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ISOLATION OF REPTILIAN CALICIVIRUS *CROTALUS* TYPE 1 FROM FERAL PINNIPEDS

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ABSTRACT: Ten virus isolates were obtained from three species of marine mammals sampled on San Miguel Island (California, USA) and 1,200 km north on Rogue Reef (Oregon, USA) during tagging operations in 1986–87. Seven of these 10 were derived from 30 sampled Steller sea lion (*Eumetopias jubatus* pups, while two of 10 were isolated from one of 19 sampled California sea lion (*Zalophus californianus californianus* pups, and the remaining isolate was derived from 30 sampled northern fur seal (*Callorhinus ursinus*) pups. All 10 isolates were identified as belonging to a single serotype, reptilian calicivirus *Crotalus* type 1 (RCV Cro-1), previously isolated from both healthy and diseased snakes and frogs in a California zoologic collection. The marine samples also showed that nine of 30 Steller sea lion pups, one of 19 California sea lion pups and zero of 30 fur seal pups were producing type specific neutralizing antibodies to RCV Cro-1. This represents the first reported instance of the isolation from marine sources of a calicivirus originally isolated from a terrestrial species.

Key words: Calicivirus, California sea lion, *Zalophus californianus californianus*, Steller sea lion, *Eumetopias jubatus*, northern fur seal, *Callorhinus ursinus*, virus isolation, neutralizing antibodies, foreign animal disease.

INTRODUCTION

In 1978–79, calicivirus was isolated from four poikilothermic species in a California zoologic collection (Smith et al., 1986). These isolates were collected from eight asymptomatic Aruba Island rattlesnakes (*Crotalus unicolor*; from cloacal swab samples), and at necropsy from four Aruba Island rattlesnakes, one rock rattlesnake (*Crotalus lepidus*), one eyelash viper (*Bothrops schlegeli*), and two Bell's horned frogs (*Ceratophrys orata*). On the basis of cross-neutralization test results, the 16 isolates were antigenically indistinguishable and were considered to represent a new calicivirus serotype, designated reptilian calicivirus *Crotalus* type 1 (RCV Cro-1). Although this virus was isolated in association with diseased tissues from dead poikilotherms it has not been causally linked to any specific disease entity. It has been shown experimentally to produce asymptomatic infections in at least one mammalian species, the domestic pig (*Sus scrofa*) (Smith et al., 1986).

During tagging operations along the coast of Oregon and California in 1986–87, samples were collected from California sea lion (*Zalophus californianus californianus*), Steller sea lion (*Eumetopias jubatus*), and northern fur seal (*Callorhinus ursinus*) pups for virus isolation and serologic examination. To date we have 26 isolates of RCV. These have been isolated from 3 reptilian, one amphibian and 3 mammalian species over a 10 yr period in three geographical locations from San Diego, California to Rogue Reef, Oregon. The purpose of the present communication is to describe the first isolation of RCV Cro-1 from marine sources, the first documented association of RCV Cro-1 with clinical illness (vesicular lesions) in a mammalian host, and the antigenic relatedness of the marine isolates to prototype RCV Cro-1.

MATERIALS AND METHODS

Sample collection

In September, 1986, blood and swab samples for virus isolation were collected in conjunction with tagging operations on San Miguel Island (California, USA; 34°01'N, 120°12'W) California sea lion and northern fur seal pups were caught in hoop nets, weighed, their sex determined and National Marine Fisheries marine mammal metal identification tags placed in the tailing edge of the pectoral flippers. Swab samples were collected from the oropharynx and rectum of each animal (19 California sea lions and 30 northern fur seals) and fluid aspirated with a 28 gauge needle syringe from a lesion found on the non-haired portion of a California sea lion pectoral flipper. Vesicular fluid was placed in and swabs were broken off into 5 ml screw-cap glass vials containing 2 ml of cell culture medium (Eagle MEM; Whittaker M.A. Bioproducts, Walkersville, Maryland, USA) with 10% fetal calf serum and 100 μg/ml gentamicin). These samples were frozen and held on dry ice for transport to the laboratory. One to 5 ml of blood was obtained from the rear flipper of the 49 tagged pups which had been sampled for virus isolation. Blood samples were allowed to coagulate at ambient temperatures for 1 to 6 hr; then the serum was separated from the clot by centrifugation at approximately 1,500 rpm for 10 min using a portable centrifuge and power supply and subsequently frozen to −20 C until tested. In the laboratory, the sera were heat-inactivated at 56 C for 30 min in preparation for serum neutralization (SN) testing.

