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RESTRICTION ENDONUCLEASE ANALYSIS OF HERPESVIRUSES ISOLATED FROM TWO PENINSULAR BIGHORN SHEEP (*OVIS CANADENSIS CREMNOBATES*)

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ABSTRACT: In 1989, herpesviruses were isolated from nasal swabs taken from two peninsular bighorn sheep (*Ovis canadensis cremnobates*) in the Anza-Borrego Desert State Park, San Diego County, California (USA). Using restriction endonuclease analysis (REA) with *Pst*1 enzyme, each isolate was found to be similar to the Cooper strain of infectious bovine rhinotracheitis virus (IBRV). The REA patterns of the two herpesviruses from bighorn sheep were typical of either field strains or vaccine strains of IBRV commonly associated with cattle in the USA.

Key words: Herpesvirus, infectious bovine rhinotracheitis, restriction endonuclease patterns, bighorn sheep.

INTRODUCTION

In recent years, peninsular bighorn sheep (*Ovis canadensis cremnobates*) populations have been studied in and around the Jacumba Mountains, McCain Valley, In-Ko-Pah Mountains, and Carrizo Gorge areas within and adjacent to Anza-Borrego Desert State Park (ABDSP) in San Diego County, California, USA. Bighorn sheep population estimates from water hole counts and helicopter or fixed-wing aircraft surveys ranged from 250 in 1976 to 50 in 1989. Lamb : ewe and yearling : ewe ratios have both been ≤15:100 since studies were initiated by ABDSP rangers in 1973 (Jorgensen, unpubl.). Four-month-old lambs have been observed with severe dyspnea, nasal discharge, coughing, unable to run from the helicopter. The cause of this problem has been difficult to determine due to the remote locations of these herds, capture techniques required, and lack of a systematic evaluation of potential pathogens in affected sheep. We document the first reported isolation of infectious bovine rhinotracheitis virus (IBRV) from one yearling and one adult free-ranging bighorn sheep.

MATERIALS AND METHODS

Nine peninsular bighorn sheep were net-gun captured by helicopter in Carrizo Gorge (32°45′N, 116°10′W) on 29 and 30 March, 1989. Capture techniques and sampling and treatment protocols have been described previously (Clark et al., 1985; Jessup et al., 1988; Clark and Jessup, 1991).

The same biological samples were collected from all bighorn sheep. Sera and whole blood were preserved in commercial blood tubes (Monoject®, Sherwood Medical, St. Louis, Missouri, USA) with the latter containing 143 USP units sodium heparin. Rayon swabs used for nasal bacterial culture were placed in Amies modified medium with charcoal (Precision®, Culture and Transport System, Precision Dynamics Corporation, San Fernando, California). Cotton swabs were placed in 4 ml of viral transport medium (VTM) that was prepared and provided by the California Veterinary Diagnostic Laboratory System (CVDLS) (University of California, Davis, California). The VTM consisted of a 1 l package of minimal essential medium (MEM) powder, 250 μg kanamycin, 4 μg Fungizone, and 10 ml sterile horse serum (Gibco Life Technologies, Inc., Grand Island, New York, USA), plus 1 g sodium bicarbonate and 3.57 g

HEPES buffer (Sigma Chemical Company, St. Louis, Missouri), plus 250 mg gentamicin sulfate (Shering Corporation, Kenilworth, New Jersey, USA), resuspended into 1 l with triple distilled water. This suspension was then filtered through a 0.22 μm Millipak 20® (Millipore Products Division, Bedford, Massachusetts, USA) disposable filter unit and aseptically stored in 4 ml quantities in 15 ml tubes at 4 C.

