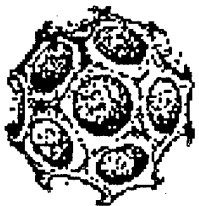
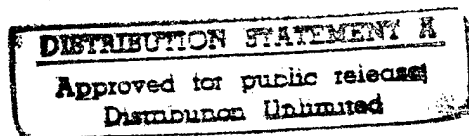


Final Report: R&T #4426703-1  
Contract between the Office of  
Naval Research and Oregon State  
University, College of Veterinary  
Medicine, Corvallis, Oregon

## Marine Mammal Health: The Interrelationship of Autochthonous Viruses and Stress



Laboratory for  
Calicivirus Studies  
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Marine Mammal Virology Program

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**Jim Carbonara,  
Director, Field Operations**

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## SUMMARY OF WORK

Our overall goal was to improve the health and operational effectiveness of the Navy's working marine mammal systems wherever they may be deployed in the oceans of the world. Our supporting objectives in combination with those of the Hawaii and Kansas State Laboratories was to define more clearly the interrelationship of viral disease and stress and the decrement both impose upon the overall health of marine mammals including specific components of the immune system. In addition, we began to assess the epidemiologic aspects of some marine mammal viral disease agents as they may impact the worldwide deployment of Navy animals and the health of personnel working in the water with these animals.

Our approach for addressing these goals was as follows. Captive marine mammals were tested for autochthonous virus shedding, and the presence of specific antibodies. These data were correlated on an opportunistic basis with a clinical assessment of the individual animal's overall health and known stresses such as recent capture, social restructuring, training, transportation, restraint, or other activities. Viruses associated with naturally occurring diseases or shed by animals with subclinical infections were isolated from both captive and wild marine mammals. The impact of the diseases caused by such agents was assessed using clinical observation, retrospective serology, experimental infection of non marine mammal modeling species such as swine, and necropsy reports. Epidemiologic studies were carried out to determine some of the oceanographic distributions of marine viruses and to determine their possible effects upon marine mammal populations under conditions of natural exposure and their possible human health effects.

We are reporting for the first time the finding that cetacean calicivirus as well as other, but not all, caliciviruses can cause hepatitis. We feel that this is an important finding because of the long history of hepatitis from unknown causes which has occurred among captive and Navy-owned cetaceans. Furthermore, we suggest that because the enterically transmitted type of human hepatitis (Hepatitis E) involves a calicivirus, there may be a linkage between this disease and our marine mammal work. To further link caliciviruses and hepatitis, a deadly hemorrhagic hepatitis of rabbits has now been shown to be caused by a calicivirus.

We have isolated a member of the Reoviridae from California sea lions, Northern sea lions, and Northern fur seals and a member of the Retroviridae from walrus. Isolation of these two virus classes from marine mammals has never been reported previously. Preliminary tests of the reovirus RNA show migration patterns similar to, but not identical with, human reovirus type 3 and the retrovirus from walrus has the morphologic features of a lentivirus. Taxonomically the AIDS virus of man is also a lentivirus. Both the retrovirus and the reovirus appear to be widespread in certain geographic locations.

New isolates of calicivirus include the re-isolation in 1987 of a walrus calicivirus from the Eastern Siberian Sea. This virus was first recovered in 1977 in the Chukchi Sea, yet one of the Pacific bottle-nosed dolphin sera tested had antibodies to this agent suggesting its wide distribution in the Pacific basin. Four new San Miguel virus types (SMSV-14, SMSV-15, SMSV-16 and SMSV-17) has been isolated from sea lions. An additional walrus calicivirus has been typed and is a reisolation of the 1977 walrus serotype. The reptilian calicivirus originally isolated from four species of poikilotherms at the San Diego Zoo in 1978 has been re-isolated from Northern fur seals and both California and Stellar sea lions in California and Oregon, respectively. Serologic studies suggest this agent is widely distributed in the North pacific. The first virus ever isolated from marine mammals and originally classified as a California grey whale enterovirus has been re-examined and shown to be a calicivirus. Furthermore, this virus proved to be the same serotype (VESVA<sub>48</sub>) as the calicivirus causing the foreign animal disease, vascular exanthema of swine and it is the prototype for the entire taxonomic family Caliciviridae.

Cetacean sera from **Tursiops** of both Atlantic and Pacific origins has been examined for antibodies to marine viruses and both have tested positive for an array of caliciviruses. We are conducting an intensive examination of this phenomena to determine whether or not caliciviruses persistently infect dolphins and whether or not they might be useful as tracer viruses indicative of stress and suppressed immunity.

Work with copy DNA (cDNA) from the genomic RNA of caliciviruses is continuing. A pilot run was made in collaboration with Dr. Harry Greenberg at Stanford University using feline caliciviruses and, more recently, the San Miguel Type 5 genome has been copied at Oregon State University. Both viruses have been cloned and hybridizations studies have been carried out by our laboratory and others.

We have cDNA clones which will hybridize with all caliciviruses tested (24 serotypes plus human Norwalk agent and human Hepatitis E) and are therefore useful as a universal calicivirus specific probe for detecting calicivirus shedding or presence of calicivirus in clinical samples. As this grant ends, we are identifying PCR primer sets to increase probe sensitivities.

Monoclonal antibodies have been generated which appear to bind to all caliciviruses of known ocean origin but not to other viruses. Antigens for immunoblot screening of sera have been assembled and used to show human and animal antibody reactions to caliciviruses.

Herpes viruses have been described in phocid seals and have been incriminated in pneumonias. We have observed a herpes virus when using direct electron microscopy to examine the vomitis of a sick California seal lion but were unable to make any causal

associations because the virus could not be replicated in available systems.

The epidemiology of caliciviruses gives a general picture of ocean "hot spots" such as the Santa Barbara Channel Islands off the coast of California with general spread throughout the entire Pacific basin into the Bering, Beaufort, Chukchi, and Eastern Siberian Seas. Various virus serotypes seem to become involved in cyclic events among susceptible marine mammals. Human infections are highly probably but are rarely documented. There is little reason to believe that caliciviruses have not been spread to all the world's oceans.

Mounting evidence shows a continuum of known human caliciviruses and caliciviruses of ocean origin, thus we can expect marine mammal pathogens to also infect animal handlers or others who work in the water with these animals.

## TECHNICAL REPORT

**Hepatitis Associated with Cetacean Calicivirus:** Some caliciviruses of ocean origin are known to be vesiculogenic in a variety of species including domestic swine, Atlantic bottlenosed dolphins (*Tursiops truncatus*), California sea lions (*Zalophus californianus*), Northern fur seals (*Callorhinus ursinus*), domestic cattle, and humans. Furthermore, reproductive failures in pinnipeds may be associated with calicivirus infections as may be pneumonia and encephalitis. Hepatitis caused by a caliciviruses is a finding which has been reported as Hepatitis E in humans and was observed in swine infected with a walrus calicivirus isolate. The cetacean calicivirus (CCV-Tur-1) was isolated on two occasions from dolphins with vesicular lesions on their bodies. Three groups of three swine each were experimentally infected with this agent intradermally, orally and by pen contact and subsequently all developed clinical vesicular exanthema thus confirming the vesiculogenic potency of this virus. In addition, eight of nine swine that had been exposed were examined for hepatitis and all eight had hepatocellular degeneration graded as mild to marked.

Intradermal inoculation of CCV caused primary vesicles 2-7mm at inoculation sites and within 2-6 days vesicles up to 1 cm in diameter at secondary sites on the tongues and noses and feet. Pigs within the orally exposed and pen contact groups developed similar nose, mouth and foot lesions within 96 hours. A total of 76 virus isolations were made from the throat, rectal swabs and a variety of tissues taken at necropsy from the nine infected pigs during the term of the experiment. Using direct electron microscopy 11 of 11 virus isolates examined were calicivirus. Furthermore, these were neutralized by CCV typing sera.

Serum neutralizing (SN) antibodies at titers of 1:40 to 1:160 were present in three of

nine pigs by day seven. By day 14 six of nine were positive with SN titers of 1:40 to 1:320 and three remained negative at the 1:20 screening level. The most outstanding feature was the occurrence of mild to marked hepatocellular degeneration. In comparing the hepatitis in the CCV infected swine to those similarly infected with other caliciviruses of ocean origin, it was noted that the walrus calicivirus caused a more severe hepatitis but that the highly vesticulogenic SMSV-13 caused little or no liver damage.

The importance of this finding is in the possible relationship between caliciviral induced hepatitis for dolphins, past losses of Navy dolphins due to a probable viral hepatitis and, finally, the recently reported finding that the etiology of human fecally transmitted hepatitis E is a calicivirus.

Recently captured *Tursiops gillii* in Hawaii were free of exposure to cetacean calicivirus, SMSV-6 and SMSV-13 but did have antibodies to the walrus calicivirus which is the most hepatotropic of the 4 viruses tested.

Over the past year we have developed a cooperative program with Dr. Dan Bradley at Centers for Disease Control specifically to explore the possible relationship between the marine calicivirus hepatitis phenomena and the so-called enterically transmitted hepatitis E which is of caliciviral origin. Microgram quantities of walrus virus were produced and inactivated and then shipped to CDC for RNA extraction and cDNA production in preparation for cloning. In addition, sera have been exchanged to determine cross reactivity between the two virus groups. To date, the only relatedness observed has been the hybridization of some cDNA probes of marine mammal caliciviruses with the hepatitis E RNA. Our calicivirus monoclonal antibodies do not appear to recognize hepatitis E protein.

**San Miguel Sea lion virus Type 14 (SMSV-14): A New Calicivirus Serotype from Marine Mammals:** This recently identified calicivirus serotype was isolated in 1987 from rectal swabs collected from a California sea lion on San Miguel Island. To date, only cetacean sera from Hawaii has tested positive for antibodies against SMSV-14. Of the 60 sera tested two were positive for SMSV-14 and these two sera collected seven days apart were from a single *Tursiops gilli* which in addition to having antibodies to 5 other serotypes of caliciviruses had elevated liver enzymes.

**San Miguel Sea Lion Virus Type 15 (SMSV-15):** In September and October of 1988 approximately 185 rectal and vesicular lesion samples were collected from seals on San Miguel Island. Several animals had numerous intact vesicles extending into the haired areas and others had healed lesions on their flippers. A total of two virus isolates were recovered from the rectal swabs and from vesicular fluid all from



*Zalophus*. All three isolates were made in pig kidney cells and then adapted to Vero cells.

None of the California Sea lion could be sampled for serum antibodies and for this reason the distribution of this virus in nature is not known although one *Tursiops gillii* sample in Hawaii did have low levels of antibody to this agent. Sera was collected from 41 fur seals during the virus sampling on San Miguel Island and none of these had detectable antibodies at the 1:10 screening dilution.

This virus is not neutralized by the sera of any established virus type and is, therefore, classed as a new marine calicivirus, San Miguel Sea lion Virus Type-15.

**San Miguel Sea lion Virus Type-16 (SMSV-16)**: In December of 1988 an outbreak of gastroenteritis passed through the Sea lion colony at NOSC, San Diego. Four sea lions from a group of 10 housed in a floating enclosure in San Diego Bay became ill in December 1988. Clinical signs, which appeared suddenly, included diarrhea of a yellow mucous consistency, mild tenesmus, lethargy, anorexia, mild conjunctivitis, and a moderate febrile response. Rectal temperatures increased from a normal of 99 degrees F to a range of 101.1 to 102 degrees F. One of the four animals vomited and exhibited increased salivation. Palpation did not reveal any tenderness of the abdomen. The animals preferred to rest on deck spending a significantly greater period of time out of the water. These clinical signs lasted from 24 hours to 5 days with an average duration of 48 hours. The one sea lion that vomited was ill for the longest period, 5 days. After this time all animals returned to normal appetite and behavior. A total of seven samples were collected from 2 animals and examined for virus. Both samples from one animal were negative and all four fecal samples plus the single vomitus sample collected from the second sick animal all yielded a calicivirus isolate.

