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This report describes the partial characterization of the first cultivable calicivirus isolated from a European rabbit (*Oryctolagus cuniculus*), named rabbit calicivirus *Oryctolagus*-1 and abbreviated RaCv *Ory*-1. RaCv *Ory*-1 was isolated from juvenile feeder rabbits displaying symptoms of diarrhea. Absence of neutralization by type specific neutralizing antibodies for 40 caliciviruses and phylogenetic sequence comparisons among the caliciviruses of partial ORF1 and complete ORF2 and ORF3 sequences demonstrate that RaCv *Ory*-1 is a novel member of the marine calicivirus sub-group. Phylogenetic evaluation of the *Caliciviridae* indicates that analyses using pooled 3D-polymerase and capsid sequences are more statistically robust than identically executed analyses of single gene sequence data. Phylogenetic analysis of pooled 3D-polymerase and capsid a.a. sequences show canine calicivirus isolate 48 (CaCv-48) to be an intermediate species which forms a node approximately equidistant to the feline, marine, and

Sapporo-like caliciviruses. RaCV *Ory*-1 is suggested as a possible cultivable model of rabbit hemorrhagic disease virus.

Isolation and Partial Characterization of a Cultivable Rabbit Calicivirus, RaCv *Ory*-1

by

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Nathan K. Keefer, Author

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Chapter 1: Literature review

Introduction

The *Caliciviridae* is a family of small non-enveloped, positive-single stranded RNA viruses characterized by a 5′ located non-structural gene and capsid composed of a single protein species. These characteristics led to the recognition in 1978 (29) that the caliciviruses are taxonomically distinct from the *Picornaviridae.*

Caliciviruses are remarkable for the breadth of their host range and associated disease conditions, and their genetic diversity. Important members of the family include San Miguel Sea Lion virus, which is indistinguishable from vesicular exanthema of swine virus, feline calicivirus, rabbit hemorrhagic disease virus, Norwalk virus, and Sapporo virus. Cultivable caliciviruses have been isolated from marine and terrestrial mammals, amphibians, fish, and reptiles. Associated disease syndromes in non-human animals include vesicular disease, hemorrhagic fever, spontaneous abortion, gastroenteritis, and persistent respiratory disease (149). In humans, caliciviruses cause sporadic and localized outbreaks of gastroenteritis (98) and vesicular disease (148). In addition, hepatitis E virus, which causes hepatitis in humans and a seemingly inapparent infection in swine (100) is currently assigned to the *Caliciviridae.* Localization of the non-structural polygene to the 5′ terminus of the genome, as well as the

presence of a small 3′ ORF encoding a protein of unknown function are common to all caliciviruses. However, genome organizations vary widely among the subgroups of *Caliciviridae.* Sequence analyses have revealed two fundamentally different relationships between the structural and non-structural coding regions of calicivirus genomes (Figure 1)(28).

Those caliciviruses sometimes identified as the "animal caliciviruses" and currently grouped as the feline and marine caliciviruses (MaCV), including the SMSV's and VESV's, are the only members of the family which reliably replicate in cell culture. *In vitro* cultivability greatly facilitates viral isolation and characterization. Investigations of the cultivable caliciviruses have best demonstrated the breadth of host range and tissue tropism associated with *Caliciviridae* (159). Many of the animal caliciviruses are of proven marine origin but have emerged periodically as terrestrial outbreaks (149). The best documented of these emergences is that of vesicular exanthema of swine virus which was also the first recognized calicivirus.

History

In 1932 the first evidence of caliciviral disease was reported in Orange County, California (4). A commercial swine herd, as was then common, was being fed raw garbage largely generated by Los Angeles area restaurant and institutional kitchens. Hogs suffering from a vesicular disease clinically indistinguishable from the officially reportable foot and mouth disease were

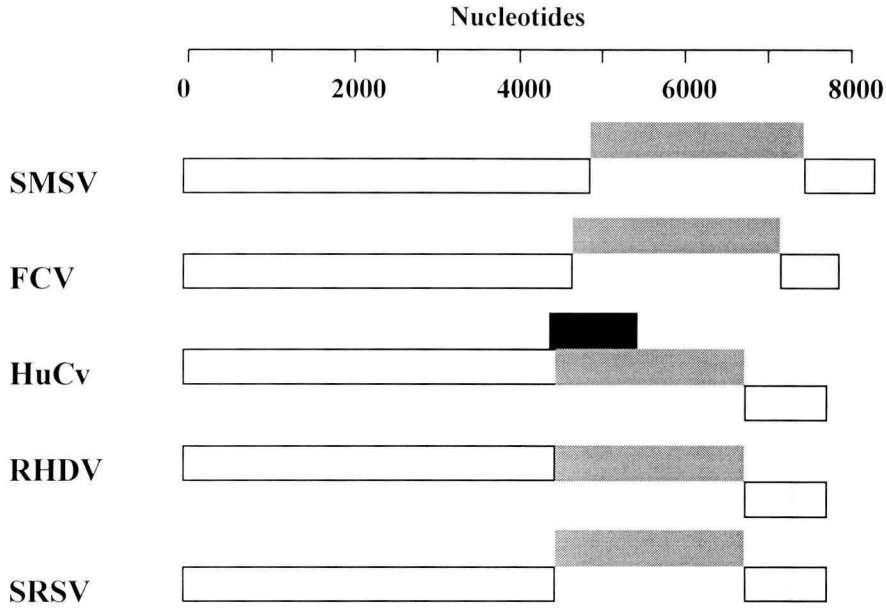


Figure 1. Genome organization and open reading frame usage, offset boxes refer to new ORF's. ▭ represents the non-structural polygene, ▒ represents the capsid gene, ▭ represents the 3' ORF, and ■ represents the 2nd HuCv small ORF.

indistinguishable from the officially reportable foot and mouth disease were described to regulatory veterinarians. The infected herd and neighboring herds of both cattle and swine, about 19,000 head, were duly destroyed. The following year, another outbreak was reported near San Diego , California and the disease agent was described as a novel pathogen of swine, given that unlike foot and mouth disease virus, it did not appear to infect cattle. Vesicular exanthema of swine(VES) repeatedly emerged and disappeared throughout California during the 1930's and 1940's. At times nearly 50% of California swine herds were concurrently affected. In 1952, the embargoes on California hogs and raw pork products were broken when a transcontinental passenger train originating in San Fransisco, California discarded garbage including raw pork scraps in Wyoming. The garbage was fed to local swine which were subsequently dispersed at auction. Within fourteen months, forty-one states had reported VES. Containment of the disease followed the expenditure of $39,000,000 in federal funds and the enforcement of legislation requiring that all garbage fed to hogs be cooked. In 1959 VES was officially declared eradicated and designated a foreign animal disease although it had been noted only once outside the United States. This single instance, in 1955, was traced to the shipment of contaminated pork from the United States to a US military base on Iceland (2). At that time, scientists could not discover the reservoirs for the initial outbreaks aside from describing the association of VES with the feeding of raw garbage.

Forty years after the initial outbreak of vesicular exanthema of swine, two viral isolates were obtained during the investigation of premature parturition in California sea lions (*Zalophus californianus*) on San Miguel Island off the coast of southern California. The agent was named San Miguel Sea Lion virus (SMSV) although it could not be distinguished physicochemically or morphologically from VESV (146, 164). Inoculation of the new isolates into hogs produced lesions indistinguishable from VES (146, 164). More than forty serotypes of these SMSV / VESV type caliciviruses have been described. Marine species from which caliciviruses have been isolated include: California sea lion (*Z. californianus*), Stellar sea lion (*Eumatopias juatus*), Northern elephant seal (*Mirounga augustirostris*), Northern fur seal (*Callorhinus ursinus*), Pacific walrus(*Odobenus rosmarus divergens*), common bottle nosed dolphin (*Tursiops truncatus*), and Opaleye perch (*Girella nigricans*). SMSV type caliciviruses have also been isolated from various terrestrial species including swine (mid-century epidemic and 1976) (4) and cattle (152). Two serotypes were isolated from various species at the San Diego Zoo including an amphibian, Bell's horned frog(*Certophus orata*), several reptile species, including Aruba Island rattlesnake(*Crotalus unicolor*), and several primate species including pygmy chimpanzee(*Pan paniscus*) (147, 161). Many of the forty serotypes of SMSV / VESV have been isolated from multiple species.

A non-mammalian primary reservoir for marine caliciviruses has been postulated (145, 155, 158, 160). Marine caliciviruses have been isolated from

the oceangoing fish species, opaleye(*Girella nigricans*), and experimental infection of captive opaleye resulted in viral replication of up to 10^7 infectious particles per gram of splenic tissue with virus persisting 31 days post inoculation (163). The habitat of the opaleye, which consists of coastal shallows and tidal pools, lies within the boundaries of the breeding range for the California sea lion, the marine mammal species most strongly associated with SMSV-type viruses (6, 149, 155). Within their shared habitat, the two species interact on multiple levels. The opaleye serves both as a food source for the sea lion and as the intermediate host and vector for sea lion lung worm (*Parafilaroides decorus*) which encysts in the fish gut (158). Pronounced differences between the antibody profiles of northern fur seals and California sea lions indicate a much lower rate of calicivirus infection among the fur seals despite the inevitable interaction between the two populations within their shared San Miguel Island rookery habitat (11, 151). Northern fur seals however, are understood to feed along the continental shelf margin where deeper, colder water sustains a different cohort of fish species which does not include the opaleye (151).

Pinnipeds have been experimentally infected via ingestion of SMSV-5 inoculated opaleye, one month post inoculation of the fish, and furthermore, opaleye infected by ingestion of calicivirus spiked sea lion lung worm larvae were shown to transmit the viral infection to northern fur seal pups (158). These experiments support the plausibility of a parasite/fish/marine mammal reinfection cycle. Fish gut encysted lungworms, which have been shown to persist for years,

could harbor virus serotypes outside the reach of either fish or mammalian immune systems, re-exposing sea lion populations after a time period sufficient to produce immunologically naive individuals (6, 162, 163).

Serological studies have further demonstrated the serotypic diversity of those caliciviruses similar to SMSV and the host diversity of single calicivirus serotypes. Hawaiian monk seals(*Monachus schauinslandi*), bowhead whales(*Balaena mysticetus*), sperm whales(*Physeter macrocephalus*), and fin whales(*Balaenoptera physalus*) have not yielded calicivirus isolates but do exhibit type specific serum neutralizing antibodies against a variety of SMSV type caliciviruses (5, 6, 155-157). Wild and feral terrestrial species from the coastal region of southern California were shown to have serum neutralizing antibodies against multiple marine isolates(151). Cetaceans and pinnipeds, including California sea lions, Hawaiian monk seals, bowhead whales, sperm whales, California gray whales, and fin whales, possess neutralizing antibodies against various SMSV types and VESV serotypes A through K (155), and domestic swine and cattle also possess neutralizing antibodies against a wide array of caliciviruses, including SMSV 4, 5 and 13 (5, 155).

A marine presence for all SMSV or VESV type caliciviruses has been demonstrated by isolation or neutralizing serology. Bovine calicivirus (BCV *Bos*-1 or unofficially Tillamook calicivirus TCV) and mink calicivirus, among others, were originally isolated from terrestrial species. In both cases, plausible, although unsubstantiated links to ocean virus sources can be described. Bovine

calicivirus was isolated from calves raised on a Tillamook County, Oregon dairy farm (152). In Tillamook County, dairy herds are concentrated near the Pacific Coast and around various small estuaries. Mink calicivirus was isolated from ranched mink in Idaho. Domestic mink are sometimes fed marine protein sources which have included ground fur seal carcasses (96, 140), and mink experimentally fed ground calicivirus infected seals became infected with SMSV (139, 181). Type specific serum neutralizing antibodies to both BCV and MCV have been found in marine species (3, 149). Serum neutralizing antibodies to SMSV-13, isolated from California sea lions in 1984, have also been detected in serum samples from domestic cattle in Oregon(11) Terrestrial mammals of the Santa Barbara Channel Island chain, including the indigenous gray fox and feral sheep, donkeys, and swine, were shown to be seropostitive for a number of calicivirus types(151). This diverse set of species does not share a common food source, so any generalized route of transmission would necessarily differ from the introductions of caliciviruses to domestic swine(VESV), dairy cattle(BCV), and mink(MCV) associated with the feeding of contaminated marine byproducts. VESV serotypes J_{56} and K_{56} were isolated only once in 1956 from a New Jersey swine herd. Twenty-four years later type specific neutralizing antibodies to those two serotypes were found expressed in Bowhead whales, a species limited to a circumpolar Arctic habitat (156).

The well described history of VESV induced disease among swine in the United States, when considered along with the evident breadth of SMSV type

calicivirus infection of marine mammals, strongly indicates that VES appearances represented multiple introductions of caliciviruses into the swine population from a marine reservoir. Marine caliciviruses probably spread to swine through the feeding of tissue from one or more infected marine species to hogs as a portion of their regular diet of uncooked garbage. The path of emergence of VESV from a marine source is reflected in other apparent transfers of marine calicivirus to cattle, mink, and wild coastal land mammals (149).

Feline Calicivirus

Feline calicivirus(FCV) is a serious pathogen of cats which can cause acute respiratory tract infections, acute and chronic stomatitis, or acute arthritis (33, 91, 123, 169, 178, 179). Despite the longtime availability and field use of both killed and attenuated FCV vaccines, feline calicivirus continues to cause widespread acute illness and establish persistent infections among both chronic carrier and disseminator animals (122). Infection with feline calicivirus occurs worldwide and apparently across the spectrum of *Felidae* species (61, 73, 105).

The disease syndrome associated with feline calicivirus most commonly presents symptoms of rhinitis, tracheitis, conjunctivitis, and ulceration of the oral cavity epithelium. Pyrexia, lethargy, and anorexia are also common and more severe cases can result in pneumonia, and yet many animals experience only inapparent infection. Lameness or limping syndrome (arthritis) has more recently been recognized as an important result of FCV infection (33, 123). The

pathogenesis of limping syndrome is not clear, although FCV has been isolated from synovial fluid of naturally affected animals and gross pathological changes including swollen and hemorrhagic synovia have been noted in experimentally infected animals (33, 91). Chronic stomatitis and faucitis have also been reported as resulting from FCV infection (81, 134). Most recently, FCV with resistence to bile salt inactivation has been linked with enteric disease (104).