Bighorn sheep sera was transported in liquid nitrogen while whole blood and nasal swabs were transported either on wet ice in a commercial ice chest or under refrigeration at 2 to 4 C and delivered to the CVDLS. Methods described by Cottral (1978) and Castro and Heuschele (1992) were used to isolate or serologically detect exposure to the following pathogens common to livestock or free-ranging wildlife: bluetongue virus (BTV), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), caprine arthritis encephalitis-ovine progressive pneumonia virus (CAE-OPPV), contagious ecthyma virus (CEV), epizootic hemorrhagic disease virus (EHDV), infectious bovine rhinotracheitis virus (IBRV), parainfluenza type 3 (PI-3) virus, *Anaplasma* spp., *Brucella* spp., *Leptospira interrogans canicola, icterohemorrhagica*, and *pomona* serovars, *Pasteurella* spp., *Chlamydia* spp., and *Mycoplasma* spp.

Specimens

Nasal swabs for virus isolation from nine bighorn sheep were received at CVDLS within 96 hr of collection. Swabs were immediately mixed (Vortex-Genie, Fisher Scientific, Santa Clara, California) for 2 to 5 min in VTM and the medium was filtered through 0.2 μm syringe filters (Acrodisc®, Gelman Sciences, Ann Arbor, Michigan, USA). Filtered fluids were stored at −70 C and inoculated within 48 hr onto cell cultures.

Cell culture and virus isolation

Fresh, 24-hr monolayers of Madin-Darby Bovine Kidney, BVDV-free (MDBK) cells, grown in Corning T25 flasks (Corning Glass Works, Corning, New York) were inoculated with 0.5 ml of the filtered swab sample fluids. Inocula were adsorbed onto cells for 1 hr at 36 C, then the medium was replaced with 4.5 ml of Dulbecco's MEM, 4 mM L-glutamine, 10 μg/ml kanamycin, and 100 μg/ml tylosin (Gibco Life Technologies, Inc., Grand Island, New York) plus 2% fetal bovine serum (Whitaker Bioproducts, Inc., Baltimore, Maryland, USA) and 5 μg/ml chlortetracycline (Sigma Chemical Company, St. Louis, Missouri). Cell controls consisted of an uninoculated flask of MDBK cells. For cytopathic virus identification by immunofluorescence, 0.1 ml samples from flasks demonstrating cytopathic effects (CPE) ([CPE]: 1+ = 25%, 2+ = 50%, 3+ = 75% and 4+ = 100% of cell monolayer affected) were inoculated onto 4-chambered slides (Lab-Tek®, Nunc, Inc., Naperville, Illinois, USA) containing monolayers of MDBK cells and subsequently fixed at 4 C with acetone when CPE was 1+ (Castro and Heuschele, 1992). Grading of CPE was based on extent of the cell monolayer affected; 1+ was ≤25%, 2+ was 26 to 50%, 3+ was 51 to 75%, and 4+ was ≥75% affected.

Flasks of viral-infected cell monolayers with CPE of 4+ from bighorn sheep SP89017 and SP89020 were placed in a −70 C freezer until the monolayer tops were frozen solid. The monolayers then were allowed to thaw at 22 C until a slush was formed. This freeze/thaw cycle was repeated two more times to maximize the abrasive effect of the forming ice crystals to lyse the MDBK cell internal membranes to release virus particles. The slurries were then centrifuged at 500 × g. The supernatant fluids were diluted 1:20 and passed (passage 2) onto 24-hr-old monolayers of MDBK cells grown in Corning T75 flasks.

These viral-infected cell cultures were collected and passed onto 24-hr monolayers of MDBK cells grown in Lab-Tek® slides (passage 3) with 0.15 ml cell culture inoculum/chamber well. Additional pools of both herpesviral isolates were prepared for both restriction endonuclease analysis (REA), and long-term storage in a virus repository.

A hemadsorption (HAD) assay (Cottral, 1978) was performed at 3 days post-inoculation (dpi) to ascertain the presence of any hemadsorbing viruses by the addition of 0.05% guinea pig erythrocytes in phosphate buffered saline (PBS), pH 7.2, to inoculated and control cell cultures which were pre-washed with PBS. After 30 min of erythrocyte adsorption at 25 C, the treated monolayers were washed three times with VTM, and moistened cell monolayers were observed using an inverted Olympus microscope, model IMT-2 (Scientific Instruments, Sunnyvale, California).