This virus is remarkable in three ways. First, it is the first time that caliciviruses have been tentatively associated with diarrhea in sea lions although various caliciviruses are known to cause diarrhea in dogs, man, calves and swine. Second, the virus was isolated in each instance in swine cell lines and a swine testicular cell line was especially sensitive. The virus replicated poorly to not at all in Vero cells which have traditionally been the most sensitive for marine calicivirus isolation and propagation. Finally, this represents a new serotype of virus and has been called San Miguel Sea lion Virus Type-16. Additional tests are in progress to gather possible evidence on the causal relationship of this virus and sea lion diarrhea.

In March 1989 three sea lions from a group of four housed in a floating enclosure became ill. Clinical signs of this illness were identical to the illness that occurred in December 1988. This group of animals was separate from the previously mentioned group and these animals were housed in a different location. However, from February

9 to March 1, one of these animals was housed with the group that became ill in December. This suggests the possibility that the agent that caused the December illness may have caused the March illness and this agent may also persist in clinically healthy animals in a carrier state.

**A New Marine Mammal Virus Isolate from the Russian Arctic Provisionally Classified as Lentivirus:** Rectal swabs collected from walrus taken in the Eastern Siberian Sea during a joint USSR/American research cruise were processed for virus isolation. From these an enveloped virus with a bar shaped nucleocapsid was isolated. Based on size and morphology, this isolate is taxonomically compatible with the Lentiviruses which are a group of non tumor producing RNA retroviruses. Feline immunodeficiency virus, Bovine leukemia virus, Caprine arthritis-encephalitis virus, Ovine progressive pneumonia virus and the human immunodeficiency viruses or AIDS virus are all Lentiviruses.

Serologic tests of walrus sera collected during the same joint cruise were examined for neutralizing antibody and approximately one-half of the 63 walrus sampled showed evidence of exposure to this agent.

Tests to determine the definitive classification of this putative Retrovirus of walrus were not conclusive and further work on this interesting isolate has been set aside.

**Eastern Siberian Sea Isolate of Walrus Calicivirus Type 1 (WCV Odb-1):** In 1977 three isolates of calicivirus were obtained from walrus feces collected from the ice in the South central Chukchi Sea. Serologic tests from 58 walrus taken in 1976 east of the Pribilofs in the Bering Sea revealed only five positive animals. Ten years later (1987) 63 walrus were sampled in the Eastern Siberian Sea (between lat 69.37N to 71.28N and Long 161.07 to 178.20W). The walrus calicivirus, WCV Odb-1, was again isolated. This second isolated, although it has the identical serum neutralization typing characteristics as the original isolated, replicates to much higher titer in the tissue culture and is much more cytolytic.

The original walrus isolate has been shown to be hepatogenic in swine and to produce a much more profound hepatitis than other more vesiculogenic caliciviruses. The hepatogenicity of the second WCV Odb-1 isolated has not been tested.

A total of 60 cetacean sera from Hawaii were tested for antibodies to walrus virus and only one of these reacted at the 1:10 screening level. This was from a *Tursiops gilli* captured in the Hawaiian Islands but subsequently released because of non adaptability. These results reaffirm the widespread nature of certain of these agents. It is noteworthy that Pacific dolphins in Hawaii can have antibodies to viruses known to

exist in the Bering, Chukchi and East Siberian Seas.

**Whale Enterovirus (W-6) Reclassified as a Calicivirus:** In 1948 the type species strain of Vesicular Exanthema of Swine Virus (VESV) was isolated from swine in Fontana, California. Just eight years later, this virus designated A<sub>48</sub> and the twelve other known serotypes of VESV were thought to have not only been eradicated from their only known natural host, domestic swine, but to have disappeared from Planet Earth as well. The epizootics of VESV-induced vesicular disease that had begun in 1932 and eventually spread throughout the United States were contained in 1956 and three years later the disease, Vesicular Exanthema of Swine, was said to have been eradicated. In 1968 interest in disease agents of ocean origin began to intensify and in that year the first virus isolate from any marine mammal was recorded. This isolate designated W-6 was recovered from a rectal swab taken from a California gray whale (*Eschrichtius robustus*) being commercially processed at the Richmond, California whaling station, in San Francisco Bay. Based on its physico-chemical properties it was classified as an enterovirus. We have now re-examined this agent and find it not to be an enterovirus but, instead, a calicivirus. More importantly, on the basis of virus neutralization serotyping isolate, W-6 is indistinguishable from the VESV A<sub>48</sub> calicivirus which is the type species for vesicular exanthema of swine virus.

Caliciviruses can be differentiated from other small round RNA viruses on the basis of three physico-chemical characteristics. First, caliciviruses have a distinctive morphology showing cuplike surface features or calyxes from which they derive their name. Secondly, those tested previously have all been more readily degraded by heat in the presence of divalent cation (MgC1++) than by heat alone, whereas MgC1++ has a sparing effect on enteroviruses in the presence of heat. Finally, caliciviruses appear to be unique in that they exhibit just one major structural protein and this latter feature has been accepted as an identifying feature for a new group of putative caliciviruses that cannot be cultivated *in vitro*. These are the Norwalk group of agents and other enterotrophic calicivirus-like agents that cause gastroenteritis of humans and animals.

The original W-6 isolation was made in Dolphin kidney cells and was characterized in 1968 as follows using a whale kidney cell line. It was heat sensitive but resistant to IUDR, chloroform, and low pH which at that time "would classify the isolate as analogous to an echovirus." In retrospect, one should be reminded that the removal of the Caliciviridae from the Picornaviridae into a separate family of small RNA viruses distinct from rhinoviruses and enteroviruses was not published until several years later.

Recently, we recovered an ampule of the W-6 agent that had been grown in whale kidney cells and remained frozen at -70 C since 1973. Initial transmission electron microscopic examination revealed a virus approximately 36 nm in diameter having the morphology of a calicivirus rather than an enterovirus. Monolayers of Vero cells, PK-15

cells, and Pilot whale kidney cells were inoculated with 0.2 ml of the reconstituted stock culture. Virus was allowed to absorb for 60 minutes at 37 C and then the monolayers were washed, re-fed and incubated at 37 C in 5% CO<sub>2</sub> and 95% air using previously described procedures. Although these preparations were blind-passaged several times, typical calicivirus CPE failed to appear. Stock virus was added to L-cystine using the methods previously reported where aliquots of 0.2 ml of virus were periodically withdrawn from the L-cystine treated stock virus and passaged in the three cell lines described above. Samples withdrawn after 35 days of L-cystine treatment caused typical calicivirus CPE in all three cell lines after 48 hours incubation. Supernatant examined under transmission EM contained virus particles with morphology typical of caliciviruses. Furthermore, when examined by immunoelectron microscopy this virus formed aggregates with A<sub>48</sub> typing serum and took on the smudged appearance characteristic of a virus coated with antibody. In addition, the W-6 isolated was neutralized by VESV A<sub>48</sub> typing sera. In other studies marine mammal sera examined in 1976 contained VESV A<sub>48</sub> type-specific virus neutralizing antibodies. Considering the broad antigenic variability (minimum of 31 serotypes) of the caliciviruses known to be of ocean origin, the antigenic stability and long-term persistence of the single calicivirus serotype A<sub>48</sub> is remarkable, especially in light of the adaptability of these agents to new host species.

It is our belief that the role of caliciviruses as pathogens is newly emerging. We find evidence that the agent is widespread in animal species of divergent phylogenetic makeup. Not only that, but a single serotype has the capability of replicating in host species as different as man and fish where body core temperatures are 37 C and 15 C respectively. That the caliciviruses are uniquely marine in their origin is well established. It is now well proven that they can cross the land-sea interface and become established in terrestrial species. This becomes especially germane because of the possible introduction of virus through improperly cooked fish products and could shed light on the many outbreaks of Norwalk-induced gastroenteritis caused by eating raw shell fish. One could postulate that the virus finds oysters and mussels to be suitable host species or mechanical collectors of caliciviruses contained in ocean reservoir species rather than simply biological filters for waters contaminated with human waste. The possibility that caliciviral diseases of man may be contained in ocean reservoirs as occurs with the caliciviral diseases of animals should be examined.

**Cetacean Serology for Viruses of Ocean Origin:** The naval Ocean Systems Laboratory in Hawaii over the past eight years has collected from marine mammals maintained at that facility a large repository of sera. In terms of retrospective serology this collection is important for several reasons. Many of the sera are from the same animal sampled over a several year period and these sera can be correlated with medical records, periods of experimental manipulation, training and other potential stresses. In some instances the sera will date from the time that specific animals were

new arrivals at the Hawaii facility thus providing the opportunity to examine them for possible antibody conversions and subsequent changes in titers over time. The geographic movements of these animals since coming into the inventories are well documented and can also be correlated with points in time when antibody conversions may have occurred. In addition to mixing of populations having differing geographic origins and experiences, there will have occurred mixing of populations of differing species. Thus, there exists the possibility that both the interpopulation and interspecies movements of viruses can be followed.

There have been very few viruses isolated from cetacean species. These include an influenza virus of pilot whales, the cetacean morbillivirus, an adenovirus from a Sei whale, and VESV A<sub>48</sub> from a California gray whale. *Tursiops* spp. Have been examined extensively for virus shedding yet, with the exception of the two pox virus types which cannot be grown *in vitro* there are documented only 2 virus isolations from this genus. Both isolates are caliciviruses of a single serotype designated cetacean calicivirus *Tursiops* type-1 (CCV Tur-1) and both were from skin lesions seen on *T. Truncatus* maintained in San Diego, California.

We have begun a systematic screening of the NOSC Hawaii serum bank starting with 60 sera collected in 1987 and have examined these at a 1:10 dilution for neutralizing activity to the following 20 viruses of ocean origin, see Table 1.

Table 1  
Marine Mammal Sera: NOSC Hawaii

Neutralization Results: 50% Endpoint, 100 TCID<sub>50</sub>, 1:10 Dilution

<u>Virus</u>	<u># of Positive Sera</u>	<u>OSU Serum ID Numbers</u> (Positive Sera)
SMSV 1	4/60	1,9,24,59
SMSV 2	2/60	11,12
SMSV 4	0/60	
SMSV 5	3/60	1,20,21
SMSV 6	1/60	49
SMSV 7	11/60	1,10,11,12,13,23,37,48,53,58,59
SMSV 8	14/60	12,16,20,23,24,30,37,40,45,48, 52,55,56,59
SMSV 9	11/60	5,18,21,31,35,37,44,48,50,52,56
SMSV 10	8/60	23,36,37,39,41,42,47,48
SMSV 11	1/60	55
SMSV 12	2/60	12,24
SMSV 13	0/60	
SMSV 14	2/60	23,37
W-6 (VESV A <sub>48</sub> )	0/60	
MV20-3	10/60	11,16,21,22,23,30,34,50,51,56
Reptile	0/60	
C420R (Rota)	0/60	
Walrus 7420	1/60	55
Cetacean CV	0/60	
T1/19		
(Retrovirus?)	0/60	

Twenty sera from ten individuals numbered one through ten were examined and all ten animals were positive for antibodies against up to six calicivirus types. The remaining sera from 40 individuals were similarly screened and 20 were positive for antibodies against up to four calicivirus serotypes. Antibodies were found for 13 of 18 calicivirus types tested whereas all 60 were negative for the Pinniped Reovirus and the Walrus Putative Retrovirus. Although CCV Tur-1 was first isolated in 1979 from two Tursiops in the NOSC inventory none of the 60 sera examined had antibody detectable at the 1:10 level.