In June, 1987, similar procedures were carried out 1,200 km to the north on Rogue Reef, three km northwest of the mouth of the Rogue River, near Gold Beach (Oregon, USA; 42°15'N, 124°16'W) where blood and swab samples (oropharyngeal and rectal) were obtained from 30 Steller sea lion pups. These samples were preserved in an identical manner except that transport to the laboratory took place within a few hours after collection.

Virus isolation, identification and characterization

Swab samples were thawed, vortexed, and clarified by low-speed (3,000 × g for 20 min) centrifugation and 0.2 ml of the supernatant was adsorbed for 60 min to monolayers of PK-15 and Vero cells (American Type Culture Collection, Rockville, Maryland, USA) in roller tubes and processed as previously described. (Smith et al., 1980). The inocula were poured off, the monolayers were rinsed with cell culture medium (Eagle MEM), the cells re-fed (media poured off and 2 ml fresh media added) with MEM and 2% fetal bovine serum, and incubated at 37 C on a roller apparatus. Each culture was examined daily for cytopathic effect (i.e., any cell culture abnormality). All samples were blind-passaged at least three times, at 7 day intervals, before being considered negative. Viruses causing cytopathic effect manifestations were purified using three plaque purified passages (Smith et al., 1983) and morphologic features of purified isolates were examined by negative-contrast electron microscopy (Smith and Latham, 1978; Skilling et al., 1985). Biochemical testing included virus stability in ether, nucleic acid (RNA) determination using 5-fluoro-2-deoxyuridine, pH stability, heat lability, and divalent cation effects (Zee and Hackett, 1967; Howatson and Whitemore, 1973; Schaffer et al., 1980). Isolates were identified serologically by serum neutralization (SN) tests using 100 tissue culture infective doses ($TCID_{50}$) of virus against 20 antibody units of typing serum (Kapikian et al., 1967). Typing sera maintained in our reference laboratory for all extant, established animal calicivirus serotypes were used including 17 serotypes of San Miguel sea lion virus (SMSV), 12 serotypes of vesicular exanthema of swine virus (VESV), feline calicivirus, primate calicivirus *Pan paniscus* type 1, mink calicivirus, walrus calicivirus, cetacean calicivirus *Tursiops* type 1, bovine (Tillamook) calicivirus *Bos*-1, canine calicivirus, (Evermann et al., 1983) and RCV-Cro 1.

Immunoelectron microscopy

Immunoelectron microscopy was completed as described previously (Smith et al., 1978) using 1 ml of clarified stock virus isolate centrifuged at 15,000 g for 20 min. The pellet was resuspended in 10 μl of distilled water mixed with 10 μl of RCV Cro-1 typing serum then incubated overnight at 4 C. A formvar-coated 300-mesh copper grid was floated on a drop of the virus-antibody mix for 2 min, blotted dry, stained with PTA and examined at an accelerating voltage of 80 KV using a Phillips CM 12 (Eindhoven, Netherlands) transmission electron microscope.

