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DIAGNOSIS OF MALIGNANT CATARRHAL FEVER BY POLYMERASE CHAIN REACTION AMPLIFICATION OF ALCELAPHINE HERPESVIRUS 1 SEQUENCE

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ABSTRACT: We derived sequence information from cloned *Hin*dIII fragment "D" of alcelaphine herpesvirus 1 strain WC11, an agent of malignant catarrhal fever (MCF). Based on this sequence, oligonucleotide primers were selected and synthesized for use in a polymerase chain reaction amplification assay. These primers were used to test samples of total nucleic acids isolated from multiple tissues taken from an Indian gaur (*Bos gaurus gaurus*) at the San Diego Wild Animal Park in San Diego, California (USA) which had clinical signs of a natural infection of MCF. Six of eight tissue samples examined had amplifiable sequences present. A nucleic acid probe complementary to the sequence of the original clone between the primer sites also was synthesized and used to confirm the identity of the amplified viral sequences, thus providing a diagnosis of MCF at the molecular level.

Key words: Alcelaphine herpesvirus, polymerase chain reaction, Indian gaur, malignant catarrhal fever.

INTRODUCTION

Malignant catarrhal fever (MCF) is a disease of high mortality in cattle and wild ruminants, with a worldwide distribution (Heuschele and Castro, 1985). A herpesvirus capable of inducing MCF (now designated alcelaphine herpesvirus 1, or AHV-1) first was isolated in Africa from a blue wildebeest (*Connochaetes taurinus*) (Plowright et al., 1960). Since that time, U.S. zoological parks including the San Diego Zoo and Wild Animal Park, San Diego, California (USA) and the Oklahoma City Zoo, Oklahoma City, Oklahoma (USA) have reported numerous losses of exotic species due to MCF (Heuschele, 1982, 1988). While the mode of MCF transmission is not precisely understood, bovids and other ruminants may contract the virus from either carrier alcelaphine antelopes such as wildebeest or possibly from wild or domestic sheep and goats (Heuschele and Castro, 1985). A reliable, rapid and specific detection assay for the viral agents of MCF would enable the monitoring of bovid and sheep populations and thereby control the spread of this disease. Polymerase chain reaction (PCR) technology (Saiki et al., 1988) has proved useful in amplifying nucleic acid sequences present in low copy number. This diagnostic method could meet the needs of scientists interested in monitoring the prevalence of MCF agents.

Hsu et al. (1990) developed a PCR assay based on primers obtained from a 5.4 kilobase-pair (kbp) *Bam*HI fragment of AHV-1 strain WC11; primers were tested against purified AHV-1 deoxyribonucleic acid (DNA) and DNA from AHV-1-infected and uninfected cells, but not against DNA isolated from bovine or alcelaphine herpesviruses other than AHV-1 isolate WC11.

Seal et al. (1990) cloned *Hin*dIII fragments of AHV-1 strain WC11 at the Center for Reproduction of Endangered Species (C.R.E.S.). We then derived a PCR primer pair from one of these clones, designated B25, representing *Hin*dIII fragment "D." The primers were tested against the isolate of origin and infected versus uninfected cell DNA as well as DNA of other herpesviruses. However, the establishment of relevance to clinical MCF re-

quired that we test the primers against tissue samples taken from a naturally infected animal under controlled conditions.

Using the same clone (B25) obtained from C.R.E.S., Katz et al. (1991) developed nested sets of primers. These primers were used to analyze lymphocyte DNA from blood samples of domestic cattle which had been intravenously injected with the AHV-1 virus. However, they did not describe use of PCR to confirm MCF in a naturally infected animal.

On 6 February 1992, an adult female Indian gaur (*Bos gaurus gaurus*) with no previous medical history, kept in a large multi-species enclosure at the Wild Animal Park (Escondido, California, USA; 33°6′N, 117°4′W), was observed with classic head tremors. The next day the animal was recumbent, and was examined. The animal had numerous oral abrasions or ulcers, had noticeable fibrin in the anterior chamber of both eyes and was salivating profusely. Based on the described clinical signs, the acute onset, the severity of illness and the species involved, MCF was suggested as a possible diagnosis. Our objective was to confirm this diagnosis by applying the PCR assay to total nucleic acids extracted from tissue samples acquired at necropsy.