While considerable antigenic diversity is evident among FCV isolates, there is a degree of serum cross neutralization common to many variants. This cross protection led to the widely held generalization that all FCV isolates belong to a single serotype (127-129). The severity of disease varies between strains of virus. Inconclusive reports have suggested that isolates associated with symptom specific disease clusters differ antigenically when analyzed with monospecific polyclonal antisera and that this diversity represents FCV biotypes that cause distinct disease syndromes (33, 79). Geissler et al found that there was no such correlation between antigenicity and biotype among a set a of German FCV isolates (43).

Many cats shed infective feline calicivirus particles for prolonged periods following acute disease. Nearly one-fifth of healthy adult cats have been shown to shed FCV in their saliva (53, 80, 169). This proportion has changed little since chronic shedding was first noted before the advent of FCV vaccines nearly three decades ago (53, 177). Viral replication appears to occur primarily in the tonsillar tissues of most chronic carrier animals, and virus is shed from the

oropharynx (34, 176). The preference of the virus for localization in these tissues has not been explained. Previously infected or immunized cats have been shown to shed significant virus when challenged with heterologous strains one to three months after the initial development of immunity although few demonstrated clinical signs of disease (74, 81, 127). Vaccination appears to effectively limit the likelihood of acute illness but not the circulation of virus from animal to animal (122). Comparative studies of the FCV capsid gene sequence indicate that the virus exists as a quasispecies in persistently infected animals and that the neutralization profile of this quasispecies significantly changes over time (133). The establishment and maintenance of persistence may depend upon this diversity and inconstancy.

Rabbit Hemorrhagic Disease Virus and European Brown Hare Syndrome Virus

Rabbit hemorrhagic disease(RHD) is an acute and usually fatal infection of European rabbits(*Oryctolagus cuniculus*) first described in China in 1984 among a group of commercially bred Angora rabbits imported 76 hours previously from Germany (95). No literature record of the disease had appeared previous to 1984, although it may have been observed in Germany at an earlier date (121). From China the disease spread to Korea and then from Asia to some continental European countries. By 1989 the virus was widespread throughout Europe (107). The disease is characterized by a short incubation period of 1 to 2 days and 100% morbidity with rates of mortality as high as 100% (120). Initial

classification of the causative agent of RHD was controversial. Picornavirus, parvovirus, and calicivirus were all suggested (173, 187, 188). In 1990, a virus was recovered from the livers of rabbits by Spanish researchers using cesium chloride(CsCl) centrifugation and band extraction and then characterized by electron microscopy, protein electrophoresis, western blot analysis, and nucleic acid determination. The results, while not fulfilling Koch's postulates, have been widely interpreted to indicate that rabbit hemorrhagic disease virus(RHDV), a calicivirus, was the etiologic agent of RHD (120).

Three disease courses associated with different epidemic stages or levels of population naiveté were originally defined in Chinese reports of RHD: the *peracute* form described rapid onset of death without exhibition of clinical signs and occurred soon after introduction of the disease agent; the *acute* form described rapid onset of death with exhibition of clinical signs and occurred within populations with an established RHD presence; and the *subacute* form described low mortality with mild exhibition of clinical signs and occurred at the latter stages of an epidemic (188). Most subsequent descriptions of RHD have been consistent with the first two of these categories (24) although the third disease course may be represented in those areas of Australia where spreading RHDV as a biological control agent has failed to eliminate feral rabbits. It has been noted that the disease is not generally fatal for rabbits younger than 2 months of age although this phenomenon has not been satisfactorily explained (24, 188).

RHD pathology is typically characterized by severe disseminated necrotic

hepatitis with multifocal petechial hemorrhages in the liver, lungs, kidneys, and heart. Pneumo-tracheitis and tracheal hemorrhaging are commonly described. Clinical signs include fever, rapid respiration, and nervous behavior characterized by "paddling" motion of the feet, ataxia, or frenetic action accompanied by squealing. Approximately 20% of affected rabbits display a bloody discharge from the nostrils (118). Disseminated intravascular coagulation is characteristic of RHD, and the associated circulatory stasis and loss of blood clotting factors contributes to generalized circulatory dysfunction and multiple organ failure (125).

A killed RHDV vaccine has been derived from virus material harvested from the livers of actively infected rabbits exhibiting symptoms of RHD (144). Immunity develops within several days of inoculation but lasts at most several months (24). The requirement for production of the vaccine antigen in rabbits is problematic. Studies of vaccination with RHDV "very light particles" self-assembled from capsid protein expressed in a baculovirus system or with recombinant canary pox virus containing RHDV capsid sequence have demonstrated alternative inductions of immunity, but have not yet led to new commercially available vaccines (40, 124). Plana-Duran et al reported protection of rabbits from challenge with 3.6×10^4 LD_{50} RHDV after intramuscular(IM) vaccination with as little as 0.5 μg of baculovirus-recombinant RHDV capsid (124). Vaccinated rabbits demonstrated no clinical signs of RHD, nor was RHDV detected in liver samples using hemagglutination tests which detected no

activity in liver samples from IM vaccinated animals while tests on negative control animals yielded HA titers from 16,384 to 65,536 HA units per 50 μl of liver extract. Oral vaccination with 3 μg of ethylenimine-treated recombinant capsids conferred protection from development of RHD to 4 of 5 animals tested although in one case liver samples showed low levels of hemagglutanizing activity (124). While baculovirus-recombinant produced protein may not represent the most economically feasible vaccine at the current time, the protection of rabbits from RHD by vaccination with RHDV recombinant protein provides further proof that RHDV is involved in the etiology of RHD.

European brown hare syndrome virus(EBHSV) has been identified as the probable etiologic agent of a disease of European brown hares(*Lepus europanus*) similar to RHD , but not associated with disseminated intravascular coagulation. EBHSV and RHDV have both been completely sequenced (46, 102, 182) and shown to share an identical genome organization (182) as well as typical calicivirus morphology and physicochemical properties (90). Nucleotide homologies between the capsid genes of the two viruses range between 52.6 and 60% (115) which is consistent with their degree of antigenic similarity . There is serologic and virologic evidence that RHDV can infect hares although no evidence of EBHSV infection in rabbits has been described (115, 168).

A third, apparently non-pathogenic calicivirus found to be infecting rabbits in 1996 is closely related to RHDV and EBHSV and yet is apparently limited to replication within the intestine (19). Examination by competitive ELISA of this

third virus indicated a closer antigenic relationship with RHDV than with EBHSV. Phylogenetic analysis of capsid protein gene sequences by the Pileup program predicted similar evolutionary distance between the nonpathogenic virus and EBHSV and between RHDV and EBHSV while also clearly separating the virulent and avirulent rabbit viruses (19). The presence of nonpathogenic or perhaps variously tissue trophic viruses similar to RHDV may explain the retrospective serological and virological studies that have indicated a European presence of RHDV-like viruses long predating the first descriptions of RHD (108) and the widespread presence of antibodies protective against RHDV among both domestic and wild rabbits (25, 63, 136). This new calicivirus of rabbits, like RHDV and EBHSV, has not been successfully cultivated in cell culture.

Recently primary cultures of rabbit hepatocytes were shown to support translation of caliciviral proteins following inoculation with cesium chloride banded RHDV (83). Hepatocytes were isolated using collagenase perfusion techniques which avoid much of the damage associated with mechanical manipulation (175). König et al inoculated preparations of rabbit hepatocytes with an undefined dose of RHDV. After 1 hour the virus suspension was removed and the cells washed and incubated with media containing an ^{35}S-label. After 5 hours, hepatocytes were treated for immunoprecipitation and found to be expressing various proteins corresponding to portions of RHDV ORF1. The quantity of protein translated suggested to the authors that replication of the genome had taken place, although replication was not assayed. Production or

release of infectious virions was not indicated.

Human Caliciviruses

The major concern for the public health raised by caliciviruses has been epidemic gastroenteritis. The disease syndrome of calicivirus linked gastroenteritis includes diarrhea, vomiting nausea, abdominal cramping, malaise, and fever(68). Enteric disease is caused by many caliciviral types belonging to two genetic clades (10) Additionally, a calicivirus derived from SMSV-5 was reported to have caused vesicular disease and possibly an influenza-like illness in a laboratory researcher (148). Along with this confirmed case, there have been occasional field reports of vesicular disease among wildlife biologists working with pinnipeds. One individual who developed painful oral blistering submitted to throat washings 30 days post onset from which was isolated a novel strain of calicivirus belonging to the marine calicivirus genogroup (148). Hepatitis E virus, a hepatitis pathogen most associated with the Third World, is tentatively and controversially classified as a calicivirus (84, 85, 97).

Caliciviruses of humans include particle morphologies both typical and atypical of caliciviruses. Norwalk virus(NV) has defined those caliciviruses, often called small round structured viruses(SRSV's), which do not consistently present under immune electron microscopy(IEM) the calices characteristic of the family. The virions of SRSV's, like those of all known caliciviruses, conform to the 3-dimensional capsid-structure model of 90 homodimeric capsomeres while being somwhat smaller at approximately 27 nm in diameter than most other

caliciviruses(35 to 40 nm) (130, 132). The Norwalk agent was first visualized from the fecal filtrate collected from volunteers administered filtered stool samples from the then unexplained 1968 Norwalk, Ohio outbreak of winter vomiting (76). Norwalk-like agents have been associated with gastroenteritis worldwide and typically bear the names of outbreak locations: Southampton, Snow Mountain, Mexico, Hawaii, and Taunton (172).

Sapporovirus was first detected in outbreaks of infantile gastroenteritis which occurred between 1977 and 1982 in Sapporo, Japan (27). IEM images of sapporovirus reveal a typical calicivirus appearance (170) and like NV, Sapporovirus has come to represent a diverse group of human pathogens sharing a similar appearance. The Sapporo-like caliciviruses, including Manchester, London, and Vanderbijlpark, are primarily associated with enteric disease of children under the age of 5 years, and more specifically infants (111).

The human enteric caliciviruses have been shown to group phylogenetically in accordance with the morphological division of Norwalk and Sapporo-like viruses (97). Complete genome sequences are available for representatives of both clades including Southampton (87, 88), Norwalk (54, 72) and Manchester (94). Human enteric caliciviruses have not proven amenable to cultivation in cell culture, nor has a suitable(chimpanzees will serve as hosts) animal model been developed. The consequent reliance on volunteer infection for production of virus has hampered investigation. Recombinant expression systems for some human caliciviral proteins have been developed (49, 67, 70, 71).

Early cross challenge studies in adults demonstrated that Norwalk-like caliciviruses were antigenically diverse, and moreover that preexisting specific anti-Norwalk serum antibodies failed to protect against not only Norwalk-like viruses but sometimes Norwalk virus itself. Further investigations have indicated that preexisting antibody to Norwalk does not generally correlate to resistence to disease upon experimental challenge to NV and that higher antibody titers correlate with greater disease susceptibility (47). Caliciviruses within the Norwalk clade share as little as 48% amino acid identity in their capsid proteins (92). Considering this diversity, lifelong susceptibility to Norwalk-like virus infection is unsurprising. Norwalk infection has been traced to feces contaminated produce, well water and shellfish (9, 50, 135). Transmission of the virus by food handling may occur after apparent recovery of infected food workers from acute illness, perhaps as a result of persistent subacute infection (65, 180). Reflecting the ease of transmission, results from large scale serologic surveys in the United Kingdom and Japan have demonstrated approximately 70% seropositivity to endemic Norwalk virus strains, with seropositivity rising with age into late adulthood (48, 116).

Methods of cooking conventionally believed to neutralize shellfish born viral pathogens have failed to protect consumers from contaminated oysters (99, 167). Consumption of commercially harvested shellfish contaminated by overboard dumping of human feces in Apalachicola Bay, Florida appeared to have caused a 1994-1995 outbreak of Norwalk virus-associated gastroenteritis.

Epidemiological analysis of the outbreak demonstrated no link between the thoroughness of oyster cooking and transmission of NV (99). This study corroborates investigations of poliovirus, which demonstrated retention of 7% of infectivity after 30 minutes of steaming of inoculated oysters(internal temperature of 94°C) (35). The infectious dose of SRSV's is thought to be quite low, and NV is considerably more heat resistant than poliovirus (37, 75, 185).

The sero-epidemiology of Sapporovirus in Japan indicates a broad distribution across geographical regions and infection early in life. In tests against HuCv Sapporo/82/Japan, 87% of young to middle aged adults were shown to be seropositive without significant differences in seroprevalence relative to age or location (109). Serologic examinations worldwide have indicated similarly high specific antibody prevalences in the adult population (110). Adult antibodies against Sapporo-like caliciviruses provide specific protection from re-infection by children actively shedding the virus (31).

Examples of both Norwalk and Sapporo-like viruses violate the respective adult epidemic and infantile diarrhea patterns of infection. High rates of seroconversion to Norwalk-like HuCv-Mexico have been demonstrated among very young children in both Mexico and the United Kingdom (69, 119). Sapporo-like HuCv-UK3 was suggested to be a zoonotic pathogen transmitted by a vomitting dog to humans of all ages who developed gastrointestional illness with fever, malaise, and myalgia (64).

Poorly Characterized and Candidate Caliciviruses

Several caliciviruses and candidate caliciviruses of animals remain largely uncharacterized. Particles displaying classic calicivirus morphology have been observed in conjunction with gastrointestinal disease in domestic fowl (16, 44, 45) and vesicular disease in a white tern (126). The calicivirus associated disease syndrome of chickens causes often permanent stunting and poor feather growth and has been described as infectious stunting of chickens, helicopter disease, palebird syndrome, and runting disease (103). Replication of candidate chicken calicivirus in cell culture was reported, but virus yields were not sufficiently high for characterization at the time of the investigation (32). A virus isolated from the feces of a dog with diarrhea was propagated in canine as well as dolphin kidney cell culture and was shown to be antigenicly similar to chicken calicivirus while there was no apparent cross-reaction with specific antibodies for other caliciviruses including FCV, SMSV, Newbury agent, and Norwalk virus (142).

