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DETECTION OF A NON-CULTIVATABLE CALICIVIRUS FROM THE WHITE TERN (*GYGIS ALBA ROTHSCHILDI*)

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ABSTRACT: In April 1992, on Tern Island, French Frigate Shoals, Hawaii (USA), researchers observed a hand-reared white tern hatchling (*Gygis alba rothschildi*) develop vesicular lesions on the webbing between its toes, 6 days after falling out of its nest. Vesicular fluid collected from the foot lesions contained virus-like particles having typical calicivirus morphology. Calicivirus RNA was detected in the vesicular fluid by dot hybridization with a group-specific calicivirus copy DNA probe. Attempts to cultivate the virus in African green monkey kidney cells and porcine kidney cells were unsuccessful. This is the first report of a calicivirus infection associated with vesicular disease in a wild avian species.

Key words: Calicivirus, white tern, *Gygis alba*, cDNA hybridization.

INTRODUCTION

The Caliciviridae is a family composed of small, non-enveloped, single-stranded RNA viruses with morphologically distinctive cup-shaped surface structures. These agents have a geographically and phylogenetically diverse host range. Characterized caliciviruses have been isolated from amphibians, fish, reptiles, marine mammals, terrestrial mammals, and humans (Barlough et al., 1986a, b). Agents that have not yet been grown in vitro, but are suspected of being caliciviruses based on capsid morphology, have been described in insects, humans, and birds (Cubitt, 1987). Pathogenic caliciviruses are known to spread through diverse host species across long distances. Migratory and wide-ranging ocean-associated species, including birds, may be involved in the spread of these agents to naive and susceptible host populations.

Calicivirus-like particles have been detected in association with gastrointestinal disease in four captive-raised avian species: chickens, Guinea fowl (*Numida meleagris*), goldfinches (*Carduelis carduelis*), and ring-necked pheasants (*Phasianus colchicus*). Bracewell and Wyeth (1981) reported stunting and poor feather growth in young chickens; particles with calicivirus-like morphology were seen in intestinal contents of affected animals, but could not be isolated in tissue culture (Wyeth et al., 1981). Particles consistent with calicivirus morphology have been observed in cecal contents of 6- to 16-day-old Guinea fowl (*Numida meleagris*) keets dying of typhlitis (Gough and Spackman, 1981). The Guinea fowl disease was of sudden onset, producing 50% mortality, and only occurred in keets raised with broiler chickens. Negative contrast electron microscopy of the intestinal contents of 3- and 4-wk-old ring-necked pheasant poults with signs of enteritis and paralysis contained calicivirus-like particles, but samples from unaffected poults did not (Gough et al., 1992). Furthermore, calicivirus particles were observed in the intestinal contents of goldfinches with hemorrhagic, necrotizing proximal enteritis (Sironi, 1994). Chicken calicivirus has been grown in vitro with great difficulty, and causes disease in specific pathogen-free day-old chicks (Cubitt and Barrett, 1985).

The white, or fairy, tern (*Gygis alba rothschildi*) is widely distributed throughout offshore and pelagic waters of the subtropical and tropical Pacific Ocean. This avian species may be observed any dis-

tance from land, but is usually found within 50 miles of islands where they breed (King, 1967). In the spring of 1992, researchers found a newly hatched white tern on the ground under its nesting tree on Tern Island, French Frigate Shoals (23°45′N, 166°10′W) in the northwest Hawaiian Islands (USA). During attempts to rehabilitate the animal, vesicular lesions developed on the webbing between the toes of the tern chick's feet. Because of the vesiculogenic disease potential of ocean-origin caliciviruses (Smith et al., 1980b; Gelberg et al., 1982a, b; Smith and Boyt, 1990), the white tern vesicular fluid was collected for virus isolation and group-specific copy DNA dot hybridization to determine whether caliciviruses may be associated with vesicular disease in wild sea bird populations.