MATERIALS AND METHODS

The viral isolates used in this study were AHV-1 isolates WC11 (Plowright et al., 1960) and C500 (Plowright et al., 1975), alcelaphine herpesvirus 2 (AHV-2) isolate 840412 (Seal et al., 1989), the Minnesota sheep-associated isolate (Hamdy et al., 1978), bovine herpesvirus 1 (BHV-1) isolate Los Angeles (Seal et al., 1985), and bovine herpesvirus 2 (BHV-2) (Sterz et al., 1973). Propagation of bovine embryonic lung (BEL) and fetal mouflon sheep kidney (FMSK) cells as well as the preparation of viral DNA were performed as described by Seal et al. (1990).

Tissue samples were removed at necropsy from the MCF-affected animal and stored at −80 C until the nucleic acids were extracted for assay. The eight sampling sites were composed of an ulcerated area on the mucosal surface of the rumen wall (rumen pillar); surface scraping of an ulcerated area on the mucosal side of the small intestine (gut 1); a mesenteric lymph node (lymph node 1); surface scraping of a grossly hyperemic section on the mucosal side of the small intestine (gut 2); an ulcerated area between buccal papillae in the oral cavity (buccal membrane); epithelium from a hyperemic area of the tongue (tongue); eye tissue between the edge of the cornea and the underlying ciliary bands (eye); and a retropharyngeal lymph node (lymph node 2).

For the preparation of total nucleic acids used as assay material, 1 to 2 mm^3 samples from each tissue were separately homogenized in microtissue grinders (VWR Scientific, Los Angeles, California) in 500 µl of water to disrupt the cells. The cell homogenates were transferred to 1.5-ml microfuge tubes (Brinkmann Instruments, Inc., Westbury, New York, USA); the total nucleic acids were released by adding an equal volume of phenol and chloroform (1:1), mixing vigorously for 5 min and centrifuging in a microfuge for 5 min at 12,000 rpm. Total nucleic acids then were precipitated from the aqueous phase by adding sodium acetate (0.3 M final concentration) and two volumes of ice-cold ethanol, and placing the solution at −20 C for ≥60 min. The isolated nucleic acids were centrifuged at 12,000 rpm for 5 min to produce a pellet, then resuspended in 20 µl of tris-EDTA (TE) buffer (10 mM tris, 1 mM ethylenediaminetetraacetic acid pH 7.6; Sigma Chemical Co., St. Louis, Missouri, USA) and frozen until evaluated.

Buffy-coat cells from EDTA-anticoagulated blood were pre-treated by hypotonic lysis of red blood cells (Mishell and Shiigi, 1980). Lymphocyte DNA was extracted from buffy-coat cells for the isolation of high-molecular-weight DNA from mammalian cells by the methods of Maniatis et al. (1982).

Polymerase chain reaction amplification employed the following primer sequences: Primer "A": 5′-ATACATGTCATTTAAGACACCCACGCACCA-3′; Primer "B": 5′-CTGGTGCAGGATGACCACAATTTTACTATC-3′, where A is adenine, T is thymine, C is cytosine, and G is guanine.

The reaction was carried out by adding 1 µg of tissue-derived DNA to 100 µl of PCR amplification mixture containing 10 µl of 10× amplification buffer (500 mM KCl; 100 mM tris, pH 8.3; 15 mM $MgCl_2$; 1 mg/ml gelatin); 20 µl deoxynucleotide triphosphate (dNTP) mix (1.25 mM of each dNTP; Aldrich Chemical Co., Milwaukee, Wisconsin, USA); 1.0 µl of each primer (0.25 µg/µl) and sterile distilled water. The reaction mixtures were heated in a boiling water bath for 3 min, cooled slowly to 50 C in a water bath, and then to 25 C for primer annealing. Two units of *Taq* DNA polymerase (Perkin-Elmer/Cetus, Norwalk, Connecticut, USA) were

AMPLIFIED SEQUENCE OF AHV-1 CLONE B25

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        10 Primer "A"          30          40          50          60
ATACATGTCA TTTAAGACAC CCACGCACCA GTGCACATGG GCCACTATTT TATTGAAGAA
TATGTACAGT AAATTCTGTG GGTGCGTGGT CACGTGTACC CGGTGATAAA ATAACTTCTT