A commercial human respiratory syncytial virus (RSV) enzyme-linked immunosorbent assay (ELISA) kit (Abbott Laboratories, North Chicago, Illinois) was used according to the manufacturer's instructions to detect BRSV antigen.

A hemagglutination (HA) assay (Castro and Heuschele, 1992) was performed using serial two-fold dilutions of harvested fluids in PBS, pH 7.2 and 0.25% chicken erythrocytes to detect hemagglutinating viruses.

Fluorescent antibody test

For fluorescent antibody (FA) assays, as described by Cottral (1978) and Castro and Heu-

schele (1992), cultures of MDBK cells grown on Lab-Tek® slides were inoculated with the original filtered medium from nasal swabs and fixed at 4 C with acetone at 6 dpi. As a control, one well on each test slide was left uninoculated.

Conjugates for FA assays were obtained for IBRV, BVDV, and PI-3 viruses from the National Veterinary Services Laboratories (NVSL), Ames, Iowa (USA). These conjugates were used for direct FA staining of the virus cultures contained on the Lab-Tek® slides. The FA conjugate for BVDV was used to detect both BVDV and the antigenically related border disease virus (BDV) of domestic sheep as described by Timoney et al. (1988). Cell culture slides containing reference antigens from each of these viruses were stained in parallel. The stained slides were viewed under a light microscope using ultraviolet illumination to detect specific fluorescence for BVDV, BDV, and/or PI-3 viral antigens.

Negative stain electron microscopy

Fluids gathered from each of the T75 infected flasks were centrifuged at 2,000 × g for 20 min to remove cellular debris. The resultant supernate was then centrifuged at 340,000 × g for 45 min in a fixed angle Beckman T55 rotor using a Beckman L8-55m preparative centrifuge (Beckman Instruments, Inc., Palo Alto, California). The supernatant fluid was discarded and sterile distilled water was added to the pellet. The pellet suspension was placed in a nebulizer (Pelco All-Glass® Model 14601, Ted Pella, Redding, California) with several drops of 4% phosphotungstic acid and applied as a mist onto a 200 mesh, carbon-coated copper grid. For each specimen, two grids were viewed by placing in a liquid nitrogen cooled column of a Zeiss 10C electron microscope (Carl Zeiss, Inc., Thornwood, New York).

DNA preparation and analysis

Two flasks, each containing 150 cm^2 of fresh MDBK cells, were inoculated with each of the peninsular bighorn sheep herpesviruses and allowed to adsorb at 37 C for 1 hr. The number of viral particles calculated to be present in the inoculum that could potentially infect each cell was ≤0.1. The medium was replaced with Earle's MEM, 0.1 mM sodium pyruvate, 0.2% lactalbumin hydrolysate, 0.02% L-glutamine, and 50 μg/ml gentamicin sulfate (Gibco Life Technologies, Inc., Grand Island, New York, USA), supplemented with 10% heat-inactivated bovine fetal serum (NVSL), and cells were incubated at 37 C. When 100% CPE occurred, the medium was centrifuged at 2,000 RPM for 10 min. The viruses of the clarified medium were concentrated by centrifugation at 100,000 × g for 1 hr through 40% glycerol in 0.2M TE (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.6) buffer. Viral DNA was extracted with sodium dodecylsulfate (0.4%) and proteinase K (0.1% mg/ml) for 1 hr at 37 C. After extraction, viral DNA was mixed at 22 C for 20 sec once with an equal volume of TE-saturated phenol, once with an equal volume of 1:1 mixture of TE-saturated phenol and chloroform (with 2% isoamyl alcohol), and twice with an equal volume of chloroform (with 2% isoamyl alcohol) and then dialyzed against two changes of TE buffer (Campbell et al., 1970). Approximately 1 μg of viral DNA, as determined by a minigel preparation of uncut DNA (Maniatis et al., 1982), was digested at 37 C with 10 units of restriction endonuclease *Pst*1 (Bethesda Research Laboratories, Gaithersburg, Maryland) in reaction buffer (60 mM Tris, 60 mM $MgCl_2$, 10 mM dithiothreitol, 600 mM NaCl, 600 μg bovine serum albumin [nuclease free]/ml). After 1 to 1.5 hr, Loening's buffer (30 mM NaH_2PO_4, 1 mM Na_2EDTA, 36 mM Tris at pH 7.7) containing 60% sucrose and 0.02% bromophenol blue was added. The DNA preparations were electrophoresed through a submerged 0.8% agarose gel (Sea Kem, FMC, Rockland, Maine, USA) in Loening's buffer for 17 to 18 hr at a constant 45 volts. The gel was stained with 1 μg/ml ethidium bromide (Gibco Life Technologies, Inc., Grand Island, New York) in Loening's buffer for 30 min and photographed under ultraviolet illumination.