These results show that *T. Gillii* at the Hawaii NOSC facility had antibody profiles ranging across an array of caliciviruses of ocean origin. Not only that but one of these

animals was shedding calicivirus and this virus was typed as San Miguel Seal lion virus type-9 which was a virus prototype originally isolated from pinnipeds in 1976 on San Miguel Island, California.

Our initial findings were confusing in that the same animal would be positive or negative for different viruses at the screening serum dilution of 1:10 in serum samples that were taken only a week or so apart. This was thought to be primarily a low dilution effect where marginal levels of neutralizing antibody present at the 1:10 dilution sometimes were sufficient to neutralize a specific virus and sometimes were not. The principle would be that a given dilution of antibody would have had a lethal effect on the virus just 50% of the time thereby giving both positive and negative results at these 50% endpoints.

These tests have been repeated using blind sampling, coded sera and an initial dilution of 1:5 instead of 1:10. In addition, all positive animals were titrated to antibody endpoint for each virus. These results were remarkably consistent with the initial tests in that the 1:5 serum dilutions were useful in filling in nearly all existing discrepancies. (See Table 2)

The importance of this work and its possible utility in explaining our stress vs. Disease model has been previously discussed.

Table 2  
Tursiops gilii: Antibody Profiles

Animal	Date	1	2	5	6	7	8	9	10	11	12	14	7420	15*	MV20-3
TG-1	4-11-87 Screen														
	4-16-87 Titer	1:20				1:20	+	+							+
	4-16-87 Screen	+				+	1:10								1:5
TG-2	4-30-87 Titer	1:5					1:20	1:10							1:40
	4-20-87 Screen					1:5								1:5	
	4-20-87 Titer					+									
TG-3	4-29-87 Screen					+									
	4-29-87 Titer					1:5									
	4-30-87 Screen								+						
TG-4	4-30-87 Titer								1:10						
	5-09-87 Screen								+						
	5-09-87 Titer								1:10						
TG-5	5-06-87 Screen														
	5-06-87 Titer														
	5-14-87 Screen								+						
TG-6	7-11-87 Screen		+			+									+
	Titer		1:10			1:10									1:20
	7-22-87 Screen		1:5												+
TG-7	7-14-87 Screen														
	7-14-87 Titer		+			+	+								1:20
	7-16-87 Screen		1:10			1:10	1:20			1:5					1:5
TG-8	7-16-87 Titer						+				+				+
	7-16-87 Screen		1:5			1:5	1:20				1:10				1:10
	7-22-87 Titer					1:40	1:10								1:5
TG-9	7-24-87 Screen														
	7-24-87 Titer	1:5		+		+		+							+
	8-06-87 Screen	+													1:10
TG-10	8-06-87 Titer			1:10				1:20							1:20
	7-25-87 Screen														
	7-25-87 Titer														+
TG-11	8-06-87 Screen														1:5
	8-06-87 Titer														1:40
	7-25-87 Screen														
TG-12	7-25-87 Titer														+
	8-06-87 Screen														1:5
	8-06-87 Titer														1:40

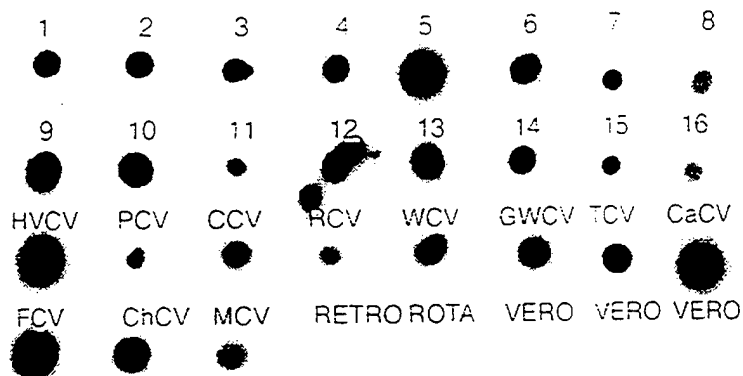
\*Screening did not include tests for SMSV-15. + = positive. All screening at 1:10 dilution.



**Copy DNA Probes for Caliciviruses:** Two methods of reverse transcribing viral genomic RNA were used. One uses the polyadenylated tail of the RNA genome to replicate possible full length genomic cDNA. The second method is random priming which provides various sizes of cDNA coding from random nick sites along the viral genome. These methods have generated a cDNA library for SMSV-5 containing some 600,000 recombinants. We have processed 200 of these and already have a cDNA oligomer of approximately 1.5 kilobases which is thought to be slightly less than 1/4 of the viral genome.

This oligomer has been amplified, cut from the plasmid by restriction enzyme and purified by gel electrophoresis. This intact oligomer has then been hybridized to self, whole purified virus and cell infected with homologous virus. In addition, it has been hybridized to 27 additional calicivirus types and the cross reactive profiling of the probe will continue (see figure 3). Further use of this clone designated 5RT is seen in its use on field samples from diverse species and sources (Table III and Figure 5) where use of the probe has established the presence of calicivirus in a migratory pelagic bird, fish from mid-Pacific reefs and shellfish filterfeeders on offshore coastal islands.

Figure 3. 5RT cDNA calicivirus probe using dot hybridization against RNA of a wide range of calicivirus serotypes.



1-16 = San Miguel sea lion caliciviruses serotypes 1 through 16

HVCV = Hepatitis virus calicivirus (hepatitis E)

RCV = Reptilian calicivirus

GWCV = Grey whale calicivirus (VESV A48)

PCV = Primate calicivirus

ChCV = Cheetah calicivirus

CCV = Cetacean calicivirus

ROTA = Rotavirus

FCV = Feline calicivirus

TCV = Bovine (Tillamook) calicivirus

CaCV = Canine calicivirus

WCV = Walrus calicivirus

RETRO = A walrus retrovirus

MCV = Mink calicivirus

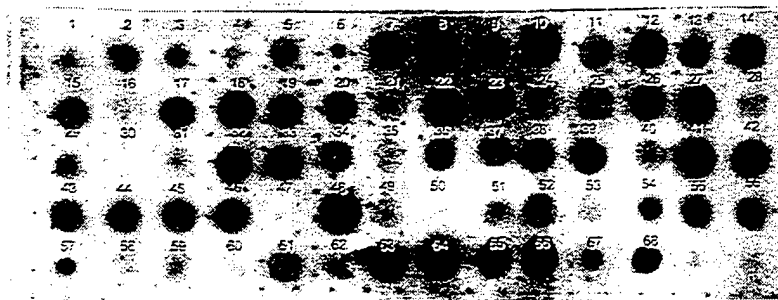
VERO = Uninfected vero cell controls

Table III. Summary of calicivirus cDNA probe analysis, \* monoclonal antibody reactions and direct electron microscopy of diverse samplings of ocean sources.

Sample and Source	cDNA Probe	Monoclonal Ab	EM
22 mussels	22/22	22/22	4/4**
Gut, gill and residual water from each mussel	62/66	63/66	4/4
19 Hawaiian monk seals***	13/19*	19/19	5/5**
Throat and rectal swabs and WBC buffy coat from each seal	22/57*	54/57	5/5
5 fish, shad mackerel*** and Hawaiian forktail	1/5*	3/5	
1 white tern chick (fluid from foot lesion)	1/1	--	1/1****

- \* cDNA probe analysis of seal and fish samples should be updated. These results were accumulated prior to improving the cDNA probe methods (compare with the results of mussel assays).
- \*\* Only a few samples were spot checked by direct EM.
- \*\*\* 19 Monk seals, 5 fish and the white tern chick with a blister on its foot were all sampled on French Frigate Shoals of the Hawaiian chain.
- \*\*\*\* The direct EM of vesicular fluid revealed many antibody coated caliciviruses and this could account for the negative Mab test.

Figure 5. cDNA probe analysis of *M. californianus* tissues (gill and gut) and residual water from 22 individual mussels using a chemoluminescent indicator



Lab #	Sample	Mussel #	Lab #	Sample	Mussel #	Lab#	Sample	Mussel #
1	water	Mc 12	26	gut	Mc 12	51	water	Mc 18
2	water	Mc 13	27	gut	Mc 14	52	water	Mc 10
3	water	Mc 14	28	gut	Mc 13	53	water	Mc 19
4	water	Mc 15	29	gills	Mc 12	54	water	Mc 4
5	water	Mc 16	30	gut	Mc 16	55	water	Mc 6
6	water	Mc 17	31	gills	Mc 14	56	water	Mc 8
7	gills	Mc 17	32	gut	Mc 15	57	water	Mc 3
8	gut	Mc 17	33	gills	Mc 13	58	water	Mc 5
9	gills	Mc 18	34	gills	Mc 15	59	water	Mc 7
10	gut	Mc 18	35	water	Mc 9	60	water	Mc 2
11	gills	Mc 19	36	water	Mc 20	61	water	Mc 22
12	gut	Mc 19	37	gills	Mc 4	62	water	Mc 1
13	gills	Mc 9	38	gills	Mc 1	63	gills	Mc 5
14	gut	Mc 9	39	gut	Mc 1	64	gut	Mc 5
15	gills	Mc 10	40	gills	Mc 8	65	gills	Mc 7
16	gills	Mc 11	41	gut	Mc 6	66	gut	Mc 7
17	gut	Mc 21	42	gut	Mc 2	67	calicivirus isolate	Mc 19
18	gut	Mc 22	43	gut	Mc 8		calicivirus	
19	gut	Mc 11	44	gut	Mc 3	68	Positive control	
20	gills	Mc 20	45	gills	Mc 3		PK cell	
21	gills	Mc 21	46	gut	Mc 4	69	Negative control	
22	gut	Mc 20	47	gills	Mc 6		Vero cell	
23	gills	Mc 10	48	gills	Mc 2	70	Negative control	
24	gills	Mc 22	49	water	Mc 11			
25	gills	Mc 16	50	water	Mc 21			

**Calicivirus Monoclonal Antibodies:** We have developed calicivirus specific and cross reactive monoclonal antibodies for the purposes of calicivirus diagnosis. The diagnostic use is to establish antigen trap types of assays to test for calicivirus shedding.

This is important for two reasons. First, we need to know if specific animals are shedding at specific times because of the importance of desciminating unwanted viruses via carrier animals and second, appropriate monoclonals provide a tool for evaluating the effects of virus shedding associated with stress. Monoclonal antibodies also provide probes to be used for identifying cross reactive and neutralizing epitopes. First, the monoclonal antibodies could be bound to protein fragments and these could be amplified using recombinant technology then used as antigen for serologic testing. The other use would be for identifying antigens that elicit a neutralizing antibody. Such antigens would then become prime candidates for vaccines for preventing caliciviral disease in exposed marine mammals.

At this juncture we have milligram quantities of purified IgG from 3 different monoclonals that recognize caliciviruses specifically. These have been used to detect calicivirus shedding fecal samples and a variety of tissue samples using immunoblot and other procedures.

**Diagnostic Immunoblot Tests for Calicivirus:** We have broadened our search for calicivirus test antigens by purifying viruses of ocean origin, absorbing them to nitrocellulose and passing various test sera over the preparations then testing for antibody binding.

In a series of studies these tests have proven very effective in detecting calicivirus antibody in sera diluted 1:250. The tests were positive for the 32 caliciviruses of known ocean origin with the exception of Tillamook calicivirus and cetacean calicivirus (see table 4). Surprisingly, a number of other sera also tested positive including feline calicivirus, canine calicivirus and several human caliciviruses. (Note--subsequent testing using additional typing sera and antigen preparations show that both Tillamook and cetacean calicivirus can be detected with these procedures.)

