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Pathogenic caliciviruses are known to spread through diverse host species across broad geographic distances. Migratory and wide ranging ocean species, such as fish, pelagic birds, and marine mammals have been suspect in the spread of caliciviruses to naive and susceptible host populations, and bivalve mollusks have been linked to the transmission of caliciviral gastroenteritis in humans. San Miguel sea lion virus, type 5, because of its broad host range, was cloned in an attempt to identify a portion of the viral genome which could be used as a calicivirus group-specific hybridization probe. A 1671 base-pair cDNA clone was identified which hybridized with cell cultures infected with 28 different calicivirus serotypes, while not recognizing nucleic acid from other RNA viruses. Using this probe, mussels (*Mytilus californianus*) collected from rocks adjacent to pinniped rookeries were shown to contain calicivirus RNA, and San Miguel sea lion virus, type 17 (SMSV-17) was isolated from one sample, thereby implicating this invertebrate species as an important ocean reservoir for caliciviral agents. Furthermore, the cDNA hybridization assay detected calicivirus RNA in vesicular fluid collected from a white tern hatchling (*Gygis alba*) and calicivirus-like particles were observed by electron microscopy. This first report of a calicivirus infection associated with vesicular disease in a wild avian species, and the isolation of a calicivirus pathogen from mollusks, establishes the order Aves and the phylum Mollusca as reservoirs in the natural history of caliciviral disease.

Development and Diagnostic Applications of a Group-Specific Caliciviridae cDNA Hybridization Probe Cloned from San Miguel Sea Lion Virus, Type 5, a Calicivirus of Ocean Origin

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PREFACE

Dr. Alvin W. Smith, as principle investigator, appears as an author on the three papers in this thesis. Douglas E. Skilling, the senior research assistant at the Laboratory for Calicivirus Studies, Oregon State University, appears on the papers as well because of his outstanding talents in tissue culture, virus isolation, and electron microscopy techniques.

Dr. Robert L. DeLong, National Marine Mammal Laboratory, NMFS/NOAA, was instrumental in the acquisition of permits for the collection of biological samples on San Miguel Island, California, and appears as an author on Chapter Three. Jennifer L. Megyesi, a field researcher at French Frigate Shoals, Hawaii, appears as an author in Chapter Four, since her observation of vesicular lesions on the foot of a white tern chick she was rehabilitating, initiated the subsequent calicivirus finding.

Development and Diagnostic Applications of a Group-Specific Caliciviridae cDNA Hybridization Probe Cloned from San Miguel Sea Lion Virus, Type 5, a Calicivirus of Ocean Origin

CHAPTER ONE

The Caliciviridae

Steven E. Poet

INTRODUCTION

The Caliciviridae is a growing family of small, non-enveloped, single-stranded, positive-polarity RNA viruses, with morphologically distinctive cup-shaped surface structures. In the early 1930's, an outbreak of vesicular disease in a southern California swine herd was found to be similar in appearance to foot and mouth disease. The outbreak was subsequently recognized as a new disease of pigs named vesicular exanthema of swine (VES), and the etiologic agent was called vesicular exanthema of swine virus (VESV) (134, 248). Type A_{48} was first isolated in 1948 and is the type species for the calicivirus family (192). Other important members of the Caliciviridae include: San Miguel sea lion virus, which is indistinguishable from vesicular exanthema of swine virus, feline calicivirus, hepatitis E virus, rabbit hemorrhagic disease virus, and Norwalk virus. The candidate caliciviruses are a group of enteric agents which are morphologically similar to caliciviruses, but are not fully characterized because they cannot be replicated in tissue culture (49). Recent molecular studies with two of the candidate caliciviruses, Norwalk virus and hepatitis E virus, have provided evidence for their inclusion in the family Caliciviridae (105, 238).

Caliciviruses have a geographically and phylogenetically diverse host range. Characterized caliciviruses have been isolated from amphibians, fish, reptiles, marine mammals, terrestrial mammals, and humans (12, 13). Candidate caliciviruses have also been detected in swine (185), cattle (257), insects (110) and birds (49). Most continents on earth and even ice flows in the Chukchi Sea are sites of calicivirus isolation (171, 216, 218, 255, 259).

History of Caliciviruses

The calicivirus story began in 1932 with the report of a vesicular disease outbreak in southern California swine (12, 237). The outbreak raised serious concerns in the food animal industry because it was clinically identical to foot and mouth disease (FMD), an animal disease exotic to the United States, and vesicular stomatitis (VS). Vesicular exanthema of swine (VES) remained a significant problem in California, involving up to 50% of the swine herds. In 1952, VES crossed the California state line and became a nationwide epizootic. With the enactment and enforcement of legislation requiring the cooking of all garbage fed to swine and the expenditure of $39,000,000 in federal funds, VES was contained. The last outbreak was reported in Secaucus, New Jersey, in 1956. In 1959, vesicular exanthema of swine was officially declared eradicated, and was designated a foreign animal disease. Vesicular exanthema of swine has never been reported outside the United States with the exception of an outbreak, in 1955, at a U.S. military base in Iceland, which was traced to a shipment of contaminated U.S. pork products (12). Aside from linking the VES epizootics with the feeding of uncooked garbage, no reservoirs for the initial disease outbreaks were ever found.

San Miguel Island is the northernmost member of the Santa Barbara Channel Island chain off the coast of Southern California. In 1972, while studying an increased incidence of premature parturition in California sea lions (*Zalophus californianus*) on San Miguel Island, two serotypes of a single agent were recovered from a number of aborting females (207, 213). This agent, named San Miguel sea lion virus (SMSV), represented the first isolation of a virus from a pinniped species, and the second isolation of a virus from a marine mammal (13). Furthermore, SMSV could not be distinguished either physicochemically or morphologically from VESV (207, 233). When the new isolates were inoculated into pigs, vesicular lesions indistinguishable from VES were produced (207, 213).

Since 1972, a series of calicivirus isolates have been recovered from a variety of marine species, including: California sea lions (*Z. californianus*), Steller sea lions (*Eumatopias jubatus*), Northern fur seals (*Callorhinus ursinus*), Northern elephant seals (*Mirounga angustirostris*), Pacific walruses (*Odobenus rosmarus divergens*), common bottle-nosed dolphins (*Tursiops truncatus*), California gray whales (*Eschrichtius robustus*), and opaleye fish (*Girella nigricans*). All of the isolates are indistinguishable from the presumably eradicated VESV (13, 18, 198, 206, 207, 213, 218, 221, 223, 226, 228). In addition to the above list of known calicivirus marine hosts, other marine mammals exhibit type specific serum neutralizing antibody titers against a variety of marine calicivirus isolates, including the VES viruses, indicating previous infection with these agents (11, 15, 218, 219, 221). They include: Hawaiian monk seals (*Monachus schauinslandi*), bowhead whales (*Balaena mysticetus*), sperm whales (*Physeter macrocephalus*), and fin whales (*Balaenoptera physalus*).

Caliciviruses of ocean origin have also been isolated from several terrestrial animal species. In 1981, the Tillamook calicivirus (bovine calicivirus, BCV Bos-1) was isolated from a dairy calf on a coastal Oregon farm (212). San Miguel sea lion virus type 4 (SMSV-4) was isolated from a California swine herd in 1976 (13). Caliciviruses were isolated from the following species at the San Diego Zoo: Bell's horned frog (*Ceratophrys orata*), Aruba Island rattlesnake (*Crotalus unicolor*), rock rattlesnake (*Crotalus lepidus*), eyelash viper (*Bothrops schlegelii*), pygmy chimpanzee (*Pan paniscus*), spider monkey (*Ateles fuscipes*), lowland gorilla (*Gorilla gorilla*), silverleaf langur (*Presbytis cristata*), and douc langur (*Pygathrix nemaeus*) (209, 215, 217, 220, 224). The single serotype isolated from Bell's horned frog and the snakes, designated reptile calicivirus Cro-1 (RCV Cro-1), has subsequently been isolated from California sea lions, northern fur seals, and Stellar sea lions (204). Primate calicivirus (PCV Pan-1), the single serotype isolated from the primates, however, has not yet been shown to have a marine link. Domestic mink populations, many of which are sometimes fed ground fur seal carcasses or other marine food products, have also been documented to be calicivirus hosts (129, 189). Although no marine organisms have had mink calicivirus (MCV) isolated from their tissues, type specific serum neutralizing antibodies against MCV have been found in bottle-nosed dolphins (204).

Serological studies have shown that many wild and feral terrestrial animal populations, inhabiting southern California coastal areas, possess type specific serum neutralizing antibodies against a variety of caliciviruses isolated from marine species (205). Furthermore, domestic swine and cattle have been shown to possess neutralizing antibody titers against marine caliciviruses (218). Conversely, California sea lions, Hawaiian monk seals, bowhead whales, sperm whales,

California gray whales, and fin whales have been shown to have serum neutralizing antibody titers against a variety of VESV serotypes (218).

There is mounting evidence to suggest that the VESV outbreak which started along the southern California coast and spread inland, originated from a marine reservoir. Marine mammals that inhabit restricted geographical regions, such as the bowhead whale's circumpolar pack ice habitat, have been found to have serum neutralizing antibody titers to VESV-J_{56} and VESV-K_{56}. These VESV serotypes were only isolated once on a New Jersey swine ranch in 1956, 24 years before the discovery of type specific neutralizing antibodies in bowhead whales (219).

The number of new serotypes of marine origin caliciviruses continues to grow. To date, seventeen serotypes of SMSV have been characterized (202, 204). Given these numbers plus many more variants which have not been propagated *in vitro*, the ocean and its inhabitants represent a vast reservoir for this important pathogenic agent.

Feline Calicivirus

Feline caliciviruses (FCV), are responsible for a significant number of upper respiratory infections in cats (32, 58, 171, 237). Along with feline herpesvirus (FHV), FCV accounts for approximately 80% of upper respiratory tract infections in cats (90). Feline caliciviruses have been isolated worldwide and are probably infectious for most species of the Felidae family (34, 67, 90, 115, 150, 255). These agents were originally called feline picornaviruses even though early observations of an unique capsid structure cast doubts regarding this classification

(31, 32, 33, 260). Although many antigenic variants occur among FCV, it has been generally accepted that all are strains of a single serotype (171).

Feline calicivirus disease is most common in cats one to twelve months of age. Infection with FCV can result in acute, chronic, or subclinical disease. The clinical signs are usually characterized by conjunctivitis, rhinitis, tracheitis, pneumonia, and vesiculation or ulceration of the oral cavity epithelium. Fever, lethargy, and oculonasal discharge are also common signs. If uncomplicated by secondary infection, recovery usually occurs within five to seven days of the onset of clinical signs. The severity of the disease varies with the strain of virus. Death has been observed during the acute disease in kittens less than twelve weeks of age (237). Chronic stomatitis, acute and chronic faucitis, and lameness have also been reported in association with FCV infection (17, 116, 117, 175). Moreover, FCV has been reported to be linked with enteric disease and these intestinal associated strains have been shown to be resistant to bile salt inactivation (149).

After recovery from the acute stage of the infection, many animals will asymptomatically shed infective virus for prolonged periods of time. Cats which shed virus for periods of 30 days or longer are defined as persistently infected carriers (58, 237). The level of virus shedding varies with individual cats, and is not influenced by natural or artificial stress (253). Recent studies have shown that animals less than one year of age are more likely to become persistent shedders (90). The site of viral shedding in the cat is the oropharynx, and the virus appears to persist almost exclusively in the tonsillar tissue (58, 253). The reasons for viral localization in the tonsillar tissue, and the mechanisms for virus persistence are speculative. During the carrier state, very few foci of infection exist at any one time. This restriction of viral replication to a very limited number of cells

decreases the amount of tissue damage and viral invasion to a level which may be insufficient to induce inflammation or a cellular immune response. This characteristic of limited cellular infectivity may be dependent on genomic mutation and antigenic variability (58).

The introduction of an attenuated vaccine over the past few years has not prevented FCV from remaining a significant clinical problem in feline veterinary medicine. While the vaccine reduces the severity and duration of initial acute infections and appears to reduce the time of persistent shedding, it does not prevent disease or spread of virus to susceptible animals (240).

Rabbit Hemorrhagic Disease and European Brown Hare Syndrome Viruses

In 1984 an apparently unique viral disease of rabbits was reported in the People's Republic of China (127). The characteristics of the outbreak included severe hemorrhage in all organ systems, 100% morbidity, and 90% mortality (163). The disease has been subsequently reported in several countries including, Korea, Germany, France, Czechoslovakia, Spain, Austria, Belgium, Holland, Greece, and Denmark (153, 163, 200, 259). Initially, the identification of the etiological agent of this economically devastating disease was controversial. Picornavirus, parvovirus, and calicivirus were all implicated (251, 259). In 1990, Parra and Prieto (163) identified and recovered rabbit hemorrhage disease virus (RHDV), from the livers of rabbits showing clinical signs of the disease from a rabbitery in Spain. The agent was purified and characterized by electron

microscopy, protein electrophoresis, western blot analysis, and nucleic acid determination. All the results indicated that the etiological agent of RHD was a calicivirus (163).

A vaccine has been developed against RHDV using the organs of infected rabbits (200). The inactivated product has successfully protected susceptible animals 3 weeks after immunization with a single dose. Antibodies could be detected 5 days post vaccination. Interestingly, naturally acquired antibodies were detected in some farms indicating exposure to less virulent strains of RHDV or other antigenically related viruses (200).

Another, closely related, pathological agent of lagomorphs was reported in the 1980's to be the cause of mortality in wild and domesticated European brown hares (*Lepus europaeus*), (170). This recently observed disease syndrome, European brown hare syndrome (EBHS), has been diagnosed in all western European countries, and has produced signs similar to that of RHDV, with the exception of the presence of disseminated intravascular coagulopathy being absent in EBHS (72, 153). The etiological agent of EBHS was determined to have similar morphological and physicochemical properties to that of the caliciviruses, and was antigenically related to RHDV when immunoelectronmicroscopy was performed with hyperimmune antisera against RHDV. Although antigenically related, RHDV and EBHS virus (EBHSV) possess distinct differences in surface epitopes, cross-protection, and transmission (43, 153, 170).

Retrospective serological and virological studies involving RHDV and EBHSV have provided evidence that sub-clinical infection by both of these agents has occurred for many years before clinical disease was observed. Particles morphologically identical to RHDV have been observed in rabbit livers collected in

1982 (153). Antibody titers against RHDV have been found in rabbit sera collected 12 years before the first reported outbreak of RHD in China (200). Furthermore, serological studies of European brown hares revealed the existence of animals seroconverting against EBHSV as early as 1971, indicating hares may have been infected with EBHSV long before RHDV was identified in rabbits (153).

Caliciviruses Infectious for Humans

Caliciviruses cause a number of disease syndromes in humans, the majority of which are enteric in nature. An electron microscopic survey of infantile gastroenteritis in the United Kingdom revealed that human calicivirus (HCV), and Norwalk agents accounted for 5% and 4% of viral diarrhea, respectively (49). Similar studies in other countries have shown that sporadic cases of calicivirus associated gastroenteritis have occurred throughout Europe, North America, Saudi Arabia, India, Japan, and Australia (24, 49, 60, 70, 84, 124, 137, 151, 152, 234).

Norwalk virus was first reported in 1968 as a disease outbreak of winter vomiting at a secondary school in Norwalk, Ohio (2). Similar Norwalk-like agents have been associated with other outbreaks of gastroenteritis. These agents, Snow Mountain, Hawaii, Sapporo, Southampton, and Tauton have been reported to cause both epidemiologically and clinically similar disease to that of Norwalk virus (118, 122, 135). Since none of the Norwalk-like agents have been cultivated in tissue culture systems, virus propagation by infecting human volunteers was necessary. These studies showed that similar 27-39 nm diameter virus-like particles were detectable and reproduced the disease when passaged fecal-orally in

volunteers (60). Norwalk virus does not always exhibit the characteristic capsid morphology of the caliciviruses. Alterations in the morphology may be due to inadvertent specimen abuse, or proteolytic digestion within the gastrointestinal tract (95). While some of these agents are immunologically cross-reactive, others are not. An array, therefore, of antigenic variants exist, making it difficult to fully characterize individual disease outbreaks. Serum antibodies as well as secretory gastrointestinal tract antibodies do not reliably protect volunteers against experimental infection with Norwalk virus. Resistance to infection does occur, but does not correlate well with specific antibody levels (24). The Norwalk-like agents are frequently associated with disease in adults and teenage children, but not in infants and young children (49). This group of viruses, most commonly transmitted by the fecal-oral route, has been implicated in gastrointestinal disease associated with contaminated food and water supplies (124, 179). Furthermore, Norwalk virus has been shown to be present in shellfish and to cause widespread outbreaks of gastroenteritis in individuals consuming shellfish raw (92, 152). Replication within the shellfish, however, has not been shown to occur.

Human caliciviruses were first observed in human fecal specimens in 1976 (133). These less understood agents have not undergone the experimental infectivity studies that occurred with Norwalk virus, and detection of the virus has depended solely upon electron microscopic observation and immunoassay techniques (24). There are at least 5 serologically distinct strains of HCV, but a group specific antigen appears to be present which is detectable by immunoassay (52). Specific antibodies against HCV has been shown to provide protection against infection (49).

Two different patterns of infection have been reported with HCV. One group of viruses commonly affect infants, causing signs of vomiting, diarrhea, fever, and sometimes upper respiratory tract disease. The duration of this disease syndrome is between one and eleven days and is transmitted by the fecal-oral route. Most adults exhibit high levels of serum and secretory antibodies against these viral strains and are protected from infection even when exposed to infected children (49).

Other HCV strains affect individuals of all age groups, causing signs of fever, malaise, aching limbs, and nausea, with limited signs of gastrointestinal origin. The duration of infection is much shorter at one or two days. One of these strains, HCV-UK3, may have originated from a vomiting dog (101). Not unlike the infection pattern of Norwalk virus, another strain, HCV-UK4, has been associated with consumption of raw oysters, contaminated cold foods, and water. It has been suggested that HCV can be spread by food handlers, and carriers may exist who periodically shed virus in a manner similar to feline calicivirus (49).

Other similarities exist between Norwalk virus and human calicivirus. Individuals associated with outbreaks involving contaminated water or foodstuffs have produced antibodies to both Norwalk virus and HCV. A rise in antibody titer to Norwalk virus following HCV disease indicates that at least minor cross-reactivity occurs between these two agents (60).

Enterically transmitted non-A non-B hepatitis (ET-NANBH), first described in India in 1980, is an acute viral hepatitis disease (111, 256). Outbreaks of ET-NANBH usually affect young to middle-age adults in developing countries around the world (27). Sporadic disease has been reported in Western countries, but travel in endemic areas has been associated with these cases (197). Although ET-

NANBH is usually a self-limiting disease, there is an extremely high, 20%, case fatality ratio in pregnant women (27, 44).

Acute hepatic disease that could not be attributed to hepatitis A or B viruses was grouped into two categories, parenterally and enterically transmitted disease. In 1983, 27 to 30 nm virus-like spherical particles were visualized, from pre-clinical and early post-clinical stool samples in experimentally infected volunteers immune to hepatitis A virus, using immunoelectron microscopy. This lead to the postulation of a virus etiology of non-A non-B enterically transmitted hepatitis (6). Bradley and co-workers (28) confirmed the presence of 32 nm virus-like particles in the stools of patients associated with outbreaks of ET-NANBH in the Soviet Union, North Africa, and North America and further characterization indicated that the virus was similar morphologically and physicochemically to Norwalk virus (28). The etiological agent associated with enterically transmitted hepatitis has been renamed hepatitis E virus (HEV). Hepatitis E accounts for one third of acute non-A, non-B, and non-C hepatitis seen in Hong Kong, and co-infection with hepatitis A can occur. As with other calicivirus disease, contaminated shellfish sources have been implicated in HEV outbreaks (128).

Pathogenesis studies of HEV have involved the use of several different species of non-human primates, most commonly cynomolgus macaques (6, 27). Studies using human volunteers have also been performed (44). In both the monkey model and man the virus can be observed in serum as well as feces before the onset of clinical signs, indicating the possibility of parenteral transmission of HEV in addition to enteric transmission in endemic areas (44). There is potential, therefore, for HEV related post-transfusion hepatitis, indicating the need for a screening test of donor blood.

As with a large number of caliciviruses, HEV has not been successfully cultivated in the laboratory. Kazachkov and co-workers (109) have reported the presence of HEV RNA and antigen, but not fully assembled viral particles, associated with serial passage in fresh fetal rhesus monkey kidney cells after co-cultivation with primary cynomolgus kidney cells from experimentally infected animals (109). Unfortunately, detection and recovery of HEV particles has not been reported. A preliminary report describing the tissue culture isolation of a calicivirus from an outbreak of HEV in China has been published (98). Immunoelectron microscopy studies using antisera from a known HEV-infected chimpanzee demonstrated viral particle aggregation at only low dilutions of antisera. In addition, the acute phase serum from affected patients reacted with this calicivirus isolate while the convalescent serum from the same patient did not, indicating that the calicivirus isolate was not responsible for the hepatitis outbreak in China (98). Even though the laboratory isolation of a calicivirus from humans is a significant finding, further work is required to definitively determine if this agent is a strain of HEV.