Serology

A microtiter (96-well) SN procedure using Vero cells was performed, first to screen sera at a dilution of 1:20 which is a significant titer, and then to titrate positive samples using 2 fold dilutions (Monto and Bryan, 1974; Smith et al., 1976; Barlough et al., 1987). Serum-virus mixtures (2.0 μl each) were incubated for 60 min at room temperature before addition of cells as previously described. The antibody titer

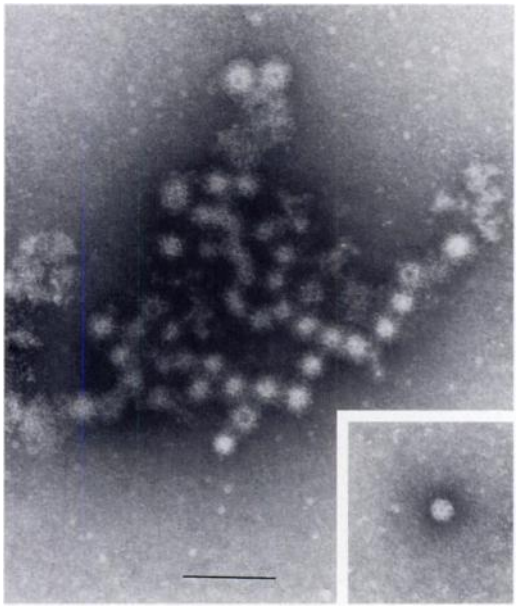

FIGURE 1. Immunoelectron photomicrograph of Reptilian calicivirus Zalophus-1 (RCV Zal-1) isolated from a California sea lion rectal swab, when reacted against hyperimmune rabbit antiserum to reptile calicivirus Crotalus-1 (RCV Cro-1) and without hyperimmune antiserum (insert). Phosphotungstic acid. Bar = 100 nm.

(as judged by cytopathic effect after 72 hr incubation at 37 C) was defined as the highest dilution of serum completely neutralizing 100 $TCID_{50}$ of virus in all four replicate test wells (100% end point). Specific rabbit antiserum was used as a positive control, and type specificities were monitored in parallel neutralization tests during end-point titrations.

RESULTS AND DISCUSSION

Rectal and oral swabs of seven Steller sea lion pups (Rogue Reef), one California sea lion pup and one northern fur seal pup (San Miguel Island) yielded virus isolates identified as members of Caliciviridae (Fig. 1) (Schaffer et al., 1980). Vesicular fluid from flipper lesions on the California sea lion pup also yielded a calicivirus isolate. All 10 isolates were neutralized only by antiserum to RCV Cro-1. Thus, all are classed as new strains of RCV.

Of 79 pup sera tested, 10 (13%) contained specific neutralizing antibodies to RCV Cro-1. Titers ranged from 1:20 to 1:80 (Table 1).

TABLE 1. Serotype-specific neutralizing antibodies to reptilian calicivirus in sea lion and fur seal pups.

Neutralizing antibody titer (100% endpoint)	California sea lions[a]	Northern fur seals[a]	Steller sea lions[b]
<1:20	18	30	21
1:20	1	0	5
1:40	0	0	2
1:80	0	0	2
Number positive/ number examined	1/19	0/30	9/30

[a] Sampled on San Miguel Island, California, USA.
[b] Sampled on Rogue Reef, Oregon, USA.

Since 1972, 20 distinct calicivirus serotypes have been isolated from several marine mammal species, including the California sea lion, Steller sea lion, northern fur seal, Pacific walrus (*Odobenus rosmarus divergens*), northern elephant seal (*Mirounga angustirostris*), and Atlantic bottlenose dolphin (*Tursiops truncatus*) (for reviews see Barlough et al., 1986; Smith and Boyt, 1990). Two of these serotypes have been isolated also from opaleye (*Girella nigricans*), an ocean fish, and one from the sea lion liver fluke *Zalophatrema* spp. (Smith et al., 1980). Both pathotypically and regarding partial sequence homologies these "marine caliciviruses," are all within one genogroup which includes the internationally listed foreign animal disease (FAD) virus VESV, said to have been eradicated from the United States in 1959 (Smith, 1981; Matson et al., 1996; Neill et al., 1995). However, VESV serotypes based on type specific serum neutralization classification still survive in marine mammal populations along the eastern Pacific rim (western margins of the United States) (Smith and Boyt, 1990). Thus, the costly outbreaks of VESV infections that affected the swine industry in the USA from 1932 to 1956 probably resulted from translittoral movement of VESV serotypes from ocean reservoirs to domestic pig populations, most likely through the food chain (Smith, 1981; Smith and Madin, 1986). The presence of VESV and bovine (Tillamook) calicivirus antibodies in feral marine mammals of the