These are exciting data because they show the yet unexploited potential of developing screening tests for calicivirus diagnosis across broad species lines. In addition, they suggested a wide ranging array of common epitopes which offer promise not only for effective diagnostic tests but also for common antigens that may form the basis for protective vaccines.

Table 4  
Antigen Reactivity

<u>Antiserum</u>	<u>Serotype</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>
1	Normal rabbit	-	-	-	-	-	-
2	Normal rabbit	-	-	-	-	-	-
3	7420 Infected Pig	-	-	-	-	-	-
4	022 Primate	++	++	++	++	++	++
5	HCV Anti Sapporo	-	-	-	-	-	-
6	F-9 Antisera	+++	+++	+++	+++	+++	+++
7	Norwalk	-	-	-	-	-	-
8	NANB Hepatitis	-	-	-	-	-	-
9	7420 Walrus	+/-	+	+	+	+	+
10	Reptile	+++	+++	+++	+++	+++	+++
11	SMSV-1	+++	+++	+++	+++	+++	+++
12	SMSV-2	+++	+++	+++	+++	+++	+++
13	SMSV-4	+++	+++	+++	+++	+++	+++
14	SMSV-5	+++	+++	+++	+++	+++	+++
15	SMSV-6	+++	+++	+++	+++	+++	+++
16	SMSV-7	+++	+++	+++	+++	+++	+++
17	SMSV-8	+/-	+++	+	++	+	++
18	SMSV-9	+++	+++	+++	+++	+++	+++
19	SMSV-10	+++	+++	+++	+++	+++	+++
20	SMSV-11	+++	+++	+++	+++	+++	+++
21	SMSV-12	+/-	+	+	++	+	++
22	SMSV-13	+++	+++	+++	+++	+++	+++
23	SMSV-14	+++	+++	+++	+++	+++	+++
24	SMSV-15	+++	+++	+++	+++	+++	+++
25	VESV-A	+++	+++	+++	+++	+++	+++
26	VESV-B	+++	+++	+++	+++	+++	+++
27	VESV-C	+	++	++	++	++	++
28	VESV-D	+++	+++	+++	+++	+++	+++
29	VESV-E	+++	+++	+++	+++	+++	+++
30	VESV-F	+++	+++	+++	+++	+++	+++
31	VESV-G	+++	+++	+++	+++	+++	+++
32	VESV-H	+++	+++	+++	+++	+++	+++
33	VESV-I	+++	+++	+++	+++	+++	+++
34	VESV-J	++	++	++	++	++	+++
35	VESV-K	+++	+++	+++	+++	+++	+++
36	HCV UK3	+/-	+	+	+	++	+
37	HCV NBL	+++	+++	+++	+++	+++	+++
38	Anti NOW	-	-	-	-	-	-
39	UK I	+++	+++	+++	+++	+++	+++
40	Anti Chick	-	-	-	-	-	-
41	HCV UK2	-	+/-	+	+	+/-	-
42	HCV Cubbit	+	++	++	+++	++	+++
43	041 Dolphin	-	-	-	-	-	-
44	Porcine	-	-	-	-	-	-
45	Newburg	-	-	-	-	-	-
46	HCV-IAS	-	-	-	-	-	-
47	Tillamook	-	-	-	-	-	-
48	Canine	+	++	++	++	+	++

**Sea lion Herpes Virus, An Electron Microscopic Finding:** While investigating the possible viral etiology of an outbreak of gastroenteritis among the California Sea lions at the NOSC San Diego facility, an electron photomicrograph from a vomitus sample showed not only calicivirus but also a herpes virus.

Attempts to propagate the agent so that more definitive tests could be run to determine possible causal relationships between virus and Sea lion gastroenteritis have failed. This has occurred, at least in part, because calicivirus has been propagated from the same sample and because caliciviruses have such a rapid replicative cycle that the slower herpes virus cannot complete. To cope with this phenomena we have produced high affinity polyvalent antiserum specific for the calicivirus and have used this in attempts to neutralize the calicivirus in culture and thus allow the herpes virus to replicate. These efforts have failed presumably because the herpes virus replicative mechanisms were not compatible with the cell lines we used for propagation.

**First Isolation of Double-Stranded RNA Virus from Marine Mammals:** Northern Sea lions on Rogue Reef, Oregon and California sea lions and Northern fur seals on San Miguel Island, California, were sampled for virus shedding using rectal swabs. These samples were stored frozen, then examined for virus. A total of 35 reovirus-like agents were isolated from 12 individual Northern fur seals (*Callorhinus ursinus*), 2 California sea lions (*Zalophus californianus*) and 21 Steller sea lions (*Eumetopias jubatus*) sampled on 1985-87. Further, all San Miguel isolates but one from a fur seal and the two California sea lion isolates were from four-month-old pups from a single rookery where the haul out areas were densely populated and contained pools of stagnant sea water contaminated with urine and feces. Nearly all non-shedding animals, both fur seals and sea lions, were from dry sand beaches where oral-fecal transmission is much less likely to occur. In addition, fur seal pups from the infected rookery were measurably smaller than others.

This class of virus is a pathogen in domestic animals and humans where it can cause severe diarrhea resulting in permanent damage to the absorptive capacity of the gut as well as respiratory disease and occasional hepatoencephalitis. The finding of this new class of virus in the wild pinniped population is especially germane to the navy's marine mammal programs where working marine mammals may inadvertently contract the disease from wild populations and possibly animal handlers. Very preliminary serologic assays are underway to determine if this virus has spread between species to sea lions and *Tursiops* and to determine how widely it has spread through fur seals and other wild marine mammal populations. Using immunoelectron microscopy to react the sera (from six pinnipeds yielding virus isolates), against the virus isolated from one fur seal, antibody was detected in three animals, see Table 5. Those which reacted negatively could have done so for three reasons. First, there may be more than one antigenic virus type and, second, these seals may have been sampled too early in the infectious

process for detectable levels of circulating antibody to have been formed and, finally, the levels of virus antigen could have been such that these animals were running an antigen excess, thus binding all available circulating antibody.

RNA has been extracted from the virus and migration patterns show 10 segments as is seen in reovirus and arbovirus. Furthermore, these segments band and migrate much like, but not identical to, a human isolate of Reovirus type 3 which was concurrently examined by polyacrylamide gel electrophoresis.

Not only is this the first isolation of marine mammal virus of the Reoviridae classification but, once again, it points to a possible close linkage between viruses of marine mammals and man.

Table 5  
Immunoelectromicroscopy of Pinniped Reovirus-like Agent

<u>Sera*</u>	<u>Aggregation</u>	<u>Remarks</u>
C222R	-	<i>C. ursinus</i> -rectal swab-Castle Rock**
C233R	+	<i>C. ursinus</i> -rectal swab-Castle Rock
C225R****	+	<i>C. ursinus</i> -rectal swab-Castle Rock
C244	-	<i>C. ursinus</i> -rectal swab-Castle Rock
C489R	-	<i>C. ursinus</i> -rectal swab-Adams Cove***
C249R	+	<i>C. ursinus</i> -rectal swab-Castle Rock

\* All sera tested were from animals yielding isolates.

\*\* Castle Rock = Primary fur seal rookery just off the Northwest tip of San Miguel Island, California.

\*\*\* Adams Cove = Sandy beach on Southwest tip of San Miguel Island

\*\*\*\* C225R = *Callorhinus ursinus* tag number used on the animal yielding the prototype virus for this series of tests.

### **Reptilian Calicivirus Crotalus Type-1 (RCV Cro-1): A New Virus Isolate from**

**Marine Mammals**: Reptilian Calicivirus was first isolated in 1978 from adult and hatchling Aruba Island Rattlesnakes (*Crotalus unicolor*) in a zoologic collection. A total of sixteen isolates all proved to be a single new calicivirus serotype which was given the name Reptilian Calicivirus Crotalus Type-1 (RCV Cro-1). Subsequently, this virus was isolated from a rock rattlesnake (*Crotalus lepidus*), an Eyelash viper (*Bothrops schlegelii*) and from an amphibian species, the Bell's horned frog (*Ceratophrys ornata*), all housed within the confines of the zoologic garden. Caliciviruses recently isolated from a flipper versicle on a wild California sea lion (*Zalophus californianus*) and rectal swabs taken from Steller sea lion pups (*Eumetopias jubata*) and Northern fur seals, (*Callorhinus ursinus*) were serologically identified. On the basis of serum neutralization testing, isolates from all three species were indistinguishable from RCV Cro-1.

A total of 69 sera were collected from the 1-4 month old pups from three species of pinnipeds at two widely separated geographic locations. Twenty northern fur seal sera and 19 California sea lion sera collected on San Miguel Island, California were tested and 30 Steller sea lion sera collected some 500 miles further north on Rogue Reef, Oregon were similarly screened for neutralizing activity at a 1:10 final dilution of serum. All the Steller sera were positive at the screening dilution indicating neutralizing antibody levels of 1:10 or greater; whereas, five of 19 California sea lion sera were positive at a 1:10 endpoint and an additional nine were positive at 1:10 or >1:10. Only one of 20 *Callorhinus* sera showed any neutralizing activity at the screening dilution and that was at a 50 percent endpoint. (See Table 6).

These results are remarkable in three respects. First, those antigenic epitopes on the virus that elicit a neutralizing antibody response in exposed species have remained stable for eight years. Next, this calicivirus is now known to infect four poikilothermic terrestrial species and three marine mammal species under conditions of natural exposure. Experimentally, the virus elicits an antibody response in inoculated swine but has not caused overt disease. Finally, this is another in a series of calicivirus isolates that has somehow bridged the land-sea interface and has become established in both aquatic and terrestrial hosts.

Table 6  
Preliminary Screen for RCV Cro-1 Antibodies\*

<u>Species</u>	<u>Location</u>	<u>1:10</u>	<u>&gt;1:10</u>
Eumetopias ***	Rogue Reef	0/30	30/30
Callorhinus**	San Miguel Island	1/30	0/30
Zalophus**	San Miguel Island	5/19	9/19

\* Tests were against the prototype strain of RCV, not the strain isolated from *Zalophus* and *Callorhinus*.

\*\* These sera were from 3 month old pups sampled in 1986 and were among the same group from which RCV was isolated.

\*\*\* All sera were from pups approximately 1 month old.



**Epidemiology of Caliciviruses in the Pacific Basin:** Because caliciviruses are proven pathogens of cetaceans and various pinniped species, their geographic distribution becomes a concern should various working marine mammals be taken into these endemic areas. At this time it is generally thought that Atlantic species are naive with regard to calicivirus exposure whereas calicivirus distribution appears to be endemic throughout the North Pacific Ocean, Bering, Chukchi, Beaufort and Eastern Siberian Seas. These data can be readily demonstrated by examining the geographic distribution of ocean species known to have been infected with various virus types.

For example, Virus Type A<sub>48</sub> was originally isolated from swine in California in 1948 but was subsequently isolated from a California grey whale in 1968. This species breeds in the Sea of Cortez and annually migrates into the Chukchi Sea as well as the Sea of Okhotsk and the Sea of Japan. Thus, type A<sub>48</sub> is thought to be widely disseminated throughout the grey whale range.

Virus types J<sub>56</sub> and K<sub>56</sub> were isolated from swine in New Jersey. Subsequently, California grey whales and California Sea lions were shown to carry antibodies to this virus type, thus J and K are thought to be disseminated through the geographic ranges of these species. Furthermore, Bowhead whales whose ranges are confined to the margins of the pack ice in the Bering, Chukchi, and Beaufort Seas also carry antibodies to J and K.