A virus with calicivirus morphology has been reported in neonatal pigs with gastroenteritis (17, 137) and was shown to replicate weakly in primary tissue culture (41). Similarly, the Newbury agent causes anorexia, diarrhea, and xylose malabsorption in 16 to 60 day old calves and has proven refractory to cultivation in cell culture (186).

Isolation of amyelois chronic stunt virus(ACSV) from the naval orange worm(*Amyelois transitella*) was reported during investigation of the insect pest in northern California almond groves (78). The virus was characterized as

displaying morphological and physicochemical traits similar to those of animal caliciviruses for some particles while others, at 28 nm, resembled small round structured viruses (60). Both particle types were shown to be infectious. The larger, 38 nm particles could be converted to the smaller SRSV-like particles by ∝-chymotrypsin degradation (60), perhaps analogous to the degradation of Norwalk-like viruses hypothesized to occur in the human gut (55).

Structure and Molecular Biology of the Caliciviruses

There are large differences in morphology and genome organization among members of the *Caliciviridae.* Calicivirus virions, of approximately 28 to 40 nm in diameter, present, with various degrees of clarity, the characteristic calyx pattern which inspired their name. Established members of the family diverge in the number and location of open reading frames along genomes no longer than 7 to 8 kb. Only two simple physical characteristics can be said to define the family: the 5′ location of the gene coding for the non-structural polyprotein and the construction of the capsid from 90 homodimeric capsomeres. While both capsid structure and genome orientation serve to differentiate caliciviruses from the phylogenetically similar picornaviruses, it is the caliciviral capsid structure which is unique among the viruses of vertebrates.

The icosahedral capsids of caliciviruses are composed of a single structural protein organized with T=3 lattice symmetry. While more common among plant viruses, capsid organization including only a single structural protein

species is unique among animal viruses to the caliciviruses and the nodaviruses of insects (56, 62). Studies of empty Norwalk virus particles(rNV) assembled from recombinant capsid protein and primate calicivirus(PCV) particles by Prasad et al using cryo-electron microscopy have begun to provide the information necessary for the illumination of the structure-function relationships of calicivirions (130-132). The most prominent features of the 3-dimensional structures of rNV and PCV particles are the 90 arch-like capsomeres which form all the local and strict two fold axes of the capsid lattice. This organization has the effect of surrounding each 5 and 3-fold axis with a ring of arches, creating 32 cup-like structures, 12 of which encompass the 5-fold axes while the remaining 20 encompass the 3-fold axes. The arch structures of caliciviruses most resemble the prominent projections characteristic of tomato bushy stunt virus (TBSV - *Tombusviridae*) and turnip crinkle virus(TCV - *Tombusviridae*). TBSV and TCV belong to the two previously described families of T=3 viruses which display similarly formed virion surface structures. However, phylogenetic examination of calicivirus capsid gene sequences has failed to identify any homology with TBSV or TCV structural genes (131).

The calicivirus genome includes 2 to 4 apparently functional open reading frames (Figure 1). Complete genome sequences have been published for members of each group with the exception of the SMSV's for which there is no published sequence of the 5′ end of the genome (22, 36, 54, 72, 87, 88, 94, 102). Sequence analyses have established two fundamentally different arrangements of

these ORF's. For the Sapporo-like human caliciviruses(HuCv) and RHDV the genes coding for the nonstructural polyprotein and the capsid protein are fused into one contiguous coding sequence more than 7kb in length (28). The Norwalk-like human caliciviruses, feline calicivirus, and SMSV/VESV caliciviruses include separate reading frames for the structural and non-structural genes. All caliciviruses include a separate small ORF of slightly more than 100 nucleotides coding for no functionally defined protein product and located at the 3' end of the genome Additionally the HuCv's possess another short ORF of about 160 nucleotides overlapping the N-terminal coding region of the capsid gene (94).

The calicivirus replication cycle involves the synthesis of at least one subgenomic RNA. Early analyses of SMSV-infected cells demonstrated four densometrically separable RNA molecules which proved to represent a 36S genomic RNA, a 22S subgenomic RNA and double stranded forms of both (38, 39). Similar species were detected in studies of VESV infected cells in 1978 (13). These approximately 22S subgenomic RNA's extending 2.2 to 2.4 kb in length have since been shown to be 3′ co-terminal with the complete genome and to include the ubiquitous small 3′ ORF and capsid protein coding sequence (57). RHDV infected tissue similarly contains genomic RNA and single species of subgenomic RNA despite the continuous non-structural and structural reading frame of RHDV (101, 102). The subgenomic RNA population of actively replicating FCV appeared to be more complex. The northern blot analysis of

Neil and Mengeling revealed 3′ co-terminal subgenomic RNA's of 4.8, 4.2 and 2.4kb while the analysis of Carter revealed RNA's of 5.3, 4.3, 3.6, 2.7, 1.9, 1.5, and 0.55 kb (21, 113). Neill and Mengeling demonstrated double stranded forms of the genomic and 2.4 kb subgenomic RNA's (113, 114). Carter found negative complementary strands corresponding to the 5.3, 4.3, 3.6, and 2.7 kb subgenomic RNA's (21). A more recent study of FCV infected cells study using [^{32}P]-orthophosphate labeling of *in vitro* synthesizing RNA detected only genomic RNA and a single subgenomic RNA of 2.4 kb (57).

The possibility remains that the earlier studies of FCV transciption revealed real evidence of a more complicated scheme of transcription and replication for FCV and other caliciviruses. For instance, the more heterogenous RNA population may be short lived and easily overlooked by studies which harvest cells at only one time post-infection. However, it seems more likely that those results indicating more heterogenous RNA populations are technical artifacts. Sequence analysis of the 5′ ends of the genomic and 2.4 kb subgenomic RNA's of FCV demonstrated conservation of an approximately 20 nucleotide section which might serve as a promoter or binding site for initiation of replication (57). Likewise, comparison of the 5′ terminal nucleotides of the genomic and subgenomic RNA's of RHDV has shown a conserved sequence motif of 16 bases (22). In neither case do the conserved motifs appear elsewhere on the viral genome where replication might also initiate. The terminal motifs found in FCV and RHDV bear similarity to one another and motifs found on the

genomes of SRSV type human caliciviruses (89). While the absence of a cell culture system and low *in vivo* copy numbers of the human enteric caliciviruses have precluded studies of viral replication during infection, a preliminary report has indicated the presence of a 2.3kb Norwalk virus subgenomic RNA (72).

The presence of double stranded RNA molecules corresponding to both the full length genome and the subgenomic RNA indicates that the smaller RNA may be replicated independently of the genome sized RNA (57). Both full length and subgenomic RNA covalently linked to a 15 kDa VPg protein have been shown to be encapsidated by RHDV. Separation of RHDV virions by sucrose density gradient fractionation resolved two virion populations containing either genomic or subgenomic RNA (101) and a second report concluded that similarly purified RHDV virions of uniform buoyant density yielded both genomic and subgenomic RNA with a molar ratio of 1 : 1 (14). Encapsidation of subgenomic RNA has not been shown for other caliciviruses (28). However, ^{125}I protein labeling has demonstrated that genomic and 2.4 kb subgenomic RNA molecules linked to a 15kDa protein (VPg) are present in feline calicivirus(FCV) infected cells (58).

The mechanism(s) for initiation of replication of the calicivirus genome and perhaps the subgenomic segment have not been elucidated. As a first step, the RNA-dependent RNA polymerase of RHDV has recently been expressed in *E. coli* by Vasquez et al (174) to yield an enzymatically active protein product. With this polymerase protein, template sized products were synthesized *in vitro*

from positively stranded, poly(A) tailed subgenomic RNA(2.2 kb) using poly(U) primers while double length products(4.4 kb) were expressed from the same templates in the absence of added primer. The double length products were shown to be resistant to treatment with RNase A and hence double stranded. Northern blot analysis demonstrated that the product hybridized with positively and negatively sensed subgenomic RNA (174). Sequence analysis was not preformed on the 4.4 kb product so the exact locus of the unaided priming event is not known, nor is it known which if either priming event represents *in vivo* initiation of replication.

Virally encoded proteins termed VPg's of 10 to 15 kDa are covalently attached to the 5′ genomic termini of VESV, SMSV, RHDV, and FCV (18, 58, 101, 141). The subgenomic RNA molecules of FCV and RHDV have also been seen to include attached VPg's (58, 101). The results of Burroughs/Brown (18) and Schaffer et al (141) indicated that attachment of VPg was necessary for the RNA infectivity of VESV and SMSV whereas Sosnovtsev and Green (165) showed successful transfection of full length RNA transcripts of FCV in the absence of VPg with the attachment of an m7G(5′)ppp(5′)G cap(eurkaryotis mRNA cap) structure analogue. However, Herbert et al (58) showed that proteinase K treatment of total FCV RNA, Oligo-T purified FCV RNA and virion RNA dramatically reduced *in vitro* translation, presumably because of the removal of VPg. The fidelity of translation, as assessed by the sizes of observed polypeptides, was apparently unaffected by the loss of the cap protein. A

competition assay of the translation of untreated FCV RNA using cap analogue 7mGTP, normally bound by cellular translation factor eIF4F, showed no effect of 7mGTP on FCV translation. Introduction of 7mGTP into an *in vitro* translation mixture of naturally capped β-globulin mRNA caused a large reduction in translation, and furthermore, translation of FCV RNA was shown to be unaffected by K^+ concentrations which abolish translation of cellular RNA.

VPg is seemingly necessary for efficient translation of FCV, and initiation of translation for FCV occurs by a mechanism independent of at least one factor required for cellular translation (58). In picornaviruses, VPg is believed to be involved as a primer in both negative- and positive-strand RNA synthesis (86). Neither a protein nor nucleotide cap structure is apparently necessary for efficient translation of picornaviruses, which initiate translation through internal ribosome binding (66, 117). Like picornaviruses, caliciviruses seem to initiate translation using a modified cellular mechanism, however the caliciviral process of translational initiation must be quite different from that of *Picornaviridae*. Whereas the genomic secondary structure formed by long picornaviral 5′ non-coding sequences facilitates internal ribosome binding, the unusually short nucleotide sequences preceding both major initiation codons for all described caliciviruses probably ensure that the ribosome complex interacts with VPg (58). The ribosomal footprint described for other translational initiation sites would easily encompass the 5 to 20 nucleotides that precede first in-frame AUG's on caliciviral genomic and subgenomic RNA (28, 58). Whether the caliciviral

VPg's play a second role in replication similar to the function of picornaviral VPg's, has yet to be elaborated.

Several recent studies have evaluated the activity of the caliciviral 3C-proteases of RHDV, FCV, and Southampton virus(SRSV, Norwalk-type). Translation from the large first ORF's of caliciviruses would hypothetically result in polyproteins including all non-structural components and, in some cases, all viral proteins with the exception of the small ORF3 protein product. These full length translation products are not found in infected cell culture or in *in vitro* translation mixtures. Instead, a more complex collection of smaller proteins is found in these systems (28). When conditions known to inhibit protease activity were shown to simplify the viral protein mixture in infected cell culture and prevent the assembly and release of viral particles, a protease processing cascade similar to that of picornaviruses was hypothesized for *caliciviridae* (12, 20, 42). Sequence analyses showed that caliciviruses did indeed possess a sequence motif analogous to the trypsin-like cysteine proteases(TCP's) of *picornaviridae* (72, 87, 102, 112).

The RHDV 3C-like protease has been subject to detailed studies which have shown the enzyme, within an *E. coli* expression system, to cleave substrates *in cis* as well as *in trans* (15, 183). Boniotti et al showed that a protein product including the RHDV protease and immediate up- and downstream amino acid sequences self cleaved at the putative NH_2 protease boundary while another construct expressed from the 3′ two-thirds of ORF1 self cleaved the RHDV

capsid protein from its COOH terminus (15). Purified protease from the smaller construct was shown to cleave the non-structural-capsid protein boundary expressed from a third plasmid *in trans*. Wirblich et al (15) demonstrated cleavage of the RHDV protease at both its NH_2 and COOH boundaries and using amino acid sequencing of the resulting protein products, localized these boundaries to amino acids 1109 and 1251 of the hypothetical ORF1 polyprotein (Figure 2). These cleavages occur within glutamic acid- glycine and glutamic acid -threonine pairs(the two amino acids of each dipeptide are referred to as P1 and P1′ with cleavage occurring between P1 and P1′). Cleavage after a glutamine or glutamate residue is characteristic of viral trypsin-like cysteine proteases (TCP's) (28). The Southampton 3C-protease was shown to cleave at two glutamine-glycine pairs which sandwich the putative 2C-helicase coding region (93). Mutational analysis of the cleavage site within the capsid protein precursor of FCV (wild type glutamic acid-alanine dipeptide) demonstrated that substitution at P1 with leucine (Leu), histidine (His), and lysine (Lys) abolished proteolysis while substitution with aspartic acid (Asp) and glutamine (Gln) reduced proteolytic efficiency. Only substitution of proline for P1′ substantially altered the end point concentrations of the cleavage reaction (166).

Sequence comparisons of calici- and picornaviral 3C-proteases reveal little extended similarity with respect to primary sequence. However, TCP's share, with the extended family of chymotrypsin-like proteases, four similarity boxes, the first three of which include the amino acids histidine (His), aspartic acid (Asp)

or glutamic acid (Glu), and cysteine (Cys), constituting what is called the catalytic triad (7, 8). Within the protease triad model the cysteine residue acts as the cleaving nucleophile while the histidine residue associates with conserved P1 glutamine or glutamate residues. The necessity of the triad for proteolytic activity has been demonstrated for various picornaviruses (26, 51, 77) and alignment of RHDV (His, Asp, and Cys), FCV (His, Glu, and Cys), and Norwalk virus (His, Glu, and Cys) protease motifs with several picornavirus 3C-proteases has indicated that calicivirus TCP's also possess this triad (15, 166). Mutational analysis of the supposed catalytic triad of RHDV suggested that the amino acids played similar essential roles in caliciviruses to those roles well established for analogous amino acids in picornaviruses (15).