        70          80          90         100         110      PROBE
GTGGCCCCGG TAAAAAGAGT ATTTAAGATT GGCAACAAGG TCGCCTCTTA GGGTGAAGAC
CACCGGGGCC ATTTTTCTCA TAAATTCTAA CCGTTGTTCC AGCGGAGAAT CCCACTTCTG

       130         140         150         160         170         180
AGCTACTAAA AACTAATTTT GTACACTAAT AATCGGGGGG GGGTGGCTGT AGATAGTAAA
TCGATGATTT TTGATTAAAA CATGTGATTA TTAGCCCCCC CCCACCGACA TCTATCATTT

       190         200
ATTGTGGTCA TCCTGCACCA G
TAACACCAGT AGGACGTGGT C
 Primer "B"
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FIGURE 1. Partial nucleotide sequence of clone B25 representing *Hin*dIII fragment "D" of AHV-1 strain WC11. The primer and probe sequences used in the assay are denoted by bold letters.

added to each tube followed by 100 μl of lightweight mineral oil (Sigma Chemical Co.) which prevented evaporation of the sample.

Amplification was performed using a PCR cycling protocol consisting of extension for 5 min at 70 C, thermal denaturation for 30 sec at 94 C, and primer annealing for 1 min at 50 C. This protocol was repeated for 34 cycles and linked to a single extension cycle of 10 min at 72 C. Amplified DNA fragments and molecular size markers (Gibco Life Technologies, Grand Island, New York) were subjected to electrophoresis through a submerged agarose gel containing 2% NuSieve agarose and 1% SeaPlaque agarose (FMC BioProducts, Rockland, Maine, USA). The gel-separated bands were made visible by staining with 1 μg/ml ethidium bromide (Gibco Life Technologies) and photographed under ultraviolet light for a permanent record (Maniatis et al., 1982).

Southern blot hybridization was used to confirm the identity of the DNA bands after PCR amplification. Deoxyribonucleic acid fragments separated by gel electrophoresis were transferred to a nylon membrane and fixed to the membrane using an ultraviolet Stratalinker set at 120,000 μjoules/cm² (Stratagene, Inc., La Jolla, California). The nylon membrane was prehybridized for 30 min at 50 C in 5 ml of hybridization buffer (5× saline sodium citrate [SSC], 1% sodium dodecylsulfate [SDS], 0.5% bovine serum albumin [BSA], where 20× SSC = 175.3 gm NaCl + 88.2 gm sodium citrate in 1,000 ml of H_2O, pH 7.0) (Sigma Chemical Co.). An oligonucleotide probe specific for the region of AHV-1 between the PCR primers [5′-TCGCCTCTTAGGGTGAAGACAGCTACT-3′] was end-labeled using T4 polynucleotide kinase and phosphorus-32-deoxyadenosine triphosphate (^{32}P-dATP; DuPont/NEN, Boston, Massachusetts, USA), then added to the hybridization buffer and allowed to hybridize for 1 hr at 50 C. The membrane was washed successively in 1× SSC plus 1% SDS for 5 min at 50 C, in 1× SSC plus 1% Triton X-100 (Sigma Chemical Co.) for 5 min at 50 C, and finally in 1× SSC for 5 min at 25 C. The membrane was air-dried and exposed to X-ray film (Maniatis et al., 1982).

In order to obtain sequence data for the AHV-1 genome, we sequenced a region of DNA from AHV-1 strain WC11 that was contained in a plasmid designated B25. Sequencing of clone B25 used in this study was carried out according to the Sanger dideoxy chain termination method (Sanger et al., 1977). Enough sequence information was derived from one terminus of the