The Reptilian calicivirus was first isolated from a zoologic collection in San Diego and subsequently isolated from both California Sea lions and Steller sea lions. Thus, the geographic distribution of this agent is assumed to be from Southern California up the Western pacific Coast into the Bering Sea and throughout the Aleutian chain to the Russian Coast.

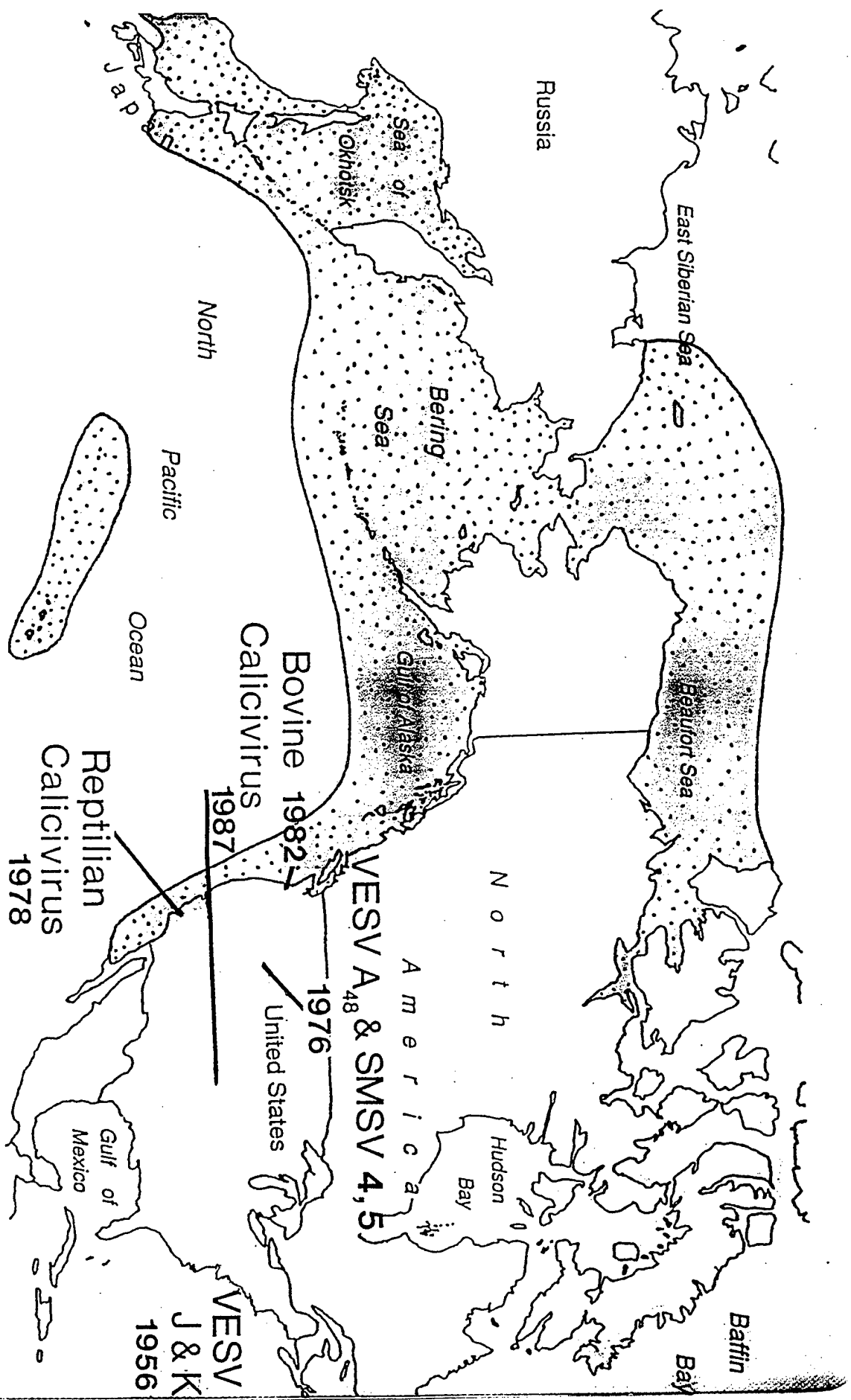
The SMSV-4 virus was isolated from California Sea lion in 1973 and appears to be confined primarily to California sea lions. Thus, the distribution ranges from the pacific shore of Baja up the US coast to Vancouver Island, BC.

The SMSV-5 virus was isolated from Northern fur seals in the Bering Sea in 1973 and has shown great plasticity in host range, thus it is assumed to be spread throughout the Pacific Basin and the adjacent coastal zone.

In 1976, the SMSV-7 virus was isolated from a Southern California fish, California Sea lion liver fluke, and a Northern Elephant seal and in 1985, from a Steller sea lion. Northern elephant seals range from Baja up the Oregon Coast and westward onto the Hawaiian leeward islands. Thus SMSV-7 is thought to be distributed throughout the Pacific Basin and the Bering Sea.

Limited tests among terrestrial species has also verified the presence of caliciviruses of

# Composite of Calicivirus Spread



ocean origin and antibodies to these. It, therefore, appears that these agents can spread from ocean to land and because some of them are classed as Foreign Animal Disease Agents it is incumbent upon responsible agencies to guard against the spread of these viruses while moving or maintaining working marine mammals in coastal or inland sites.

Finally, an overlay of all the areas of distribution for calciviruses show that they are widespread throughout the entire Pacific Basin and contiguous waters.

#### **Zoonotic Ocean Viruses Infecting both Marine Mammals and Humans:**

Caliciviruses of ocean origin which cause disease in animals can also cause clinical disease in humans. In addition, caliciviruses of unknown, but possible ocean origin cause hepatitis E and gastroenteritis in humans. We have examined only ten sera from women experiencing problems of repeated spontaneous abortion, but all ten showed moderate to high levels of calicivirus antibodies when compared to a control group of individuals known to have had calicivirus gastroenteritis (Tables 7 & 8). These preliminary data suggest that caliciviruses, which are known potent abortogenic agents of animal, could be a cause of undiagnosed spontaneous abortion in women.

Evidence exists which demonstrates the convergence of pathogenic animal caliciviruses that are maintained in ocean fish with the caliciviruses causing human gastroenteritis which are also contained in seafoods, especially raw or lightly steamed shellfish. For the past two decades, and independent of the animal calicivirus work cited above, information has been building on what was a newly discovered viral agent of human diarrhea and vomiting called Norwalk virus. Over the years, other viruses causing similar disease and sharing this same morphology were catalogued and given names such as Small round structured viruses (SRSV), Snow Mountain agent, Ditchling agent, Hawaii agent, Sapporo calicivirus, human calicivirus, United Kingdom 1-4 (UK 1-4), Minireovirus, Desert Shield virus, Southampton virus, and Toronto virus. All of these were generally lumped together as Norwalk or Norwalk-like viruses, none of them could be cultivated *in vitro*, and they were an antigenically diverse group. In 1991, all were tentatively classified as caliciviruses. As a group, they are highly transmissible through fecal contamination of food or water and often cause explosive epidemics of diarrhea and vomiting. Consumption of lightly steamed or raw shellfish has been a frequent source of Norwalk-like calicivirus food borne gastroenteritis, yet the shellfish beds implicated have not always been shown to be contaminated with human fecal waste. Oliver, publishing from the Collaborating Center for Food Virology on behalf of the World Health Organization, cites Norwalk virus as fifth in causes of food borne disease during the period of 1983-1987 and molluscs as the most common vehicle. He further states there are a lack of applicable and sensitive detection methods for monitoring foods.

One reason for the lack of sensitive methods for testing seafoods for calicivirus contamination has been the failure by researchers in this area to appreciate the diversity of human calicivirus antigenic types that were for many years simply lumped together as Norwalk viruses or human caliciviruses. Now a flurry of activity using molecular probes based on Norwalk sequencing and Norwalk capsid protein has shown that there is great diversity in the caliciviruses causing diarrhea and vomiting in humans. Although a few instances of true "Norwalk" disease do occur, very often the molecular probes available which are "Norwalk" specific, fail in part or altogether when used to identify caliciviral agents causing gastroenteritis including those from molluscan shellfish. These results have caused scientists at the Centers for Disease Control to point out the need for additional and improved group reactive probes for caliciviruses. This diversity of calicivirus types has been well documented in animal work where repeat infections routinely occur because of the lack of cross protection between calicivirus types, and clearly shows a convergence of our knowledge of the pathogenic caliciviruses infecting humans and those infecting animals.

# Human Antibody to Calicivirus Antigen

Table 7. Preliminary Test--Repeat spontaneous abortion--human sera tested against various calicivirus antigens using an immunoblot technique.

<u>Sample</u>	<u>Dil</u>	<u>Species</u>	<u>Antigen Reactivity Pool</u>					
			<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>
OHS-1	1:5	Human	++	+	+++	+++	+++	+++
OHS-2	1:5	Human	++	+	+++	+++	+++	+++
OHS-3	1:5	Human	+	+++	+	+	+/-	++
OHS-4	1:5	Human	+/-	+	+	+	+/-	+
OHS-5	1:5	Human	+/-	+/-	+/-	+	+	+
OHS-6	1:5	Human	+	+	+	+	+	+
OHS-7	1:5	Human	+	+	+	+	+	+
OHS-8	1:5	Human	+++	++	+++	+++	+++	+++
OHS-9	1:5	Human	++	+/-	++	+++	+	+++
OHS-10	1:5	Human	+++	++	+++	+++	+++	+++

Table 8. Preliminary Tests--Human calicivirus diarrhea and NANB hepatitis sera tested against various calicivirus antigen preparations using an immunoblot technique.

<u>Sample</u>	<u>Dil</u>	<u>Species</u>	<u>Antigen Reactivity Pool</u>					
			<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>
21721	1:10	Human	+	+	+	+	+/-	+
1209	1:10	Human	+	+	+	+	+	+
Chiba B	1:10	Human	+/-	+/-	+/-	+	+/-	+
20879	1:10	Human	+/-	+/-	+/-	+	+/-	+
20878	1:10	Human	++	+/-	++	++	+	++
1204	1:10	Human	++	+/-	++	+++	+	++
1506	1:10	Human	+/-	+/-	+/-	+	+	+
2018	1:10	Human	+	+	+	++	+	++
1680	1:10	Human	+/-	+/-	+/-	+/-	+/-	+/-
1206	1:10	Human	+/-	+/-	+/-	+/-	+/-	+/-
21720	1:10	Human	++	+/-	++	++	+	++
23141	1:10	Human	+	+/-	+	++	++	++
1908	1:10	Human	+	+/-	+	++	+	++
22155	1:10	Human	+	+/-	++	++	++	++
2459	1:10	Human	-	-	+/-	+/-	+/-	+/-
Chiba A	1:10	Human	-	-	+/-	+/-	-	+/-
Chiba F	1:10	Human	-	-	+/-	+/-	-	+/-
Chiba E	1:10	Human	+/-	-	+/-	+/-	+/-	+/-
Chiba C	1:10	Human	-	-	+/-	+/-	-	+/-
Chiba D	1:10	Human	-	-	+/-	+/-	-	+/-
NANBH	1:10	Human	+/-	+/-	+/-	+	+	+

Calicivirus Antigen Pools (whole virus)

A contains SMSV-1, SMSV-2, SMSV-3, SMSV-4

B contains SMSV-5, SMSV-6, SMSV-7, SMSV-8

C contains SMSV-9, SMSV-10, SMSV-11, SMSV-12

D contains SMSV-13, SMSV-14, SMSV-15, SMSV-16

E contains Cetacean, Bovine, Reptile and Walrus

F contains all of the above of the 20 serotypes

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# CALICIVIRUSES OF OCEAN ORIGIN: A REVIEW

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## CALICIVIRUSES OF OCEAN ORIGIN: A REVIEW

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**Abstract:** A disease of caliciviral etiology was first described in swine in 1932. The virus was host specific and naturally infected only swine. Later, a calicivirus of cats was discovered, and again, it was thought to be host specific. In 1972, caliciviruses were the first viruses to be isolated from pinnipeds (California sea lions [*Zalophus californianus*]). Shortly thereafter, additional calicivirus types were isolated from northern fur seals (*Callorhinus ursinus*) and other marine species. During this time, a complex picture of antigenic diversity and host nonspecificity emerged for the marine caliciviruses. These agents infected and produced disease in marine mammals as well as terrestrial animals. The viruses causing the devastating epidemics of caliciviral disease in swine, which occurred subsequent to 1932, were eventually shown to be present in the ocean. The caliciviruses of ocean origin are known to naturally infect at least one ocean fish (opaleye fish [*Girella nigricans*]), 12 species of marine mammals, and 18 species of terrestrial animals, including humans.