Interestingly, the protease motifs of RHDV and FCV are both considerably shorter than the 3C-protease motifs of picornaviruses, and the spacing between the caliciviral catalytic residues reflects that size constraint. The size and residue spacing of FCV and RHDV proteases is more obviously similar to the 2A-proteases which some picornaviruses possess in addition to a 3C-protease. However, the location of FCV and RHDV proteases (of all caliciviruses for which sequence data is available) relative to their 2C-helicase and RNA-polymerase motifs is nearly identical to the organization of helicase, 3C-protease, and RNA-polymerase motifs in picornaviruses (Figure 2). Moreover, the protease motifs of Norwalk-like viruses are sized and possess residues spaced more similarly to

the picornavirus 3C-proteases than to the FCV protease, RHDV protease, or the 2A-proteases (15, 28, 93).

The earliest descriptions of protein synthesis in caliciviruses originated from studies of cultivable viruses from the VESV, SMSV, and FCV calicivirus subgroups. Work by Fretz and Schaffer (42) demonstrated 6 virus specific proteins in SMSV-2 infected cells of 135 kDa, 80 kDa, 60 kDa, 40 kDa, 35 kDa, and 29 kDa. The 60 kDa protein corresponded in size to the already recognized capsid protein of caliciviruses. Growth of infected cells at 42°C, which is known to inhibit proteolytic activity, resulted in greater accumulation of the 135 kDa protein and a seventh protein of 86 kDa. The proteins of 80 and 135 kDa shared some amino acid homology, while the heat induced 86 kDa protein displayed a pattern of tryptic digestion similar to that of the 60 kDa protein. Komolafe et al (82) reported the presence of FCV strain F9 induced proteins of 80kDa, 68kDa, 40kDa, and 14 kDa. Two proteins of 80kDa were noted at 2 to 3 hours and 3.5 to 10.7 hours post infection respectively, leading the authors to hypothesize temporal regulation of caliciviral protein synthesis.

Analysis of the protein synthesis of the same strain of FCV utilizing both post vaccination(attenuated FCV-F9) feline anti-serum and anti-capsid murine monoclonal antibody resulted in the detection of a more complex protein population (20). Comparison of western blots developed using polyclonal or anti-capsid monoclonal antibodies allowed for the clear differentiation of structural and non-structural protein products. Carter, like Komolafe et al,

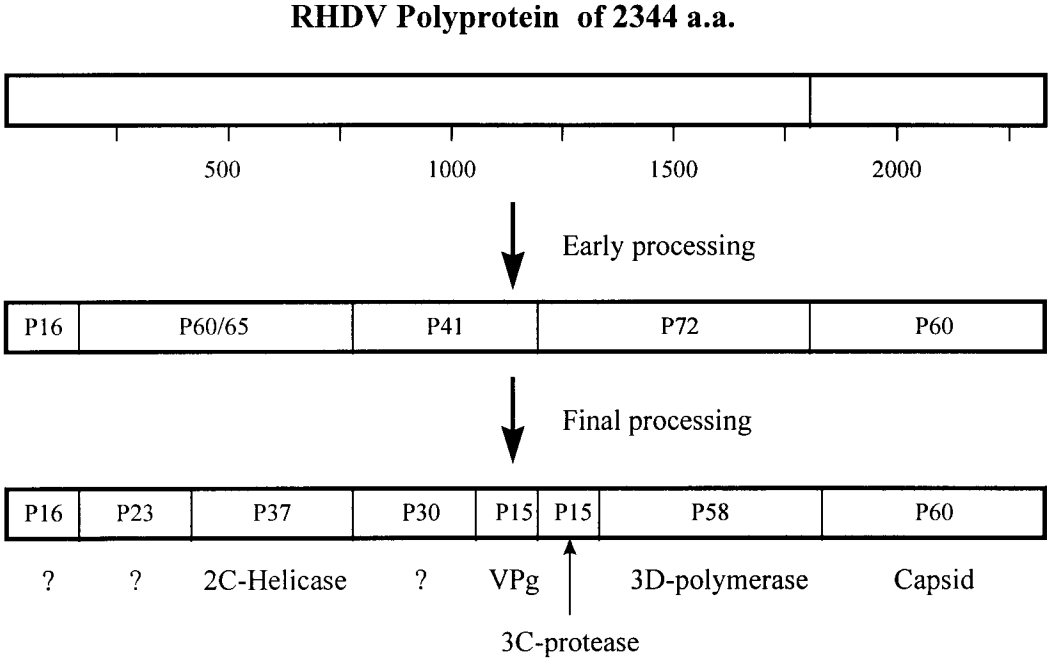


Figure. 2. Proteolytic processing cascade as defined for RHDV. Similar gene order within the non-structural polygene has been shown for all other investigated caliciviruses. Cleavage pointssurrounding the putative 2C-helicase protein have been demonstrated for Southampton calicivirus and feline calicivirus has been shown to cleave its VPg and 3C-protease proteins.

concluded that FCV protein synthesis is bi-phasic. Protein mixtures collected at 1, 2, 3, 4, and 5 hours demonstrated dramatic increases of viral protein accumulation after 3 hours post infection. Bands of 75 kDa and 62 kDa were plainly visible at 1 and 2 hours post infection while by 4 hours post infection bands appeared of 98 kDa, 96 kDa, 84 kDa, 75 kDa, 70 kDa, 49 kDa, 39 kDa, 36 kDa, and 27.5 kDa. Only the P62 band was visible after development with anti-capsid monoclonal antibody(P62 also visible with polyclonal antibody). The P62 and P75 proteins accounted for the most visible bands at each time point, hence the hypothesis of differential expression of viral proteins must be weighed against the possibility that the less readily perceptible proteins exist early in infection at concentrations below this study's sensitivity.

Carter also analyzed timecourse interrupted infections at 42°C. The general pattern of viral protein accumulation was similar to that shown at 37°C. At this elevated temperature, a second protein of 72 kDa was bound by both polyclonal and anti-capsid antibodies while the polyclonal antibody bound two more new proteins of 125 and 123 kDa. These proteins probably represent precursors of structural and non-structural proteins respectively (20).

More recently, studies of RHDV protein synthesis have been undertaken utilizing panels of genome-region specific monoclonal antibodies raised against recombinant peptides expressed in *E. coli*. Wirblich et al (184) generated *in vitro* translation products from purified virion RNA in rabbit reticulocyte lysate, and König et al (83) successfully described caliciviral translation in rabbit

hepatocytes isolated by liver perfusion and inoculated with cesium chloride banded RHDV. In reticulocyte lysate, ORF1 of the RHDV genome was translated and processed into seven non-structural proteins and the single capsid protein (184). Analysis with a 14 member panel of genome-region specific antibodies, allowed the authors to localize translational products along the genome. Proteins of 16 kDa, 60 kDa, 117 kDa, and 60kDa(capsid protein) demonstrated no apparent overlap, were ordered 5′ to 3′, and seemed to constitute the entire ORF1. Addition processing sites exist within the regions which encode P60 and P117. P60 is apparently further cleaved into NH_2 and COOH fragments of 23 kDa and 37 kDa while two cleavage sites within P117 apparently result in NH_2 and COOH fragments of 41 kDa and 69 kDa or of 30 kDa and 87 kDa. Two of these smaller non-structural proteins neatly encompass regions of the RHDV genome that have been associated with specific functional motifs. P37 corresponds to the viral helicase motif while P69 includes the regions shown to code for the *in vitro* active RNA polymerase and 3C protease(both active enzymes when expressed in *E. coli)* (15, 143, 183). Putative cleavage sites including the amino acid pairs glutamic acid-glycine(EG) or glutamic acid-threonine(ET) are located at the predicted boundaries between P60 and P117, P41 and P69, and P117 and P60(capsid protein) (184).

Analysis of ^{35}S radioactively labeled protein from RHDV inoculated rabbit hepatocyte culture resolved three sites of further protein processing (83). Proteins of 29 kDa and 14 kDa correspond to NH_2 and COOH fragments of P49,

and proteins of 15 kDa and 58kDa correspond to NH_2 and COOH fragments of P69. P14 apparently represents VPg of RHDV as it is of appropriate size, and analysis of virion purified VPg with an antibody panel localizes its coding region to the NH_2 terminal region of P49 (184). P15 and P58 correspond to the 3C protease and RNA-polymerase enzyme motifs of RHDV.

Experiments utilizing monoclonal antibody panels have not been undertaken for FCV and SMSV-type caliciviruses, so the processing of their non-structural proteins remains less completely described. However, the proteolytic processing of the capsid protein precursor, which does not exist for RHDV, has been investigated in a bacterial expression system (166). Cleavage was shown to occur within a typical target amino acid pair of glutamate-alanine located at positions 124 and 125 of the precursor molecule. The protease was shown to be located on a 78 kDa fragment which also included the RNA-polymerase motif. This placement is analogous to that described for RHDV and Southampton virus (166), and is in agreement with sequence data which similarly locate the protease and RNA polymerase motifs (10). P78, again analogous to the protease of RHDV, demonstrated self cleavage from sequence downstream of its NH_2 boundary and cleavage at several other undefined positions further downstream. Interestingly, there are several other glutamate-alanine pairs located near the site of capsid precursor cleavage (166). No evidence of cleavage at these additional sites was found, and substitution of amino acids surrounding P1 and P1′ did not obviously effect cleavage efficiency, leading the authors to suggest that the

tertiary structure of the precursor molecule as a whole may help determine the site of cleavage (166).

Phylogenetics of the Caliciviruses

The first and only thorough phylogenetic analysis of the *Caliciviridae* was completed by Berke et al (10). Their analyses of partial sequences from the 3D-RNA-dependent-polymerase region as well as the capsid gene showed that the caliciviruses can be grouped into four clades (1: SMSV, VESV, FCV; 2: Sapporo-like caliciviruses; 3: RHDV and EBHSV; 4: NV-like caliciviruses) which reflect the differences among caliciviruses in genome organization and open reading frame usage shown in Figure 2. Their analysis of capsid gene sequences resulted in a considerably more statistically robust phylogenetic tree than did their or other previous analyses of partial RNA-polymerase gene sequence. The caliciviral polymerase coding region of the genome is highly conserved which facilitates its amplification, but it does not appear to provide an ideal substrate for phylogetic analysis (10).

Objectives

The caliciviruses, once regarded as an obscure subset of the *Picornavirdae*, important only as pathogens of cats and as the shining example of the total defeat of a disease by public veterinary health measures, have been necessarily reassessed since the first isolation of San Miguel sea lion virus (SMSV) in 1973 (146). The vesicular exanthema of swine viruses (VESV),

effectively declared eradicated in 1959, were found to be examples of the marine caliciviruses, widespread among marine vertebrates along the North American Pacific Coast and perhaps beyond (149). The emergences of marine caliciviruses (MaCV's) onto land as evidenced by VES has since been shown to fit into a larger pattern of repetitive caliciviral penetration and then recession across the land-sea barrier (148). The sequencing of Norwalk virus in 1990 (72) and the resulting firm classification of the SRSV human pathogens as caliciviruses focused specific human public health concern on caliciviruses. Furthermore, the phylogenetic analyses of Berke et al (10) demonstrated that the Sapporo-like human caliciviruses were more closely related to the those caliciviruses associated with infection of non-human animals than to the small-round-structured-virus (SRSV) human pathogens. Finally, the rabbit hemorrhagic disease epidemic which swept Asia and Europe beginning in 1984, the description of rabbit hemorrhagic disease virus (RHDV) as the etiologic agent of RHD, and the subsequent investigation and release of RHDV as a biologic control agent in Australia and New Zealand combined to bring a worldwide urgency to bear on the study of caliciviruses(24). The *Caliciviridae* have become important.

Unfortunately the study of this emerging virus family has lagged behind that of its close brethren among the *Picornaviridae.* The study of caliciviral molecular biology has progressed remarkably in recent years (28), but the limitations inherent to the investigation of those caliciviruses partially or completely refractory to *in vitro* cultivation have hindered these advances. The

mechanism(s) employed by caliciviruses for cell adhesion and entry are undescribed as are many possibly related aspects of caliciviral pathogenesis, including widely variable tissue trophism and virulence. Of particular interest is the description of the interaction of the non-cultivable and devastating hemorrhagic fever virus, RHDV, with its host(s) and environment.

In 1995, the first cultivable calicivirus of rabbits was isolated in a porcine kidney(PK) cell line from fecal samples (Figure 3) taken from five dead or diseased New Zealand / California domestic crossbreed European rabbits (*Oryctolagus cuniculus*). This novel viral isolate which we, according to the past nomenclature conventions of the Working Group on Viral Taxonomy, 6th report of the International Committe on the Taxonomy of Viruses, call rabbit calicivirus, RaCV *Ory*-1, may provide a model for calicivirus behavior in the rabbit host that could be invaluable in the study of RHD. We have shown that RaCV can be typed as a novel member of the marine calicivirus subgroup by antigenic and phylogenetic characterization. Bolstering RaCV's viability as an authentic RHDV model, some marine caliciviruses have been shown to display hepatic trophism in hogs (150). Walrus calicivirus in particular, but also SMSV-6 and cetacean calicivirus, have been shown to cause moderate disseminated hepatocellular degeneration in experimentally infected swine (Figure 4).

RaCV is another example of the revealed presence of marine caliciviruses in terrestrial hosts. Like primate calicivirus, PCV *Pan*-1, RaCV cannot be directly linked with a transmission pathway from ocean to land, but it is probable,

although not yet proven that RaCV, like PCV, will prove to have an ocean presence (154). We have partially characterized the antigenicity of RaCV, sequenced 3.5 kb of the 3′terminus of its genome, and determined its phylogenetic positioning relative to other caliciviruses.