Avian Caliciviruses

Calicivirus-like particles have been observed in association with gastrointestinal disease in three avian species: chickens, pheasants, and guinea fowl (26, 80, 81). One to two week old broiler chickens exhibited signs of stunting and poor feather growth, and never obtained the body condition of unaffected chicks, resulting in significant economic loss to the producer (26). A variety of pathological changes associated with growth inhibition have also been

reported including, lowered plasma carotenoid levels, increased plasma alkaline phosphatase levels, distended gall bladder, osteoporosis, and skeletal angular deformities. Infectious stunting of chickens, helicopter disease, pale bird syndrome, and runting disease have been used to describe this suspected calicivirus disease (146). Furthermore, guinea fowl chicks hatched with broiler chickens exhibited 50% mortality due to a sudden onset typhlitis associated with central nervous system signs. Individuals hatched apart from chickens did not show any signs of disease. Calicivirus-like particles were observed upon electron microscopic examination and the disease could be experimentally reproduced from affected cecal contents (80). Calicivirus-like particles have also been observed in the intestinal contents and feces of three to four week old pheasants with low-grade enteritis (81). Presumptive evidence does exist for the implication of calicivirus infection in enteric disease of young birds.

Cubitt and Barrett (51), reported growing chicken calicivirus in tissue culture with the addition of trypsin to the medium. Unfortunately, virus yields from *in vitro* cultivation were extremely low and did not produce enough virus for biochemical characterization.

Enteric Caliciviruses of Livestock

Viruses with calicivirus morphology were first reported in association with diarrhea in neonatal pigs in 1980 (29, 185). This virus was antigenically different from vesicular exanthema of swine virus and feline calicivirus. Saif and co-workers (185) demonstrated that porcine enteric calicivirus replicates in villous

epithelial cells of the proximal small intestine, producing villous atrophy and subsequent diarrhea in gnotobiotic piglets (69).

Porcine enteric calicivirus (PECV) is difficult to grow in tissue culture. Flynn and Saif (68) used a preparation of large intestine contents to successfully propagate PECV in primary porcine kidney cells. The growth factor in the intestinal contents preparation was not determined. Medium supplements, such as trypsin, which allowed the propagation of human and chicken caliciviruses did not enhance PECV replication in cell culture (50, 51, 165).

The Newbury agents are calicivirus-like particles which have been shown to cause anorexia, diarrhea, and xylose malabsorption in 16 to 60 day old calves (257). Like porcine enteric calicivirus, the Newbury agent will induce villous atrophy in the anterior small intestine (89). There are two antigenically distinct Newbury agents, SRV-1 and SRV-2, which fail to cross-protect experimentally infected animals (30). Efforts to replicate Newbury agents in cell culture have been unsuccessful.

Canine Caliciviruses

Caliciviruses have been observed or isolated from dogs with glossitis, gastroenteritis, and vesicular genital disease (47, 65, 66, 194). The viruses isolated from dogs exhibiting signs of gastrointestinal disease varied antigenically. One isolate, which readily grew in canine cell lines and a dolphin cell line was shown to be most antigenically similar to the avian calicivirus associated with stunting disease in chickens (194). Other canine enteric calicivirus isolates, which grew in feline cell lines were shown to be most antigenically similar to feline calicivirus

(66). In both instances, a definitive etiological link between calicivirus presence and canine gastrointestinal disease could not be made.

Vesicular lesions and ulcerations have been reported in dogs infected with calicivirus. Vesicular exanthema of swine virus intradermally injected into the tongues of experimental dogs produced erosions at the site of inoculation and fever (10). Crandell (47), reported the isolation of two antigenically related caliciviruses from genital vesicles of two different dogs, sampled four years apart. These agents were shown to be antigenically distinct from other characterized feline and canine caliciviruses. As with feline calicivirus, canine caliciviruses are thought by some to remain subclinical until the host is stressed, leading to a decreased resistance against the preexisting viral infection, resulting in glossitis (65, 184, 236). Calicivirus infection in the dog appears to span all categories of pathogenesis reported for this virus family, involving vesicular, respiratory, reproductive, and enteric disease.

Insect Caliciviruses

Calicivirus-like particles have been reported in association with a variety of insect species. A group of five small isometric viruses that infect larvae of saturniid and limacodid moths, the nudaurelia ß group, were found to possess one major capsid polypeptide and are morphologically similar to caliciviruses, but have differences in capsomer architecture and genome size (130). Cricket paralysis virus, although classified as an insect picornavirus due to its four major capsid polypeptides, was found to have a genome organization more similar to

caliciviruses than picornaviruses, with the capsid protein genes located at the 3' end of the RNA molecule (120).

Kellen and Hoffman (110), reported the isolation of a chronic stunt virus from the navel orange worm (*Amyelois transitella*), from almonds in northern California. This virus could be orally transmitted to other larvae, was highly virulent to neonate larvae, and produced chronic infections in third and fourth stage larvae causing severe stunting, anorexia, and death. Sublethal infections, although rare, produced undersized pupae and subsequently stunted adult moths. Amyelois chronic stunt virus (ACSV), was characterized further and Hillman and co-workers (95) found not only a virus-like particle with morphological and physicochemical similarities to mammalian caliciviruses, but also a second, and more numerous particle which lacked calicivirus morphology. This second, 28 nm, virus could be produced by *in vitro* α-chymotrypsin degradation using the 38 nm calicivirus-like particle, demonstrating that the calici-like virus could be converted to the equally infectious smaller virus. Many human enteric caliciviral-like agents, including Norwalk virus, Hawaii agent, and Montgomery agent, have also been reported as small, round viruses with particle diameters of approximately 28 nm (53, 54, 59, 242). Due to protease degradation of viral particles in fecal extracts, a definitive diagnosis of calicivirus infection by electron microscopy is difficult.

Calicivirus Proteins

One of the hallmark characteristics of the Caliciviridae family is the presence of only one major capsid polypeptide (35, 191). One hundred and eighty copies of this peptide are arranged in an icosahedral pattern of T=3 lattice symmetry to

produce a virus particle with the unique, cup-shaped surface structure that is seen with negative stained transmission electron microscope preparations (37). Amino acid analysis of the capsid polypeptide of three serotypes of San Miguel sea lion virus revealed statistically significant differences in composition (232), linking caliciviral immunogenic diversity and viral capsid protein structure. A wide variety of major structural protein molecular weights have been reported for different members of the family (Table 1.1). Most of the values are within the 58 to 62 kilodalton range. Some, however, considerably less than 58 kilodaltons, have been reported in small round structured (Norwalk-like) viruses, ameliosis chronic stunt virus, and porcine enteric calicivirus (82, 95, 161, 166). Hillman and co-workers (95) postulated that proteolytic degradation may have caused the production of the smaller protein, since *in vitro* digestion of the native virus could produce the smaller constituent. Because the other viruses reported to have similar protein size properties are also located in the gastrointestinal tract, proteolytic digestion may explain the smaller molecular weights or presence of a smaller, soluble protein. Oishi and co-workers, however, could not demonstrate the production of smaller proteins with *in vitro* proteolytic digestion when Norwalk-like small round structured viruses with major structural polypeptides of 33 kilodaltons were seen in 3 of 14 human acute gastroenteritis samples containing characteristic calicivirus morphology. The other 11 samples contained virus with a 63 kilodalton major structural protein. The two proteins shared antigenic determinants, indicating an as yet undetermined structural relationship (161). Rodak and co-workers (181) have reported the presence of a 38kD structural protein, based on immunological studies in RHDV as well. A recent study, using electron cryomicroscopy of primate calicivirus has shown that the structure of the viral capsid is made up of

protein dimers arranged in 90 units to form the calicivirus shape (173). The dimer forms a loop which may correspond to the characteristic projections often seen on the virus capsid using conventional electron microscopy.

Picornaviruses and caliciviruses both possess a protein (VpG), which is covalently linked to the 5' end of the viral genome. This small protein, approximately 14-15 kilodaltons, is required for genome infectivity only in the caliciviruses and has been shown to exist in VESV, SMSV-type 2, SMSV-type 4, and RHDV (36, 144, 193).

Attempts to elucidate the properties of the intracellular proteins synthesized by calicivirus infection have been mostly limited to viruses which can be grown in cell culture. Most information on nonstructural proteins of caliciviruses has been generated using feline calicivirus (FCV)-infected cells (38). A limited amount of work has been done with VESV, SMSV, and PECV (21, 71, 166), while Rodak and co-workers (181) and Greenberg and co-workers (82) have reported virus specific proteins in clinical samples.

Black and Brown (20) showed that VESV-infected cells did not contain large precursor polypeptides, unlike the monocistronic translation and post-translational cleavages in the picornaviruses. Later studies by Black and Brown (21) demonstrated that three polypeptides (100kD, 80kD, and 65kD) were produced simultaneously by VESV- and FCV-infected cells. The larger viral proteins were shown not to have a precursor product relationship with the predominate 65kD viral capsid polypeptide. Using protease inhibitors, it was shown that only the production of the 100kD polypeptide was inhibited and the accumulation of a 120kD polypeptide was observed, indicating that the 120kD

polypeptide is a precursor to the 100kD product and that the 80kD and 65kD products were produced from different messages.

Fretz and Schaffer (71) showed that SMSV, type 2-infected cells produced six virus-specific proteins: 135kD, 80kD, 60kD (capsid protein), 40kD, 35kD, and, 29kD. Temperature-shift experiments to inhibit proteolytic cleavage of precursor proteins enhanced the production of the 135kD polypeptide and produced a new product with the molecular weight of 86kD. Tryptic peptide maps of the 86kD and 60kD proteins were similar, indicating that the 86kD polypeptide is a precursor for the viral capsid polypeptide. In addition, the 135kD protein was found to have amino acid sequences in common with the 80kD protein.

Komolafe and co-workers (119) reported the presence of five virus specific proteins (early 80kD, late 80kD, 68kD capsid, 40kD, and 14kD) in FCV-infected cells. Because the 14kD protein reacted weakly with feline immune serum, it was postulated that a minor capsid protein may exist for caliciviruses. It is more likely, however, that the feline antiserum may have been reacting to the VPg protein known to be covalently bound to the calicivirus genome (36, 192). No precursor proteins of approximately 100kD were seen in this study, but Komolafe and co-workers suggested that the 80kD and 40kD proteins were enzymes required for the replication and assembly of the virus. Furthermore, they suggested the possibility of temporal regulation of protein synthesis with the observation of two 80kD proteins appearing at different times in the replication cycle of the virus, one at 120-180 minutes post-infection and one at 210-640 minutes post-infection.

Carter (38) employed western blotting with feline polyclonal antiserum and murine anticapsid monoclonal antibodies to further investigate the synthesis of

feline calicivirus induced protein synthesis, and suggested that caliciviruses have two phases of protein synthesis. The first phase of protein synthesis begins with the production of a 75kD protein which accumulates throughout the entire infection cycle, and a transient 73kD protein which disappears by 3 hours post-infection. The second phase of protein synthesis begins with the production of large precursor proteins (125kD, 123kD, 98kD, and 76kD capsid precursor) at 3 hours post-infection. Due to their accumulation as the infection progresses, the 96kD, 75kD early protein, 39kD, 36kD, and 27kD proteins are postulated to be mature calicivirus proteins. Only the capsid protein, however, can be proven definitively to be a mature protein since the function of the other calicivirus polypeptides are unknown.

Since subtle gel and voltage parameter differences can alter electrophoretic mobilities of identical proteins, data from the cultivatable caliciviruses studied have described similar protein synthesis processes. This process of a temporally regulated, rapid, proteolytic cleavage maturation of virus induced proteins is far from being definitively characterized, however. Candidate calicivirus protein synthesis is virtually unknown.

Using liver homogenates of uninfected and infected rabbits, Rodak and co-workers (181) determined the presence of up to 14 viral antigens, ranging in size from 61kD to 28kD, using western blot analysis and hyperimmune polyclonal rabbit antisera from three different sources. Of the 14 antigens, three proteins of 61kD (capsid), 52kD, and 38kD, were dominant. Parwani and co-workers (166) reported the existence of four virus-induced proteins (82kD, 58kD capsid, 32kD, and 28kD) in PECV, and these values correlate well with protein sizes reported for the easily grown caliciviruses.

Because of their narrow host range, their presence in low titers in clinical specimens, and their unadaptability to tissue culture, the human caliciviruses and their induced proteins are virtually unstudied with the exception of the viral capsid protein. Greenberg and co-workers (82) reported the presence of a 30kD soluble protein in fecal specimens, in addition to the 59kD viral capsid protein, containing Norwalk virus. The function of the 30kD protein was not determined. Another Norwalk-like, small round structured virus detected in Japan was found to have a single major structural protein of 33kD (161). Terashima and co-workers (239) reported the existence of a 62kD protein in the fecal material of an acute gastroenteritis patient infected with human calicivirus.

Most of the information on caliciviral-induced proteins takes the form of the number and size of the different entities rather than their function and fate in viral replication. Very few of the caliciviruses have been studied at the molecular level. A summary of the known size and function information of all viral proteins examined are provided (Table 1.2).

The Calicivirus Genome

The understanding of the nature, coding, and replicative strategy of the calicivirus genome is still in its infancy. Wawrzkiewicz and co-workers (254) reported that VESV contained single-stranded RNA which was infectious. This conclusion was confirmed by other researchers in 1971 (159). Physicochemical investigations of SMSV by Schaffer and Soergel (190) reported the presence of single-stranded RNA, as well. In addition, the molecular weight of the SMSV RNA was reported to be 2.6×10^6 daltons (190). Love (131) reported

the presence of a single 32-35S RNA in FCV infected cells which was sensitive to RNase degradation. Love also noted a RNase resistant fraction sedimenting at 18S, and assumed this fraction to be double-stranded RNA (dsRNA) similar to the replicative intermediates seen in picornavirus.

Ehresmann and Schaffer (63) first reported the presence of multiple classes of RNA synthesized within cells infected with either SMSV-type 2, VESV-type A_{48}, or FCV, suggesting that molecular evidence exists for the removal of the caliciviruses from the family Picornaviridae. Utilizing glycerol sedimentation experiments, two major RNAs, 36S and 22S, were found in cells infected with calicivirus. They also identified a polyadenylic acid (Poly A) tract within the viral genome by demonstrating that the RNA would bind to oligo (dT) cellulose. Double-stranded as well as partially double-stranded RNA was also observed, using RNase digestion protection, in virus-infected cell cultures which was postulated to be replicative intermediate and replicative forms of the viral genome. In addition to the major RNA molecules detected, Ehresmann and Schaffer (63) also noted the presence of smaller, minor RNA components which could not be adequately resolved under the electrophoretic methods used.

Black and co-workers (22) studied calicivirus RNA structure and replication using VESV as a model. As with picornaviruses, no unusual nucleotide grouping, or cap, was found at the 5' end of the VESV RNA. Burroughs and Brown (36) , in conjunction with Black and co-workers (22) , reported the presence of a covalently linked protein at the 5' end of the viral genome, mimicking the general structure of picornaviral RNA. Black and co-workers (22) also reported the presence of three distinct species of induced RNA in VESV infected cells. These single-stranded RNAs, (37S, 22S, and 18S) were all shown to have poly (A) tracts

and would hybridize to viral-specific dsRNA in RNase protection assays. Because of the number of other RNA molecules with Poly (A) tracts at their 3' ends, Black and co-workers (22) assumed this was the location of the tract in calicivirus RNA as well. By using *in vitro* translation of the calicivirus-induced RNA in a reticulocyte cell-free assay, it was shown that the 22S RNA was translated into a polypeptide which had a similar electrophoretic mobility to the caliciviral capsid protein. The 37S and 18S RNA components did not seem to act as mRNAs in this experiment. Later experiments by Ehresmann and Schaffer (64) confirmed the absence of a 5'-cap on the RNA of SMSV-type 2. They also observed two double-stranded RNA components of the viral induced nucleic acid. These dsRNA species corresponded to the replicative forms of the 36S genomic RNA and the 22S subgenomic RNA. Early studies, therefore, placed the caliciviruses in the Baltimore Class IV group of animal viruses, but with a unique replication strategy (7).

With the arrival of gene cloning techniques, came an increase in information regarding the molecular genetics of caliciviruses. These techniques, while extremely useful in confirming and broadening the information about cultivatable calicivirus replication strategy, have greatly benefited researchers working with the subgroup of caliciviruses that cannot be grown in cell culture. Neill and Mengeling (157) used a cDNA clone of FCV to probe poly(A)-containing northern blots of FCV infected cells and found four viral specific RNA species (8.2Kb genomic, 4.8Kb, 4.2Kb, and 2.4Kb). The 8.2 and 2.4 Kb molecules correspond to the 36-37S and 22S molecules observed in earlier studies (22, 63, 64), and were also shown to be associated with double-stranded RNA structures. Although replication of the 2.4 Kb RNA, which codes for the viral capsid protein (22), was

speculated to be a unique property of calicivirus replication, it could not be shown that the subgenomic dsRNA was actually replicating (157). Studying RHDV, Boga and co-workers (25) were able to show a similar phenomena in which the 2.4 kb subgenomic viral RNA could be translated *in vitro* to produce the viral capsid protein. Using cDNA probes derived from different locations within the FCV genome, Neill and Mengeling (157) determined that the viral RNAs were nested co-terminal transcripts with common 3' poly(A) ends.

Carter (39) reported the existence of eight RNA molecules induced by FCV. By using negative sense and positive sense single-stranded cDNA probes, it was shown that five viral induced RNA molecules (7.1 Kb, 5.3 Kb, 4.3 Kb, 3.6 Kb, and 2.7 Kb) were present as negative sense RNA within the cell, indicating production of subgenomic messages during calicivirus replication, the most significant being responsible for the translation of viral capsid protein, which is similar in strategy to coronaviruses, toroviruses, and arteriviruses (187, 196, 231). The five viral RNA's were also present as positive sense molecules. Also reported as exclusively positive sense viral RNA were molecules of 1.9, 1.5, and 0.55 Kb in length.

Furthermore, subgenomic RHDV RNA has been reported to be present in infected rabbit livers (144, 160) and subgenomic HEV RNA in the livers of experimentally infected cynomolgus macaques (238). In addition to the 8 Kb genomic RNA, a 2.2 Kb RNA molecule was also observed in liver homogenates of rabbits which died from rabbit hemorrhagic disease. In HEV infected monkey livers, three viral-induced RNA's could be observed, the 7.6 Kb genomic, 3.7 Kb, and 2.0 Kb molecules, in which the 2.0 Kb RNA was the predominant subgenomic transcript.

Other minor RHDV-induced RNA molecules were sometimes seen, but could not be confirmed since RHDV does not grow in cell culture systems (144). Interestingly, Meyers and co-workers (144) also observed that the 2.2 Kb subgenomic RNA was also packaged into viral particles. This has never been seen before in caliciviruses. Differences in properties of the viral RNA among different caliciviruses point out the need for comparative sequence analysis among pathogenic calicivirus strains and between the many different caliciviruses known to exist in marine and terrestrial environments throughout the world.

Sequencing of all or almost all of the viral genome has been accomplished for FCV, RHDV, three strains of HEV, and the Norwalk-like Southampton virus (40, 99, 122, 145, 238, 250). In all cases the size of the genome was between 7.2 and 7.7 Kb in length. All caliciviruses studied to date , with the exception of RHDV, contain three open reading frames (ORF), two large ORF's corresponding to the nonstructural proteins and the viral capsid protein, and one small ORF of unknown function (Figure 1.1A), but is speculated to play a role in the packaging of the viral genome, or other nucleic acid binding activity, and may represent the VPg protein gene (99, 156). Rabbit hemorrhagic disease virus contains only two ORF's (Figure 1.1B), one large ORF encompassing both non-structural and structural proteins and the small ORF seen in other caliciviruses (145). The genomic organization for all caliciviruses is consistently 5' non-structural/3' structural (Figure 1.1), which is similar to togaviruses, coronaviruses and opposite to picornaviruses (183, 238). Differences in genomic sequence and organization do exist, however, especially between HEV and the other caliciviruses (Figure 1.1C), where the small ORF, which is located at the 3' end of the viral genome in all other caliciviruses, is located between, and overlapping, the nonstructural and

structural ORF's, in HEV (99). Interestingly, a small, fourth ORF has been described in FCV which is located in a similar position as the small ORF in HEV, but this ORF is in addition to the ORF 3 at the 3' end of the viral genome (40). A similar fourth ORF has also been described in the Norwalk-like Southampton virus (122).

The non-structural protein region (Figure 1.1) of the calicivirus genome has been found to have similarities with picornaviruses and other positive sense single-stranded RNA viruses (121). An RNA dependent RNA polymerase motif, glycine-aspartate-aspartate (GDD, Figure 1.1), which can be found in all positive sense single-stranded RNA viruses, has been shown to exist at the 3' end of the non-structural gene in FCV, HEV, RHDV, Norwalk virus, and the Norwalk-like Southampton virus (105, 122, 145, 155, 176, 177, 238, 247). In addition, other picornavirus-like protein motifs involved in viral replication have been demonstrated by nucleic acid sequence translation within the non-structural protein gene of many calicivirus members. These protein motifs include, 2C-like helicase and nucleotide binding site (2C, Figure 1.1), and a 3C-like cysteine protease (3C, Figure 1.1), which are translated in the same order, along with the RNA polymerase, as picornaviruses (122, 145, 155, 238). Unfortunately, the function of much of the non-structural protein gene of caliciviruses is not well understood. Gaps within the nucleic acid sequence have an unknown function and post-translational processing mechanisms have not been determined (Figure 1.1).