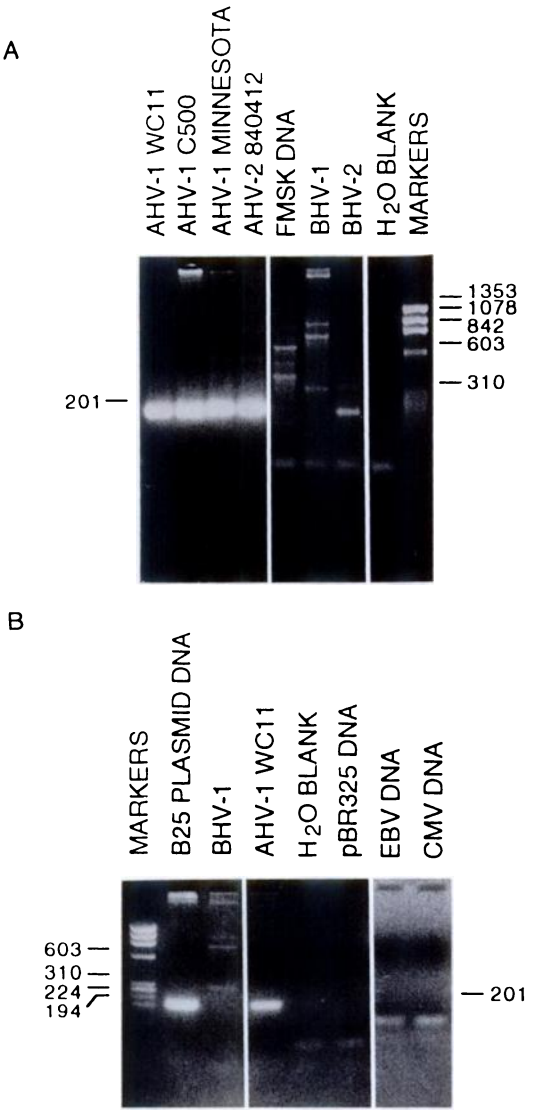

FIGURE 2. Detection of PCR-amplified sequence using AHV-1-derived primers shown in Figure 1. Tested samples were purified viral DNA of several alcelaphine herpesvirus isolates, bovine herpesviruses (Panel A), human cytomegalovirus, and Epstein-Barr virus (Panel B). Negative controls were DNA obtained from uninfected fetal mouflon sheep kidney cells (used to propagate the alcelaphine and bovine herpesviruses) and pBR325 plasmid DNA. Positive controls were DNA from clone B25 and AHV-1 isolate WC11. Molecular size markers are *Hae*III fragments of ϕX174 RF DNA, given in number of base pairs.

fragment of WC11 (European Molecular Biology Laboratory [EMBL] accession number: X55290) to select PCR primers that would amplify a 201-base-pair segment of strain WC11. In addition, an oligonucleotide probe sequence located between the primers also was selected.

RESULTS

The amplified sequence, primers and probe are shown in Figure 1. When either AHV-1 isolate WC11 DNA or plasmid DNA containing the B25 clone was used as a template in a PCR assay, this primer pair dictated the synthesis of an amplified DNA fragment of the predicted size as shown in Figure 2B. We also found that the primers amplified nucleic acids from AHV-1 isolate C500, AHV-2 isolate 840412, and the Minnesota sheep-associated isolate, and that the relative mobilities of the amplified products were indistinguishable on an agarose gel (Fig. 2A) indicating that the primers recognized other AHV isolates. In contrast, purified DNA samples from other herpesviruses including BHV-1, BHV-2, human cytomegalovirus (CMV), and Epstein-Barr virus (EBV) did not contain the amplifiable sequence (Fig. 2B). We also found that amplification does not occur using DNA from uninfected fetal mouflon sheep kidney (FMSK) cells or from the cloning vector, plasmid pBR325 (Fig. 2B).

Six of eight DNA samples obtained from a clinically affected gaur yielded positive results following PCR amplification with the AHV-1-derived primers (Fig. 3). A band of the expected size was also amplified from purified AHV-1 isolate WC11 DNA included in this experiment as a positive control. The two tissue-derived DNA samples which did not amplify were acquired from portions of the intestine. No amplification of a sample of DNA obtained from lymphocytes of a clinically normal gaur occurred when tested by PCR; thus, such sequences are not represented in the normal gaur genomic DNA. Finally, an amplification reaction without any template DNA did not generate the 201-base-pair DNA band.

