collected at different test occasions, be tested.

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