All or part of the structural polypeptide coding region within the viral genome has been cDNA cloned and studied in FCV (40, 87, 158, 244), SMSV-type 1 and -type 4 (156), RHDV (145, 148), Norwalk-like Southampton virus (122), and HEV (99, 238, 250). In all cases, the region coding for the capsid

protein or immunoreactive components is located at the 3' end of the genome. The large ORF suspected of coding for the capsid polypeptide was determined to encode a polypeptide of a size similar to the reported capsid precursor protein molecular weight of 76 KDa (39, 158, 244). This coding region also lies within the abundant, 2.2 - 2.7 Kb, subgenomic RNA found in calicivirus infected cells and provides further evidence that caliciviruses use subgenomic messages as a replication strategy (40, 145).

Sequence comparison studies have been carried out for most of the cloned caliciviruses. Early hybridization studies with two serotypes of VESV, SMSV-type 1, and FCV led researchers to conclude that SMSV was closely related to VESV and not related to FCV (37). This conclusion was supported by immunodiffusion and tryptic peptide analysis of the three caliciviruses, as well. Smith and co-workers (229), using immunoelectron microscopy, found that 8 of 9 VESV serotypes showed common antigenicity with SMSV, while 2 of 9 showed common antigenicity with FCV type F-9. Conversely, Smith and co-workers (229) reported that 12 marine calicivirus isolates displayed common antigenicity with VESV while not reacting with FCV. Burroughs and co-workers (37) also noted that one serotype of VESV (type D) was more closely related to FCV than VESV-type E, yet VESV-type E shared antigenic determinants with SMSV. These findings suggested that a complex array of similarities and differences, involving structural, physicochemical, antigenic , and genetic properties, may exist throughout the entire calicivirus family.

As the nucleic acid sequences of caliciviruses began accumulating in the various data banks, comparisons of different agents both cultivatable and non-cultivatable, revealed areas of significant genome homology as well as

hypervariable regions. Studies comparing the genomic sequences of two different strains of FCV indicated that the extreme 3' end of the RNA molecule is highly conserved (244). Strong homology was also seen among FCV strains studied in the non-structural protein region of the genome. Tohya and co-workers (245) also reported significant amino acid sequence homology of the capsid precursor protein with the VP3 capsid protein of picornaviruses. Carter and co-workers (40) reported a similar nucleotide identity between both non-structural and structural genes in two strains of FCV. The translated amino acid sequences were even more similar, and sites of potential antigenic variation among FCV strains were suggested to occur in the central region of the mature capsid protein.

Milton and co-workers (148) found, when comparing the 3' ends of RNA from different geographical isolates of RHDV that all the isolates were very closely related to one another. When these isolates were compared to sequence data from similar regions of the FCV genome it was found that capsid protein sequences of RHDV differed markedly from FCV. Interestingly, the extreme 3' ends of both RHDV and FCV were conserved, indicating that the small ORF 3 may have a functional role in calicivirus replication.

Neill (156) studied the amino acid sequences of the capsid protein gene in two serotypes of SMSV. The capsid proteins of the two serotypes were reported to be 73% homologous with one another. When the capsid protein sequences were compared with FCV and RHDV, regions of similarity and hypervariability were found. Three regions of similarity existed for the three different viruses while one region was well conserved between the SMSV serotypes but not with FCV or RHDV. A hypervariable region was identified with the capsid protein which may contain the serotype-specific antigenic determinants. One of the well conserved

regions displayed sequence similarity to the picornavirus VP3 capsid protein as reported by Tohya and co-workers (245). The ORF 3 of SMSV also displayed a significant similarity in amino acid sequence to FCV.

Lambden and co-workers (122) reported that the capsid encoding region of the Norwalk-like Southampton virus displayed marked amino acid sequence similarity when compared with a small region of Norwalk virus genome known to be immunogenic (138). Additionally, there was limited capsid protein amino acid sequence homology between this virus and FCV and RHDV. A fourth ORF, overlapping ORF 2 and ORF 3, has also been reported for Southampton virus, but has a different location in FCV and HEV, where it overlaps ORF 1 and ORF 2. This newly recognized, uncharacterized region in the genome may play an important role in how the Caliciviridae are organized phylogenetically.

Sequence analysis has also been performed on the region encoding the non-structural proteins for FCV, RHDV, Norwalk virus, Norwalk-like Southampton virus, and HEV (40, 99, 105, 121, 122, 145, 250). Sequence similarity studies within the region encoding for the RNA polymerase have shown that FCV and RHDV are more closely related to one another than to Norwalk virus and HEV (145). Norwalk virus, and the closely related Southampton virus, are more similar to FCV and RHDV than to HEV. A hypervariable region has been identified within the nonstructural protein region of HEV despite significant homology, among three different geographic isolates, within this entire gene (99). This non-structural hypervariable region has not been identified in other caliciviruses. Hepatitis E virus appears to be the most distantly related member of the caliciviruses and has led some researchers to hypothesize that it is the prototype

member of a new virus family (121), or a separate genus within the calicivirus family (238).

While data on the molecular biology of caliciviruses is increasing, most of the information generated describes the number, size, and sequence of the various viral messages, genes and proteins. Little definitive work has been carried out to elucidate the nature and function of the various RNA molecules and amino acid motifs that have been reported. In addition to morphological and physicochemical properties, molecular evidence now exists to group the caliciviral members that have grown in cell culture systems together with the non-cultivatable candidate caliciviruses, RHDV and the Norwalk-like viruses. While HEV has properties which are morphologically, structurally, and molecularly similar to the caliciviruses, a greater understanding of the caliciviral genetic organization and replication is required before definitive conclusions can be made about the taxonomic structure of this unique family of viruses.

Diagnosis of Caliciviral Infection

Critical to any prevention and control program for infectious disease is the ability to determine if a specific disease is present in a given population, or in a particular population's food or water supply. The first outbreaks of caliciviral disease in swine displayed easily observed gross clinical signs and provided clinical specimens, vesicular coverings, which enabled the straight forward isolation, and characterization, of VESV up to 35 years after the field samples were collected (9, 62). Diagnosis of feline calicivirus and the many serotypes of SMSV and other ocean origin caliciviruses has been made easier because these biotypes can be

isolated in cell culture systems (12, 14, 48). Isolation of caliciviruses from clinical samples and morphologic confirmation by electron microscopy is considered the diagnostic gold standard when coupled with evidence that the host was mounting a type-specific immune response to the recovered virus. Unfortunately, a large number of known caliciviral infectious agents cannot be grown in the laboratory, and for that reason, various researchers have developed other methods of detecting the virus in clinical samples and food supplies.

The diagnosis of the non-cultivatable agents has relied on the expensive, time consuming, and unreliable method of screening clinical samples using electron microscopy to visualize the unique morphology of caliciviruses. This method, in conjunction with limited physical parameters such as particle diameter and buoyant density, has been taken to its extreme with the diagnosis of human nonbacterial gastroenteritis, in which an entire classification system has been established based on the electron microscopic appearance of negatively stained viral particles within the feces of patients. The small round structured viruses (SRSV) of nonbacterial gastroenteritis in humans include the astro-, calici-, and Norwalk-like viruses (42, 54, 118, 132, 151, 234). In an attempt to reduce the confusion of assigning cause and effect relationships to viruses seen by EM in stool samples from disease outbreaks, criteria were established to help diagnose non-bacterial gastroenteritis. Guidelines include: outbreaks being studied must be in a closed community to avoid complicating transient viruses, material must be collected to assess clinically normal as well as affected individuals, samples must be collected from post recovery patients, and paired sera from affected and unaffected individuals be collected to assess rises in acute and convalescent antibody titers to the suspected etiological agent (53, 167, 241). Additional criteria for the Norwalk-like viruses

has been established which include, vomiting must occur in 50% of the patients, duration of illness is 12 to 60 hours, and the incubation period is 24 to 48 hours (107). Non-cultivatable animal caliciviruses such as the PECV, RHDV, European brown hare syndrome virus, Newbury agent in cattle, chicken calicivirus, pheasant calicivirus, Amelyois chronic stunt virus, and grey seal calicivirus have all relied on electron microscopy for their identification within clinical samples (81, 110, 170, 185, 235, 257, 258). Although useful in making diagnoses of caliciviral infection, the electron microscope cannot accurately identify morphologically altered virus-like particles, which appear smooth because of proteolysis. Caliciviruses have been shown to undergo enzymatic degradation which will alter their morphology, resulting in diagnostic error if only electron microscopic methods are employed to detect their presence (49, 95).

Serological methods have enabled researchers to more accurately and sensitively detect calicivirus infection. Using specific, pre-immune and immune antisera to known caliciviruses, diagnostic samples can be screened with electron microscopy, and aggregates of viral particles or antibody covered virions are an indication of specific calicivirus infection, if the same phenomenon cannot be observed using the pre-immune reagents. This technique is called immunoelectron microscopy (IEM) and is commonly used in the diagnosis of Norwalk-like virus, HEV, and human calicivirus in gastroenteric disease (100, 107, 108, 124, 137, 141, 164, 241, 242). A variation of IEM, immuno-gold staining of viral antigen in stool specimens has also been used to detect human caliciviruses and Norwalk virus (114, 126). By using paired, acute and convalescent patient sera, rises in specific anti-Norwalk-like antibodies can be detected using type specific antigens in the IEM assay (107). Unfortunately, diagnostic reagents to perform this and

other immunologically based assays require the experimental infection of human volunteers, which limits the use of these tools to only a few laboratories.

More efficient immunodiagnostic methods, such as enzyme linked immunosorbent assay (ELISA) and radioimmuno assay (RIA) have been established as specific tests for Norwalk-like and human caliciviral gastroenteritis, as well as HEV, which enable rapid testing of a larger number of samples than can be accommodated by IEM (28, 52, 56, 83, 94, 100, 154). These assays are based on the increase in binding of virus antigen in fecal samples to a microtiter well coated with convalescent phase specific antisera as compared to acute phase antisera obtained from human volunteers. Use of experimentally infected human volunteers enables researchers to also develop assays which can detect the presence of antibody titers to specific viruses in patient sera. These blocking assays require a specific antibody bound to a microtiter plate which captures the low titer virus from stool samples of infected volunteers. Suspect patient sera is then allowed to react with the bound virus. A second, labeled specific antibody is then added and is blocked from binding to the viral antigen if the patient serum has bound the available antigen (23). A western blot immunoassay has also been reported useful in the diagnosis of gastroenteritis outbreaks in Japan, using clinical specimens and the appropriate paired patient sera (93). Because only a few laboratories make the effort to overcome the problems of not being able to grow the virus in tissue culture, having no animal model to grow large quantities of virus, and having extremely low titer clinical samples when human volunteers are infected, standardization of how diagnostic reagents are prepared has not occurred, leading to confusing results when interpreting the immunobiological relationships among the human gastroenteric calici-like viruses (24, 52).

A similar ELISA assay has been developed for RHDV (181). This assay relies on the RHDV antigen which can be obtained in relatively high amounts from experimentally infected rabbits which have died from the disease. Rodak and co-workers (181) have used this assay to serologically screen for anti-RHDV in various rabbit populations. This assay is more sensitive than a hemagglutination assay developed by Pu and co-workers in China (174). Immunoelectron microscopy has also been used to detect RHDV in liver samples and anti-RHDV antibodies in rabbit sera (162, 200, 251). Immunohistology has aided in the determination of organ and cellular distribution of RHDV in experimentally infected animals utilizing affinity purified hyperimmune anti-RHDV rabbit sera (1). Moreover, immunohistology has detected FCV in formalin-fixed tissue sections (57).

Monoclonal antibodies have been developed for a variety of caliciviruses and have been used to diagnose infection mainly in the non-cultivatable caliciviruses. Their use in cultivatable caliciviruses has been mainly devoted to the elucidation of protein function and antigenic site studies (38, 41, 147, 244, 246). Monoclonal antibodies, however, have been developed against Norwalk virus, and the Norwalk-like Snow Mountain agent, and should prove invaluable as diagnostic reagents, and tools for virus purification (126, 249).

As the calicivirus genome became known, nucleic acid hybridization probes and polymerase chain reaction (PCR) primers have been developed and used to detect calicivirus in clinical samples. Most of the work reported to date has involved the non-cultivatable members of the Caliciviridae, such as, Norwalk virus and HEV. Using PCR primers constructed from the published sequence in the RNA-dependent RNA polymerase region of the HEV genome (177), McCaustland

and co-workers (139) reported that reverse transcriptase-PCR (RT-PCR) on known positive HEV fecal samples yielded inconsistent results. By utilizing glass powder in the extraction of the RNA from the clinical samples, the sensitivity of the diagnostic procedure improved. Using the cationic detergent cetyltrimethylammonium bromide to remove RT-PCR inhibitory factors, Jiang and co-workers (106) found that the RT-PCR assay to detect Norwalk virus was two orders of magnitude more sensitive than dot blot hybridization. DeLeon and co-workers (55) have also developed a RT-PCR based diagnostic assay for Norwalk virus. This assay incorporated the use of two sets of primers which were specific for the polymerase and immunoreactive regions of the viral genome. In addition, an internally specific oligoprobe for each amplicon was hybridized to the PCR products and detected non-radioactively. Inhibitory components in the clinical specimens were removed and structural integrity of the virus capsid, and genome, have been maintained using Sephadex G-200 gel chromatography, thus conserving structure for more reliable reverse transcription (55). In all PCR based assays developed to date, the primers and probes are specific for only one member of the calicivirus family (55, 106, 139). No reliable group-specific diagnostic assay exists for caliciviruses.

Caliciviruses can infect a diverse array of animal species, ranging from insects to humans. These viruses have a history of emerging rapidly and causing serious disease in livestock and human populations, yet reservoirs for the agents are difficult to pinpoint (27, 107, 153, 203). A large number of ocean origin caliciviruses have been isolated and characterized. These same viruses have been traced serologically to a wide variety of ocean and terrestrial species, some displaying interspecies, interorder, and interphyla infectivity (204, 205, 228). A

single serotype of ocean origin calicivirus has been shown to be extremely host non-specific, capable of infecting, and causing an immune response in a wide variety of marine and terrestrial mammals, fish, and possibly helminths (204, 222, 227). Fortunately, a large number of ocean origin caliciviruses can be grown in cell culture systems. Significant numbers of samples collected from marine mammals, however, contained calicivirus-like particles by electron microscopy, but failed to yield virus isolates in cell culture (202).

Outbreaks of calicivirus-induced human gastroenteritis are commonly linked to contaminated shellfish consumption, providing an obvious link between caliciviruses pathogenic to humans and their ocean origin counterparts (78, 85, 88, 152). In order to further study the relationship between ocean origin and terrestrial caliciviruses, a group-specific diagnostic reagent is required to study the prevalence of calicivirus infection in marine as well as terrestrial hosts, and the prevalence of calicivirus contamination within the food supply. By coupling group- and type-specific diagnostic reagents, decisions can be made concerning the pathogenic potential of calicivirus encountered in domestic animals, threatened wild animal populations, and food products for livestock and human consumption.

OBJECTIVES

Caliciviruses have the potential to cause serious disease in a wide variety of animals, including humans, whenever they are introduced into a susceptible population. This family of viruses has been associated with many disease states, indicating unusually diverse tissue trophisms. Vesicular (47, 73, 74, 207, 248), oral, (117, 175), respiratory (172), reproductive (47, 213), hepatic (27, 170, 210),

hemorrhagic, (153), enteric (26, 59, 66, 137, 257), ophthalmic (184), encephalitic (73), cardiac (74, 75), myositic (48), and arthritic (17, 125) disease has been shown to be caused by caliciviral infections. Furthermore, these agents have been isolated from, or observed in, phylogenetically diverse species of marine and terrestrial animals, and serotype-specific neutralizing antibodies in these species suggests that many of these agents can move freely, over great distances, between ocean and land (11, 15, 16, 49, 201, 208, 211, 215, 218). Probably the best example of the broad caliciviral host-range and geographic distribution is illustrated in the bowhead whale, an Arctic species associated with the margins of the pack ice, and, therefore, rarely moves south of the 50^{o} Northern latitude (241). Sera, collected from bowhead whales harvested at Barrow, Alaska, in 1980, contained type-specific neutralizing antibodies to two serotypes of VESV (J_{56} and K_{56}), which had been isolated only once, 24 years previously, from domestic swine in New Jersey in 1956 (221). The mechanism of VESV transmission from rural New Jersey to the Alaskan pack ice was never determined. The transmission of caliciviral disease through contaminated marine or terrestrial food products, or other routes, puts at risk virtually all animals: domestic, captive exotic, wild endangered, and human. Reliable, group-specific, diagnostic reagents, which do not depend on *in vitro* isolation, identification, and cultivation, are needed to better understand caliciviral transmission. These same reagents are needed to detect caliciviral contamination of food supplies. Such contamination has been historically linked to the spread of caliciviral disease among humans and livestock (152, 203).

San Miguel sea lion virus, type 5 is a single calicivirus serotype with an exceptionally broad host range. It was first isolated from a Northern fur seal

(*Callorhinus ursinus*) on St. Paul Island, Alaska, in 1973 (214). In subsequent experimental infectivity studies, virus-contaminated fish was fed to Northern fur seal pups as follows. Nematode larvae of the sea lion lung worm (*Parafilaroides decorus*) were dipped in SMSV-5 suspensions, then washed and fed to the parasite's teleost intermediate host, opaleye (*Girella nigricans*). Thirty one days later, when the larvae become infective, ground whole fish was fed to fur seal pups where it caused disease, and SMSV-5 was reisolated from a vesicular lesion. This work demonstrated a possible mammal - poikilotherm - mammal cycle for caliciviruses (222). In 1985, SMSV-5 was proven to be zoonotic when a research worker developed clinical signs identical to caliciviral vesicular disease in other animals. The worker developed blisters on the thick skinned areas of the hands and feet, and SMSV-5 was isolated from the vesicular fluid and a sharp rise in type-specific antibody occurred (19). Furthermore, specific serum neutralizing antibodies to SMSV-5 have been found in numerous marine and terrestrial animals, including pinnipeds, toothed whales, baleen whales, feral and domestic swine, cattle, musk oxen, and gray foxes (204). Moreover, this same serotype has also been isolated from a calf with diarrhea (202).

Caliciviruses have a relatively simple genomic structure. By utilizing the genomic blueprint of a calicivirus such as SMSV-5 which is proven to infect and replicate in a wide array of marine and terrestrial organisms and tissues, it is hypothesized that a group-specific cDNA hybridization probe incorporating conserved caliciviral sequences can be constructed.

Because of the need for a group-specific caliciviral diagnostic reagent which does not depend on virus isolation, the main objective of this project was to develop a hybridization probe from the cloned cDNA library of a calicivirus with a

proven broad host range. San Miguel sea lion virus, type 5 fit this criteria and could be grown in large quantities to facilitate the cDNA cloning. The group specific nature of the resultant cDNA clones could be tested using dot hybridization assays against 28 different serotypes of calicivirus grown in cell culture and a large number of non-caliciviruses, as well. Finally, the sensitivity of the probe in detecting the template RNA as well as caliciviral RNA in human and animal clinical samples could also be determined using dot hybridization, sample dilutions and total RNA assays. In order to better characterize the selected cDNA probe, the translated amino acid sequences were compared to other published caliciviral sequences. In this way, its genetic similarity to other caliciviruses and genomic location could be shown.

Smith and Akers (203) suspected that marine mammal populations may not be the primary reservoir for ocean-origin caliciviruses, and suggested that fish or other components in the marine mammal food chain may harbor these agents. This hypothesis was confirmed with the isolation of two, marine mammal SMSV serotypes from the opaleye perch (223). Furthermore, one of these serotypes, SMSV-7, was also isolated from a parasitic trematode (*Zalophatrema* sp.) recovered from the liver of a moribund California sea lion and this isolate produced classic VES in exposed swine (223). The marine environment encompasses a larger variety of organisms than just marine mammals and fish. Caliciviruses have been isolated from invertebrate species and have been shown to contaminate shellfish, which can lead to gastroenteritis, and possibly other diseases, in humans. Domestic avian species have also been shown to develop disease associated with caliciviral infections.

The second objective of this project addresses the problem of identifying marine reservoirs capable of transmitting calicivirus to terrestrial hosts. Specific mechanisms of geographical caliciviral transmission have not been identified, largely because diagnosis has been dependent on virus isolation. By utilizing a cDNA probe, birds (*Gygis alba rothschilbi*) and mussels (*Mytilus trossulus)* closely associated with marine mammal populations, were studied and shown to harbor calicivirus RNA. Such data will help assess the potential of these, and perhaps other, diverse species for calicivirus transmission among populations of wild and domestic animals and humans.

Table 1.1: Summary of the known major capsid polypeptide molecular weights for selected members of the Caliciviridae.