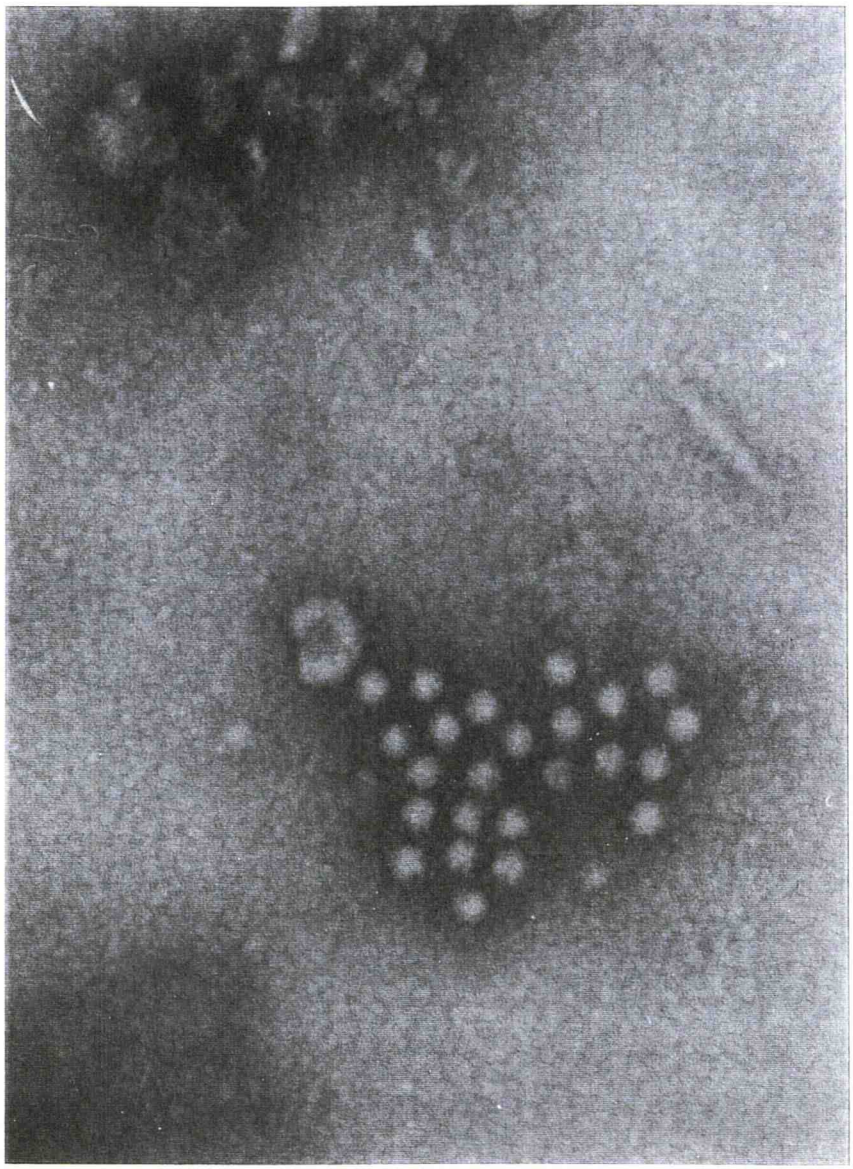
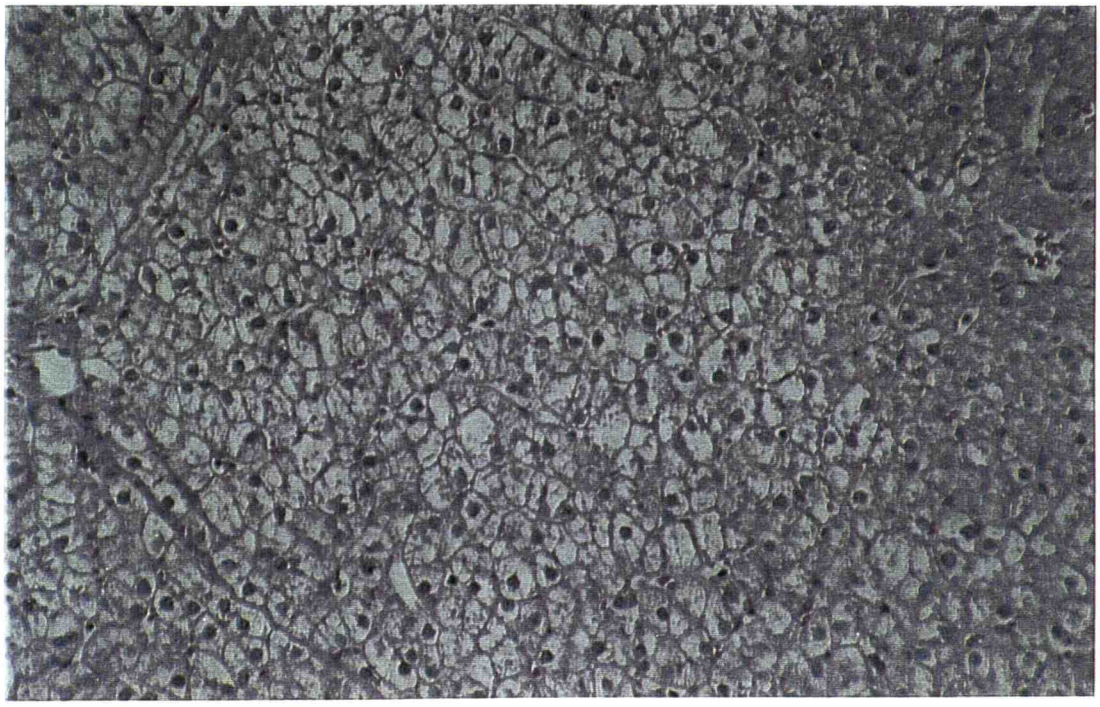
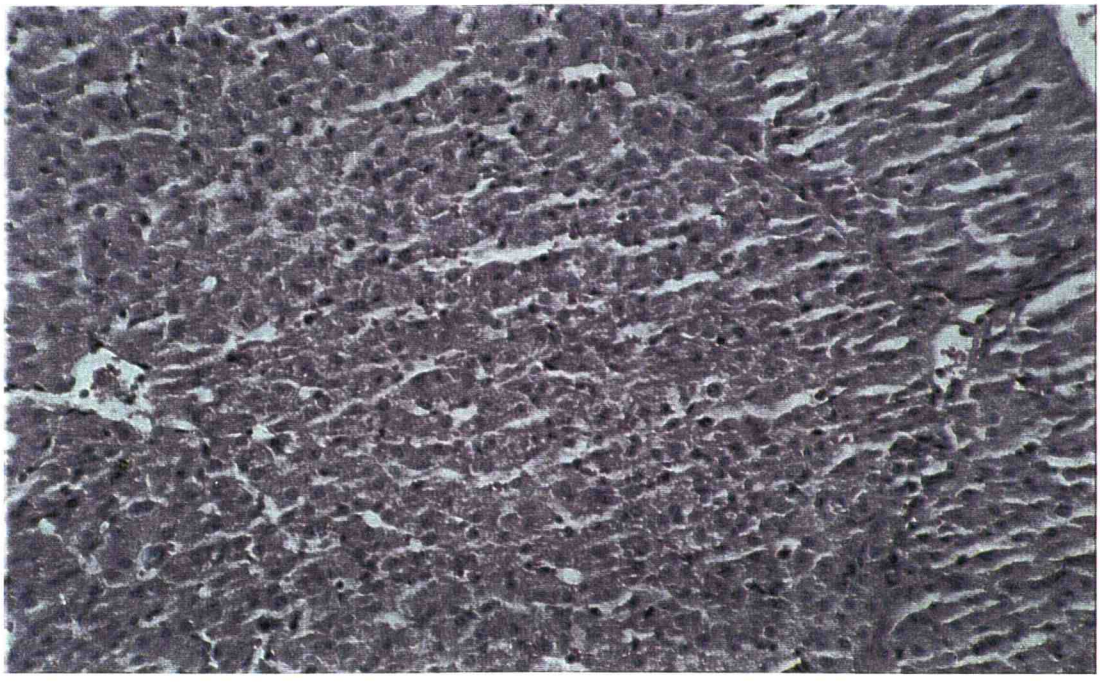


Figure. 3. Electron micrograph of rabbit fecal sample included in pooled sample from which RaCv *Ory*-1 was isolated. Magnification = × 162,000



A.)



B.)

Figure. 4. Swine liver tissue sections after experimental infection with A.) Walrus calicivirus - 7420 and B.) SMSV-13. Note disseminated hepatocellular degeneration in A.).

Chapter 2: Materials and Methods

Sample acquisition: In 1995 six New Zealand / California domestic crossbreed European rabbits (*Oryctolagus cuniculus*), belonging to small Oregon rabbit producer and all 11 weeks of age and younger, were presented to the Veterinary Diagnostic Laboratory at Oregon State University College of Veterinary Medicine. Three of the rabbits were dead, two diseased, and one apparently healthy. Of the five dead or diseased animals all were undersized and displayed mild to severe diarrhea. All affected animals displayed intestinal pathology, while the livers of three affected animals displayed rare white linear foci. Two dead rabbits displayed parasitism by coccidial organisms while there was heavy growth of *E. coli* evident in pooled intestinal contents of dead rabbits and *E. coli* was isolated from liver tissue derived from same animals. Disease etiology was difficult to ascertain given the presense of multiple possible diease agents. The pooled intestinal contents of dead rabbits were shown to contain calicivirus-like particles by electron microscopy (Figure 3), and this same sample yielded the first cultivable calicivirus isolate from a rabbit which we call rabbit calicivirus, RaCV *Ory*-1.

Virus propagation: Pooled fecal samples from necropsied rabbits were clarified for 10 minutes at 400 × g, supernatant was removed, and re-clarified 10 minutes at 7700 × g. Supernatant was centrifuged at 21,000 × g for one hour and resulting

pellet resuspended in 1:1000 L-cysteine hydrochloride and then incubated at 8 °C. Aliquots were removed and passed to Vero monkey kidney(VMK) and porcine kidney(PK) cells every two weeks and then examined daily for cytopathic effect. Isolate was plaque purified as previously described(153).

Serum neutralization: Neutralization for typing and determination of antibody titers in rabbit sera was completed with use of Vero cells in 96-well plates and replicates of 4 wells with two-fold dilution of test serum against 100 $TCID_{50}$ of virus (153). The rabbit isolate was typed with use of 20 antibody units of heterologous typing serum from each of 40 calicivirus types (153).

ELISA for antibody for SMSV's: Assays were completed within 96-well microtiter plates. The test antigen consisted of CsCl banded SMSV-13, RaCV *Ory*-1, and McAllister calicivirus. The test antigen was absorbed to the plate for 2 hours at 37°C and then washed twice with Tris-buffered saline containing Tween 20 (TBST) and blocked overnight at 4°C with TBST containing 0.25% BSA. After two TBST washes, each serum sample (diluted 1:20 in blocking buffer) was added and incubated for 2 hours at 37°C and then washed six times with TBST.

Anti-rabbit antibody (whole molecule) IgG-alkaline phosphatase (A3687 Sigma-Aldrich Corp., St. Louis, MO), diluted 1 : 35,000 in blocking buffer, was added and incubated for 2 hours at 37°C. The wells were then washed six times

with TBST and twice with TBS without Tween. The chromogenic substrate pnpp (Sigma-Aldrich Corp.) was added (200 μl per well at 1 mg/ml) and incubated overnight at 37°C. Optical densities (OD's) were read at 405 nm on an ELISA plate reader (Tiertek Multiskan, ICN Biomedicals, Costa Mesa, CA). The reported OD value was arrived at by subtracting the OD of a serum control well from the experimental OD. The OD values of control wells were $\leq$ 0.005, and most were 0.000. Values above an OD of 0.2 were defined as positive.

RNA extraction: Vero cells grown to confluence in two 75 cm^2 cell culture flasks were inoculated with plaque purified virus and allowed to progress to 4+ CPE in 10 ml growth media. Flasks were frozen briefly and rapped sharply to dislodge any remaining attached cells then media was cetrifuged at 400 × g for 10 minutes to pellet cell debris. Supernatant was centrifuged for 2 hours at 29,000 × g. Each resulting pellet was resuspended in 250 μl of sterile distilled water. Virus suspension of 250 μl was treated with 750 μl of Trizol reagent(Gibco-BRL, Gaithersburg, MA). This mixture was vortexed for 45 seconds at high speed and incubated at room temperature for 5 to 10 minutes and then centrifuged at 12,000 × g and 4°C for 10 minutes. Supernatant including neither pellet nor red gel was removed to a sterile microcentrifuge tube. Supernatant with 200 μl added chloroform was inverted gently and repeatedly for 30 seconds and then incubated at room temperature and centrifuged for 15 minutes at 12,000 g at 4°C. 500 μl of clear top layer (aqueous phase) was removed and 1.2 ml of isopropanol was

added, mixed by inversion, incubated overnight at -20°C, and centrifuged at 12,000 × g and 4°C for 30 minutes. Resulting pellet was then washed twice with 75% EtOH, air dried, and re-suspended in 20 μl sterile distilled DEPC water. RNA concentration and purity were assayed on a Beckman DU-64 spectrophotometer using "Warburg program".

RT-PCR: Approximately 1 μg of total RNA was reverse transcribed using AMV-RT (Invitrogen, Carlsbad, CA) and 0.5 to 1.0 μl 0.2 nmol/μl of first primer in a reaction volume of 20 μl with incubation at 45°C for 45 to 90 minutes. Polymerase chain reaction was carried out with Elongase © (Gibco-BRL, Gaithersburg, MA) or *Taq* polymerase (Perkin Elmer, Norwlk, CT) using 1 to 2 μl RT-mixture and 0.5 to 1.0 μl of both first and second primers in a reaction mixture of 100μl. The parameters for PCR for long fragments: were 17 × cycles of denaturation for 20 seconds at 94°C, annealing for 45 seconds at 42°C, and elongation for 4 minutes at 72°C and 18 × cycles with additive 30 second extensions to elongation steps and for short fragments: were 40 × cycles of denaturation for 20 seconds at 94°C, annealing for 45 seconds at 42°C, and elongation for 1 minute at 72°C.

Primers: Primers were purchased from Gibco-BRL, and included PrCv35 and PrCv36 (97) as well as the complement of PrCv35 (5′-GTT TGG TCT GAA ACC GAC CCG-3′), referred to as PrCv35-RV, PrCv8300. (5′-CCT AAT GCA ACC

TAC CAA TTA-3′), based of the genomic sequence of primate calicivirus, David O. Matson, personal communication, and RCV-5300 (5′-GGA CTA TTG TCC GCT TGA GAA-3′), based on intitial unidirectional sequencing of the PrCv35-RV / PrCv8300 PCR product.

Sequencing: PCR products were sequenced directly from both terminal primers by automated sequencing procedures. When necessary for completion of sequencing from both directions, internal primers were synthesized.