MATERIALS AND METHODS

A white tern (*Gygis alba rothschildi*), approximately 1 day old, was found on the ground at Tern Island, French Frigate Shoals, Hawaii, on 9 April 1992. One member of the field research team attempted to rehabilitate the hatchling. It was hand-reared in a small box, and fed freshly netted small fish (approximately 5 cm in length) of various unknown species for 5 days. On the sixth day of rehabilitation, two vesicles, each approximately 3 mm in diameter, were observed on the dorsal foot webbing of the bird. The vesicles contained clear serous fluid, and approximately 10 μl of the transudate was collected with a 30 G needle and syringe, frozen in liquid nitrogen, and transported back to the Laboratory for Calicivirus Studies, Oregon State University, Corvallis, Oregon (USA), where it was stored at −70 C.

One half of the vesicular fluid (5 μl) was diluted 1:5 with Eagle minimal essential medium with Earle's salts (Gibco Bethesda Research Laboratories [BRL], Life Technologies, Gaithersburg, Maryland, USA) and 12.5 μl of the diluted sample was adsorbed onto separate monolayers of African green monkey kidney (Vero) cells (American Type Culture Collection [ATCC] Certified Cell Line [CCL] 81; ATCC, Rockville, Maryland, USA) and porcine kidney (PK-15) cells (ATCC CCL 33) in 96-well microtiter plates (Corning Incorporated, Corning, New York, USA). Each sample was given four blind passages with one freeze-thaw cycle between each pass.

The remaining vesicular fluid was applied to a grid for examination by negative stain electron microscopy (Skilling et al., 1985). The 5 μl drop of vesicular fluid was placed on Parafilm (American National Can, Greenwich, Connecticut, USA), and a formvar-covered, carbon-coated, glow-discharged copper electron microscopy grid (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) was floated on it for 2 min at 25 C; the drop was retained for cDNA dot hybridization. The grid was touched to a drop of sterile-filtered double-distilled water, blotted dry with filter paper, and floated on 1.5% phosphotungstic acid, pH 7.0, for 1 min at 25 C. Excess stain was removed by blotting on filter paper, and the grid was placed under germicidal ultraviolet light (280 nm) for 15 to 20 min to inactivate possible pathogens. The grid was examined with a transmission electron microscope (Phillips CM 12/STEM Analytical Electron Microscope, Phillips, Eindhoven, Netherlands) at an accelerating voltage of 80 kV.

The RNA was extracted with RNAzol B (Cinna/Biotecx, Houston, Texas, USA), using of the phenol/guanidinium isothiocyanate procedure of Chirgwin et al. (1979), with the following modifications. The remaining vesicular fluid was transferred to a sterilized 1.5 ml microcentrifuge tube, 800 μl of RNAzol B was added to disrupt the tissue, solublize nucleic acid, and inactivate ribonucleases present in the sample mixture. The solution was briefly mixed in a vortex mixer before 80 μl of chloroform was added. The sample was stored on ice for 5 min before it was mixed vigorously for 15 sec and again allowed to stand on ice for an additional 15 min. The solution was centrifuged at 14,000 × *G* at 4 C for 15 min and the top aqueous layer was removed to another microcentrifuge tube. An equal volume of cold (−20 C) isopropanol was added and the RNA was precipitated at 4 C for 45 min. The RNA was reduced to a pellet in a microcentrifuge for 15 min and washed once with 1 ml of 75% ethanol. After another centrifugation for 5 min the supernatant was discarded, and the sample was centrifuged again to drive the residual ethanol to the bottom of the tube so the final traces could be removed. The RNA pellet was dissolved in 10 μl of 0.1% diethylpyrocarbonate (Sigma Chemical Company, St. Louis, Missouri, USA)-treated double-distilled water (Sambrook et al., 1989).