Virus	Mol. Weight (Daltons)	Reference
Vesicular Exanthema of Swine	70,000 61,000	37 5
Feline Calicivirus	61,000 62,000 65,000	5 38 247
San Miguel Sea Lion Virus	60,000 61,000	71 5
Porcine Enteric Calicivirus	58,000	166
Rabbit Hemorrhagic Disease Virus	61,000	181a
Norwalk-like Viruses	33,000 59,000 63,000	161 82 161
Human Caliciviruses	62,000	239
Canine Calicivirus (Enteric)	58,000	194
Amelois Chronic Stunt Virus	70,000 29,000	95

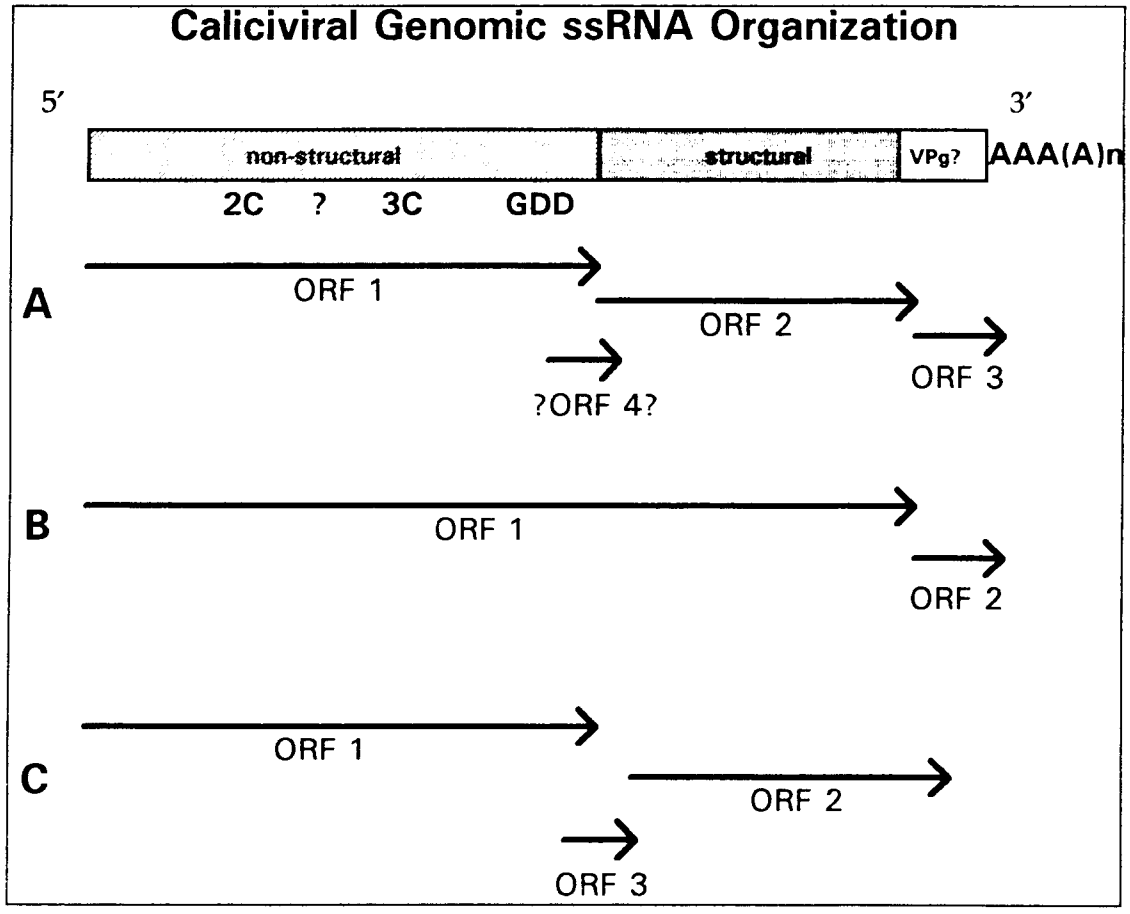
Table 1.2: Molecular weights and probable function of reported caliciviral-induced polypeptides. Italicized values in the precursor protein category have an associated final product protein italicized in the same row. Degradation products of mature proteins are also indicated.

Virus	Precursor Proteins (KDa)	Early Non-structural Proteins (KDa)	Other Non-structural Proteins (KDa)	Structural Proteins (KDa)	Reference
VESV	***120***	?	***100*** 80	65	21
SMSV	***135*** ***86***	?	***80*** 40 35 29	***60***	71
Norwalk	?	?	30	59	82
PECV	?	?	82 32 28	58	166
RHDV	?	?	52	61 38	181

Table 1.2: Continued

Virus	Precursor Proteins (KDa)	Early Non-structural Proteins (KDa)	Other Non-structural Proteins (KDa)	Structural Proteins (KDa)	Reference
FCV	?	80	80 40	68 14	119
FCV	125 123 98 ***76***	73	96 75 39 36 27	***62*** 40 (Degraded)	38

Figure 1.1: Organization of the calicivirus genome with respect to the structural and non-structural protein genes. Polypeptide function, determined from sequence analysis, within the non-structural protein gene is also indicated. **2C** = picornavirus-like helicase and nucleotide binding site; **?** = unknown function; **3C** = picornavirus-like cysteine protease; **GDD** = RNA dependent RNA polymerase. **A)** Open Reading Frames (ORF) of FCV and Norwalk-like Southampton Virus. The ORF 4 has been suggested to exist in FCV. **B)** ORF's of RHDV. **C)** ORF's of HEV. The ORF 3 of HEV lies between the structural and non-structural ORF's and not at the 3' end of the viral genome as is the case with other caliciviruses.



CHAPTER TWO

Identification and Characterization of a Group-specific Caliciviridae cDNA Hybridization Probe Cloned from San Miguel Sea Lion Virus, Type 5, a Calicivirus of Ocean Origin

Steven E. Poet, Douglas E. Skilling, and Alvin W. Smith

ABSTRACT

San Miguel sea lion virus, type 5, because of its broad host range, was cloned into an *E. coli* plasmid vector in an attempt to identify a portion of the viral genome which could be used as a group-specific probe in dot hybridization assays. A 1671 base-pair cDNA viral insert, p5RT73, was selected, and from this clone, a 300 base-pair restriction endonuclease fragment (5RT73xr-300) was hybridized with RNA extracted from 28 different calicivirus-infected cell culture lysates, including the prototype calicivirus, vesicular exanthema of swine virus, type A_{48}. Moreover, RNA extracted from sixteen picornaviruses, a rotavirus, togavirus, and a putative retrovirus did not hybridize with the probe. Using nucleic acid extracted from density gradient purified SMSV-5, the probe could detect approximately one nanogram of viral RNA. Norwalk virus, a calicivirus responsible for outbreaks of human gastroenteritis, could be detected in feces from an experimentally infected human volunteer, but not from the paired pre-infection stool sample. Preliminary studies with other non-cultivatable caliciviruses, human calicivirus and hepatitis E virus, indicate this probe may detect these agents in fecal specimens, as well. Sequence analysis of the translated amino acid residues of p5RT73 revealed a 3C-like cysteine protease motif, located within the probe segment (N-terminus), and

the RNA dependent RNA polymerase motif at the C-terminus. The amino acid sequence of p5RT73 was compared with the sequences of feline calicivirus, rabbit hemorrhagic disease virus, and the Norwalk-like Southampton virus. This group-specific probe should prove useful in studying calicivirus reservoirs in the marine environment, and in evaluating livestock and human food supplies for calicivirus presence.

INTRODUCTION

The family Caliciviridae comprises a group of morphologically distinct viruses with an unusually diverse host spectrum, both phylogenetically and geographically. Caliciviruses are small (29-37 nm), non-enveloped, icosahedral viruses with T = 3 lattice symmetry, that possess unique cup-shaped surface structures which are readily identifiable under the electron microscope (134). The genome of the caliciviruses is comprised of a single molecule of polyadenylated, single-stranded RNA of positive polarity (159, 190). These agents have been isolated from, or observed in, a phylogenetically diverse variety of marine and terrestrial organisms, and serotype specific neutralizing antibody titers suggest that many of these agents move freely, over great distances, between ocean and land (11, 15, 16, 49, 211, 218).

Caliciviruses have the potential to cause serious disease in a wide variety of animals, including humans, whenever they are introduced into a susceptible population. Foremost in any prevention and control program for infectious disease is the means to determine if a specific disease is present in a given population, or in a particular population's food or water supply. The major methods currently used

to detect calicivirus presence include virus isolation, electron microscopic visualization, and retrospective serology (11, 48, 81, 107, 110, 185, 207, 257). All of these methods lack sensitivity, are expensive, and time-consuming. Some caliciviral agents have been shown to undergo enzymatic degradation which will alter their morphology, resulting in diagnostic error if only electron microscopic methods are used (49, 95). Recently, cloned sequences of the Norwalk virus, a human gastroenteritis-causing calicivirus, have been used to develop polymerase chain reaction (PCR) reagents for detection of Norwalk virus in stool samples (55, 106). These new, nucleic acid-based diagnostic methods have been shown to be reliable and sensitive, but no group specific test has been reported to date.

Caliciviruses can infect a diverse array of animal species, ranging from insects to humans. These viruses have a history of emerging rapidly and causing serious disease in livestock and human populations, yet reservoirs for these outbreaks are difficult to pinpoint (27, 107, 153, 203). A large number of ocean origin caliciviruses have been isolated and characterized, and these same viruses have been traced by serological methods to a wide variety of ocean and terrestrial species, some displaying interspecies, interorder, and interphyla transmission (204, 205, 228). A single serotype of ocean-origin calicivirus, San Miguel sea lion virus, type 5 (SMSV-5), has been shown to be extremely host non-specific, capable of infecting, and causing an immune response in a wide variety of marine and terrestrial mammals as well as fish, and possibly helminths (204, 222, 227). Fortunately, a large number of ocean origin caliciviruses can be cultivated in cell culture systems, but significant numbers of marine mammal field samples containing calicivirus-like particles when viewed under the electron microscope, yielded no virus isolate (202).

Outbreaks of calicivirus-induced human gastroenteritis are commonly linked to contaminated shellfish consumption, providing an intuitive link between caliciviruses pathogenic to humans and their ocean-origin counterparts (78, 85, 88, 152). In order to further study calicivirus contamination of foods, as well as the relationship and prevalence within and between ocean-origin and terrestrial caliciviruses, a group-specific diagnostic reagent is needed. By coupling group-specific and type-specific diagnostic tools, decisions can then be made on the pathogenic potential of calicivirus encountered in domestic animals, threatened wild animal populations, and food products bound for livestock or human consumption.

San Miguel sea lion virus - type 5 (SMSV-5) is a single calicivirus serotype with an exceptionally broad host range. It was first isolated from a Northern fur seal (*Callorhinus ursinus*) on St. Paul Island, Alaska, in 1973 (214). In subsequent experimental infectivity studies, a possible mammal - poikilotherm - mammal cycle was demonstrated for calicivirus transmission (222). In 1985, SMSV-5 was proven to be zoonotic when a research worker exhibited clinical signs identical to caliciviral vesicular disease in other animals. The worker developed blisters on the thick skinned areas of the hands and feet, SMSV-5 was isolated from the vesicular fluid, and a sharp rise in type-specific neutralizing antibody occurred (19). Furthermore, specific serum neutralizing antibodies to SMSV-5 have been found in numerous marine and terrestrial animals, including pinnipeds, toothed whales, baleen whales, feral and domestic swine, cattle, musk oxen, and gray foxes (204).

The use of group-specific hybridization probes to detect small RNA viruses was demonstrated to be reliable for detecting enteroviruses in environmental and clinical samples (46, 61, 102, 103, 104, 182). Moreover, uncharacterized

caliciviruses are being isolated regularly from or identified in wild animal, domestic livestock, and human clinical samples. A need exists, therefore, for a screening method which is capable of detecting both known and new caliciviral agents. The objective of this study was to identify a group-specific cDNA hybridization probe for caliciviruses.

MATERIALS AND METHODS

Virus Purification

The procedure was developed from the methods of Oglesby and co-workers (159), Schaffer and Soergel (190), and Soergel and co-workers (233). Vero Monkey Kidney cells were grown to confluency in 850 cm^2 roller bottles using 100 ml of 10% fetal calf serum (FCS) in Eagle Minimal Essential Medium (MEM) with Earle's Salts. The tissue culture medium was reduced to approximately 20 ml and San Miguel Sea Lion Virus, type 5, (SMSV-5) was inoculated into each vessel. The roller bottles were incubated about 18 to 36 hours with rolling until 75-100% of the cells were rounded, refractile, and detached from the bottle interior, indicative of calicivirus cytopathic effect (168). The roller bottle tissue culture medium was decanted into 50 ml plastic centrifuge tubes, and each roller bottle was rinsed with approximately 10 ml of sterilized, double distilled water (ddH_2O). The rinse was combined with the tissue culture fluid. The fluid was centrifuged at 1,700 x *g* for 10 minutes to remove large cell debris . After clarification, the supernatant and pellet were separated and retained for 1,1,2-trichloro-1,2,2-trifluoroethane (Uvasol) extraction (77). The pellet was resuspended in ddH_2O in a 10 ml glass tube and an equal amount of Uvasol was

added. The mixture was alternately mixed vigorously for one minute and chilled on ice for one minute for a total of ten minutes and then centrifuged at 1,700 x *g* for 20 minutes at 4°C. This extraction was repeated three times.

The supernatant was decanted and centrifuged in a SW 27 rotor (Beckman) at 120,000 x *g* for 2 hours at 4°C to pellet the virus. A drop of glycerol was added to the bottom of each centrifuge tube to simplify resuspending the pellet. The viral pellets were resuspended in the uvasol extracted cell debris mixture and the suspension was uvasol extracted three more times. A final virus pellet was obtained by centrifuging the aqueous fraction of the uvasol extraction in a SW 50.1 rotor (Beckman) at 150,000 x *g* for 2 hours at 4°C. The virus pellet was resuspended in, and layered onto 1.38 g/ml CsCl in ddH_2O and centrifuged in a SW 50.1 rotor at 150,000 x *g* for at least 15 hours at 4°C. The virus, visualized as a light scattering band approximately half way down the centrifuge tube, was removed by side puncture using a 23 G needle and a 1 ml tuberculin syringe. The virus was placed in another centrifuge tube, diluted with ddH_2O, and pelleted in a SW 50.1 rotor at 150,000 x *g* to remove the CsCl. The viral pellet was resuspended in 100 μl of ddH_2O.

Viral RNA Extraction

The viral RNA was extracted using a modification of the phenol/ guanidinium isothiocyanate procedure of Chirgwin and co-workers (45). RNAzol B (Cinna/Biotecx) was used to extract all RNA samples. The 100 μl SMSV-5 suspension above was transferred to a sterilized 1.5 ml microcentrifuge tube, 800 μ l of RNAzol B was added to disrupt the viral capsid, release the genomic RNA,

and inactivate ribonucleases present in the mixture. The solution was mixed briefly before 80 μl of chloroform was added. The sample was stored on ice for 5 minutes before it was mixed vigorously for 15 seconds and again allowed to stand on ice for an additional 15 minutes. The solution was microcentrifuged (14,000 x *g*) at 4^{o}C for 15 minutes and the top aqueous layer was removed to another microcentrifuge tube. An equal volume of cold (-20^{o}C) isopropanol was added and the RNA was precipitated at 4^{o}C for 45 minutes or overnight. The RNA was pelleted in a microcentrifuge for 15 minutes and washed once with 500 μl of 75% ethanol. After another microcentrifugation for 5 minutes the supernatant was removed and the sample was centrifuged again to drive all the residual ethanol to the bottom of the tube so the final traces could be removed. The viral RNA pellet was dissolved in 50 μl of diethylpyrocarbonate (DEPC)-treated ddH_2O, the concentration and purity of the viral RNA were determined by ultraviolet spectroscopy (136), and cDNA synthesis was carried out immediately.

Synthesis of Viral cDNA

The synthesis of cDNA from the SMSV-5 RNA template was performed using a modification of the protocol described by Gubler and Hoffman (86). All reagents, buffers, and enzymes were provided in kit form by Invitrogen, Inc. as The Librarian II cDNA Library Construction System.

The dissolved RNA pellet was placed on ice and 12.5 μl of 0.1M methyl mercury hydroxide was added. The contents were mixed lightly, spun momentarily in a microcentrifuge and allowed to incubate at room temperature for 7 minutes. Following denaturing with methyl mercury hydroxide, 25 μl of ddh2O and 11 μl of

0.7M β-mercaptoethanol was added, mixed, spun and allowed to incubate at room temperature for an additional 5 minutes, after which the tube was placed on ice.

To initiate first strand DNA synthesis, 1 μl of the kit supplied oligo dT solution and 10 μl of the hexanucleotide random primer were added and incubated at 65°C for 2 minutes and placed on ice. The following were added in order: 2 μl of placental RNase inhibitor, 50 μl of 5X reverse transcriptase buffer, 5 μl of 25 mM dNTP solution, 6 μl of reverse transcriptase, and ddH_2O to bring the reaction volume to 250 μl. The mixture was gently mixed, spun and allowed to incubate at 42°C for 1 hour after which the reaction was placed on ice and stopped by the addition of 10 μl 0.5M EDTA. The reaction mixture was then extracted with an equal volume of buffered phenol:chloroform and the RNA:DNA hybrid was precipitated twice in the presence of 2M ammonium acetate with 2 volumes of cold, 100% ethanol. The pellet was washed in 80% ethanol and all traces of ethanol were removed by the respinning method described above.

The RNA:DNA hybrid pellet was resuspended in 33.5 μl ddH_2O and placed on ice. The following was added in order: 5 μl of 10X second strand reaction buffer, 2.5 μl 1.0 mg/ml Bovine serum albumin, 1 μl 10 mM β-NAD^+, 2 μl 5.0 mM dNTP solution, 4 μl RNase H/*E. coli* DNA ligase, and 2 μl DNA polymerase I. The reaction was incubated at 15°C for 1 hour and room temperature for an additional hour. The reaction was stopped by heating to 70°C for 10 minutes. The tube was incubated at room temperature for 2 minutes and chilled on ice for 2-3 minutes before 3.5 μl of T4 DNA polymerase was added and allowed to react for 10 minutes at 37°C. The double stranded cDNA was phenol:chloroform extracted, ethanol precipitated and washed once, and ethanol traces removed before linker addition was carried out.

BstX I non-palindromic linkers were blunt-end ligated to the cDNA to ensure efficient insertion into the plasmid vector pcDNA II. The cDNA was resuspended in 22 μl of ddH_2O and the following were added in order: 3 μl 10X ligation buffer, 3 μl BstX I linkers, and 2 μl T4 DNA ligase. The reaction was incubated overnight at 15^{o}C, stopped by the addition of 2 μl 0.5M EDTA and ethanol precipitated once with the removal of ethanol traces.

The cDNA was sized on a 1% agarose gel in tris-acetate (TAE), pH 7.5 buffer (40 mM tris-acetate, 2 mM EDTA). The cDNA fragments 1600 bases and larger were electroeluted from the gel and resuspended in 61 μl of ddH_2O after two ethanol precipitations.

The sized cDNA was ligated into the plasmid vector pcDNA II (Invitrogen). To the cDNA suspension the following was added in order: 15 μl 10X ligation buffer, 8 μl BstX I cut, gel purified pcDNA II vector (0.2 μg/μl), 2 μl (5 units) T4 DNA ligase, and ddH_2O to a final volume of 150 μl. The reaction mixture was incubated at room temperature for 1 hour then 15^{o}C overnight. The DNA was ethanol precipitated once and washed once with traces of ethanol being removed before it was resuspended in 25 μl of ddH_2O.

Transformation of *E. coli* with the cDNA Containing pcDNA II Plasmids

The protocol was performed according to kit instructions (Invitrogen, Inc.). Fifty microliters of 0.5 M β-mercaptoethanol was placed into the bottom of a 50 ml plastic centrifuge tube, prechilled on ice, and 1.0 ml of competent INVαF' *Escherichia coli* was added. The mixture was placed on ice and gently swirled every 2 minutes for 10 minutes. The 25 μl suspension of cDNA containing plasmid

was added to the mixture containing the bacterium, swirled gently and allowed to incubate on ice for 30 minutes. The transformation mixture was incubated for exactly 120 seconds without swirling at 42°C and immediately placed on ice for 2 minutes. Nine milliliters of prewarmed (37°C) SOC medium was added to the transformation tube and the mixture was incubated at 37°C with moderate shaking (225 rpm) for exactly 1 hour. The bacterium was pelleted at 3000 x *g* for 10 minutes and the pellet was resuspended in 2.5 ml of SOC medium. The resulting cDNA library was frozen at -70°C in 15% glycerol.

Screening of the cDNA Library

Dilutions of the frozen cDNA library were spread out on Luria broth (0.5% tryptone, 0.25% yeast extract, 0.5% NaCl) agar (LBA) plates containing 200 μg/ml of ampicillin at 100 μl of bacterial dilution per plate and incubated overnight at 37°C. The ampicillin resistant colonies were streaked onto another antibiotic containing plate and allowed to incubate overnight at 37°C. A loop of each colony was suspended in 20 μl of TE buffer, pH 8.0 and phenol extracted. The aqueous top layer was transferred to another microcentrifuge tube, 3 μl of loading buffer (50% sucrose, .025% bromophenol blue) was added and the mixture was loaded onto an ethidium bromide containing 1% agarose tris-acetate buffer gel and electrophoresed at 100V until the dye had migrated half way down the gel. A supercoiled DNA ladder (BRL) was co-electrophoresed with the samples. Plasmids were visualized under UV illumination and the largest insert containing plasmids were identified, grown in LB broth with ampicillin, and frozen in 15% glycerol at -70°C.

Plasmid Purification

Bacteria containing plasmids of interest were grown overnight at 37°C in LB broth containing ampicillin. The bacterial cell suspension was transferred to a microcentrifuge tube and spun momentarily to pellet the organisms. The supernatant was discarded and the bacteria were resuspended in 250 μl of STET buffer (8 g sucrose, 5 ml NP-40, 10 ml 0.5M EDTA, 5 ml 1.0M tris, pH 8.0, and ddH_2O to 100 ml). The suspension was placed on ice and 10 μl of 10 mg/ml lysozyme in 50 mM tris, pH 8.0 was added and incubated for 1 minute. The bacteria were then placed in a boiling water bath for exactly 1 minute and subsequently microcentrifuged for at least 10 minutes at 4°C. The mucus-like pellet was removed with a sterile wooden applicator stick, 250 μl of cold isopropanol was added and the mixture was allowed to incubate on ice for 10 minutes. After microcentrifugation for 15 minutes at 4°C, the plasmid pellet was washed in 75% ethanol and traces of ethanol were removed with recentrifugation. The plasmid p5RT73 was resuspended in 20-50 μl of TE buffer and frozen at -20°C until it was utilized for the production of the hybridization probe (5RT73xr-300), and for nucleic acid sequencing.