Sequence analysis: Nucleotide and predicted amino acid sequences were analysed using the GCG genomics software package, version 9.0. Distance and parsimony trees were assembled and to subjected to bootstrap analysis using Dnadist or Protdist and Dnapars or Protpars respectively. Rudimentary alignments were assembled using CLUSTAL, Version 3.0 (59) and completed by hand and eye. Sequence homology searches were preformed using GAPPED BLAST[National Center for Biotechnology Information(NCBI)] (1). All sequences included in phylogenetic analysis were accessed through GenBank (Table. 1) with the exception of primate calicivirus, PCV *Pan*-1, which was kindly provided by David O. Matson.

Table. 1. Sequences utilized in phylogenetic analyses

Name	Genbank Number	Abbreviation
San Miguel sea lion virus-1	U14676, M87481	SMSV1
San Miguel sea lion virus-2	U18730, U76882, U76881	SMSV2
San Miguel sea lion virus-4	U14674, M87482,	SMSV4
San Miguel sea lion virus-5	U18731, U76884, U76883	SMSV5
San Miguel sea lion virus-6	U18732, U76886, U76885	SMSV6
San Miguel sea lion virus-7	U18733, U76888, U76887	SMSV7
San Miguel sea lion virus-13	U52087, U76878, U76876	SMSV13
San Miguel sea lion virus-14	U18735, U76880, U76879	SMSV14
San Miguel sea lion virus-17	U52005, U52094	SMSV17
Bovine calicivirus *Bos* - 1	U18741, U76875, U76876	BCV
Primate calicivirus *Paniscus* - 1	not yet submitted	PCV
Vesicular exanthema of swine virus - Type A48	U76874, U18737	VESVA48
Feline calicivirus - F4	M86379	FCV-F4
Feline calicivirus - F9	Z11536	FCV-F9
Feline calicivirus - Urbana	L40021	FCV-Urb
Feline calicivirus - CFI6	U13992	FCV-CFI6
Rabbit hemorrhagic disease virus	M67473	RHDV
European brown hare syndrome virus	U09199	EBHSV
Human calicivirus - Norwalk	M87661	NV
Human calicivirus - Southampton/91/UK	L07418	Southapmton
Human calicivirus - MX/89/Mexico	U22498	Mexico
Human calicivirus - Lorsdale	X86557	Lorsdale
Human calicivirus - Houston/27/90/US	U67859	Houston
Human calicivirus - Manchester/93/UK	X86559	Manchester
Human calicivirus - Sapporo/82/Japan	S77903	Sapporo

Chapter 3: Results

Serum neutralization:

There was no evidence of neutralization of 100 $TCID_{50}$ RaCV *Ory*-1 by 20 antibody units of typing serum for any of 40 calicivirus types. Neutralizing antibodies to individual SMSV and VESV serotypes have been shown to be type specific (151).

Rabbit serology:

Serologic evaluation of rabbits housed in the same facility which yielded the four apparently identical isolates of RaCV revealed that 40.9 % of rabbits examined had antibody titers by ELISA greater than 0.2 OD (optical density) at a 1 : 20 dilution to a mixture of CsCl banded marine calicivirus including McAll virus, SMSV-13, and RaCV (Table. 2) The presence of marine calicivirus specific antibody in the serum of co-resident rabbits indicates that the rabbit herd in question was indeed infected with at least one calicivirus.

Table. 2. ELISA of rabbit sera diluted 1:20 against combined antigen of McAllister, SMSV-13 and RaCV

Serum #	OD w/ antigen	Serum control	Corrected OD
1	0.202	0	0.202
2	0.155	0	0.155
3	0.237	0	0.237
4	0.358	0.086	0.272
5	0.274	0	0.274
6	0.390	0.002	0.388
7	0.473	0.132	0.341
8	0.392	0.005	0.387
9	0.283	0	0.283
10	0.011	0	0.011
11	0.032	0	0.032
12	0.409	0.032	0.377
13	0.059	0.016	0.043
14	0.062	0.002	0.060
15	0.039	0	0.039
16	0.024	0	0.024
17	0.178	0	0.178
18	0.025	0	0.025
19	0.141	0	0.141
20	0.007	0	0.007
21	0.030	0	0.030
22	0.096	0.015	0.081

Primary nucleotide sequence and predicted protein products:

Sequence was determined for 3531 nucleotides of the 3′ end of the RaCV *Ory*-1 genome, extending from the 3′ end of the non-structural polyprotein coding region through 164 nucleotides of the incompletely sequenced 3′ untranslated region(UTR). Reverse transcription-polymerase chain reaction amplification of the RaCV genome utilizing poly-T primer proved to be problematic, so primer PrCv-8300, which complements the 21 nucleotides preceding the poly-A tail of the primate calicivirus (PCV *Pan*-1) genome , was synthesized and used to amplify RaCV RNA. PCR amplification was preformed using three primer sets, PrCv-35 / PrCv-36 (97), PrCv-36-RV / PrCv-83, and PrCv-35 / PrCv5300 yielding PCR products of approximately 500, 550, and 3000 nucleotides respectively, which were sequenced bidirectionally using nested sets of internal primers.

The assembled sequence encodes one partial and two complete open reading frames (ORF's). The partial ORF (ORF1) contains 914 nucleotides coding for 304 amino acids. The partial sequence of ORF1 contains the three 3′-most motifs of the four motifs characteristic of caliciviral (102, 171) as well as picornaviral (52) RNA-dependent-RNA-polymerases. A five nucleotide gap separates the stop codon of ORF1 from the start codon of the second included open reading frame (ORF2), which is 2112 amino acids in length encoding a protein of 704 amino acids. ORF2 contains the PPG motif associated with the capsid proteins of caliciviruses and the VP3 capsid proteins of picornaviruses.

The start codon of the third included open reading frame (ORF3) overlaps the stop codon of ORF2 by one nucleotide . ORF3 includes 330 nucleotides encoding a protein of 110 amino acids.. The ORF organization of the available RaCV nucleotide sequence, like those of other partially sequenced marine caliciviruses, indicates a genome organization analogous to that of the SRSV's and feline caliciviruses. Unlike RHDV and the Sapporo-like human caliciviruses the major nonstructural and structural coding regions are not fused into a single open reading frame, and additionally there is no second small ORF overlapping the juncture of the RNA-polymerase and capsid precursor protein coding regions as is the case for Sapporo-like human caliciviruses.

Predicted capsid protein:

The lengths of predicted capsid precursor proteins among the caliciviruses vary widely, from the longest, among the marine caliciviruses, of more than 700 amino acids to the smallest, among the SRSV's, of closer to 500 amino acids. Alignment of the capsid precursor amino acid sequences indicates that most of this disparity lies at the NH_2 terminal end of the protein (Figure 5). Furthermore, the capsid precursor proteins of both the FCV's and SMSV's have been shown to undergo rapid post translational cleavage (23, 42) by a specific viral protease (166). Carter et al (23) localized the cleavage site of FCV-F9 between amino acids 124 (glutamic acid) and 125 (alanine) while Sosnovtsev et al(166) demonstrated that mutation of the P1 (a.a.-124 ; glutamic acid) position of

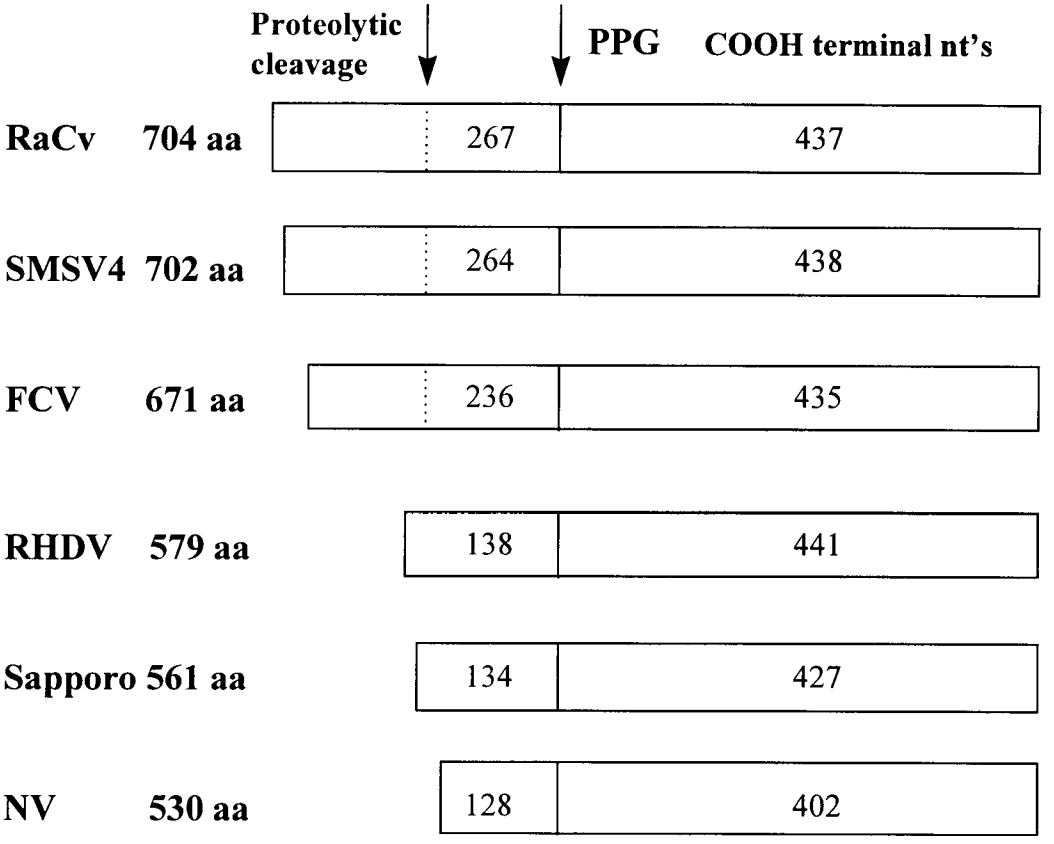


Figure. 5. Alignment of capsid precursor proteins of six caliciviruses around conserved PPG motif. Putative proteolytic cleavage sites of the feline and marine caliciviruses are designated by dotted lines. Number of amino acids located up or downstream of the PPG motif are indicated for each protein.

the FCV-F9 capsid cleavage site limited or eliminated cleavage depending on the nature of the substitution. The P1 position of all currently identified caliciviral cleavage sites is occupied by either glutamic acid (Glu) or glutamine (Gln) (28). Sosnovtsev et al noted that there are several alternative Glu / Ala pairs near the cleaved pair which apparently do not represent alternative targets for cleavage. Alignment of the predicted capsid precursor a.a. sequence of FCV-F9 with that of several other animal caliciviruses indicates that the known capsid precursor cleavage site of FCV-F9 is mirrored by closely homologous sites in marine caliciviruses, including RaCV, and the Japanese canine calicivirus # 48 (Figure 6).

These sites may represent precursor cleavage sites for these other viruses. Between positions P5 and P6′, there are three positions, P3, arginine (R), P1, glutamic acid (E), and P2′, aspartic acid (D), which are conserved among the feline, San Miguel, and canine isolate #48 caliciviruses. Position P4 is conserved as phenylalanine (F), with the exception of FCV-F9, which includes P4 isoleucine (I). The putative cleavage site of RaCV occurs between a.a.'s 150, glutamic acid (E) and 151, serine (S). If the 3C-protease of RaCV does cut between those residues, then the processing product, at 554 a.a.'s, would be within the range of lengths among the capsid proteins of RHDV, Sapporo virus and Norwalk virus (Figure 5).