RESULTS

Cell monolayers from two flasks inoculated with nasal swabs from either bighorn sheep SP89017 or SP89020 showed 4+ CPE at 5 dpi. Other uninfected flasks did not show any CPE after 6 days of incubation. Passages 2 and 3 of both isolates resulted in an amplification of virus by replication as a 4+ CPE was seen ≤24 hr on each passage.

The HAD and HA tests were uniformly negative for both viral isolates. Fluid harvests of each virus were also negative for BRSV by ELISA. Fluorescent antibody tests on infected cells at 6 dpi, using 10-fold dilutions of inoculum, were negative for antigens of PI-3 virus, BVDV, and BDV; however, cell cultures contained specific fluorescence for antigens of IBRV.

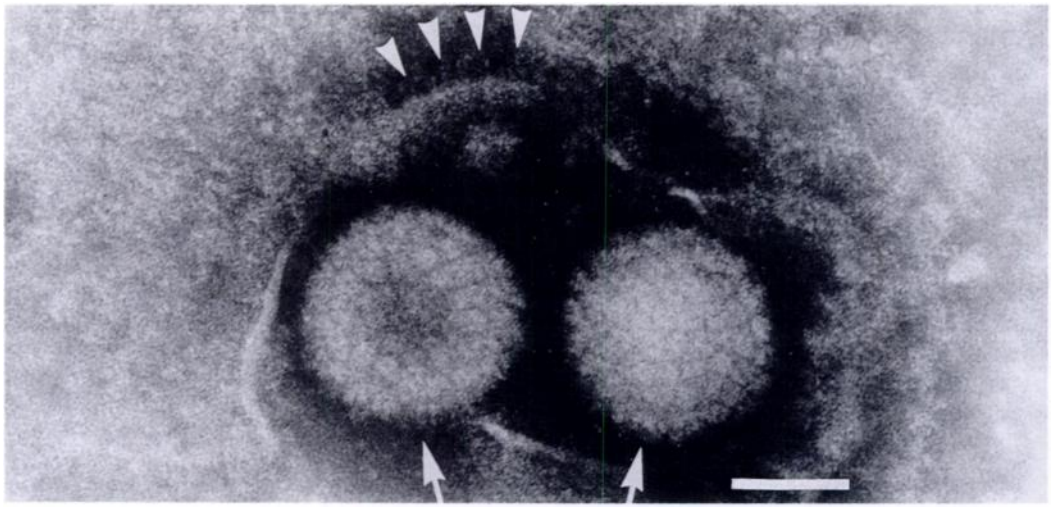

FIGURE 1. Electron micrograph of two herpesvirions, enclosed in a common envelope, that were isolated from a peninsular bighorn sheep captured in Anza-Borrego Desert State Park, California, USA, in 1989. Arrows indicate the two virus nucleocapsids and arrowheads indicate the common envelope with spike projections on the surface. Bar = 50 nm.

Herpesviral particles were observed in the supernate of inoculated cell cultures by negative stain electron microscopy (EM) (Fig. 1). Other viruses, including BTV and EHDV, were not isolated from seven other nasal swabs or any of the blood specimens drawn from bighorn sheep in this study.