Restriction Endonuclease Digestion and Purification of the Hybridization Probe

The plasmid containing the cDNA hybridization probe, p5RT73, was digested with the restriction endonucleases, *Xba* I and *Rsa* I (IBI, Inc.). Several reactions were carried out simultaneously and the resulting reaction products were separated on a preparative 1% agarose gel in tris-acetate buffer.

The reaction mixture is as follows: 5 μl of purified plasmid, 2 μl 10X restriction endonuclease buffer A (IBI, Inc.), 10 μl ddH_2O, 1 μl *Xba* I, 1 μl *Rsa* I, and 1 μl RNase A (10 μg/ml). The reaction was incubated at 37°C for 1 hour and stopped by incubating at 70°C for 10 minutes. Four microliters of loading buffer were added to each reaction vial and the contents were pooled and electrophoresed on a preparative 1% agarose gel in tris-acetate buffer at 100V until the dye migrated half way down the gel. A section of the gel was stained in ethidium bromide and the band corresponding to the 300 base-pair hybridization probe fragment (5RT73xr-300) was cut out and electroeluted from the gel. The purified probe was stored in TE buffer at -20°C.

Nick Translation Labeling of the Hybridization Probe

The protocol used to label the probe was provided by BRL in their Nick Translation System kit. Approximately 1 μg of probe was transferred to a 1.5 ml microcentrifuge tube and the following were added: 5 μl of the solution containing dATP, dGTP, and dTTP, 156 pmoles of (α-^{32}P) dCTP, and ddH_2O to 45 μl. The mixture was mixed gently and 5 μl of DNA polymerase/DNase I was added. The reaction was mixed and spun briefly and incubated at 15°C for 1 hour. The reaction was stopped by the addition of 5 μl of 0.5 M EDTA.

Unincorporated nucleotides were removed using a Sephadex G-50 spin column gel chromatography (IBI, Inc.). The radioactive probe was stored at -70°C until needed, or for a maximum of 2 weeks.

RNA Preparation

All cultivatable caliciviruses used in the dot hybridization assay were obtained from the Laboratory for Calicivirus Studies, Oregon State University College of Veterinary Medicine. These caliciviruses include: San Miguel sea lion virus (SMSV) serotypes 1 - 17, walrus calicivirus (WCV), cetacean calicivirus (CCV), gray whale calicivirus (GWCV), human vesicular calicivirus (HVCV), reptile calicivirus (RCV), Tillamook calicivirus (TCV), primate calicivirus (PCV), feline calicivirus, strain F-9 (FCV), cheetah calicivirus (ChCV), canine calicivirus (CaCV), and mink calicivirus (MCV).

Several non-Caliciviridae viruses were also assayed. Viruses obtained from the Laboratory for Calicivirus Studies include: a rotavirus isolated from a Northern fur seal (*Callorhinus ursinus*), and a putative retrovirus isolated from a Pacific walrus (*Odobenus rosmarus*), (202). An enterovirus isolated from human stool, and adapted for growth in Vero cell culture, was provided by Dr. D.O. Matson, Baylor University, College of Medicine, Houston, Texas. Equine viral arteritis virus (EVA) was obtained from Dr. D. Mattson, Oregon State University, College of Veterinary Medicine Diagnostic Laboratory, Corvallis, Oregon. In addition, 15 picornaviruses listed in Table 2.1, were obtained from the American Type Culture Collection.

Each calicivirus was inoculated onto separate 25 cm^2 monolayers of Vero monkey kidney cells. When 75% of the cell monolayer displayed cytopathic effect, the cells were solublized with 800 μl RNazol B (Biotecx, Inc.), and immediately placed in a 1.5 ml microcentrifuge tube and vortexed. As with the preparation of SMSV-5 RNA for cDNA cloning, the various calicivirus-infected Vero cells were guanidinium thiocyanate-phenol-chloroform extracted, isopropanol precipitated,

75% ethanol washed, and dissolved in DEPC H_2O, according to the conditions of the manufacturer (Biotecx, Inc.), to yield total, calicivirus-infected, cellular RNA. The non-calicivirus stocks (100 μl), not grown in Vero cells, were extracted directly with RNazol B. The amount and purity of the RNA was determined by ultraviolet spectroscopy. Uninfected Vero cell RNA was also extracted for use as a negative control.

RNA from density gradient purified SMSV-5 was extracted using the RNazol B procedure in order to determine the hybridization probe's approximate minimum threshold for detecting homologous nucleic acid. The RNA was quantified using UV spectroscopy and diluted logarithmically. Known quantities of SMSV-5 RNA were spotted on a nylon membrane and hybridized against the 5RT73xr-300 cDNA probe as described below.

Pre- and post-infected Norwalk virus stool specimens from a human volunteer, and a fecal specimen containing human calicivirus, were kindly provided by D.O. Matson, Baylor University, College of Medicine, Houston, Texans. Two hepatitis E virus-containing human stool specimens were kindly provided by D. Bradley, Center for Disease Control, Atlanta, Georgia. These stool samples along with gray whale stool, stored at -70^{o}C for 24 years, from which the gray whale calicivirus was isolated, were diluted 1:5 in TBS and 100 μl of the fecal suspension was extracted with RNazol B according to the method used to extract virus-infected cellular RNA.

cDNA:RNA Dot Hybridization

Approximately 1 μg each of the virus-infected cellular RNA, or fecal sample RNA was spotted onto a nylon membrane (Nylon 66 Plus, Hoeffer Scientific Instruments, Inc.). After the membrane was allowed to air dry, it was exposed to germicidal ultraviolet radiation (280 nm) for 20 minutes in order to link the sample RNA to the hybridization support medium. In addition, a known amount of RNA from density gradient purified SMSV-5 was serially log diluted and similarly applied to a nylon membrane support in order to determine the detection level of the cDNA probe.

Dot hybridization was carried out in a Hoeffer PR 800 Hybridization Chamber according to the Hardy and co-workers (91) modification protocol provided by the company (Hoeffer Scientific Instruments, Inc.). The membrane was soaked in hot (65^{o}C) prehybridization solution (1.0M NaCl, 1.0% SDS) and placed in the hybridization chamber. The chamber was filled with prehybridization solution and incubated at 65^{o}C for 3 hours. The prehybridization solution was removed and hot hybridization solution (10% dextran sulfate, 1.0M NaCl, 1% SDS, and 100 μg/ml denatured heterologous DNA) and approximately 100 ng of boiled radioactive probe was placed in the hybridization chamber and incubated 48 hours at 65^{o}C.

After overnight incubation the radioactive hybridization solution was removed and the membrane was washed within the chamber with 2X SSPE(300 mM NaCl, 20 mM Na-PO_4, 2 mM EDTA, pH 7.4)/1% SDS, and washed under high stringency conditions with 0.1X SSPE/1% SDS for one hour at 65^{o}C. The membrane was removed from the chamber and wrapped in plastic film.

Samples hybridizing to the probe were visualized by autoradiography. X-ray film (Kodak X-Omat Xs-5) was placed over the wrapped membrane and intensifying screens (Pickering, Inc.), were incorporated to reduce exposure times to 1 to 3 days at -70^{o}C.

Sequencing of the p5RT73 Viral Insert

Dideoxy sequencing (186) of caliciviral cDNA was carried out using the Taq Dye-Primer Cycle Sequencing Core Kit (Applied Biosystems, Inc.). T7 and SP6 polymerase primers, and synthesized internal downstream primers were utilized to initiate the PCR reaction directly from the recombinant pcDNAII plasmid (Invitrogen, Inc.). The sequencing was automated using an Applied Biosystems, Inc. DNA Sequencer Model 373A.

Searches for sequence similarities, sequence translation, and other computer analysis was performed using Intelligenetics Suite, version 5.3 (Intelligenetics, Inc.) and the GenBank, EMBL, and Swiss-Prot data banks.

RESULTS

The SMSV-5 cDNA library synthesized, using random and oligo-dT primers in combination, yielded approximately one million ampicillin resistant *E. coli* colonies per milliliter. The pcDNA II plasmid vector (Invitrogen, Inc.), contains the gene for ampicillin resistance for the selection of recombinant DNA-containing bacteria. A 300 base pair Xba I/Rsa I restriction endonuclease fragment of one of these clones, p5RT73, was used as a cDNA hybridization probe (5RT73xr-300) against a variety of cultivatable caliciviruses and the results are shown in Figure

2.1A. Strong hybridization is evident with SMSV-5 infected cells while uninfected cellular RNA does not react. Furthermore, 5RT73xr-300 reacts, at differing intensities, with all of the calicivirus-infected cells while excluding the non-Caliciviridae samples. In a different experiment, SMSV-17-infected pig kidney cellular RNA also reacts with the hybridization probe (Figure 2.1B), while uninfected cellular RNA does not. Serial dilutions of RNA extracted from gradient purified SMSV-5 show that the probe is capable of detecting approximately one nanogram of target nucleic acid (Figure 2.1C). No virus tested to date, belonging to a family other than the Caliciviridae, has given a positive result when tested with the 5RT73xr-300 hybridization probe. Table 2.2 lists the viruses which have been negative in hybridization assays against 5RT73xr-300.

Preliminary assays using a limited number of non-cultivatable caliciviruses have provided encouraging results. As seen in Figure 2.2, fecal RNA from a human volunteer infected with Norwalk virus hybridizes with the probe while feces collected from the same volunteer prior to Norwalk virus infection are negative. In addition, while no pre-infection samples were provided, stool samples of hepatitis E virus-infected patients and a patient infected with human calicivirus appear to react with this hybridization probe constructed from the genome of a calicivirus of ocean origin (Figure 2.2).

The gray whale calicivirus, isolated recently out of feces collected from a gray whale in 1969, has been neutralized by type specific antisera produced against the prototype calicivirus, VESV A_{48}, providing additional evidence that vesicular exanthema of swine virus is a calicivirus of ocean origin (225). The hybridization probe readily reacts with this agent in cell culture and is capable of detecting viral nucleic acid contained within the sample of 24 year old whale feces (Figure 2.3).

Sequencing studies showed the p5RT73 viral insert to be 1671 bases in length, with two Rsa I restriction endonuclease cut sites which produce three fragments: 50, 299, and 1322 bases (Figure 2.4A). Comparison of p5RT73 with other known caliciviral sequences (40, 105, 122, 145, 155, 177), indicated the presence of the 3C cysteine protease motif of picornaviruses and the RNA dependent RNA polymerase motif of most positive polarity RNA viruses (Figure 2.4B, 2.4C). The sequence of p5RT73 places the origin of this cDNA within the coding region for the caliciviral non-structural proteins.

DISCUSSION

A rapid, reliable method is needed to detect caliciviral contamination in animal populations and food supplies. Diagnosis of many members of the calicivirus family has been accomplished by isolation of these agents in cell culture systems, a procedure which can take months, but unfortunately, even then a large number of known caliciviruses cannot be grown in the laboratory. Other detection methods, therefore, have been used and are being developed which do not depend on *in vitro* cultivation of the suspected viral contaminant (4, 107). The hybridization probe developed in the present study offers a rapid and definitive diagnostic tool with sensitivity at the nanogram level for viral RNA.

Amino acid residue analysis of the SMSV-5 p5RT73 cDNA insert places it approximately 3300 bases from the 5' end of the viral genome, within the non-structural protein region, when feline calicivirus is used as a comparison sequence (155). Evidence for this assumption is based on the presence of a picornavirus 3C-

like cysteine protease amino acid motif and two RNA-dependent RNA polymerase amino acid motifs (GLPS and YGDD) contained within the cDNA insert (Figure 2.4A).

A cDNA hybridization probe has been identified which will only detect caliciviral RNA, by taking advantage of the numerous calicivirus serotypes stored at the Laboratory for Calicivirus Studies at Oregon State University, and several non-caliciviral agents from different sources (Figure 2.1, Table 2.2). From preliminary sequence analysis, the probe (5RT73xr-300) corresponds to a non-structural protein coding region and contains the catalytic sites of the 3C-like cysteine protease motif which is similar to the picornaviruses (155). By comparing the translated nucleotide sequence of the SMSV-5 cDNA with the published amino acid sequences of feline calicivirus (FCV), rabbit hemorrhagic disease virus (RHDV), and the Norwalk-like, Southampton virus (SV), in the region of the 3C-like cysteine protease motif, amino acid residue similarity is easily observed (Figure 2.4B), (122, 145, 155). Because this protein motif is also present in the picornavirus genome, a large number of enterovirus-infected cell lysates were probed to determine the specificity of the calicivirus cDNA insert. In addition, rotavirus-, retrovirus-, and togavirus-infected cell lysates did not react with the probe. No cross reactivity was observed with any virus that was not a calicivirus (Table 2.2).

The variation in hybridization signals seen in Figure 2.1A may represent differences in the amount of viral RNA in each extracted sample, due to different cell culture titers, as well as slight differences in nucleic acid sequence among the tested caliciviral agents within the conserved 3C-like cysteine protease region of the genome. The *in vitro* replicative potential of caliciviruses vary considerably.

Many Caliciviridae members cannot be grown in tissue culture, while others grow to high titer (49, 206). Ocean origin calicivirus cell culture titers range from 10^4 to $10^{8.5}$ TCID50/ml (206, 207, 216, 223). In addition, slight genomic sequence heterogeneity will give rise to a decrease in hybridization efficiency, resulting in less stable probe/target duplex formation (142). Very little sequence analysis has been carried out with the ocean-origin caliciviruses. Neill (156), reported that the translated, immunologically different, capsid protein amino acid sequences of SMSV-1 and SMSV-4 were 73% homologous, and that regions of similarity exist among the two marine caliciviruses, FCV and RHDV as well. The 3C-like cysteine protease region within the non-structural protein gene may represent another conserved region within the calicivirus genome.

Hybridization of 5RT73xr-300 with the gray whale calicivirus, first isolated from gray whale (*Eschrichtius robustus*) rectal contents in 1969, is significant because type-specific antisera to VESV-A_{48} will neutralize the whale virus (225). Reciprocal neutralization studies conducted at Plum Island Animal Disease Center with gray whale calicivirus type-specific antisera and VESV-A_{48} confirmed that gray whale calicivirus was a strain of VESV-A_{48} which is the type species for the family Caliciviridae (192). This virus type was believed to have been eradicated from swine, prior to being declared a foreign animal disease agent in the United States, in 1959 (225). Probe 5RT73xr-300 is capable of detecting all caliciviruses first isolated from marine animals, as well as a representative agent, also of marine host lineage, from the economically significant outbreaks of vesicular exanthema of swine which occurred from 1932 to 1956 (12).

Many of the ocean-origin caliciviruses have been isolated from host feces (207, 216, 226). In addition, many non-cultivatable caliciviruses have been

implicated in gastroenteric disease in mammalian livestock, poultry, and humans (49). Preliminary studies were done to assess the hybridization probe's ability to detect caliciviral RNA in stool samples. The probe appears to detect caliciviral RNA in the original gray whale rectal contents from which VESV-A_{48} was isolated 24 years previously (Figure 2.3). Unfortunately, no known calicivirus-free gray whale feces was available as a control and non-specific hybridization cannot be ruled out in this case.

Human clinical stool samples, collected from patients infected with Norwalk virus, human calicivirus, and hepatitis E virus, gave similar hybridization results. As with the gray whale feces, the human calicivirus and hepatitis E virus samples did not have corresponding negative control samples. The Norwalk virus stool sample, however, was paired with a pre-infection stool sample from the same volunteer, and clearly indicates that the probe is capable of detecting at least one calicivirus responsible for gastroenteritis outbreaks in human populations (Figure 2.2). The low signal of the post-infection Norwalk virus sample indicates that this virus is near the minimum detection level for this assay, as determined by Figure 2.1C, of approximately one nanogram. This finding corresponds with other researcher's observations that Norwalk virus has been extremely difficult to diagnose due to refractory growth in tissue culture and minimal viral shedding in the feces (24, 52).

The cDNA hybridization assay, using a probe cloned from SMSV-5, a calicivirus of ocean origin with a broad host range, was capable of detecting all cultivatable caliciviruses tested, including the type species for the Caliciviridae family, VESV-A_{48}. Furthermore this group-specific probe was capable of detecting a non-cultivatable calicivirus, Norwalk virus, in a paired stool sample

from an experimentally infected human volunteer. Further studies are required to assess the probe's sensitivity, and specificity for detecting caliciviral contamination in clinical and field samples, as well as human and animal food supplies. This group-specific cDNA hybridization assay for caliciviruses will provide another tool for evaluating the marine calicivirus hypothesis which states that serotypes of VESV, SMSV, FCV, and perhaps other members of the Caliciviridae, encompass antigenic variants of a single agent which can be contained in marine host reservoirs.

Table 2.1: A list of picornaviruses obtained from the American Type Culture Collection which were used to assess the specificity of the calicivirus-specific cDNA hybridization probe (5RT73xr-300).

Virus	Strain	ATCC #
Echo 6	D'Amori	#VR 36
Echo 2	Cornelis	#VR 32
Echo 8	Hall	#VR-1049
Echo 9	Vispo	#VR-1051
Coxsackie A-22	Chulman	#VR-177
Coxsackie A-5	G.S. (Swartz)	#VR-164
Coxsackie A-4	ALA-CDC	#VR-318
Coxsackie A-7	W.P. (Parker)	#VR-166
Enterovirus 68	Fermon	#VR-561
Enterovirus 69	Toluca-1	#VR-785
Enterovirus 71	BRCR	#VR-784
Enterovirus 70	J670/71	#VR-836
Coxsackie B-5	Faulkner	#VR-185
Coxsackie B-6	Schmitt	#VR-155
Coxsackie B-4	JVB	#VR-184

Table 2.2: A list of viruses, their taxonomic family, and source, that were negative in a cDNA dot hybridization assay using a calicivirus-specific probe (5RT73xr-300). All RNA samples were extracted from virus-infected cell culture lysates. All ATCC samples were isolated from humans.

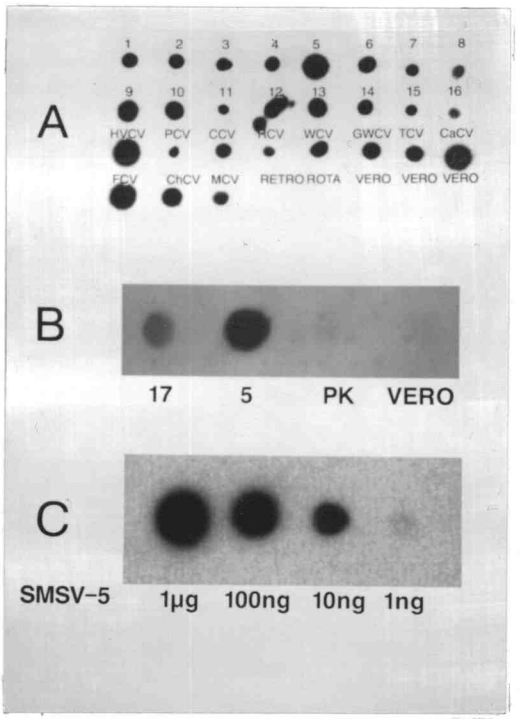
Virus	Family	Source
Echo 6	Picornaviridae	ATCC
Echo 2	Picornaviridae	ATCC
Echo 8	Picornaviridae	ATCC
Echo 9	Picornaviridae	ATCC
Coxsackie A-22	Picornaviridae	ATCC
Coxsackie A-5	Picornaviridae	ATCC
Coxsackie A-4	Picornaviridae	ATCC
Coxsackie A-7	Picornaviridae	ATCC
Enterovirus 68	Picornaviridae	ATCC
Enterovirus 69	Picornaviridae	ATCC
Enterovirus 71	Picornaviridae	ATCC
Enterovirus 70	Picornaviridae	ATCC
Coxsackie B-5	Picornaviridae	ATCC
Coxsackie B-6	Picornaviridae	ATCC
Coxsackie B-4	Picornaviridae	ATCC
Entero-1	Picornaviridae	Human Feces

Table 2.2: Continued.

Virus	Family	Source
C-2222	Rotaviridae	Fur Seal
T2/19	Retroviridae?	Walrus
Equine viral	Togaviridae	Diagnostic

Figure 2.1: Dot hybridization with calicivirus cDNA probe 5RT73xr-300. **A)**. Hybridization against RNA extracted from calicivirus-infected Vero monkey kidney cell lysates. Samples are as follows: **1 - 16**, San Miguel sea lion virus (SMSV) types 1 - 16; **HVCV**, human vesicular calicivirus (SMSV-5 isolate recovered from human vesicular lesions on the hands and feet); **PCV**, primate calicivirus; **CCV**, cetacean calicivirus Tursiops-1; **RCV**, reptile calicivirus Cro-1; **WCV**, walrus calicivirus; **GWCV**, gray whale calicivirus W-6 (vesicular exanthema of swine virus type A_{48}); **TCV**, Tillamook calicivirus (bovine calicivirus Bos-1); **CaCv**, canine calicivirus; **FCV**, feline calicivirus F-9; **ChCV**, cheetah calicivirus; **MCV**, mink calicivirus. Non-calicivirus-infected Vero cell lysates include: **Retro**, putative retrovirus isolated from a walrus; **Rota**, rotavirus isolated from a Northern fur seal. Uninfected Vero monkey kidney cells (**Vero**) were used as a negative control. **B)**. Hybridization against RNA extracted from SMSV-17-infected porcine kidney (PK-15) cell lysates (**17**), and SMSV-5-infected Vero cell lysates (**5**). Uninfected cells (**PK** and **Vero**) were used as negative controls. **C)**. Hybridization against serial log dilutions of RNA extracted from density gradient purified SMSV-5. Approximate amounts of RNA, as determined by ultraviolet spectroscopy, are indicated beneath the dot.