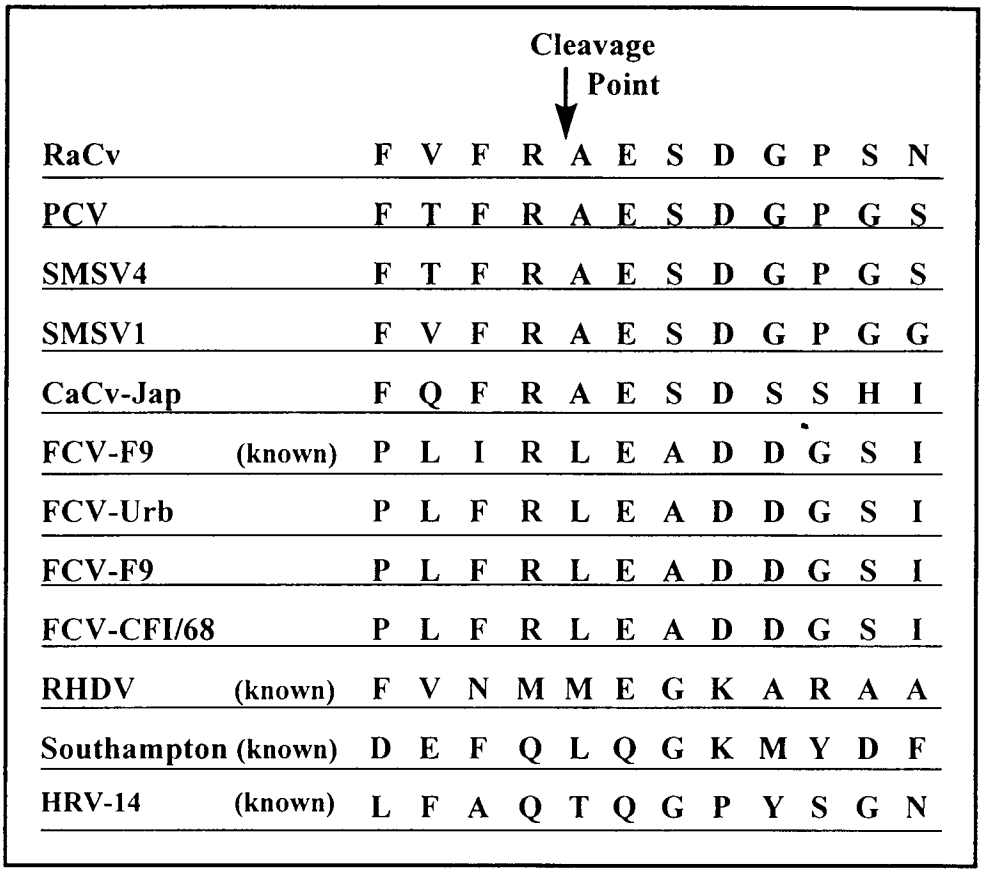


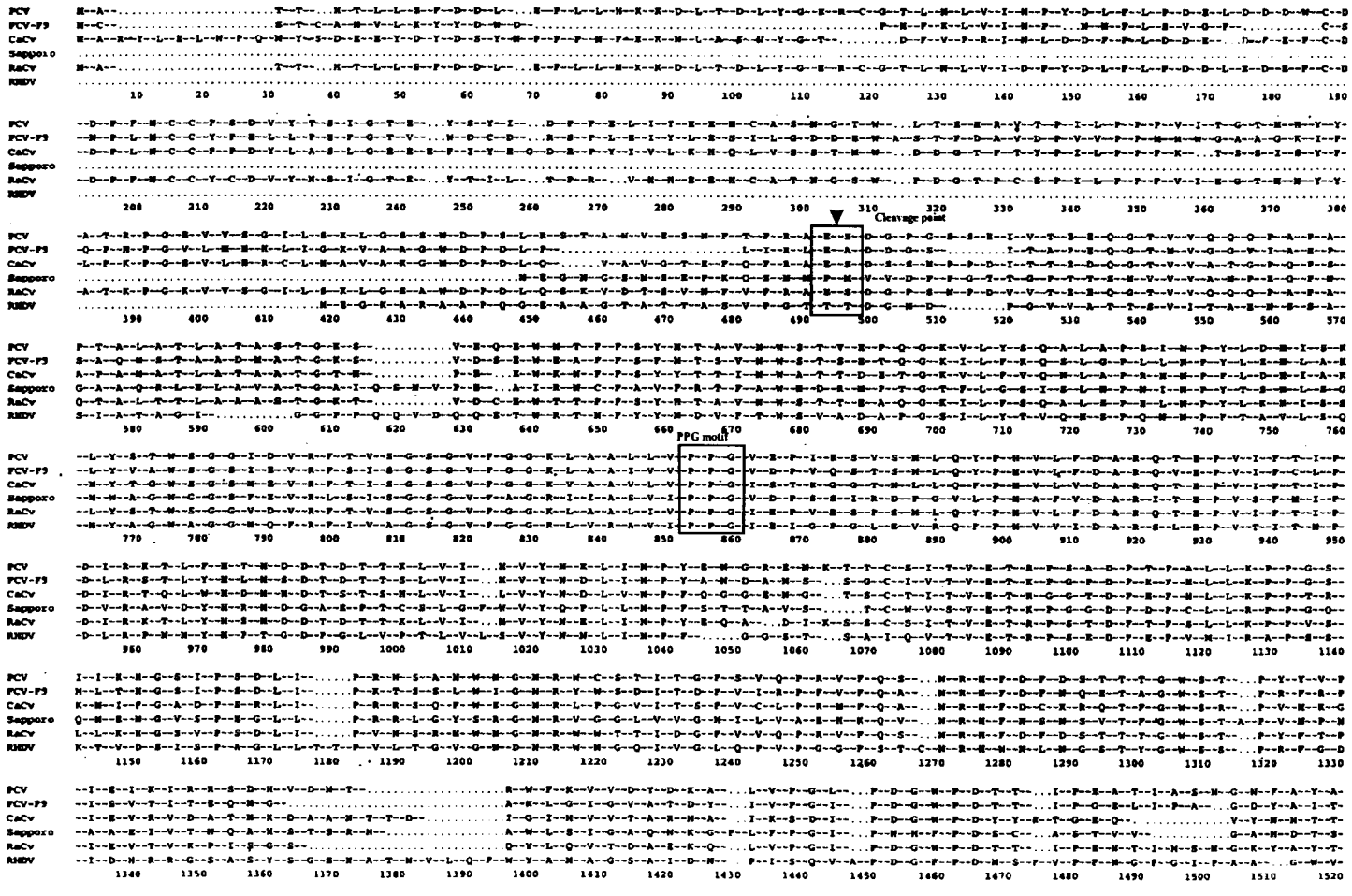
Figure 6. Alignment of identified cleavage site of FCV-F9 capsid precursor with the possible capsid precursor cleavage sites of RaCv, PCV (Primate calicivirus), SMSV-4, SMSV-1, CaCv-Jap (Japan isolate), FCV Urb (Urbana), F4, and CFI/68; the known capsid cleavage site of RHDV, the known helicase cleavage site from HuCv-Southampton, and the known 3A-3B cleavage site from human rhinovirus-14 (HRV-14).

Comparison of the identified capsid cleavage site of RHDV or the helicase cleavage sites of Southampton calicivirus with the known cleavage site of FCV-F9 revealed little similarity beyond the P1 / P1′ pair (Figure 6). This reflects the finding in human rhinovirus type 14(HRV-14), a picornavirus, that 2C-3A (non-structural protein processing site) cleavage sites from closely related picornaviral species will serve as efficient substrate alternatives for the HRV 3C -protease while 2C-3A cleavage sites from more distantly related picornaviruses are not only refractory to cleavage by the HRV protease but also display little homology to the cleavage site of HRV-14 aside from the P1 / P1′ pair (30).

Alignment of ORF2 structural gene of RaCV *Ory*-1 with those of other caliciviruses

The alignment of the putative translation product of RaCV ORF2 with the ORF2 amino acid sequences from PCV *Pan*-1, FCV-F9, CaCv-48, RHDV, and Sapporovirus is shown in Figure 7. The significant divergence of these sequences required that the alignment be completed largely by hand. The conserved capsid protein PPG motifs are shown, along with the putative capsid precursor cleavage sites for RaCV, PCV, and CaCv-48. The ORF2 translation products of PCV, FCV-F9, CaCv-48, Sapporo virus, and RHDV display 69%, 46%, 37%, 18%, and 18% a.a. identity respectively with ORF2 of RaCV. A partial sequence of CaCv-48 was recently directly submitted to Genbank by Roerink et al (Accession # - AF053720). ORF2 of this sequence codes for a protein of 692 a.a.'s, similar in length to the ORF products of FCV-F9, PCV, and

RaCV. The organization of the capsid coding sequence of CaCv-48 into an open reading frame separate from the non-structural polygene and the coding of additional preprocessing NH_2 - terminal a.a.'s and a putative 3C-protease cleavage site by the CaCv-48 ORF2 indicate evolutionary similarity to the marine and feline caliciviruses. However, ORF2 of CaCv-48 displays a.a. identity of only 37% and 34% to RaCV and FCV-F9 respectively, indicating less similarity to the feline or marine caliciviruses than exists between the marine and feline caliciviruses. The a.a. identities of Sapporo virus, a human calicivirus with classic morphology, to CaCv, FCV-F9, and RaCV are nearly equal at 21%, 20% and 18%, respectively.



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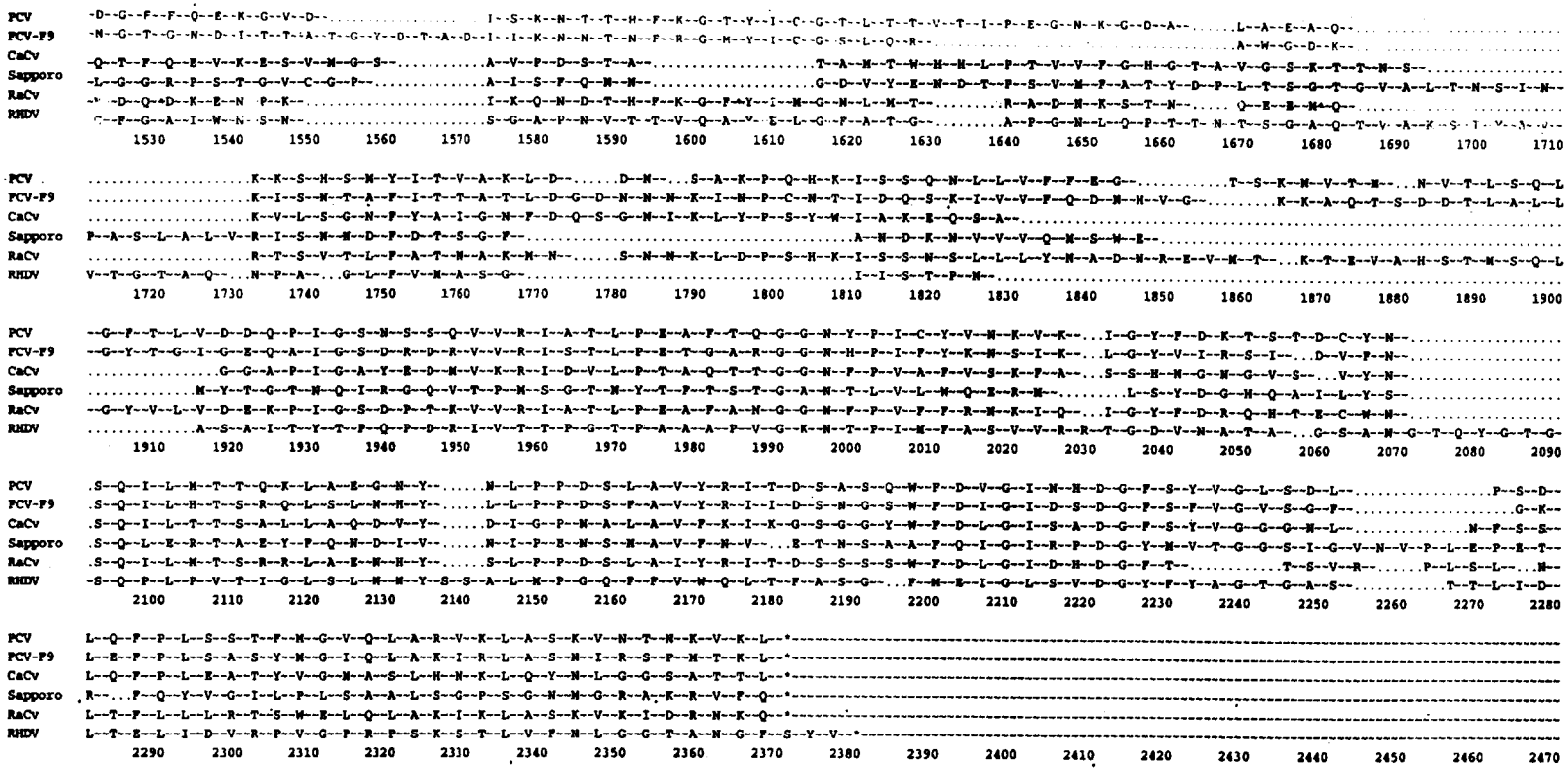

Figure. 7. Alignment of primate calicivirus, PCV*Ory*-1; feline calicivirus F9, FCV-F9; human calicivirus-Sapporo, Sapporo; rabbit calicivirus, RaCv; and rabbit hemorrhagic disease virus, RHDV.

Phylogenetic analysis of RaCV *Ory*-1 and other caliciviruses

Distance and parsimony methods were separately applied with bootstrapping to partial nucleotide and amino acid sequences of the 3D-polymerase gene and the capsid protein coding region. For distance analyses, branch lengths were calculated using the Fitch-Margoliash algorithm, and phylogenetic trees were assembled using the neighbor joining method. The analysed segment of 3D-polymerase included 300 a.a.'s for RaCV and contained the 3′ end of the non-structural polygene. The complete capsid gene sequences of those caliciviruses not known to undergo proteolytic cleavage of an extensive segment of the capsid precursor polypeptide, including viruses antigenically similar to NV, RHDV, and Sapporo virus, were aligned with the putative mature capsid protein sequences resulting from likely 3C-proteolytic cleavages of marine, feline, and canine-48 caliciviruses. The processed and unprocessed sequences are of similar lengths (ranging from the longest, 579 a.a.'s from RHDV, to the shortest, 530 a.a.'s from NV) and display significant homology throughout the alignment.

Bootstrapped phylogenetic trees resulting from distance and parsimony analyses of both the nucleotide and amino acid partial 3D-polymerase sequences were not statistically robust (data not shown). Similar analyses of partial capsid coding sequence resulted in phylogenetic trees with identical major nodes (those separating the five major branching clusters) to those generated by analysis of the partial polymerase gene sequences (Figure 8). Nucleotide and amino acid comparisons of capsid sequence generated branching patterns with conservation

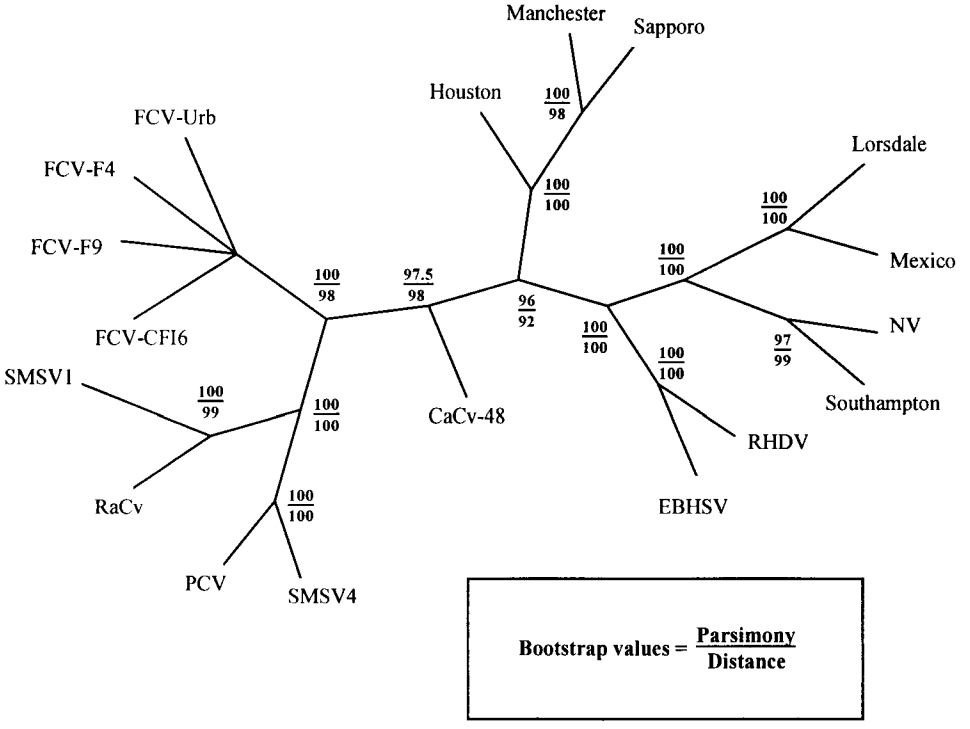

Figure. 8 Unrooted tree constructed independently with parsimony and distance methods from amino acid sequence of caliciviral capsid protein. All species included in analyses are shown and branches shared by both distance and parsimony trees are retained as bifurcating.

of both minor and major nodes, albeit with unequal statistical validity. Amino acid comparisons of capsid sequences were more statically robust than nucleotide comparisons (data not shown). This observation probably reflects the considerable divergence of structural gene sequence within the family, which disallows useful comparisons of primary nucleotide sequence.