The 27-base oligonucleotide sequence located between the primers (Fig. 1) was used as a detection probe to hybridize in

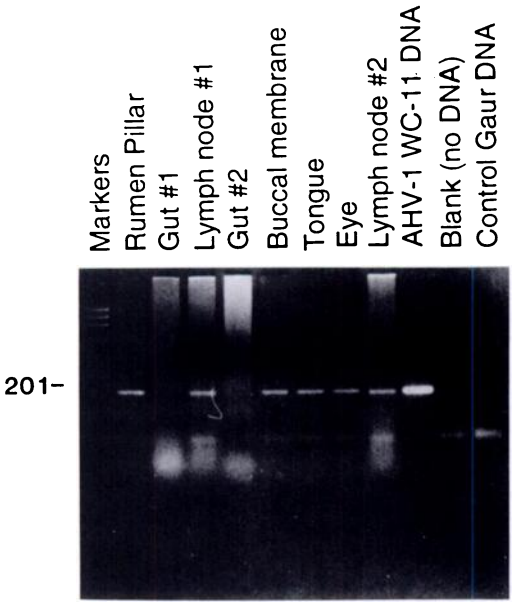

FIGURE 3. Detection of PCR-amplified sequence using AHV-1-derived primers shown in Figure 1. Samples tested were isolated DNA from post-mortem tissue samples of an infected Indian gaur. Origins of test specimens and controls are indicated above each lane. Molecular size markers are *Hae*III fragments of ϕX174 RF DNA, given as numbers of base pairs.

a Southern blot analysis of the amplified bands. The DNA bands amplified from the six gaur samples contained the AHV sequence as shown by specific hybridization of the WC11 probe (Fig. 4).

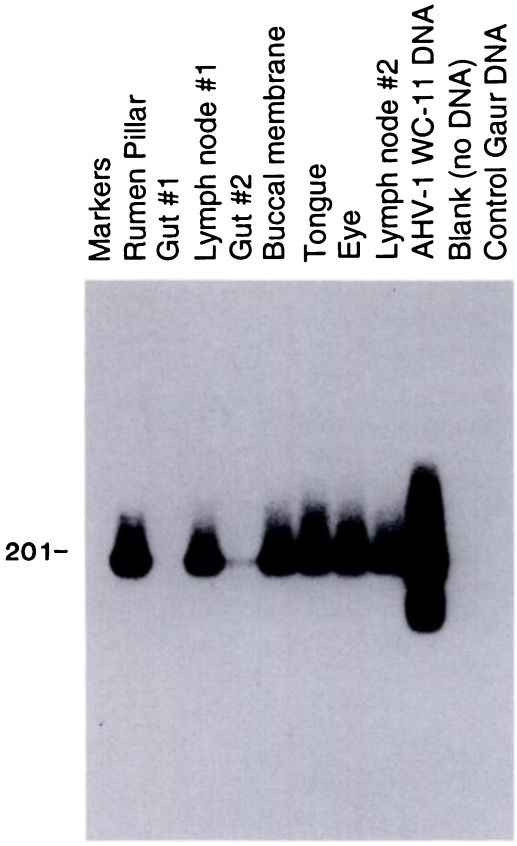

FIGURE 4. Southern blot hybridization. The gel-separated DNA fragments shown in Figure 3 were transferred to a nylon membrane. The blot was hybridized with the AHV-1 oligonucleotide probe (Fig. 1) end-labeled with ^{32}P-dATP. Origins of test specimens and controls are indicated above each lane.

DISCUSSION

The polymerase chain reaction is well established as a technique for amplification of nucleic-acid sequences which are difficult to detect by other means, but the applicability of the general method to a particular infectious agent should be empirically validated. While others have used PCR to detect AHV-1 sequences, this is the first known use of PCR to confirm clinical MCF resulting from natural infection. This application is indispensable in verifying the efficacy of any diagnostic assay, but the occasion to examine a naturally occurring MCF infection and to obtain appropriate specimens is extremely rare in the wild and in zoos because the disease progresses rapidly.

We now have successfully used nucleic acid amplification, and molecular hybridization with an AHV-1 isolate WC11-derived probe, to demonstrate that various tissues obtained from an MCF-affected animal harbored the viral sequence. The lymphocyte DNA from a clinically normal gaur did not produce an amplified band by PCR, demonstrating absence of the sequence in the genomic DNA of this species. These data support the preliminary diagnosis of MCF based on clinical signs and pathologic lesions in the affected animal. These preliminary results suggested that PCR might be useful in surveillance for early evidence of infection.

We conclude that at least in the acute

phase of MCF, the etiologic agent can be identified by PCR amplification. It remains to be determined whether PCR is capable of reliably detecting the presence of a latent AHV viral genome in multiple species of exotic or domestic ruminants.

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