The plasmid containing the cDNA hybridization probe, p5RT73, was digested with the restriction endonucleases, Xba I and Rsa I (IBI, Inc., Rochester, New York, USA). The reaction digest was extracted with 25:24:1 v/v phenol : chloroform : isoamyl alcohol (Gibco BRL), and

ethanol-precipitated in preparation for biotinylation using random octamer priming (Sambrook et al., 1989). The protocol and reagents used to label the probe were supplied in kit form by Gibco BRL as their BioPrime DNA Labeling System. Approximately 100 ng of denatured restriction digest was biotinylated by incubating at 37 C for 4 hr in a solution containing a mixture of 50 mM tris(hydroxymethyl)aminomethane (tris), pH 6.8; 5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 0.1 mM biotin-14-dCTP; 0.1 mM dCTP; 0.2 mM dATP; 0.2 mM dGTP; 0.2 mM dTTP; 15 μg random octamer deoxyribonucleotide primers; and 40 units of the Klenow Fragment of DNA polymerase I. The reaction was stopped by the addition of 0.2 M ethylenediaminetetraacetic acid (EDTA), pH 7.5. Unincorporated nucleotides were removed using Sephadex G-50 spin column gel chromatography (IBI, Inc.). The biotinylated probe was stored at −20 C until needed.

One half of the vesicular fluid RNA was spotted onto an uncharged nylon membrane (PhotoGene Nylon Membrane, Gibco BRL). After the membrane was allowed to air dry, it was exposed to germicidal ultraviolet radiation (280 nm) for 20 min in order to link the sample RNA to the hybridization support medium. In addition, RNA extracted from SMSV-5 infected cell culture lysates and uninfected cells was also applied to the membrane as controls.

Dot hybridization was carried out according to the Hardy et al. (1985) modification of a protocol provided by Gibco BRL in the PhotoGene Nucleic Acid Detection System. The membrane was soaked in hot (65 C) prehybridization solution (1.0 M NaCl, 1.0% sodium dodecyl sulfate [SDS]) and placed in a hybridization roller bottle (National Labnet Company, Woodbridge, New Jersey, USA). Prehybridization solution was added to the bottle and the membrane was incubated at 65 C for 3 hr at six rotations per min. The prehybridization solution was removed and approximately 500 ng of boiled biotinylated probe was added to hot (65 C) hybridization solution (10% dextran sulfate, 1.0 M NaCl, 1% SDS, and 100 μg/ml denatured heterologous DNA) and incubated 48 hr at 65 C. After hybridization, the membrane was washed under high stringency conditions with 5× salt sodium citrate (SSC) solution (750 mM NaCl, 75 mM sodium citrate, dihydrate), 0.5% SDS at 65 C for 5 min, 2× SSC, 1% SDS at 65 C for 5 min, and 0.5× SSC, 1% SDS at 65 C for 30 min. The membrane was then rinsed in tris-buffered saline (TBS; 100 mM tris, pH 7.5, 150 mM NaCl), 0.05% Tween 20 (Sigma Chemical Company) and blocked with 3% bovine serum albumin (BSA), TBS, 0.05% Tween 20 for 1 hr at 65 C. A 1:1000 dilution of the provided streptavidin:alkaline phosphatase conjugate was applied to the membrane at 25 C for 10 min. The membrane was then washed twice with TBS, 0.05% Tween 20 at 25 C for 15 min and washed three times with Gibco BRL-provided final surfactant solution at 25 C for 20 min. The detection reagent (4-methoxy-4-[3-phosphatephenyl]spiro [1,2-dioxetane-3,2′-adamantane]; Gibco BRL) was applied to the washed membrane, and allowed to react for 3 hr at 25 C in the dark. Samples hybridizing to the probe were visualized by exposing X-ray film (X-Omat Xs-5; Eastman Kodak, Rochester, New York, USA) to the membrane for approximately 5 min.