Distance and parsimony comparisons of capsid a.a. sequences resulted in phylogenetic trees supported by bootstrap values of 90% or greater for all nodes outside the feline calicivirus cluster (Figure 8). Given that analyses of polymerase and capsid sequences resulted in trees of identical major topography, alignments of polymerase and capsid sequence were combined and analyzed. The resulting distance and parsimony trees displayed identical branching patterns to those developed from capsid sequence alone. However, bootstrap values were shown to increase for many nodes such that all nodes outside the feline calicivirus cluster were described by bootstrapped statistics of 95% or greater, and in most cases, were shown to appear in 100% of 500 bootstrapped iterations (Figure 9).

The topography of the 3D-polymerase / capsid tree shown in Figure 9 is similar to the parsimony tree constructed by Berke et al (10) from an amino acid alignment of a segment of the caliciviral capsid coding region including approximately 400 a.a.'s. Berke et al included two viruses in their analysis, hepatitis E virus and human rhinovirus 14 (*Picornaviridae*), which were not considered in this data set. I could find no significant homology between the hepatitis E virus capsid gene and other calicivirus capsid genes, or for that matter,

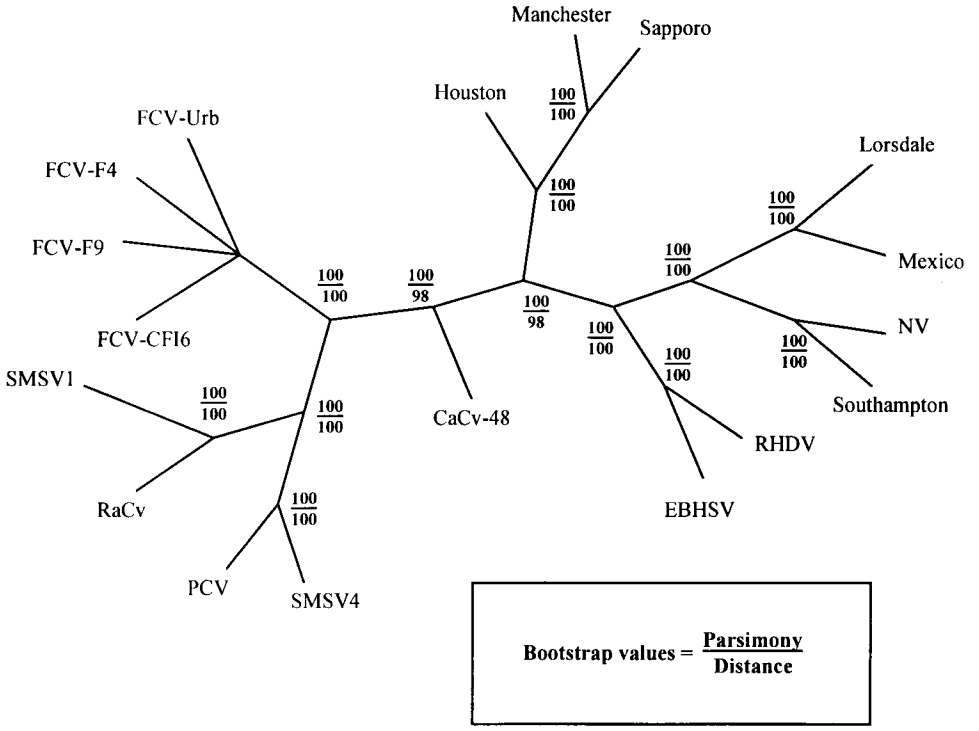

Figure. 9 Unrooted tree constructed independently with parsimony and distance methods from amino acid sequences of caliciviral capsid and 3D-polymerase proteins. All species included in analyses are shown and branches shared by both distance and parsimony trees are retained as bifurcating.

with any predicted translation products in Genbank. Furthermore, phylogenetic analyses of the caliciviruses have typically been rooted with one or more picornavirus(es). The identifiable homology between the VP3 structural genes of picornaviruses and the capsid genes of caliciviruses does not extend the length of the caliciviral capsid gene (171). If phylogenetic analysis of these structural genes is confined to sequence with recognizable homology, only a portion of homologous (within the family) caliciviral structural gene sequence can be considered, effectively limiting the number of informative data points and possibly the resolution of the analysis. The 3D-polymerase / capsid tree is statistically more robust than previous phylogenetic analyses of the caliciviruses partly because of the co-consideration of two genes, but also because of inclusion of the entire putative mature capsid protein sequences.

Five primary branch points appear in the 3D-polymerase / capsid tree (Figure 9). Four of these nodes have been previously described (10). They are the origins of four species clusters: the feline and marine caliciviruses which include RaCV, the Sapporo-like human caliciviruses, RHDV and EBHSV, and the SRSV's (small round structured viruses). These clusters are recognizable as reflecting differences in genome organization and more loosely, virion morphologic and physicochemical charcteristics. The fifth and previously undescribed major branch point apparent in this analysis belongs to the recently described canine calicivirus isolate 48. This virus, which appears to possess a genome organization most similar to the marine and feline caliciviruses, is clearly

separated from that cluster and branches at a point approximately midway between marine/feline calicivirus and the Sapporo-like human calicivirus clades.

Phylogenetic analysis of the marine caliciviruses

Complete capsid gene sequences are available for only four of the forty described marine caliciviruses. Partial sequences have been published for many others, but the described segments do not necessarily overlap, so the effective set of available sequences for phylogenetic comparisons within this subgroup is limited. Thirteen marine caliciviruses have been sequenced sufficiently to allow inclusion in a phylogenetic analysis. The analysed segment of 3D-polymerase translated to only 180 a.a.'s for RaCV from the middle of the 3D-polymerase coding region. Two short segments of the capsid gene which included amino acids 80 to 230 and 421 to 611 of RaCV were considered. As with analysis of the wider calicivirus family, phylogenetic trees resulting from analyses of polymerase and capsid sequence yielded trees of similar topography, so the gene segments were combined into a single analysis (Figure 10). Two major subgroups are apparent. The grouping which includes RaCV is fairly well defined and includes marine caliciviruses initially isolated from both terrestrial and marine hosts between 1972 and 1995 (149). Within this very preliminary analysis, no obvious patterns emerge relating phylogenetic relationships to host species or geography of initial isolation, virulence, or pathogenicity.

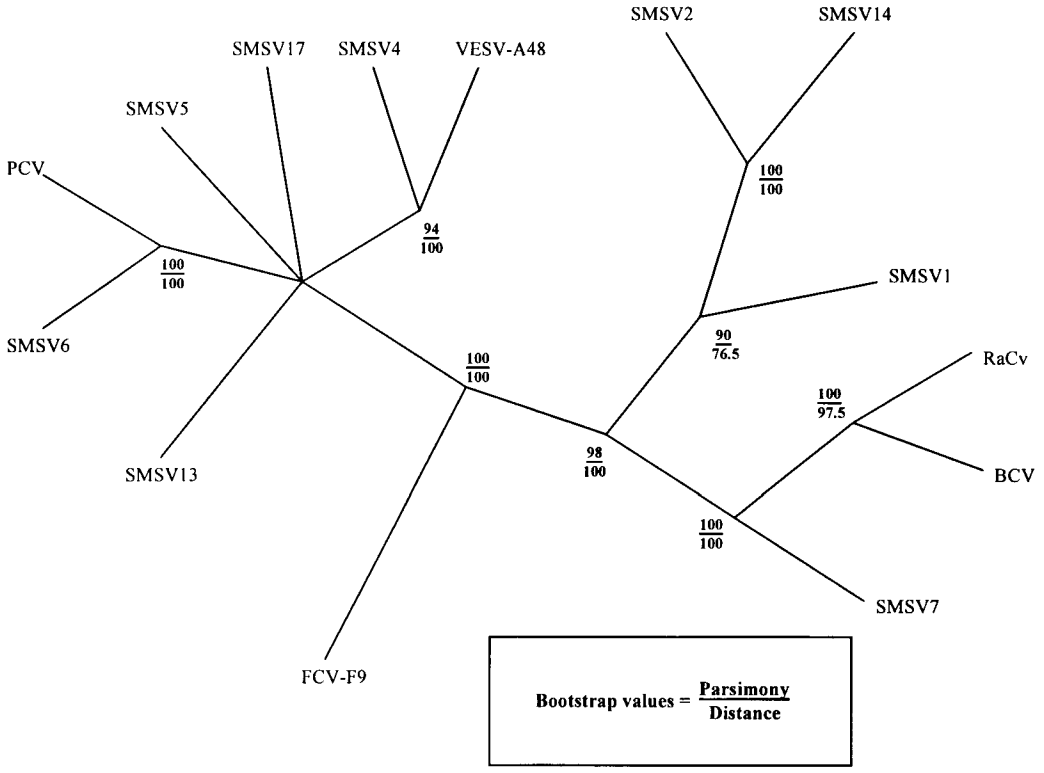

Figure. 10 Tree constructed independently with parsimony and distance methods from nucleotide sequence of partial ORF1 and complete ORF2 and ORF3. FCV-F9 (feline calicivirus) is designated root. All species included in analysis are shown, and branches shared by both distance and parsimony analyses are retained as bifurcating.

Chapter 4: Discussion

We have partially characterized rabbit calicivirus *Oryctolagus*-1, RaCV *Ory*-1, the first calicivirus isolated from rabbits and sequentially propagated *in vitro*. We have abbreviated rabbit calicivirus, RaCV, in order to distinguish it from reptilian calicivirus (RCV *Cro*-1), first isolated from an Aruba Island rattlesnake (*Crotalus unicolor*) in 1978 (147). RaCV is a novel serotype of the marine calicivirus subgroup. The marine caliciviruses are perhaps most remarkable for their range of host species including marine and terrestrial mammals, birds, reptiles, and fish (149). Additionally, infections across this broad host range are not uniform in virulence, tissue trophism, and pathogenicity. Marine caliciviruses have been shown to cause disease conditions as disparate as vesicular blistering, pneumonia, premature partuition, encephalitis, myocarditis, diarrhea, and hepatitis (149, 159). Experimental infectivity studies have not been preformed on RaCV, and the presence of multiple possibly pathogenic agents in its rabbit hosts precludes the automatic association of the virus with the diarrhea and hepatic degradation seen in the dead and dying rabbits from which RaCV was isolated.

We have presented the first evaluation of the phylogenetic relationships among caliciviruses based upon information from multiple genes. The definition of a singular gene is somewhat unclear among the genomes of the *Caliciviridae* which not only include coding sequences for multiple enzymes within single polygenes, but which also seem to have fused or separated unique coding

sequences into different open reading frame organizations over the course of the evolution of the family. There is some danger in combining information from multiple coding sequences for phylogenetic analysis given the possibility of recombinatorial coupling of genes of divergent histories. Initial comparison of single gene analyses should be undertaken so genome segments reflecting dual evolutionary histories are not inadvertently pooled into a single data set. As analysis of the polymerase and capsid protein coding regions indicates similar phylogenetic relationships among the caliciviruses, the pooling of information available from both genes to improve the resolution and statistical viability of phylogenetic analyses is justified.

The inclusion of canine calicivirus-48, isolated by Mochizuki et al in 1993 (106, 138), in the phylogenetic analysis of the caliciviruses complicates the sub-classification of the family. It has been suggested that the diversity of the *Caliciviridae* is such to warrant division of the family into genera, along the lines of the four geno-groups defined by genome organization and morphology and supported by phylogenetic analysis (10): the feline/marine caliciviruses, the Sapporo-like caliciviruses, RHDV and EBHSV, and the SRSV's. Phylogenetic evaluation of CaCv-48 clearly fails to neatly place this virus within any of the established geno-groups. If CaCv-48 is placed into the feline/marine calicivirus geno-group the possible genera of *Caliciviridae* become less well defined. However, creation of yet another sub-grouping for CaCv-48, alone, foretells an over-split and complicated system of classification. The caliciviruses that have

been completely or partially sequenced have been those already recognizable as members of subgroups defined by morphologic, antigenic, or physicochemical characteristics. The somewhat unusual caliciviruses defined as "candidate", such as porcine enteric calicivirus, canine calicivirus-1, Newbury diarrhea virus, amyelois chronic stunt virus, chicken calicivirus, etc., remain for the most part poorly characterized and uniformly unsequenced. The candidate caliciviruses may be the defining members of new genera or they may be "intermediate" species which link now seemingly distinct calicivirus subgroups. By concentrating research effort on those caliciviruses which are already somewhat defined by association with other obviously and closely related viruses, we may artificially bolster the perhaps inaccurate conception of an orderly and well defined caliciviral evolution.

RaCV requires further investigation. The fact of its shared host species with RHDV may make RaCV somewhat singular among the marine caliciviruses. RHDV has been recognized worldwide for its destructive capacity: in Europe and Asia as a threat to both wild native rabbit populations and domestic rabbits raised for human consumption and in Australia and New Zealand as a prospective and now actively disseminated biological control agent (24). The challenges inherent to the study of non-cultivable viruses render difficult the investigation of such issues as tissue trophism and mechanisms of pathogenesis which are important in the evaluation any pathogen, but especially one which is actively utilized as an agent of biological control. If RaCV can indeed be developed as a

model for RHDV, it may present an opportunity for thorough illumination of the relationship between the rabbit and the calicivirus.

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