**Key words:** Pinnipeds, caliciviruses, cetacean, viruses, marine mammals.

### INTRODUCTION

Caliciviruses have a distinctive morphology characterized by 22 calyces or cup-like depressions on their surfaces. These depressions are arranged to give a 2-, 3-, or 5-fold axis pattern, depending upon virion orientation at time of fixing (Fig. 1). These viruses differ sufficiently from other small, round, nonenveloped single stranded RNA viruses to be placed in a separate family, the Caliciviridae. They are 36 nm in diameter and have a molecular weight of  $14 \times 10^6$  daltons, a buoyant density in cesium chloride of about 1.38, and a sedimentation coefficient of 183 S in a sucrose gradient.<sup>17,48,55,103</sup>

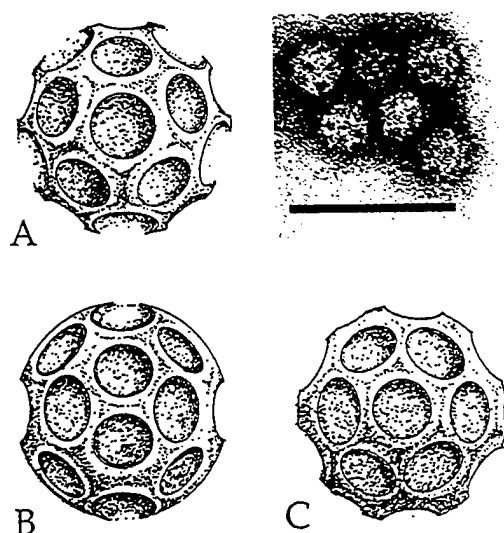
Marine caliciviruses resist in vitro propagation on initial isolation, often requiring three to six blind passages, but once adapted to cell culture, frequently replicate to  $10^9$  tissue culture infective particles per ml of stock culture. Marine mammal cell lines have been uniformly disappointing for calicivirus isolation and propagation, but primate cell lines (Vero, African green monkey

[*Cercopithecus aethiops*] kidney) and pig kidney (PK-15) cells have been especially sensitive for this purpose.<sup>11</sup> Members of the Caliciviridae remain viable in buffered solutions at 4°C for indefinite periods and survive in seawater at 15°C for more than 14 days.<sup>48,55,90,103</sup>

Virus types presumed to belong to the Caliciviridae have, for the purposes of this paper, been divided into three groups (Tables 1-3). The first is a group of caliciviruses or caliciviruslike viruses that are generally associated with enteric diseases and can be propagated in vitro either with great difficulty or not at all (Table 1). Members of this group are the porcine enteric virus, Newbury calf virus, the chicken calicivirus, and the human pathogens, which include the Norwalk agent, human enteric calicivirus, and the enterically transmitted non-A, non-B hepatitis virus.<sup>19,20,28,56</sup>

The second group is made up of those caliciviruses isolated from terrestrial animals. The presence of the viruses in the ocean has neither been observed nor sought (Table 2). This group includes the feline caliciviruses, and there are multiple antigenic variants of these but they all fall within a single serotype.<sup>37,50</sup> Feline caliciviruses sometimes infect dogs,<sup>31</sup> and there are ca-

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**Figure 1.** Schematic drawings showing calicivirus surface structure and 5-, 2-, and 3-symmetry in A, B, and C, respectively. Inset (upper right) is an electron photomicrograph of cetacean calicivirus. Bar = 100 nm.

nine caliciviruses that have been isolated only from dogs.<sup>26</sup> The last virus included in this group is the primate calicivirus.<sup>81,83,88</sup>

The third group, the caliciviruses believed to be of ocean origin, are the focus of this review (Table 3). This group includes at least 34 distinct serotypes. Each separate serotype is defined as an antigenic variant that is not neutralized by antiserum containing 20× the antibody concentration needed to neutralize the homologous virus type.

We will discuss the isolation, host spectrum, geographic distribution, and disease potential for this third group of caliciviruses. These agents are uniquely of ocean ori-

gin, are highly unusual in their host species versatility, carry broad tissue tropisms, move great distances in the environment, and are the first known viral zoonosis of ocean origin.

### EARLY HISTORY

We will spend considerable time discussing the introduction of the marine caliciviruses into swine and the character of the disease these viruses produce in swine. The purpose is to gain additional insights into the effects of these viruses on pinnipeds, cetaceans, and the other species in which detailed studies have not been possible. We preface these remarks by cautioning that interspecies extrapolations can be risky and misleading, but the available information on marine calicivirus infection in marine mammals closely parallels what has been observed and reported in swine.

The first caliciviruses were detected in swine and were called vesicular exanthema of swine virus (VESV). Several detailed accounts of the history of VESV in swine exist.<sup>2,29,96</sup> In retrospect, the first outbreak of VESV probably occurred on 22 April 1932 in Orange County, California, on a farm where raw garbage was fed to swine. This disease outbreak was characterized by vesicular disease of low mortality, high morbidity, and lameness, and was assumed to be foot-and-mouth disease (FMD). The affected swine and adjacent exposed livestock of all species were quarantined, slaughtered, and disposed of by deep burial. Each farm was thoroughly cleaned and disinfected in accordance with accepted FMD eradication

**Table 1.** Caliciviruses causing enteric disease.\*

Serotype	Species	Location	Year	Reference
Norwalk virus	Human	United States	1972	28
Human calicivirus (HCV)	Human	United Kingdom	1976	28
Newbury agent of cattle	Cattle	United Kingdom	1978	28, 101
Porcine enteric calicivirus	Swine	United States, United Kingdom	1980	28, 22
Chicken calicivirus (CCV)	Chicken	United Kingdom	1981	28, 102
Non-A, non-B hepatitis virus	Human	Asia, Africa, Mexico	1981-1986	20

\* Without exception, these agents are propagated in vitro either with great difficulty or not at all.

**Table 2.** Caliciviruses isolated from terrestrial species and not yet known to have an ocean presence.

Serotype	Species	Location	Year	Reference
Feline calicivirus (FCV)	Cat	United States	1957	32
		Australia		
Primate calicivirus (PCV Pan-1)*	Dog	United States	1981	31
	Pygmy chimpanzee ( <i>Pan paniscus</i> )	San Diego, California, USA	1978	88
	Spider monkey ( <i>Ateles fuscipes</i> )	San Diego, California, USA	1978	83
	Lowland gorilla ( <i>Gorilla gorilla</i> )	San Diego, California, USA	1978	83
	Silverleaf langur ( <i>Presbytis cristata</i> )	San Diego, California, USA	1979	83
	Douc langur ( <i>Pygathrix nemaeus</i> )	San Diego, California, USA	1979	81
Canine calicivirus (CaCV)	Dog	United States	1982	26

\* The primate calicivirus is suspected but not proven to be of ocean origin.

guidelines. No infected material for diagnostic confirmation was saved because of the FMD eradication procedures. Ten days after the outbreak started, the disease was believed to have been eradicated, and all infectious material was destroyed.

Eleven months later, a second outbreak of vesicular disease in pigs occurred in San Diego County, California, on another farm where raw garbage was fed to swine. During this outbreak, lesion material was examined, and FMD virus was not identified. All eradication procedures employed previously were again instituted. A third outbreak occurred 15 mo later 500 mi north of San Diego. This time, the disease was not FMD but was instead a new disease, vesicular exanthema of swine (VES). With this diagnosis, the Bureau of Animal Industry of the U.S. Department of Agriculture suspended its eradication efforts and support in California. State quarantines were imposed, and affected animals were allowed to recover before going to slaughter. Embargoes were placed on shipping raw pork out of California.

Epidemiological investigations failed to uncover any identifiable physical links among the first three outbreaks. Meat, meat scraps, and other infective material from

swine did not appear to play a role in the first three outbreaks, and the original sources of infection remained a mystery. After the disease was determined to be VES and not FMD, infected swine tissues became a probable means of transmission because clinically recovered pigs were allowed to be slaughtered and processed for food.

Subsequent outbreaks occurred in 1935 and 1936, followed by a 3-yr period when no disease was detected. However, in 1939, VES erupted in northern California swine herds, and within 6 mo, one-quarter of the herds in the state were infected. For the next 16 yr, the disease continued unabated. Quarantine did not provide effective control.

Garbage feeding was recognized as an important factor in the transmission of VES. All of the 1932, 1933, and 1934 outbreaks occurred on farms feeding raw garbage, and after 1939, there was a much higher incidence of VES on these farms compared with operations feeding grain. Regulations were drafted requiring that garbage be cooked before feeding it to swine. The swine industry resisted, the regulations were not enforced, and the practice of feeding raw garbage continued.

In 1952, the first case of VES was reported



Table 3. Calicivirus isolates occurring in the ocean.