Figure 2.1: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PCV
CCV
RCV
WCV
GWCV
TCV
CaCV
FCV
ChCV
MCV
RETRO
ROTA
VERO
VERO
VERO
B
17
5
PK
VERO
C
SMSV-5
1µg
100ng
10ng
1ng

Figure 2.2: Dot hybridization with calicivirus cDNA probe 5RT73xr-300 against RNA extracted from stool samples taken from humans with caliciviral disease. Samples are as follows: **NV** and **P-NV**, paired stool samples from an experimentally infected human volunteer (**P-NV** is the stool collected prior to experimental Norwalk virus exposure; **NV** is the post-exposure sample); **HCV**, human calicivirus-containing stool as determined by direct electron microscopy; **HEV**, **left** stool sample from an outbreak of hepatitis E virus (non-A non-B enterically transmitted hepatitis) in Mexico; **HEV**, **right** stool sample is pooled feces from a hepatitis E virus outbreak; **SMSV-5**, positive control, San Miguel sea lion virus type 5.

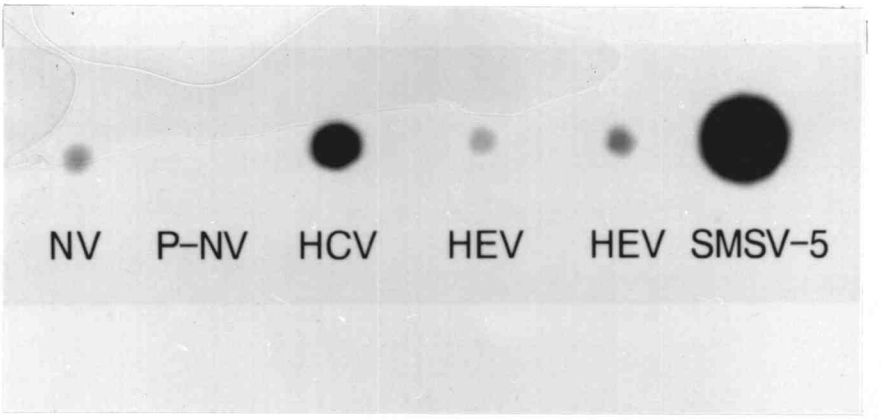


Figure 2.3: Dot hybridization with calicivirus cDNA probe 5RT73xr-300 against RNA extracted from infected Vero monkey kidney cell lysates: **SMSV-5**, San Miguel sea lion virus type 5; **GWCV**, gray whale calicivirus W-6 (VESV-A48). Extracted gray whale rectal contents, from which the original gray whale calicivirus isolation was made in 1969, were also probed (**Fecal**). Uninfected Vero cell RNA is also shown (**Vero**).

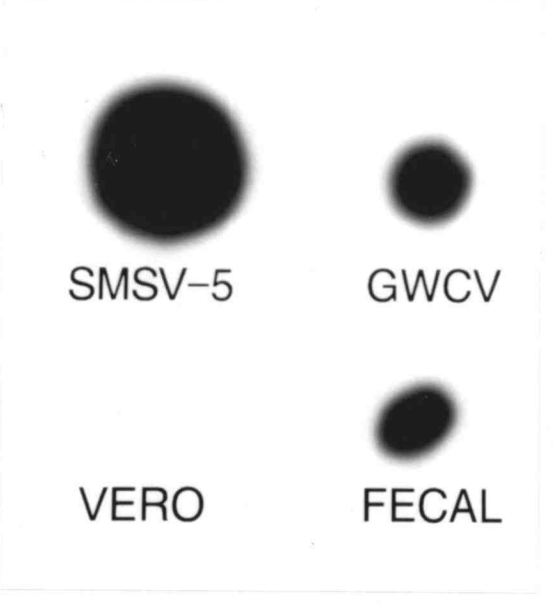
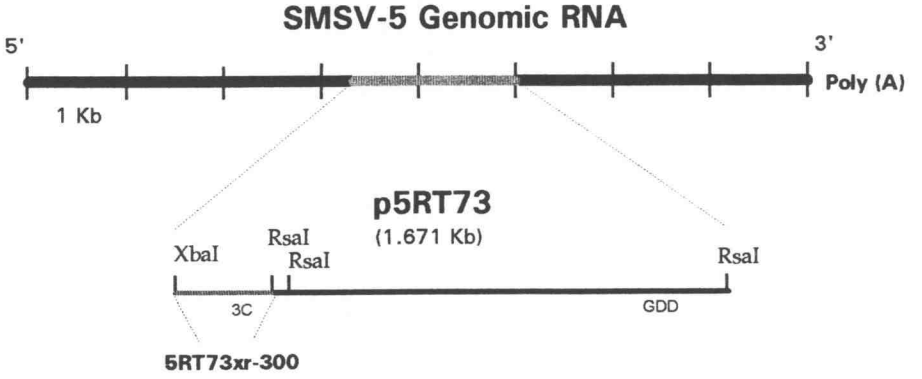


Figure 2.4: Physical map, genomic location, and translated amino acid sequence comparison of p5RT73. Feline calicivirus was used as a reference standard calicivirus genome (40). **A)**. Genomic location and size of p5RT73 within the calicivirus genome, as well as the restriction map and location of the 5RT73xr-300 probe used in the hybridization experiments. The 3C-like cysteine protease region (3C) and the RNA-dependent RNA polymerase region (GDD) are also indicated. **B)** Translated amino acid sequence comparison of SMSV-5 to other published calicivirus amino acid sequences within the 3C-like cysteine protease region of the non-structural protein of caliciviruses. SMSV-5 was compared to: **FCV**, feline calicivirus (155); **RHDV**, rabbit hemorrhagic disease virus (145); and **SV**, Southampton virus, a Norwalk-like virus, (122). Carats (^) above amino acid residues represent the putative catalytic sites of the protein. Asterisks (*) under the sequences represent the number of caliciviruses which have identical amino acid residues to SMSV-5. **C)**. Translated amino acid sequence comparison of SMSV-5 to other published calicivirus amino acid sequences within the RNA-dependent RNA polymerase region of the non-structural protein of caliciviruses. The symbols, and origin of comparison sequences and are as in Figure 2.4B.

Figure 2.4:

A



B

3C - Like Protease Region

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                  ^                                                 ^
SMSV-5    P G D C G L P Y T D E H G - - - - V V V G L H A G
FCV       P G D C G L P Y I D D N G - - - - R V T G L H T G
RHDV      H G D C G L P L Y D S S G - - - - K I V A I H T G
SV        P G D C G A P Y V Y K R A N D W - V V C G V H A A
          * * * * * * * *   *     *           * * * * * * *
          * * * * * * * *   *     *           * *   *   * *
            * * * *   *             *                   *
```

C

RNA Polymerase Region

```
SMSV-5    RYCVDYSKWDSTQPPKVTSQSIDILRHFTDK-SPIVDSACATLKSNPVGIFNGVAFK
FCV       VFAVDYSKWDSTQSPRVSAASIDILRYFSDR-TPIVDSATNTLKSPPIAVFNGVAVK
RHDV      FLCLDYSKWDSTMSPCVVRLAIDILADCCEQ-TELTKSVVLTLKSHPMTILDAMIVQ
SV        HFDADYTAWDSTQNRQIMTESFSIMCR--LTASPELASVVAQDLLAPSEMDVGDYVI
          ********** * *   ****** * *  ******* ***** *  ****** *
            ********* * *   *****        *   *   **** *    *
              **  ****        *            *   *

                ^^^^^                                           ^^^^
SMSV-5    VAG-GLPSGMPLTSIINSLNHCLMVGCAVTKALEDSGVQVTWNIFDSMDLFTYGDDG
FCV       VSS-GLPSGMPLTSVINSLNHCLYVGCAILQSLEARNVPVTWNLFSTFDMMTYGDDG
RHDV      TKR-GLPSGMPFTSVINSICHWLLWSAAVYKSCAEIGLHCS-NLYEDAPFYTYGDDG
SV        RVKEGLPSGFPCTSQVNSINHWLITLCALSEVTGLSPD-V---IQSMSYFSFYGDDE
          *   ********** ******** ***** * ** *** ******    *  ******
              ******* ** *** ** *   **          *  *        ******
              ***** * **  **  * *    *                      ****

SMSV-5    VYIVPPLIS-SVMPKVFAN
FCV       VY-MFPTMYASISDQIFAN
RHDV      VYAMTPMMV-SLLPAIIEN
SV        IVSTDIEFDPAKLTQVLRE
          **   *    *  * ****
          **   *    *    *
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CHAPTER THREE

Detection and Isolation of a Calicivirus from a Bivalve Mollusk (*Mytilus californianus*) Collected from Rocks Adjacent to Pinniped Rookeries

Steven E. Poet, Douglas E. Skilling, Robert L. DeLong, and Alvin W. Smith

ABSTRACT

Bivalve mollusks have been epidemiologically linked to the transmission of caliciviral gastroenteritis in humans. In order to study shellfish as potential reservoirs of calicivirus disease in pinniped populations, 22 mussels (*Mytilus californianus*) were collected from the rocky intertidal zone of Point Bennett, San Miguel Island, California in the Spring of 1992. Gill and intestinal tissue, and residual water, from the shellfish, were processed separately for virus isolation and calicivirus cDNA hybridization. A calicivirus was isolated from one mussel intestinal sample, and was identified by serum neutralization assay as San Miguel sea lion virus type 17 (SMSV-17), first isolated from a nasal swab of a dead, premature sea lion pup (*Zalophus californianus*) taken on San Miguel Island in the Spring of 1991. Negative stain electron microscopic examination of the original tissue sample revealed recognizable calicivirus particles with smudged capsid morphology compared to calicivirus particles passed in cell culture. Of the 66 samples assayed with the calicivirus cDNA hybridization probe, 59 were positive, including the intestinal sample yielding the SMSV-17 isolate. These findings suggest that many of the caliciviruses in the marine environment may be difficult or impossible to cultivate. Mussels collected from Puget Sound, Washington and Yachats, Oregon, regions not associated with high densities of pinnipeds, were all

negative for virus isolation and cDNA hybridization. Mussels, and possibly other bivalve mollusks appear to be an important ocean reservoir for caliciviral agents.

INTRODUCTION

The Caliciviridae family comprises a group of morphologically distinct viruses which are capable of causing disease in a wide variety of marine and terrestrial animals, including humans. Caliciviruses have been arranged into three groups for descriptive purposes. The first group, the candidate caliciviruses, are generally associated with enteric disease, including hepatopathies, of terrestrial animals and humans, and are either extremely difficult to, or have never been, propagated in cell culture systems. Norwalk virus, human calicivirus, hepatitis E virus, porcine enteric calicivirus, Newbury agent, chicken calicivirus, and rabbit hemorrhagic disease virus are examples of candidate caliciviruses (27, 49). The second group, the terrestrial caliciviruses, have not been observed to infect marine organisms, mainly due to the absence of studies designed to look for them (204). Examples of terrestrial caliciviruses include: feline calicivirus, canine calicivirus, and primate calicivirus. The third, and largest, group, the caliciviruses of ocean origin, have an unusually broad host spectrum and diverse tissue trophism. These marine agents have been shown to move great distances in the environment, bridging the land - sea barrier. One pathogen from this last calicivirus group, San Miguel sea lion virus, type 5 (SMSV-5), is the first known zoonotic virus with an ocean origin. Examples of ocean origin caliciviruses include: thirteen serotypes of vesicular exanthema of swine virus (VESV), seventeen serotypes of San Miguel sea lion virus (SMSV), Tillamook (or bovine) calicivirus (BCV Bos-1), primate

calicivirus (PCV Pan-1), and mink calicivirus (MCV). While a large number of ocean-origin caliciviruses have been isolated in cell culture systems, many resist initial isolation attempts, sometimes requiring three to six blind passages (204). It is probable that many more marine caliciviruses exist which are refractory to isolation in available laboratory cell lines.

Despite their name, ocean-origin caliciviruses are also capable of causing severe disease in terrestrial animal species. The eradication of VESV from United States swine herds, cost the Department of Agriculture $39,000,000 from 1954 to 1956. Unfortunately, in 1994, it was shown that VESV still existed in non-porcine reservoirs ten years after its declared irradication in 1959. This came about when the first virus isolated from any marine mammal, a 1969 gray whale enterovirus (W-6), was reclassified as VESV-A_{48}, the prototype virus for the Caliciviridae family (225). In 1972, Smith and co-workers (207), isolated the first San Miguel sea lion virus. This first virus isolate from pinnipeds was morphologically, physicochemically, and pathogenically indistinguishable from VESV. As additional serotypes of caliciviruses with ocean lineages were isolated from a wide variety of marine mammals, and terrestrial animals, it became apparent that these agents were widespread in the marine and terrestrial environment. The theory that the numerous serotypes of VESV, SMSV, other marine caliciviruses, and possibly terrestrial caliciviruses, represent antigenic variants of a single caliciviral agent whose major reservoirs are ocean based, has been hypothesized (201).

Smith and Akers (203) suspected that marine mammal populations may not be the primary reservoir for ocean origin caliciviruses, and suggested that fish or other components in the marine mammal food chain may harbor these agents. In pursuing evidence to support the marine calicivirus hypothesis, Smith and co-

workers (223), isolated SMSV-6 and SMSV-7 from the opaleye (*Girella nigricans*), a food source of California sea lions (*Zalophus californianus*). Furthermore, SMSV-7 was also isolated from a California sea lion parasitic trematode (*Zalophetrema sp.*). This virus was capable of producing clinical vesicular exanthema of swine, and spreading by pen contact, in exposed pigs (223).

The possibility of an invertebrate - teleost - marine mammal cycle for caliciviruses was studied with the sea lion lung worm (*Parafilaroides decorus*) and its intermediate host, the opaleye (222). The nematode parasite larvae were placed into stock cultures of SMSV-5 and rinsed before being fed to opaleye fish. Thirty one days later, when the encysted larval nematodes were infective, the opaleye were ground and fed to Northern fur seal pups (*Callorhinus ursinus*). Vesicles were observed on the fur seals, and SMSV-5 was reisolated from the vesicular fluid (222). It was postulated that a single opaleye, could become the intermediate host to many different serotypes of caliciviruses, by way of multiple infestations with the parasitic nematode, and subsequently transmit multiple calicivirus serotypes to predator marine mammals.

Bivalve mollusks are also capable of filtering and concentrating viral agents from polluted water. Human viral pathogens, such as Norwalk virus, picornaviruses, hepatitis A virus, and rotaviruses have been responsible for thousands of shellfish-associated viral gastroenteritis cases since 1980 (123, 178). Interestingly, shellfish are one of the few foods still commonly consumed raw or lightly cooked, and have remained an important vector in the transmission of viral disease within the human population, despite attempts to regulate water quality where shellfish are harvested. It has been reported that shellfish may be an

important vector in the transmission of viral agents up the food chain by concentrating potential pathogens in their feces which are subsequently consumed by helminth organisms which, in turn, are common food sources for fish (142).

San Miguel Island lies off the coast of Southern California, and Point Bennett, located on the Northeast end of the island, is a major breeding and pupping rookery for California sea lions, Northern fur seals, Northern elephant seals (*Mirounga angustirostris*), and harbor seals (*Phoca vitulina*). In addition, this Northern-most island of the Santa Barbara Channel Island Chain is an occasional haul out site for Steller sea lions (*Eumatopias jubatus*), making it the most diversified pinniped rookery in the world and a common site from which ocean-origin caliciviruses have been isolated (12, 13). The mussel (*Mytilus californianus*), is an abundant inhabitant of the rocky intertidal zone along the Pacific coast of the United States. On San Miguel Island, they are found in close proximity to the pinniped rookeries and, therefore, could have the potential, to accumulate and transmit viral disease agents within pinniped populations, as occurs with Norwalk virus in shellfish grown in close proximity to human populations. The objective of this study was to use virus isolation and cDNA hybridization with a recently identified group-specific probe, to determine if caliciviruses were present in mussels located on San Miguel Island, adjacent to areas of high pinniped density. Furthermore, mussels collected from regions not associated with high densities of pinnipeds were examined to determine if calicivirus contamination in mussels was independent of marine mammal presence.

MATERIALS AND METHODS

Collection of Mussels

Twenty two mussels (*Mytilus californianus*) were collected from the rocky intertidal zone on the Southeast and West shorelines of Point Bennett, San Miguel Island, California in the Spring of 1992. Shellfish were collected near California sea lion (*Zalophus californianus*) haul out sites. The mussels were individually packaged in dry ice, transported to the mainland, and shipped over night to Oregon State University, Laboratory for Calicivirus Studies (LCS), where they were stored at -70°C until processed.

Thirty two mussels were collected from nets of a salmon net-pen farm located in Puget Sound along the Northern coast of the Olympic Peninsula, Washington. Sixty mussels were collected from rocks near Yachats, on the central Oregon Coast. Both mussel groups were frozen at -70°C, transported to Oregon State University, LCS and stored at -70°C until processed. While pinnipeds are frequently seen around the two mussel collection sites in Washington and Oregon, these sites are not areas of tremendously high animal population densities, which is typical of breeding and pupping rookeries such as San Miguel Island.

Individual mussels from San Miguel Island were thawed and the gill, intestinal tissue, and residual water were separated. Gill and intestinal tissue from the Puget Sound mussels were also separated and tissue from four individuals was pooled. Residual water from all Puget Sound mussels was treated as one sample. Whole Oregon Coast mussels were pooled into twelve groups of five individuals.

Tissue samples or whole mussel pools were ground in Tris buffered saline (100 mM Tris, pH 7.5; 150 mM NaCl) to a final dilution of approximately 1:5 (w/v), and frozen at -70°C until further processing was carried out.

Virus Isolation and Identification

Tissues collected for virus isolation were thawed, mixed, and clarified in a Beckman TJ-6 centrifuge at 1,500 x *g* for 10 minutes. Aliquots (200 μl) of each sample were absorbed onto separate monolayers of African green monkey kidney (Vero) cells and porcine kidney (PK-15) cells in roller tubes. Samples were passaged, at 37°C, one to three times in Eagle minimal essential medium with Earle's salts (MEM) containing 5% iron supplemented calf serum. All virus isolates were, purified by three plaque passages in PK-15 cells, using agarose overlays, and their morphology was determined by negative stain electron microscopy (199, 207).

Virus isolates were screened against specific typing antisera to approximately 37 calicivirus serotypes by standard cross-neutralization testing, using 100 $TCID_{50}$ units of test virus against 20 antibody units of typing serum, where one antibody unit is defined as the dilution of typing serum required to neutralize 100 $TCID_{50}$ of its corresponding viral serotype (208, 226).

RNA Extraction

The RNA within the mussel tissue was extracted using a modification of the phenol/ guanidinium isothiocyanate procedure of Chirgwin and co-workers (1979). RNAzol B (Cinna/Biotecx) was used to extract all samples. Residual water

samples were first pelleted at 12,000 x *g* for 20 minutes in a microcentrifuge and the resultant pellet was resuspended in 100 μl of TBS. Each water sample and approximately 100 μl of ground mussel tissue was transferred to a sterile 1.5 ml microcentrifuge tube, 800 μl of RNAzol B was added to disrupt the tissue, solublize nucleic acid, and inactivate ribonucleases. The solution was mixed briefly before 80 μl of chloroform was added. The sample was stored on ice for 5 minutes before it was mixed vigorously for 15 seconds and again allowed to stand on ice for an additional 15 minutes. The solution was microcentrifuged (14,000 xg) at 4°C for 15 minutes 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 minutes or overnight. The RNA was pelleted in a microcentrifuge for 15 minutes and washed once with 500 μl of 75% ethanol. After another microcentrifugation for 5 minutes the supernatant was removed and the sample was centrifuged again to drive all the residual ethanol to the bottom of the tube so the final traces could be removed. The RNA pellet was dissolved in 50 μl of diethylpyrocarbonate (DEPC)-treated ddH_2O, and the concentration and purity of the viral RNA were determined by ultraviolet spectroscopy (136).