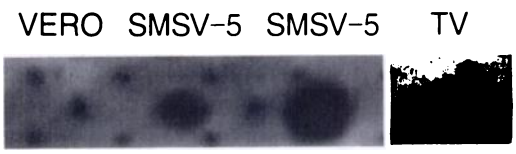

FIGURE 1. Dot hybridization with calicivirus group-specific cDNA probe 5RT73xr, against RNA extracted from 5 μl of vesicular fluid recovered from the foot of a white tern (*Gygis alba rothschildi*) on Tern Island, French Frigate Shoals, Hawaii. TV: RNA extracted from the tern vesicular fluid. Vero: RNA extracted from uninfected African green monkey kidney cells. SMSV-5: RNA extracted from Vero cells infected with San Miguel sea lion virus type 5 (SMSV-5). The left spot is a 1:5 dilution of the right spot.

RESULTS

Viral cytopathology was not observed in either cell line through four blind passages; however, the cDNA hybridization assay had a strong signal with the RNA extracted from the vesicular fluid (Fig. 1). Under negative stain electron microscopy, the vesicular fluid contained aggregates of virus-like particles with indistinct edges and cup-shaped surface depressions typical of antibody bound caliciviruses (Fig. 2).

DISCUSSION

This is the first report of a calicivirus associated with vesicular disease in a bird, and infecting a wild avian species. Caliciviruses, however, have been observed in various domestic avian species exhibiting signs of enteritis (Gough and Spackman, 1981; Wyeth et al., 1981; Gough et al.,

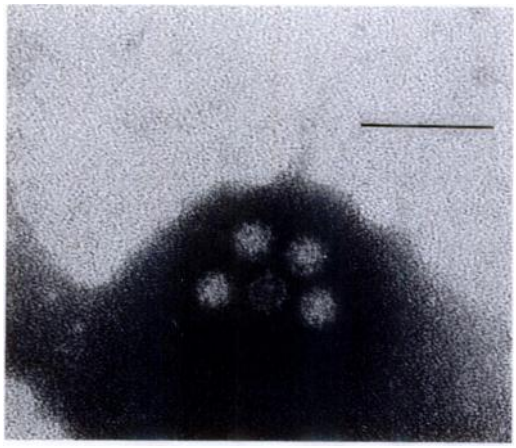

FIGURE 2. Negative stain electron micrograph of the vesicular fluid recovered from the foot of a white tern (*Gygis alba rothschildi*) on Tern Island, French Frigate Shoals, Hawaii. Virus-like particles with capsid projections and cup-like surface depressions can be morphologically identified as caliciviruses. Bar = 100 nm.

1992), and vesicular disease has been reported in wild sea birds (Kirkwood et al., 1995),

Antibody coating the capsid may explain the indistinct edge of the viral particles when viewed under the electron microscope. This was seen in immunoelectron microscopic studies with Norwalk virus and other caliciviruses (Smith et al., 1978; Kapikian and Chanock, 1990). Furthermore, an antibody coating may have contributed to the viral particles being refractory to in vitro growth. Unfortunately, a blood sample for serum antibody testing was not taken due to the small size of the tern.

Many caliciviruses are very difficult or impossible to adapt for growth in tissue culture. Rabbit hemorrhagic disease virus and European brown hare syndrome cause serious disease in their respective hosts, yet neither can be grown continuously in cell culture (Wirblich et al., 1994). Of the several caliciviruses associated with diarrhea in cattle, pigs, chickens, and humans, only porcine enteric calicivirus and chicken calicivirus can be grown in the laboratory (Flynn and Saif, 1988; Cubitt, 1987).