Serotype	Source	Location	Year	Refer- ence
VESV 1-34	Swine	San Jose, California, USA	1934	27
VESV 101-43	Swine	San Francisco, California, USA	1943	23
VESV A <sub>48</sub>	Swine	Fontana, California, USA	1948	44
VESV B <sub>51</sub>	Swine	Davis, California, USA	1951	8
VESV C <sub>32</sub>	Swine	San Francisco, California, USA	1952	8
VESV D <sub>53</sub>	Swine	Riverside, California, USA	1953	5
VESV E <sub>54</sub>	Swine	Warm Springs, California, USA	1954	6
VESV F <sub>55</sub>	Swine	San Mateo, California, USA	1955	7
VESV G <sub>55</sub>	Swine	San Mateo, California, USA	1955	7
VESV H <sub>54</sub>	Swine	San Mateo, California, USA	1954	4
VESV I <sub>55</sub>	Swine	San Mateo, California, USA	1955	4
VESV J <sub>56</sub>	Swine	Secaucus, New Jersey, USA	1956	40
VESV K <sub>56</sub>	Swine	Secaucus, New Jersey, USA	1956	40
SMSV-1	California sea lion ( <i>Zalophus californianus</i> )	San Miguel Is., California, USA	1972	65, 75
	Northern fur seal ( <i>Callorhinus ursinus</i> )	St. Paul Is., Alaska, USA	1972	75
SMSV-2	California sea lion	San Miguel Is., California, USA	1972	75
SMSV-3	California sea lion	San Miguel Is., California, USA	1972	65, 75
SMSV-4	California sea lion	San Miguel Is., California, USA	1973	76
	Swine	Sonoma County, California, USA	1976	77
SMSV-5	Northern fur seal	St. Paul Is., Alaska, USA	1973	76
	Animal feed	St. Paul Is., Alaska, USA	1974	54
	Human	Corvallis, Oregon, USA	1985	61
SMSV-6	California sea lion	San Miguel Is., California, USA	1975	64, 92
	Northern fur seal	San Miguel Is., California, USA	1977	87, 92
	Opaleye fish ( <i>Girella nigricans</i> )	San Nicolas Is., California, USA	1976	87
	Stellar sea lion ( <i>Eumetopias jubatus</i> )	Rogue Reef, Oregon, USA	1985	57
SMSV-7	Opaleye fish	San Nicolas Is., California, USA	1976	87, 92
	Northern elephant seal ( <i>Mirounga angustirostris</i> )	San Nicolas Is., California, USA	1976	87, 92
	Sea lion liver fluke ( <i>Zalophatrema</i> sp.)	San Diego, California, USA	1976	87, 92
SMSV-8	Northern fur seal	St. Paul Is., Alaska, USA	1975	89
SMSV-9	California sea lion	San Miguel Is., California, USA	1975	89, 92
	Pacific dolphin ( <i>Tursiops gillii</i> )	Honolulu, Hawaii, USA	1987	68
SMSV-10	Northern fur seal	St. Paul Is., Alaska, USA	1977	89
SMSV-11	Northern fur seal	San Miguel Is., California, USA	1977	89
SMSV-12	California sea lion	San Miguel Is., California, USA	1977	89
	Northern fur seal	San Miguel Is., California, USA	1977	89
SMSV-13	California sea lion	Fort Cronkhite, California, USA	1984	16
SMSV-14	Stellar sea lion	Rogue Reef, Oregon, USA	1987	68
	California sea lion	San Miguel Is., California, USA	1987	68
SMSV-15	California sea lion	San Miguel Is., California, USA	1988	68
SMSV-16	California sea lion	San Diego, California, USA	1988	68
Walrus (WCV)	Walrus ( <i>Odobenus rosmarus</i> )	Chukchi Sea	1977	78
	Walrus	Bering Sea	1987	68
Cetacean (CCV Tur-1)	Atlantic dolphin ( <i>Tursiops truncatus</i> )	San Diego, California, USA	1979	91
	California sea lion	San Diego, California, USA	1979	91

Table 3. Continued.

Serotype	Source	Location	Year	Reference
Reptile (RCV Cro-1)	Aruba Island rattlesnake ( <i>Crotalus unicolor</i> )	San Diego, California, USA	1978	67
	Rock rattlesnake ( <i>C. lepidus</i> )	San Diego, California, USA	1978	67
	Eyelash viper ( <i>Bothrops schlegelii</i> )	San Diego, California, USA	1978	67
	Bell's horned frog ( <i>Ceratophrys orata</i> )	San Diego, California, USA	1978	67
	California sea lion	San Miguel Is., California, USA	1986	68
	Northern fur seal	San Miguel Is., California, USA	1986	68
	Stellar sea lion	Rogue Reef, Oregon, USA	1987	68
	Mink ( <i>Mustela vison</i> )	Idaho, USA	1977	31, 42
Bovine (BCV Bos-1)	Cattle	Cloverdale, Oregon, USA	1981	74

outside California. The infection was traced to a herd in Cheyenne, Wyoming, where pigs had been fed raw garbage from a trans-continental train originating in San Francisco, California. The disease spread rapidly throughout the U.S.; 42 states became involved, and the Bureau of Animal Industry declared a national state of emergency. Thus, the federal government embarked upon an eradication effort based on FMD procedures and began enforcing laws that required the cooking of raw garbage used for swine food. By 1953, 46 states were enforcing garbage-cooking laws, the disease was contained, and the last reported outbreaks occurred in New Jersey in 1956. On 22 October 1959, the U.S. Secretary of Agriculture announced that VES had been eradicated and, at that time, designated it a reportable Foreign Animal Disease (FAD).<sup>2,29,96</sup> The direct cost of eradication was set at \$39,000,000.<sup>11</sup>

Through the entire history of VES, the disease was found in only two places outside the continental U.S. Outbreaks occurred in shipments of hogs originating from the U.S. mainland and received at the port of Honolulu, Hawaii, in 1946 and 1947. Another incidence occurred in 1955 on a swine farm in Hrafnarfjord, Iceland, where raw garbage from a nearby U.S. military base had been fed to swine.<sup>2</sup>

During the relatively short course of VES in swine, 13 antigenic types were identified by cross-immunity animal inoculation and virus neutralization testing. Four antigenically distinct types were collected during the outbreaks of 1933 and 1934 alone.<sup>27</sup> These four serotypes were lost, and their relationship to the 13 serotypes preserved in repositories is not known.<sup>2</sup> Based on the typing of hundreds of isolates, it is believed that, with the exception of VESV types J and K found in New Jersey, VESV B<sub>51</sub> was the only serotype involved in outbreaks outside of California.<sup>30</sup> Bankowski<sup>2</sup> suggested that mutation or genetic instability could explain the emergence of the large number of VESV serotypes found in California. Supporting his hypothesis was the finding that new serotypes seemingly arose "de novo" in a recurrently infected herd despite attempts to thoroughly cook the garbage being fed. Likewise, it was believed that the appearance of VESV J<sub>56</sub> and VESV K<sub>56</sub> types in New Jersey was due to a rapidly mutating VESV B<sub>51</sub> serotype.<sup>7,30</sup> Subsequent data have challenged this seemingly well thought out and well supported hypothesis.<sup>63</sup>

Vesicular exanthema of swine was characterized as a disease of variable morbidity, usually 30–80%, and low mortality, especially in older animals.<sup>3,29</sup> Clinical disease in swine was variable and could be pro-



louse of swine, was shown to be infective but may have been only a mechanical vector.<sup>3</sup> Transmission through intact hair follicles was attempted but not achieved.<sup>35</sup>

Subclinical infections of VES have been demonstrated in swine. These infections may be the source of repeated outbreaks of VES involving single virus serotypes among the replacement swine on individual farms where only cooked garbage was fed.<sup>6,46</sup>

The vesicular exanthema of swine viruses, the numbered series of San Miguel sea lion viruses (SMSV), and several other caliciviruses are indistinguishable from each other, differing only with respect to the time and species of original isolation. Serotyping of all the marine caliciviruses is based on the serum neutralization test. New serotypes are defined when 20 antibody units of known hyperimmune serum fail to neutralize 100 infective doses of the challenge virus.<sup>59,62</sup> The first isolations of SMSV were made in 1972; additional serotypes are presently being isolated and characterized (Table 3). The first isolates of SMSV occurred during an investigation into the causes of reproductive failure in pinnipeds. Over a 2-yr period, this investigation yielded four serotypes that were indistinguishable from the VESV's on the basis of physiochemical and morphological properties and animal infectivity profiles, including the ability of the virus to produce VES in exposed swine.<sup>39,65,75,76</sup>

#### VIRUS ISOLATES FROM THE OCEAN

San Miguel sea lion virus type one (SMSV-1) was recovered in 1972 from rectal swabs taken from two California sea lions (*Zalophus californianus*) that had recently aborted on San Miguel Island, California. This serotype was also recovered from the nose of an emaciated northern fur seal pup (*Calorhinus ursinus*) on St. Paul Island, Alaska, in the same year. A second serotype, SMSV-2, was also isolated in 1972 from the throat and rectum of a California sea lion aborting 30–60 days before term on San Miguel Is-

land. A third serotype, SMSV-3, was recovered in the same year from the nose of the fetus aborted by one of the female sea lions yielding the prototype isolate of SMSV-1. SMSV-3 cross-reacted with antiserum to the first two SMSV types and was thought, therefore, to be a mixed infection of SMSV-1 and SMSV-2.

The SMSV-4 and SMSV-5 serotypes have been recovered from several different species. The SMSV-4 type was first isolated in 1973 from the throats of two aborted California sea lion fetuses on San Miguel Island.<sup>76</sup> This serotype was isolated again in 1976 from throat and rectal swabs of three garbage-fed swine from a *Brucella*-infected herd in Sonoma County, California, where many swine exhibited lameness of unknown cause.<sup>58,77,82</sup> SMSV-5 was initially recovered from vesicular lesions on the un haired region on the flipper of a northern fur seal in 1973 on St. Paul Island, Alaska.<sup>76</sup> This same serotype was also isolated from mink food consisting of coarsely ground northern fur seal carcasses processed during the 1974 annual fur seal harvest in Alaska.<sup>54</sup> In addition, SMSV-5 was isolated from blisters on the hands and feet of a human experiencing flulike symptoms in 1985. In this case, infection was thought to have occurred from accidental exposure of a laboratory researcher working with the SMSV's.<sup>61</sup>

The SMSV-6 serotype was initially recovered from small vesicular lesions on the un haired portion of the flippers of two California sea lion pups on San Miguel Island in 1975. A throat swab of a northern fur seal sampled on San Miguel Island in 1977 also yielded SMSV-6.<sup>64,87,92</sup> This same serotype was recovered from the spleen of an opaleye fish (*Girella nigricans*) collected from a tidal pool on San Nicolas Island, California, in 1976<sup>87</sup> and in 1985 from a rectal swab of a Stellar sea lion pup (*Eumetopias jubatus*) on Rogue Reef, Oregon.<sup>57</sup>

The SMSV-7 serotype was isolated in 1976 from throat and rectal swabs of four clinically normal northern elephant seal pups (*Mirounga angustirostris*) on San Miguel Is-



bovine calicivirus (BCV Bos-1), was isolated in 1981 from dairy calves in a Tillamook County, Oregon, dairy herd with a history of persistent respiratory disease among the calves. The virus was first isolated from rectal and throat swabs from three calves and then reisolated 3 wk later from one calf. Although the virus was found in calves with respiratory disease, the causal relationship between virus recovery and calf pneumonia was unclear. This agent has been isolated only from cattle, but type-specific antibodies to it are widely distributed in marine mammals.<sup>74</sup>

The final virus in this group, the primate calicivirus (PCV Pan-1), was isolated from five species of primates (pygmy chimpanzee [*Pan paniscus*], lowland gorilla [*Gorilla gorilla*], silverleaf langur [*Presbytis cristata*], spider monkey [*Ateles fusciceps*], and douc langur [*Pygathrix nemaeus*]) at the San Diego Zoo from 1978 to 1980. The presence of this virus has only been studied in primates, and although an ocean presence is suspected, this possibility has not been investigated.<sup>81,83,88</sup>

These case histories provide strong evidence that natural infections with SMSV's and other marine caliciviruses result in vesicular lesions among California sea lions, northern fur seals, dolphins, and humans. In 1978 during the annual harvest of northern fur seals on the Pribilof Islands, Alaska, an attempt was made to quantitate the number of animals exhibiting vesicles. Of 25,000 seals examined, 0.1% had visible lesions.<sup>34</sup> From an earlier study done in 1974, it was estimated that 2% of the fur seals killed during harvest had vesicular lesions.<sup>54</sup> Lesions from natural infection in marine mammals were characterized as being up to 3 cm in diameter, filled with clear fluid or pus, and located on both the dorsal and ventral surface of the flippers. The vesicles usually ruptured, leaving an area of eroded epithelium. Regression of vesicles without erosion was observed in some cases.<sup>62</sup>

Other clinical syndromes associated with natural infection include abortion and diar-

rhoea in California sea lions, hemorrhagic pneumonia in mink, and persistent respiratory disease in calves. In other cases of natural infection, SMSV's have been isolated from clinically normal animals. This may represent clinically recovered animals, asymptomatic persistently infected animals, or animals experiencing self-limiting subclinical infection. It is noteworthy that several SMSV serotypes have been isolated from aborting sea lions and aborted fetuses and that the SMSV's are indistinguishable from the VESV's, which were frequently associated with abortion in swine. The causal relationship between SMSV infection and abortion in marine mammals remains highly probable but unproven.<sup>11,75,76</sup>