Preparation of the cDNA Probe

The plasmid containing the cDNA hybridization probe, p5RT73, was digested with the restriction endonucleases, Xba I and Rsa I (IBI, Inc.). The reaction digest was extracted with phenol:chloroform, and ethanol precipitated in preparation for biotinylation using random octamer priming. The protocol used to

label the probe was provided by BRL in their BioPrime DNA Labeling System. Approximately 100 ng of denatured restriction digest was biotinylated by incubating at 37°C for 4 hours in a solution containing a mixture of: 50 mM 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 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.

cDNA:RNA Dot Hybridization

Approximately 2 μg each of the mussel tissue RNA was spotted manually, or by vacuum filtration, onto uncharged nylon membrane (PhotoGene Nylon Membrane, BRL). After the membrane was allowed to air dry, it was exposed to germicidal ultraviolet radiation (280 nm) for 20 minutes 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 and co-workers (91) modification of a protocol provided by BRL in the PhotoGene Nucleic Acid Detection System. The membrane was soaked in hot (65°C) prehybridization solution (1.0M NaCl, 1.0% SDS) and placed in a hybridization roller bottle. Prehybridization solution was added to the bottle and the membrane was incubated

at 65°C for 3 hours at six rotations per minute. The prehybridization solution was removed and approximately 500 ng of boiled biotinylated probe was added to hot hybridization solution (10% dextran sulfate, 1.0M NaCl, 1% SDS, and 100 μg/ml denatured heterologous DNA)and incubated 48 hours at 65°C. After hybridization, the membrane was washed under high stringency conditions with 5X SSC (750 mM NaCl, 75 mM sodium citrate, dihydrate)/0.5% SDS at 65°C for 5 minutes, 2X SSC/1% SDS at 65°C for 5 minutes, and 0.5X SSC/1% SDS at 65°C for 30 minutes. The membrane was then rinsed in TBS/0.05% Tween 20 and blocked with 3% BSA/TBS/0.05% Tween 20 for one hour at 65°C. A 1:1000 dilution of the provided streptavidin:alkaline phosphatase conjugate was applied to the membrane at room temperature for 10 minutes, and the membrane was washed twice with TBS/0.05% Tween 20 at room temperature for 15 minutes and washed three times with BRL-provided final surfactant solution at room temperature for 20 minutes. The detection reagent (4-methoxy-4-[3-phosphatephenyl]spiro[1,2-dioxetane-3,2'-adamantane]) was applied to the washed membrane, and allowed to react for 3 hours at room temperature, in the dark. Samples hybridizing to the probe were visualized by exposing X-ray film (Kodak X-Omat Xs-5) to the membrane for approximately 5 minutes.

Biotinylated vector (pCDNA II, Invitrogen, Inc.) was used to probe the same samples, after stripping the nylon membranes by boiling in 0.1X SSC/0.1% SDS for 15 minutes, to ensure that non-specific hybridization reactions had not occurred.

RESULTS

A single virus isolate with typical calicivirus morphology was recovered from the intestinal tissue of San Miguel Island mussel #19 on the first pass in PK-15 cells. This isolate was designated Mc-19Gt (*Mytilus californianus*, #19 gut). To confirm viral presense in the original tissue sample, it was examined under the electron microscope and calicivirus-like particles were observed. The particles, however, appeared to be smudged or blurred when compared with sample material that had been passaged five times in PK-15 cells (Figure 3.1A, 3.1B). Subsequent to triple plaque passage, the purified stock Mc-19Gt was examined in a standard microtiter cross-neutralization assay and was inactivated only by SMSV-17 type-specific antiserum. This most recent isolate in the San Miguel sea lion virus series was designated as a strain of serotype SMSV-17. The prototype strain of SMSV-17 was first isolated from a nasal swab, taken from of a dead, premature California sea lion pup, on San Miguel Island in the Spring of 1991 (202). No isolations were made from the Oregon or Washington mussels.

The group-specific cDNA calicivirus probe (5RT73xr) hybridized, at differing intensities, with 59 of 66 San Miguel Island mussel samples (Figure 3.2). Each of the 22 mussels had at least one tissue which reacted with the probe (Table 3.1). The Oregon Coast and Puget Sound mussels, however, were all negative when assayed in the cDNA dot hybridization assay (Figure 3.3). Moreover, extracted RNA from fifth passage Mc-19Gt-infected PK-15 cell culture lysates hybridized with the group-specific calicivirus probe (p5RT73xr), while RNA from uninfected cell lysates did not (Figure 3.1). When the mussel samples were similarly probed using the vector plasmid without calicivirus cDNA, there was no significant hybridization (results not shown).

DISCUSSION

Ocean-origin caliciviruses have been exclusively identified by virus isolation methods, since their discovery in 1972 (12, 13). Caliciviruses which do not grow in cell culture, and , therefore, cannot be isolated, have been observed in a wide variety of domestic animals and humans (49). With the application of a calicivirus group-specific cDNA hybridization probe which does not rely on virus isolation, it appears that the ocean environment contains non-cultivatable members of the Caliciviridae, as well. Dot hybridization analysis indicated that at least one tissue from all the mussel samples collected at San Miguel Island contained caliciviral RNA (Figure 3.2, Table 3.1), while only one sample in 66 contained calicivirus that was successfully grown *in vitro*. Blurring and smudging of the capsid projections is evident when particles contained within the original tissue sample (Mc-19Gt) are compared with particles which were passed five times from that same sample in PK-15 cells (Figures 3.1A, 3.1B). This alteration in virus capsid morphology may be inhibiting viral replication in cell culture. Hillman and co-workers (95) described a similar phenomenon in amyelois chronic stunt virus, a non-cultivatable insect calicivirus, in which the capsid structure was altered in a large fraction of viral particles observed in infected larvae. This capsid structure alteration may also be occurring in mussels.

San Miguel Island does not have a human population and is the Northern most island in the Santa Barbara Channel Island chain. This island's remoteness from human impact has enabled four species of pinnipeds to maintain a major breeding rookery, despite its location off the coast of Southern California (12, 13). It is unlikely that the source of non-cultivatable calicivirus within the mussel population is due to human waste, as is thought to occur in Norwalk virus

gastroenteritis associated with the consumption of contaminated shellfish. Mussels from Puget Sound and Oregon, not associated with high population densities of marine mammals, but closer to human sewage outfall, did not contain calicivirus RNA (Figures 3.3A, 3.3B).

Ocean-origin caliciviruses have been isolated regularly from the marine mammal population on San Miguel Island, California since 1972 (204). Virus isolation and serological studies conducted throughout the North Pacific Ocean, Bering Sea, and Arctic Ocean, have shown that marine mammals, terrestrial animals, and at least one species of fish are being exposed to a wide variety of ocean-origin calicivirus serotypes. The presence of serotype-specific neutralizing antibodies establishes a trail for that specific agent within and among different populations of animals. Significant differences in the diversity of calicivirus-specific serum neutralizing antibodies exists between species of pinnipeds. Smith and Latham (205) reported that California sea lions at San Miguel Island had a high prevalence of antibodies to multiple serotypes of VESV and SMSV, while Northern fur seals, sampled from the same location, tested negative for the same VESV antibody types, and had antibodies to only one serotype of SMSV. They also reported that neutralizing antibodies to several VESV serotypes, including VESV-A_{48}, were present in 4 month old sea lion pups suggesting that VESV was still active along the California Coast. The recent reclassification of the gray whale enterovirus (W-6), which was isolated in 1969, to VESV-A_{48}, establishes VESV presence in the ocean a decade after it was thought to have been eradicated from the United States, and confirms the serological findings within the pinniped population (225).

Sea lions and fur seals frequently intermix on San Miguel Island, and direct contact between the two species is common. Furthermore, susceptibility to calicivirus infection appears to be similar between the two species (208). Fur seals feed offshore at the margin of the continental shelf, while sea lions feed close to shore (112). The differences, therefore, in the feeding habits of the two species may result in differing exposure and explain the serological profile differences, particularly if calicivirus reservoirs exist among near-shore marine organisms. The possibility of the sea lions themselves being the primary reservoir is unlikely because of their small population (208). Smith and co-workers (223) were able to identify one possible nearshore reservoir when they isolated two serotypes of SMSV from the opaleye, a fish which inhabits tide pools along the Southern California Coast.

Caliciviruses, which can be pathogenic for humans, sea lions, and other mammals, may have similar ocean-based reservoirs, in lower vertebrates and invertebrates, where the viruses are primarily maintained. This enables the agent to escape mammalian immunological pressures, which drive antigenic drift, and periodically emerge antigenically unaltered into a naive mammal population, through the food chain. The isolation, from a mussel, of SMSV-17, a calicivirus previously recovered from an aborted California sea lion pup, establishes bivalve mollusks as a possible reservoir for ocean-origin caliciviruses, and perhaps other members of the Caliciviridae family. Shellfish have been implicated as reservoirs in a number of human gastrointestinal diseases of viral etiology (123, 178). Norwalk virus, and other calicivirus-like agents, are responsible for many cases of acute nonbacterial gastroenteritis. Caliciviral associated gastroenteritis outbreaks are often epidemiologically linked to the consumption of raw or undercooked shellfish

(78, 88, 92). It is widely believed that virus contamination of shellfish beds occurs by human or animal sewage runoff during times of heavy rainfall (78, 85) Morse and co-workers (152), in studying a Norwalk virus gastroenteritis epidemic in New York State, associated with the consumption of raw oysters and clams, found that contamination of shellfish beds by heavy spring runoff could explain some of the disease episodes. Other outbreaks, however, during periods without flooding, suggested that virus was also endemic within the shellfish populations (152).

A single serotype of calicivirus has been isolated from the walrus (*Odobenus rosmarus*) on three different occasions, from feces on sea ice in the Chukchi Sea (216). The walrus lives mainly on ice flows in the Northern waters of the planet and has very little contact with other pinnipeds known to be infected with caliciviruses (112). Interestingly, the diet of walruses is made up of a variety of benthic marine organisms, a significant portion of which are bivalve mollusks. Migrating marine mammals known to be infected with caliciviruses, such as the gray whale, could conceivably shed virus into the water, during bottoming feeding, where it is concentrated within benthic marine organisms. The virus would then have the potential to infect another susceptible species by gaining access to the host through the food chain.

The isolation of SMSV-17 (a calicivirus known to infect California sea lions), from the mussel, has established this species of bivalve mollusk as another possible reservoir for ocean-origin caliciviruses. The presence of Norwalk-like caliciviruses within mussel tissues, as detected by cDNA hybridization, also provides evidence that ocean-origin caliciviruses may have similar transmission cycles as that seen with human caliciviral disease agents. Further studies of calicivirus transmission routes within various mammalian food chain organisms

may help develope strategies for controlling caliciviral disease in domestic livestock, wild animals, and humans.

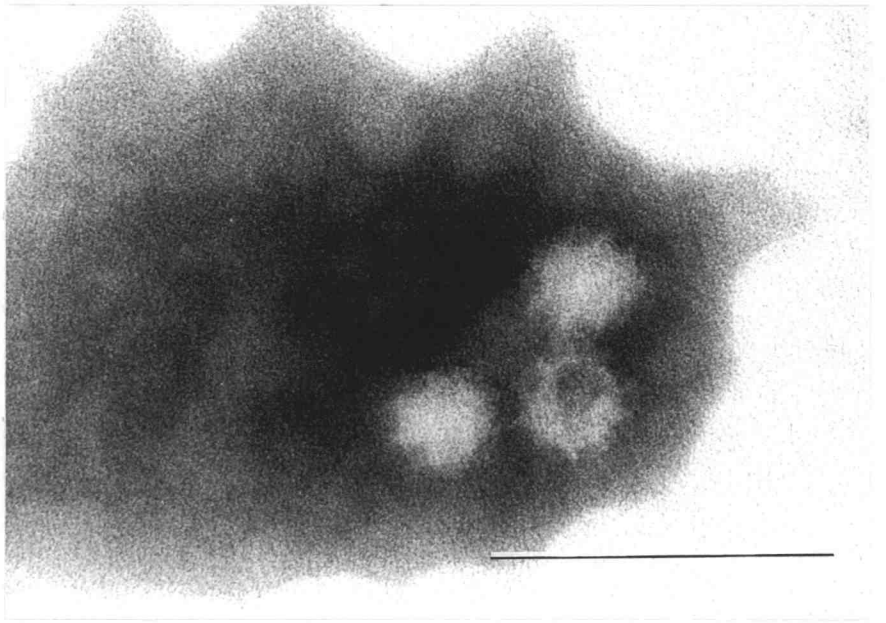
Table 3.1: Sample code for Figure 1. List of mussel samples, their corresponding cDNA dot blot sample number (Figure 1), the tissue origin of the sample, and hybridization result. The asterisk (*) represents the mussel intestinal tissue from which SMSV-17 was isolated.

Table 3.1:

Mussel/Blot No.	Tissue	Result	Mussel/Blot No.	Tissue	Result	Mussel/Blot No.	Tissue	Result
Mc1 / 62	Water	+	Mc9 / 35	Water	+	Mc17 / 6	Water	+
Mc1 / 38	Gill	+	Mc9 / 13	Gill	+	Mc17 / 7	Gill	+
Mc1 / 39	Gut	+	Mc9 / 14	Gut	+	Mc17 / 8	Gut	+
Mc2 / 60	Water	-	Mc10 / 52	Water	+	Mc18 / 51	Water	+
Mc2 / 48	Gill	+	Mc10 / 15	Gill	+	Mc18 / 9	Gill	+
Mc2 / 42	Gut	+	Mc10 / 23	Gut	+	Mc18 / 10	Gut	+
Mc3 / 57	Water	+	Mc11 / 49	Water	-	Mc19 / 53	Water	+
Mc3 / 45	Gill	+	Mc11 / 16	Gill	-	Mc19 / 11	Gill	+
Mc3 / 44	Gut	+	Mc11 / 19	Gut	+	Mc19 / 12	Gut	+*
Mc4 / 54	Water	+	Mc12 / 1	Water	+	Mc20 / 36	Water	+
Mc4 / 37	Gill	+	Mc12 / 29	Gill	+	Mc20 / 20	Gill	+
Mc4 / 46	Gut	+	Mc12 / 26	Gut	+	Mc20 / 22	Gut	+
Mc5 / 58	Water	-	Mc13 / 2	Water	+	Mc21 / 50	Water	-
Mc5 / 63	Gill	+	Mc13 / 33	Gill	+	Mc21 / 21	Gill	+
Mc5 / 64	Gut	+	Mc13 / 28	Gut	+	Mc21 / 17	Gut	+
Mc6 / 55	Water	+	Mc14 / 3	Water	+	Mc22 / 61	Water	+
Mc6 / 47	Gill	-	Mc14 / 31	Gill	+	Mc22 / 24	Gill	+
Mc6 / 41	Gut	+	Mc14 / 27	Gut	+	Mc22 / 18	Gut	+
Mc7 / 59	Water	+	Mc15 / 4	Water	+			
Mc7 / 65	Gill	+	Mc15 / 34	Gill	+			
Mc7 / 66	Gut	+	Mc15 / 32	Gut	+			
Mc8 / 56	Water	+	Mc16 / 5	Water	+			
Mc8 / 40	Gill	+	Mc16 / 25	Gill	+			
Mc8 / 43	Gut	+	Mc16 / 30	Gut	-			

Figure 3.1: **A)** Negative stain electron micrograph of ground mussel intestinal tissue, showing viral particles with smudged capsid projections, and depressions similar to typical caliciviral morphology. **B)** Negative stain electron micrograph of PK-15 cell culture supernatant from the fifth passage of ground mussel tissue shown in Figure 3.1A. Capsid projections and depressions are more recognizable as typical calicivirus morphology. Bar = 100 nm.

A)



B)

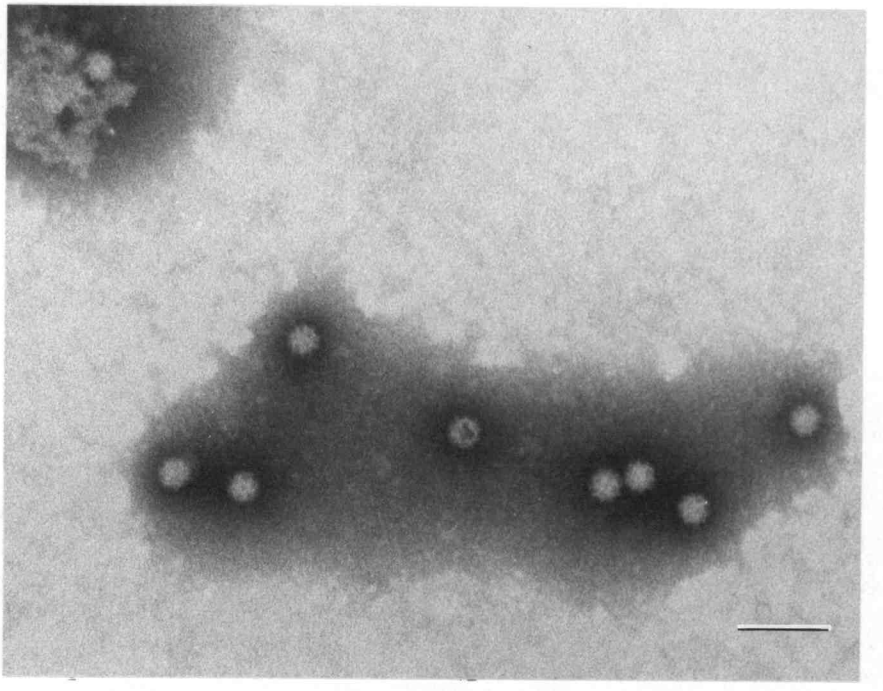


Figure 3.2: Dot hybridization with calicivirus group-specific cDNA probe 5RT73xr, against RNA extracted from Mussels (*Mytilus californianus*) collected from rocks adjacent to pinniped rookeries on San Miguel Island, California. Samples are as follows: **1 - 66**, RNA extracted from mussel gill, intestinal tissue, and residual water. See Table 3.1 for sample code; **S17**, RNA extracted from PK-15 cells infected with the SMSV-17 mussel isolate (fifth passage); **S5**, RNA extracted from Vero cells infected with SMSV-5; **PK** and **V**, RNA extracted from PK-15 and Vero cells, respectively.

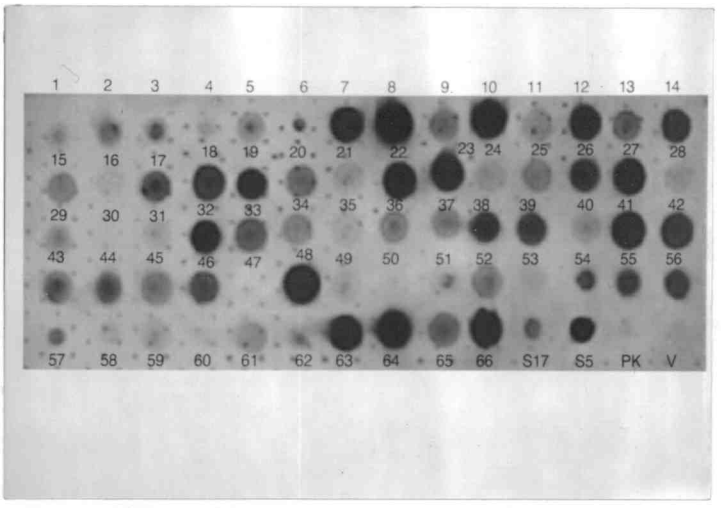
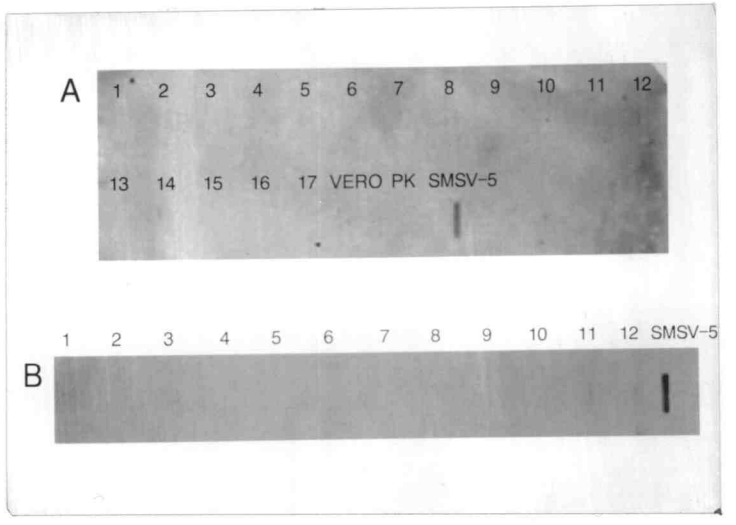


Figure 3.3: Dot hybridization with calicivirus group-specific cDNA probe 5RT73xr, against RNA extracted from Mussels (*Mytilus californianus*) collected from Puget Sound, Washington (**A**) and Yachats, Oregon (**B**). **A)**. Samples are as follows: **1, 3, 5, 7, 9, 11, 13, and 15**, RNA extracted from mussel pools each consisting of gill tissue from 4 individuals; **2, 4, 6, 8, 10, 12, 14, and 16**, RNA extracted from mussel pools each consisting of intestinal tissue from 4 individuals; **17**, RNA extracted from residual water from all mussels collected; **Vero**, RNA extracted from uninfected Vero cells; **PK**, RNA extracted from uninfected PK-15 cells; **SMSV-5**, RNA extracted from SMSV-5-infected Vero cell lysates. **B)** Samples are as follows: **1 - 12**, RNA extracted from 12 mussel pools each consisting of 5 whole-ground mussels; **SMSV-5**, RNA extracted from SMSV-5-infected Vero cell lysates.

Figure 3.3:


A
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 VERO PK SMSV-5
1 2 3 4 5 6 7 8 9 10 11 12 SMSV-5
B

CHAPTER FOUR

Detection of a Non-cultivatable Calicivirus, from the White Tern (*Gygis alba rothschilbi*)

Steven E. Poet, Douglas E. Skilling, Jennifer L. Megyesi, and Alvin W. Smith

ABSTRACT

Pathogenic caliciviruses are known to spread through diverse host species across broad geographic distances. Migratory and wide ranging ocean associated species, including birds, therefore, become suspect in the spread of these agents to naive and susceptible host populations. While studying a Hawaiian monk seal (*Monachus schauinslandi*) failure to thrive syndrome in the population on Tern Island, French Frigate Shoals, Hawaii, researchers observed a hand reared white tern hatchling (*Gygis alba rothschilbi*) develope vesicular lesions on the webbing between its toes, six days after falling out of its nest. The bird was being fed a variety of small fish of unknown species. Vesicular fluid collected from the foot lesions contained virus-like particles typical of calicivirus morphology. Calicivirus RNA was detected within the vesicular fluid by dot hybridization with a group-specific calicivirus cDNA 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. Sea birds may be important in the transmission of ocean-origin caliciviral disease.