Pacific coast (Smith and Latham, 1978; Barlough et al., 1987), the presence of SMSV antibodies in domestic livestock (Soergel et al., 1978; Barlough et al., 1986; Berry et al., 1989; Boyt, 1989), and the actual isolation of SMSV-4 from a swine herd in California in 1976 (Smith and Boyt, 1990) and from a Pacific fisheries mink food product (Sawyer et al., 1978) have provided further support for our hypothesis that VESV and SMSV are simply variants of the calicivirus causing the Foreign Animal Disease VES. Now given its marine presence, RCV should be included as one of these variants.

Serologic evidence for a marine presence of two ostensibly land-based calicivirus serotypes has been previously shown. In 1981, Tillamook calicivirus was found in a dairy herd with chronic respiratory disease problems in northern Oregon (Smith et al., 1983). Serotype-specific neutralizing antibodies to this bovine calicivirus were found in sera collected from California sea lions in 1983 and 1984 and in sera collected from Steller sea lions in 1976 and 1985 (Barlough et al., 1987). Mink calicivirus was collected in 1977 from an Idaho ranch mink (Long et al., 1980; Evermann et al., 1983) and antibodies to it have been identified in bottlenose dolphins housed at a government facility in Hawaii (USA) (Smith and Boyt, 1990). This report of the isolation of RCV from three species of marine mammals is the first instance of a calicivirus originally identified as terrestrial and then subsequently documented by in vitro isolation as a calicivirus also infecting marine species. The reverse movement (sea to land) of the SMSV's, the VESV's, bovine calicivirus, and possibly mink calicivirus represent diverse serotypes of a single genogroup, (Matson et al., 1996; Neill et al., 1995) which, based on serologic evidence, have repeatedly crossed from the sea to infect terrestrial animal populations (Sawyer et al., 1978; Smith et al., 1978, 1980; Smith, 1981; Smith and Madin, 1986; Barlough et al., 1986; Smith and Boyt, 1990).

The in vitro isolation of RCV from marine mammals demonstrates the remarkable host-range versatility of some caliciviruses and highlights their capacity for infecting both poikilothermic and mammalian hosts under natural conditions (Smith et al., 1977, 1980; Smith and Boyt, 1990). It also shows that along with the great serotypic variety of the Caliciviridae and the common appearance of new serotypes, the neutralizing epitopes of RCV Cro-1 have survived in nature for ten years or more among hosts having phylogenetic diversity crossing class level taxonomy. This same preservation involving a neutralizing epitope has been demonstrated also for SMSV-6 (Skilling et al., 1987). Previous information on the persistence of individual serotypic determinants within naturally exposed populations has been restricted to serologic studies without concurrent in vitro virus isolation. (Smith and Latham, 1978).

This report documents the first observed association between RCV infection and clinical illness (vesicular disease) in a mammalian host. Although it is not possible to assess the overall impact of RCV infection, other marine caliciviruses are also known to cause vesicular lesions, and be associated with neonatal mortality and premature parturition, encephalitis and pneumonitis, in several species of pinnipeds (Smith, 1981; Smith and Madin, 1986; Barlough et al., 1986). Finally, our knowledge of the geographic distribution of RCV in nature has been broadened considerably in that an agent whose presence once presumed to occur only in captive snakes and frogs in a southern California zoologic collection has now been found to infect three species of free ranging marine mammals 1,200 kilometers apart on the eastern Pacific rim.

In keeping with the international nomenclature currently used for the prototype strain of reptilian calicivirus *Crotulus*-1 (RCV Cro-1) and some other calicivirus types, we now propose giving strain designations to these new RCV isolates as fol-

lows: reptilian calicivirus *Zalophus*-1 (RCV Zal-1), reptilian calicivirus *Callorhinus*-1 (RCV-Cal-1) and reptilian calicivirus *Eumetopias*-1 through 7 (RCV-Eum-1-7).

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