Single serotypes of ocean-origin caliciviruses can move great distances. In 1980, two of four bowhead whales (*Balaena mysticetus*), an Arctic species associated with the margins of the pack ice, had type-specific serum-neutralizing antibodies to two serotypes of vesicular exanthema of swine virus. These were VESV-J_{56} and VESV-K_{56}, that have been isolated only once, 24 yr previously, from pigs in New Jersey in 1956 (Holbrook et al., 1959; Smith et al., 1987). Furthermore, VESV-J_{56}- and K_{56}-neutralizing antibodies were found in 100% of adult California sea lions (*Zalophus californianus*) tested along the California coast in 1970 to 1972 and in 41% of pups tested in 1975 (Smith and Latham, 1978). The mechanism of transmission of VESV among mammals associated with Arctic pack ice, the southern California coast, and rural New Jersey swine farms was never determined.

Based on serological evidence, calicivirus serotypes have also been transmitted to monk seals (*Monachus schauinslandi*), a geographically isolated, endangered pinniped. The seal population is restricted almost exclusively to the northwest Hawaiian Islands (King, 1983). Serum samples from four animals collected in 1978, contained type-specific serum-neutralizing antibodies to SMSV-1 and VESV I_{55} (Smith et al., 1986). San Miguel sea lion virus type 1 (SMSV-1) was first isolated on San Miguel Island, California, from California sea lions in 1972 and reisolated from northern fur seals (*Callorhinus ursinus*) on St. Paul Island in the Bering Sea in 1973 (Smith et al., 1973). Based on a retrospective serological study of SMSV-1 antibody prevalence in sea lions, there was a dramatic increase from 1970 and 1971 to 1972. By 1975, however, the prevalence of serum-neutralizing antibody titers to SMSV-1 in 4-mo-old pups had dropped below the 1970 and 1971 values (Smith and Latham, 1978). While the high prevalence of SMSV-1 antibodies was short-lived, a mechanism existed by which monk seals,

over 5,000 km away, were exposed to this same virus.

Smith and Latham (1978) have suggested that a reservoir for ocean-origin caliciviruses may be a component of the sea lion diet. The isolation of SMSV-6 and SMSV-7 from a marine fish, the opaleye (*Girella nigricans*), with subsequent successful experimental transmission of SMSV-5 to fur seals (*Callorhinus ursinus*), using experimentally infected opaleye as a vector, is evidence for that hypothesis (Smith et al., 1980a, b).

Calicivirus-like particles that could not be grown in tissue culture as well as calicivirus RNA, determined by cDNA hybridization, were found in rectal and nasal swabs collected from monk seals at French Frigate Shoals during the same time as the tern vesicular fluid was obtained (Poet et al., 1993). A common food-chain-based reservoir may exist for the caliciviral agent that is infecting the tern and seal populations on French Frigate Shoals. The white tern eats an extremely wide variety of fish prey species (Ashmole and Ashmole, 1967). The monk seal on French Frigate Shoals has experienced a failure-to-thrive syndrome and a decrease in available food resources has been postulated as one of the causes (Gilmartin et al., 1993). This proposed shortage of food may have caused the seals to eat a wider variety of fish, and a common food source may have infected both birds and pinnipeds.

It is possible the tern acquired the infection iatrogenically. Fish used for feeding the tern were captured at night, and the bird was most likely infected by eating an unusual prey species. Transmission by direct spread from monk seals is also a possibility, but only one seal was sampled on Tern Island. Moreover, proper sanitation protocols were practiced after handling the seals. The tern, however, was found on the ground, after falling out of its nest. Thus, the animal probably was not behaving normally prior to human contact. Additional tern chicks were not examined for similar lesions.

The finding of a calicivirus associated with vesicular disease in the white tern is evidence that wild sea bird species may be an important mechanism for the transmission of caliciviruses over large areas. The potential for birds to transmit viruses pathogenic for marine mammal species is supported by the influenza A virus of probable waterfowl origin, that caused epizootics with high mortality in the harbor seal (*Phoca vitulina*) and pilot whale (*Globuicephala melaena*) on the east coast of North America (Geraci et al., 1982; Hinshaw et al., 1986). Further epizootiological studies involving sea birds of the eastern Pacific Ocean may shed light on the mechanisms by which caliciviruses are distributed throughout the world.

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