INTRODUCTION

The Caliciviridae is a family 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 (12, 13). 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 (49).

Calicivirus-like particles have been detected in association with gastrointestinal disease in three farm raised avian species: chickens, Guinea fowl, and pheasants. Bracewell and Wyeth (26) reported stunting and poor feather growth in young chickens, and particles exhibiting calicivirus-like morphology were seen in intestinal contents of affected animals, but could not be isolated in tissue culture (258). Particles consistent with calicivirus morphology have been observed in cecal contents of six to 16 day old Guinea fowl keets (*Numida meleagris*) dying of typhlitis (80). 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 intestinal contents, in three and four week old pheasant polts displaying signs of enteritis, and paralysis, contained calicivirus-like particles, but samples of unaffected polts did not (81). Chicken calicivirus has been grown *in vitro* with great difficulty, and was shown to cause disease in specific pathogen free day old chicks (51)

The white, or fairy tern (*Gygis alba rothschilbi*) is widely distributed throughout offshore and pelagic waters of the subtropical and tropical Pacific Ocean. This avian species can be observed any distance from land, but is usually

found within 50 miles of breeding islands (113). In the Spring of 1992, while studying a failure to thrive syndrome of the monk seal (*Monachus schauinslandi*) population at Tern Island, French Frigate Shoals in the Northwest Hawaiian Islands, researchers found a newly hatched white tern on the ground under its nesting tree. During attempts to rehabilitate the animal, vesicular lesions developed on the webbing, between the toes of the tern chick's feet.

Five different serotypes of San Miguel sea lion virus (SMSV), an ocean-origin calicivirus, have been isolated from vesicular lesions observed on pinniped flippers and lips (13, 18). Experimental infection has confirmed that SMSV is capable of producing cutaneous lesions in pinnipeds, cattle, swine, and humans (19, 74, 75, 223). Because of the proven vesiculogenic disease potential of ocean-origin caliciviruses, the white tern vesicular fluid was collected for virus isolation and group-specific cDNA dot hybridization to determine if caliciviruses may be associated with vesicular disease in wild sea bird populations.

MATERIALS AND METHODS

Animal History and Sample Collection

A white tern (*Gygis alba rothschilbi*), approximately one day old was found on the ground at Tern Island, French Frigate Shoals, Hawaii in the Spring of 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 five days. The fish were netted at night with the aid of lights as attractants. On the sixth day of rehabilitation, two vesicles, approximately 3 mm in diameter, were observed on the 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, where it was stored at -70°C.

Virus Isolation and Visualization

One half of the vesicular fluid (5 μl) was diluted 1:5 with Eagle minimal essential medium with Earle's salts (MEM) and 12.5 μl of the diluted sample was adsorbed onto separate monolayers of African green monkey kidney (Vero) cells and porcine kidney (PK-15) cells in 96 well microtiter plates. Each sample was blind passaged four times with one freeze-thaw cycle between each pass.

The remaining vesicular fluid was applied to a grid for examination by negative stain electron microscopy (199). The 5 μl drop of vesicular fluid was placed on Parafilm, and a formvar covered, carbon coated glow discharged, copper electron microscopy grid was floated on it for two minutes at room temperature, and the drop was retained for cDNA dot hybridization. The grid was touched to a drop of sterile-filtered double distilled water and blotted dry with filter paper, and floated on 1.5% phosphotungstic acid, pH 7.0, for one minute at room temperature. Excess stain was removed by blotting on filter paper, and the grid was placed under germicidal ultraviolet light for 15 to 20 minutes to inactivate possible pathogens. The grid was examined with a transmission electron microscope at an accelerating voltage of 80 kV.

RNA Extraction

The RNA within vesicular fluid was extracted using a modification of the phenol/ guanidinium isothiocyanate procedure of Chirgwin and co-workers (45). RNAzol B (Cinna/Biotecx) was used to extract all samples. 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 vortexed briefly before 80 μl of chloroform was added. The sample was stored on ice for 5 minutes before it was vortexed vigorously for 15 seconds and again allowed to stand on ice for an additional 15 minutes. The solution was microcentrifuged (14,000 x *g*) at 4^{o}C for 15 minutes and the top aqueous layer was removed to another microcentrifuge tube. An equal volume of cold (-20^{o}C) isopropanol was added and the RNA was precipitated at 4^{o}C for 45 minutes or overnight. The RNA was pelleted in a microcentrifuge for 15 minutes and washed once with 500 μl of 75% ethanol. After another microcentrifugation for 5 minutes the supernatant was removed and the sample was centrifuged again to drive all 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 diethylpyrocarbonate (DEPC)-treated ddH_2O.

Preparation of the cDNA Probe

The plasmid containing the cDNA hybridization probe, p5RT73, was digested with the restriction endonucleases, Xba I and Rsa I (IBI, Inc.). The reaction digest was extracted with phenol:chloroform, and ethanol precipitated in preparation for biotinylation using random octamer priming. The protocol used to

label the probe was provided by BRL in their BioPrime DNA Labeling System. Approximately 100 ng of denatured restriction digest was biotinylated by incubating at 37°C for 4 hours in a solution containing a mixture of: 50 mM 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 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.

cDNA:RNA Dot Hybridization

One half of the vesicular fluid RNA was spotted onto an uncharged nylon membrane (PhotoGene Nylon Membrane, BRL). After the membrane was allowed to air dry, it was exposed to germicidal ultraviolet radiation (280 nm) for 20 minutes 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 and co-workers (91) modification of a protocol provided by BRL in the PhotoGene Nucleic Acid Detection System. The membrane was soaked in hot (65°C) prehybridization solution (1.0M NaCl, 1.0% SDS) and placed in a hybridization roller bottle. Prehybridization solution was added to the bottle and the membrane was incubated at 65°C for 3 hours at six rotations per minute. The prehybridization solution was

removed and approximately 500 ng of boiled biotinylated probe was added to hot hybridization solution (10% dextran sulfate, 1.0M NaCl, 1% SDS, and 100 μg/ml denatured heterologous DNA) and incubated 48 hours at 65^{o}C. After hybridization, the membrane was washed under high stringency conditions with 5X SSC (750 mM NaCl, 75 mM sodium citrate, dihydrate)/0.5% SDS at 65^{o}C for 5 minutes, 2X SSC/1% SDS at 65^{o}C for 5 minutes, and 0.5X SSC/1% SDS at 65^{o}C for 30 minutes. The membrane was then rinsed in TBS/0.05% Tween 20 and blocked with 3% BSA/TBS/0.05% Tween 20 for one hour at 65^{o}C. A 1:1000 dilution of the provided streptavidin:alkaline phosphatase conjugate was applied to the membrane at room temperature for 10 minutes, and the membrane was washed twice with TBS/0.05% Tween 20 at room temperature for 15 minutes and washed three times with BRL-provided final surfactant solution at room temperature for 20 minutes. The detection reagent (4-methoxy-4-[3-phosphatephenyl]spiro[1,2-dioxetane-3,2'-adamantane]) was applied to the washed membrane, and allowed to react for 3 hours at room temperature, in the dark. Samples hybridizing to the probe were visualized by exposing X-ray film (Kodak X-Omat Xs-5) to the membrane for approximately 5 minutes.

RESULTS

Viral cytopathology was not observed in either cell line through four blind passages. The cDNA hybridization assay, however, provided different results. As can be seen in Figure 4.1, a strong hybridization signal exists with the RNA extracted from the small amount of vesicular fluid. Negative stain electron microscopy of the same sample of vesicular fluid contained aggregates of "hazy"

virus-like particles displaying cup-shaped surface depressions typical of the morphology observed when antibody is bound to caliciviruses (Figure 4.2).

DISCUSSION

While caliciviruses have been observed in partially or fully domesticated birds with signs of enteritis, this is the first report of a calicivirus infecting a wild avian species with vesicular disease (80, 81, 258). Calicivirus vesicular disease has also been reported in swine, cattle, pinnipeds, and humans (19, 206, 214, 226, 248). Antibody coating the capsid may explain the "hazy" appearance of the viral particles when viewed under the electron microscope, as is seen in immunoelectron microscopic studies with Norwalk virus and other caliciviruses (107, 229). This humoral immune response by the bird may be contributing 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.

Single serotypes of ocean-origin caliciviruses have been shown to move great distances. In 1980, the bowhead whale (*Balaena mysticetus*), an Arctic species associated with the margins of the pack ice, was found to contain type-specific serum neutralizing antibodies to two serotypes of VESV (J_{56} and K_{56}) which had been isolated only once, 24 years previously, from pigs in New Jersey in 1956 (96, 221). Furthermore, VESV J_{56} and K_{56} neutralizing antibodies were found in a large percentage of California sea lions (*Zalophus californianus*) tested along the California Coast in 1970 - 1972 and 1975 (205). 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.

Monk seal serology also demonstrates the broad geographic distribution of single calicivirus serotypes. This endangered pinniped has a population distribution which is restricted almost exclusively to the Northwest Hawaiian Islands (112). Serum samples from a limited number of animals, sampled in 1978, were shown to contain type-specific serum neutralizing antibodies to SMSV-1 and VESV I_{55} (79). 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 (207). A retrospective serological study of SMSV-1 antibody prevalence in sea lions showed a dramatic increase from 1970 - 1971 to 1972. By 1975, however, the prevalence of serum neutralizing antibody titers to SMSV-1 in 4 month old pups had dropped below the 1970 - 1971 values (205). While the high prevalence of SMSV-1 antibodies was short-lived, a mechanism existed by which monk seals, over 3000 miles away, were exposed to this same virus.

Smith and Latham (205) have suggested that a component of the sea lion diet may be a reservoir for ocean-origin caliciviruses. 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, appears to confirm that hypothesis (222, 223).

Calicivirus-like particles that could not be grown in tissue culture as well as calicivirus RNA, determined by cDNA hybridization, was 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 (169). 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 has been shown to eat an extremely wide variety of fish prey species (3). The monk seal on French Frigate Shoals is experiencing a failure to thrive syndrome and a decrease in available food resources has been postulated as one of the causes.(8). This shortage of food may be causing the seals to eat a wider variety of fish, and a common food source may have infected both birds and pinnipeds.

The finding of a calicivirus associated with vesicular disease in the white tern suggests that wild sea bird species may be an important mechanism for the transmission of caliciviruses over large geographical areas. The potential for birds to transmit viruses pathogenic for mammalian species has been realized with the influenza A virus epizootic, involving migratory water fowl transmission, which caused high mortalities in harbor seal populations on the East Coast of North America (76). Sea birds, along with migratory marine mammals may play an important role in the transmission of caliciviral disease. Further virological and serological studies involving sea birds of the Eastern Pacific Ocean may shed light on the mechanisms by which caliciviruses are distributed throughout the world.

Figure 4.1: 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 rothschilbi*) 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.

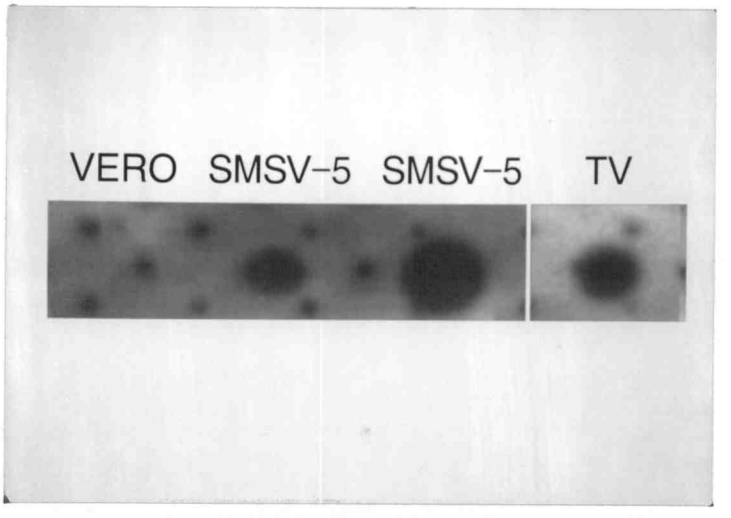
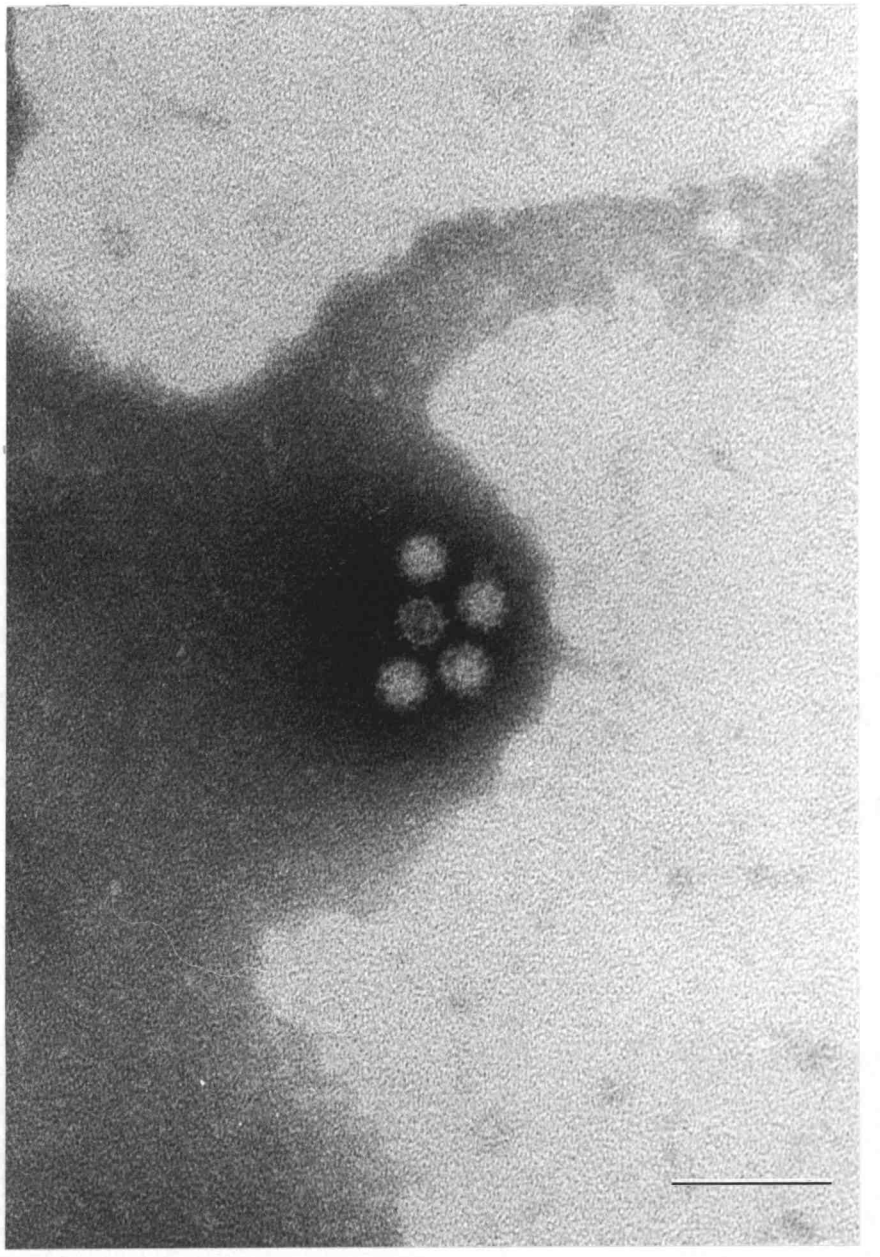


Figure 4.2: Negative stain electron micrograph of the vesicular fluid recovered from the foot of a white tern (*Gygis alba rothschilbi*) on Tern Island, French Frigate Shoals, Hawaii. Virus-like particles with capsid projections and cup-like surface depressions can be morphologically identified as caliciviruses.
Bar = 100 nm.



CHAPTER FIVE

General Discussion and Summary

Steven E. Poet

The known members of the family Caliciviridae are proven pathogenic agents which are known to disseminate over wide geographic regions and infect phylogenetically diverse host organisms. These morphologically distinct viruses are grouped based on success or failure in attempts at growing the agent in cell culture systems (49). Early molecular findings suggest that the genomic structure of caliciviruses, whether they can be grown in the laboratory or not, is relatively constant throughout the entire virus family (40, 122, 145, 155). Preliminary sequence analysis of SMSV-5 provides evidence that this ocean-origin calicivirus shares significant genome structure with the other known calicivirus sequences, irrespective of whether they can or cannot be replicated *in vitro* (Figure 1.4).

Caliciviruses which can be grown in the laboratory, using cell monolayers with agarose overlays, display variations in plaque morphology within single serotypes. This demonstrates that even the cultivatable viruses present a spectrum of replicative characteristics and potentials and suggest that the Norwalk-like caliciviruses may be at one extreme of this continuum where they are difficult, or impossible, to isolate in cell culture (140, 207). The plaque size varients observed with VESV and SMSV, range from large, clear, and circular to minute, opaque, and irregular areas of cytolysis within the cell monolayer (140, 168). Infectivity studies with purified minute plaque varients of VESV showed that they grow in a host, and readily revert to the pathogenic large plaque variants (252). In addition, Madin (134) reported that virus isolated from the lymph nodes of pigs clinically

recovered from VES, was predominantly the minute plaque variant. These findings suggest that minute plaque variant, or cell associated, agents may be important in establishing calicivirus reservoirs within animal populations.

The identification of a calicivirus group-specific cDNA hybridization probe provides a means by which caliciviruses, independent of their *in vitro* cytolytic properties, can be detected in reservoir animal populations. Identifying calicivirus reservoirs is important in both human and veterinary medicine. After the eradication of VESV from the United States, and the subsequent isolation from California sea lions of SMSV, a virus indistinguishable from VESV, Smith and Akers (203), suggested that components of the marine mammal food chain may be the primary reservoir for VESV. Furthermore, Morse and co-workers (152), found that water runoff contaminated with sewage could not explain all New York State outbreaks of human gastroenteritis involving Norwalk virus-contaminated shellfish, and suggested that Norwalk virus may be endemic within the bivalve mollusk population. The isolation of SMSV-17 from one mussel, and the detection of non-cultivatable calicivirus RNA, by cDNA probe, within several other mussels from the same sample site, provides evidence that ocean-origin caliciviruses can have reservoirs and transmission cycles in bivalve mollusks that would help explain some of the outbreaks of human caliciviral disease associated with the consumption of shellfish.

By establishing reservoirs in the food chain, caliciviruses may be able to escape immunological pressures present in higher vertebrate populations, and periodically emerge, antigenically unaltered, into naive animal populations, thereby explaining the persistence of VESV serotypes, in marine mammal populations, for decades after their supposed eradication (208, 225). Experimentally infected

opaleye (*Girella nigricans*), a component of the California sea lion diet, and intermediate host for the sea lion lung worm, were shown to transmit caliciviral disease to Northern fur seals (222). Two SMSV serotypes (types 6 and 7) were shown to naturally infect the opaleye, and SMSV-6 was also isolated from a Northern elephant seal and a sea lion liver fluke (223). While bivalve mollusks are not a common sea lion food item, they may play an important role in transmitting caliciviruses up the food chain by concentrating these agents in their feces which are subsequently consumed by helminth organisms which, in turn, are common food sources for fish (142). Shellfish are a component of the walrus diet, however, and may play a direct role in the transmission of walrus calicivirus in this pinniped species (112, 216). Norwalk virus has been successfully transmitted to human volunteers through contaminated oysters, and this suggests that reservoirs and transmission mechanisms may be similar among the pathogenic ocean-origin and human caliciviruses (85, 88).

The detection of a calicivirus associated with vesicular disease in a white tern suggests that wide ranging tropical sea birds transmit caliciviruses to susceptible animal populations throughout their range. While caliciviruses have not been reported in wild avian species until now, these agents have previously been reported in association with enteric disease in farm-raised chickens, Guinea fowl, and pheasants (80, 81, 258). The migration of marine mammal populations along the Eastern edge of the Pacific Ocean has been suggested as a mechanism for caliciviral transmission (218). The Hawaiian monk seal (*Monachus schauinslandi*), however, has serotype-specific serum neutralizing antibody titers to at least two caliciviral agents, VESV I_{55} and SMSV-1, despite being isolated from most migratory marine mammal populations (79). This first report of a

calicivirus in a wild avian species may have revealed the mechanism by which monk seals were exposed to caliciviruses thought to only infect United States swine or marine mammals of the North American Pacific Coast.

The group-specific cDNA hybridization probe for caliciviruses developed in this study, has been successfully used to detect both known and new caliciviral agents. This probe, in conjunction with virus isolation of SMSV-17, established the mussel (*Mytilus californianus*) as a reservoir for ocean-origin caliciviruses. Moreover, the identification of a calicivirus in association with vesicular disease in a sea bird, recognizes avian populations as additionl potential reservoirs and vectors in the transmission of caliciviral agents over broad geographical areas. This group-specific diagnostic tool may be used in conjunction with type-specific tools, when decisions are required in determining the pathogenic potential of caliciviruses encountered in domestic animals, threatened wild animal populations, and food products bound for livestock or human consumption.

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