Restriction endonuclease patterns, using *Pst*1 enzyme digestion, of the bighorn sheep herpesviral isolates were similar to that seen with the Cooper strain of IBRV (Fig. 2).

The herpesviral isolate from bighorn sheep SP89017 was from a female in late gestation that had a completely normal physical examination. The other herpesviral isolate, SP89020, was from a yearling ram in excellent condition.

Sera from bighorn sheep SP89017 and SP89020 were negative for antibodies against the respiratory pathogens BRSV, PI-3 virus, and IBRV; however, sera from bighorn sheep SP89020 had antibodies against BTV.

No pathogenic bacteria, *Mycoplasma* spp., or other viruses were isolated. Serological evidence of exposure to BVDV, CAE-OPPV, CEV, EHDV, *Brucella* spp., *Leptospira interrogans* serovars, *Chlamydia* spp., and *Anaplasma* spp. was not present.

DISCUSSION

The REA of the herpesviral isolates from two bighorn sheep in this study were similar to the Cooper strain of IBRV. Although some minor differences in band pattern was noted between the bighorn sheep isolates and IBRV Cooper strain, these changes were typical of what is observed during in vivo passage of IBRV (Whetstone et al., 1989a, b). Based on southern blot analysis using *Hind*III DNA probes of BHV-1 strain Cooper (Mayfield et al., 1983; Whetstone et al., 1989b), those differences in band patterns occur within the internal repeat regions at the left terminus of the IBRV genome. These are extremely active regions of the bovine herpesvirus genome during in vivo replication; band pattern differences are common (Hammerschmidt et al., 1988; Whetstone et al., 1989a, b).

In vitro restriction patterns remain stable over passage (Whetstone, unpubl.). The Cooper strain of IBRV is typical of both

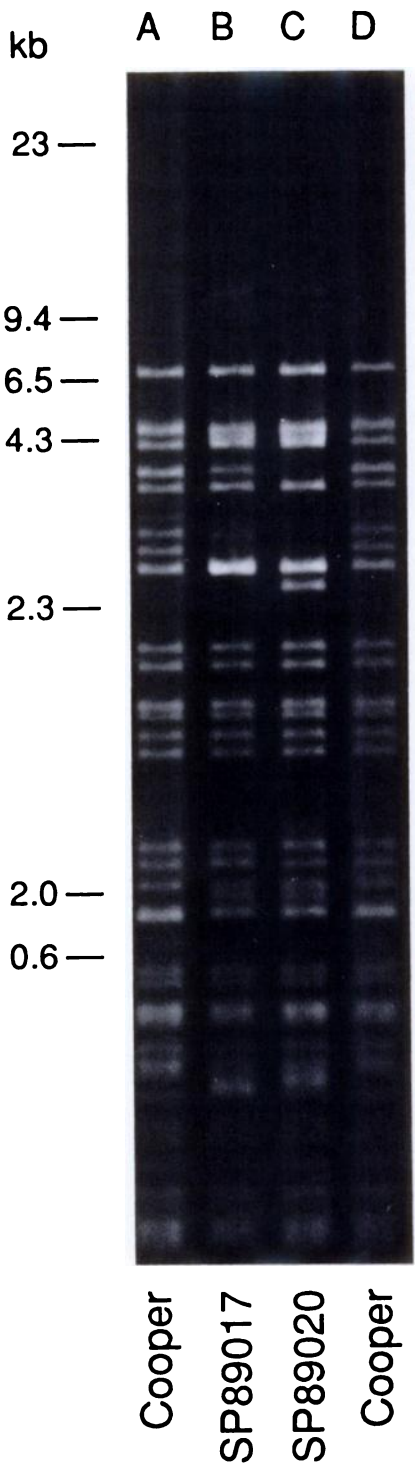

FIGURE 2. Restriction endonuclease *Pst*1 digests of herpesviruses isolated from two peninsular bighorn sheep captured in Anza-Borrego Desert State Park, California, USA, in 1989. The restriction endonuclease pattern of the Cooper strain of bovine herpesvirus type 1 is in row A and D, bighorn sheep SP89017 in row B, and bighorn sheep SP89020 in row C. Molecular weight markers are indicated to the left side of the gel.

viral field strains (Seal et al., 1985; Whetstone, unpubl.) and vaccine strains commonly associated with cattle in the USA (Whetstone et al., 1989b).

In recent years, the bighorn sheep population in the ABDSP has declined and lambs have been seen with respiratory disease at approximately 4 mo of age. As part of routine survey and biological sampling efforts, 17 bighorn sheep from the Carrizo Gorge herd have had biological samples (serum, whole blood, nasal swabs, feces, and skin scrapings) taken since 1984. Thirteen (76%) of 17 of the sera were AGID positive for antibodies against BTV including 6 of 9 in the 1989 effort (Clark, unpubl.). There was one isolation of BTV Type 11 in 1984 (Clark et al., 1985). Antibodies were found in bighorn sheep sera against EHDV (9 of 10), BVDV (1 of 17), and CEV (2 of 4). Bighorn sheep seroprevalence also has been documented for PI-3 virus (4 of 17) and BRSV (1 of 17). To date, there has been no detection of serum neutralizing antibodies against IBRV in any of the 17 serum specimens (Clark, unpubl.). Since the two IBRV isolates came from apparently healthy bighorn sheep, it could reflect a recent exposure to the virus prior to mounting a measurable humoral antibody response. The inability to detect IBRV neutralizing antibodies in serum samples also could have been due to either undetectable antibody levels in the bighorn sheep, the sensitivity of the test used (Riegel et al., 1987), or both. Future specimens from ill or convalescing bighorn sheep need to be tested to validate whether IBRV neutralizing antibodies are present in this species.

In April 1987, 115 feral cattle were captured, had biological specimens taken, and were then removed from ABDSP. Ten of 41 (25%) of these feral cattle were sero-

logically positive for antibodies against IBRV (Jorgensen, 1988). Feral cattle and bighorn sheep have been seen watering in Carrizo Gorge in the specific areas where these two bighorns from which IBRV was isolated had been captured. Two weeks following the 1989 bighorn sheep capture, ABDSP rangers observed cattle at a water source shared with bighorn sheep on a Bureau of Land Management grazing allotment near the capture sites (Jorgensen, unpubl.).

In cattle, IBRV, whether from vaccine or natural exposure, can become latent in the host and be spontaneously reactivated over the lifetime of the animal (Wyler, 1989). During those episodes of reactivation, the herpesvirus can be readily spread to other animals (Whetstone et al., 1989b). Respiratory disease resulting from BHV-1 infection has been described in domestic sheep by Kimberling (1988) and BHV-1 has been isolated from domestic sheep and goats as described by Whetstone and Evermann (1988). The IBRV can cause abortion and infertility in bovids (Miller et al., 1991). Other pathological conditions induced by BHV-1, including encephalitis in bovids, also have been reported by Bagust (1972), Bagust and Clark (1972), Brake and Studdert (1985), Engles et al. (1986), Metzler et al. (1986), and Weiblen et al. (1989).

The REA is a useful technique for epidemiological studies of IBRV to ascertain type strains found in free-ranging ungulates (Whetstone et al., 1986). To our knowledge, this is the first documented case of an isolation and REA characterization of IBRV in *O. canadensis* spp. However, the role of this herpesvirus in reproductive or respiratory diseases in this bighorn sheep population is unknown. Since IBRV has not been described previously as a causal agent of clinical disease in bighorn sheep, these findings may be incidental. Nevertheless, we suggest an extensive investigation on IBRV pathogenicity in bighorn sheep; its prevalence in bighorn herds with respiratory or reproductive diseases; and on temporal relationships between virus infection, respiratory or reproductive diseases, and development of humoral antibodies. We recommend further field evaluations of herds to clarify whether IBRV strains of cattle are transmissible to bighorn sheep.

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