Experimental infectivity studies with the SMSV's and other marine caliciviruses have yielded insights into susceptible hosts and resultant diseases produced by these agents. With respect to marine mammals, northern fur seals and harp seals (*Phoca groenlandica*) have been experimentally inoculated with SMSV-2. Resultant disease ranged from small-plaque lesions at intradermal inoculation sites to inapparent infection.<sup>34,36</sup>

Swine have been inoculated experimentally with several marine calicivirus serotypes. Intradermal exposure to SMSV-2 has produced mild vesicular disease, and SMSV-2 has been recovered from swine brain tissue, indicating that this serotype may cause encephalitis.<sup>35,59</sup> Swine fed coarsely ground whole carcasses from seals exhibiting SMSV-5-induced lesions developed a severe vesicular disease; virus was isolated from both blood samples and rectal swabs. Clinical disease was observed 2 days after consumption of contaminated meat and was characterized by severe depression, loss of appetite, lameness, a fever of 41°C, diarrhea, and loss of weight in addition to the formation of vesicles on the feet and snout.<sup>99</sup> Calicivirus morphology was compared before and after transmission from pinnipeds to swine, and no changes were noted.<sup>19</sup> Swine inoculated experimentally with SMSV-7 developed severe clinical ve-

sicular exanthema, with the formation of secondary vesicular lesions. Horizontal spread to animals by pen contact also occurred.<sup>87</sup> Swine infectivity studies with several SMSV serotypes demonstrated that some types are hepatotropic and others are not. Walrus calicivirus, for instance, from intradermal, oral, and pen-contact exposure in swine produced marked hepatocellular degeneration, whereas SMSV-13, a virus highly vesiculogenic in swine, caused little or no histocellular degeneration. Pneumonitis and enteritis have been observed in experimentally infected pigs; however, a causal relationship has not been established.<sup>68,71</sup> Swine inoculated with bovine calicivirus developed lesions only at injection sites; however, extension of these lesions occurred, producing blisters indistinguishable from those caused by VESV. Even in the absence of extensive vesicular disease, this BCV serotype produced a more serious disease in swine than did some of the VESV serotypes.<sup>74</sup> Experimental inoculation of swine with the reptile calicivirus serotype failed to produce visible lesions; however, seroconversion occurred in all subjects, including pen-contact animals.<sup>67</sup>

Calves have been inoculated experimentally with SMSV-5, SMSV-13, and BCV. The SMSV-5 serotype failed to produce visible lesions; however, SMSV-13 produced severe lesions at the site of intradermal inoculation. By the fourth day postinoculation, secondary vesicular lesions developed, and disease spread to a pen-contact calf, producing large vesicles on the feet.<sup>61</sup> Cattle inoculated with BCV developed lesions at intradermal injection sites without secondary vesicle formation. In a comparative disease study, this serotype was shown to produce less severe disease in cattle than in swine.<sup>74</sup>

Experimental inoculation of horses with SMSV types 1-5 caused erosive tracts at the sites of intradermal inoculation. A temperature rise was detected; however, none of the lesions spread by extension.<sup>100</sup>

Mink fed ground carcasses of fur seals

exhibiting SMSV-5-induced lesions developed an inapparent infection, and virus was isolated from blood samples and rectal swabs.<sup>99</sup> Mink kits inoculated with MCV did not develop clinical disease. Even so, MCV may predispose mink to severe cases of hemorrhagic pneumonia, as this virus type has been isolated during outbreaks of such disease.<sup>42</sup>

The African green monkey has been exposed experimentally to SMSV-4, SMSV-5, and VESV C<sub>54</sub>. All three virus serotypes produced lesions at intradermal injection sites, and these lesions spread by extension. A rise in temperature occurred within 72 hr. The lesions regressed and were essentially healed by the seventh day postinoculation.<sup>77</sup>

Two adult rattlesnakes have been experimentally inoculated with reptile calicivirus. Both snakes failed to develop recognizable calicivirus-induced lesions; however, virus was recovered at necropsy many weeks later.<sup>67</sup>

Opaleye fish held in a saltwater aquarium at a temperature of 15°C were inoculated experimentally with SMSV-5 by the oral and intraperitoneal routes. Generalized infection resulted. The virus replicated up to 10<sup>7</sup> infective particles per g of spleen, and the fish remained infected for at least 31 days. Despite active infection, clinical disease in the fish was not noted.<sup>90</sup>

Testing for susceptibility of common laboratory animals such as rabbits, hamsters, rats, mice, and guinea pigs to SMSV-4 and SMSV-5 has been carried out. None of the species tested demonstrated detectable infection.<sup>73</sup>

Tissue cultures from phylogenetically diverse host species supported replication of the marine caliciviruses. For example, the SMSV types 1, 2, 4, and 5 and VESV A<sub>48</sub> have grown well in feline, canine, porcine, human, and primate cell lines. These serotypes demonstrate variable growth in herbivore and rodent cell lines and no observable replication in the avian and marine mammal cell lines tested.<sup>65,73</sup> In another

study, SMSV cell line,<sup>1</sup> an reptile calic cells.<sup>31,42,67,74</sup> but not other to adapt to or respect to a SMSV propa severe disea gated in vivo the SMSV's pathic effect inoculation. a high titer trastructure has been cha went a loss and became infection pro

Pathogenic is variable ar but pathoty of pathogenic also been no example, in s was produce disease was marine enviro seems to oc tained from other seroty mals with no sizes produce en serotype variable.<sup>66</sup> P SMSV-5 and ated and ma plaque varia been compa poly C obser printing of S ants possesse did the mino gests that the serotype cou pathogenicity

There are fection. Swi SMSV-5 by in

study, SMSV-2 replicated in a whale kidney cell line,<sup>1</sup> and mink, walrus, bovine, and reptile caliciviruses replicated in Vero cells.<sup>31,42,67,74,78</sup> The ability of some SMSV's but not others to infect specific cell lines and to adapt to others has been reported.<sup>73</sup> With respect to adaptability, a specific strain of SMSV propagated in vitro has produced less severe disease in swine than virus propagated in vivo.<sup>99</sup> In tissue culture, many of the SMSV's produce high titers and cytopathic effects (CPE) within 8–10 hr post-inoculation. Other types have replicated to a high titer without causing CPE.<sup>80</sup> The ultrastructure of calicivirus-infected Vero cells has been characterized. Infected cells underwent a loss of microvilli and pseudopodia and became rough and shrunken as viral infection progressed.<sup>79</sup>

Pathogenicity of the marine caliciviruses is variable and depends on not only serotype but pathotype within serotype. Variability of pathogenicity among SMSV serotypes has also been noted in swine.<sup>21,59,61,65,74</sup> As an example, in swine, severe vesicular disease was produced by SMSV-13, but only mild disease was produced by SMSV-2. In the marine environment, the same variability seems to occur. Some serotypes were obtained from severely diseased animals, yet other serotypes were recovered from animals with no observable disease. The plaque sizes produced on tissue culture for any given serotype of the marine calicivirus are variable.<sup>66</sup> Purified large-plaque variants of SMSV-5 and SMSV-8 are less cell associated and may be more virulent than minute-plaque variants. In addition, plaque size has been compared to the length of genomic poly C observed on oligonucleotide fingerprinting of SMSV RNA. Large-plaque variants possessed longer poly C oligomers than did the minute-plaque variants. This suggests that the size of the poly C of any given serotype could be used as a measure of its pathogenicity.<sup>33,49</sup>

There are several possible routes of infection. Swine have been infected with SMSV-5 by intradermal, intranasal, and oral

routes,<sup>99</sup> and biting lice of swine are thought to be mechanical vectors. Cetacean calicivirus reportedly was shared between a California sea lion and two dolphins held at separate holding facilities; common water and animal handler contact were suggested mechanisms for spread of the virus.<sup>91</sup> Fecal contamination and direct contact from animal handlers were implicated in the transmission of the reptile calicivirus at the San Diego Zoo; however, the virus source for the reptiles and amphibians in this occurrence was never found.<sup>67</sup> Direct contact transmission may have been responsible for a "blisters on the eyes" syndrome of suspected calicivirus etiology that was experienced by a biologist after working on the Pribilof Islands and being in direct contact with fur seals exhibiting vesicular lesions on their flippers.<sup>62</sup> Transmission by direct contact from ruptured virus-laden vesicles has been shown to be an important route of exposure in several species.<sup>61,74</sup> In addition, direct contact transmission has been reported repeatedly in pen-contact animals during experimental infectivity studies.<sup>67,87</sup>

Transmission through the consumption of contaminated feedstuffs has been suggested for the widespread exposure of mink to MCV. It is known that whole northern fur seal carcasses produced from the Pribilof Islands harvest had, between the years 1963 and 1978, been ground up as "sealburger" and shipped frozen to mink farms in the U.S. Experimental transmission studies with SMSV-5 have shown that this sealburger can infect both swine and mink.<sup>54,99</sup> Mink producers commonly use swine to scavenge feed scraps that drop through mink cages, and this practice could enhance the spread of marine calicivirus to other species.

Experimental transmission of SMSV-5 to northern fur seals from infected opaleye fish held at 15°C in a seawater aquarium for 31 days has been accomplished. One fur seal pup consumed infected fish and developed characteristic vesicular disease. In this same experiment, a nematode intermediate in the transmission cycle was examined. Sea lion



lungworm (*Parafilaroides decorus*) larvae were passed through stock cultures of SMSV-5, rinsed three times, then fed to opaleye fish. Thirty-one days later, the infected fish were fed to fur seal pups who in turn became infected and developed vesicles, and SMSV-5 was isolated from these lesions. This study supports the concept that a nematode–teleost–marine mammal cycle exists for SMSV-5 and perhaps other marine calicivirus types. The entire sequence of events, from lungworm inoculation to fur seal infection and disease, covered a span of 54 days.<sup>86</sup> A single opaleye could transmit multiple serotypes of calicivirus to predator marine mammals by carrying several lungworm larvae infected with different SMSV serotypes.<sup>13,58</sup>

Vertical transmission within a species may occur; SMSV-2 has been isolated from the testicle of an experimentally infected northern fur seal.<sup>34</sup> SMSV-5 can survive for 14 days in seawater held at 15°C, suggesting transmission of virus through contaminated water.<sup>90</sup>

A carrier state for marine caliciviruses has been described in several species such as northern fur seals infected with SMSV-2 and SMSV-5,<sup>34,86</sup> mink inoculated with SMSV-5,<sup>42</sup> and calves infected with BCV.<sup>74</sup> Shedding of BCV by calves was observed for the full extent of a 45-day observation period.

The immunological response to marine caliciviruses is type specific and occurs soon after exposure. Neutralizing antibody generally appears within 3 to 5 days postinoculation.<sup>34,36</sup> Experimental SMSV infection in monkeys produced a significant antibody response on the second day postinoculation, with peak levels being reached by the fourth week. In this case, antibody was maintained at a high titer for 90 days, at which time the study was terminated.<sup>77</sup> Neutralizing antibody titers to the SMSV's in naturally or experimentally infected species range from a screening dilution of 1:10 up to 1:640.<sup>27,61,64,67,72,78</sup> Some titers as high as 1:2,560 in marine populations have been recorded.<sup>66</sup> In contrast, calves natu-

rally infected and shedding BCV have demonstrated neutralizing antibody titers no higher than 1:10.<sup>74</sup>

Several immunological tests are used for detecting caliciviruses and calicivirus antibodies. The complement fixation test, using hyperimmune polyvalent antisera, is the official assay for detecting VESV and SMSV antigens in tissues and vesicular fluid. The virus neutralization test is employed for VESV and SMSV serotype differentiation as well as for detecting type-specific antibody. The specificity of the neutralization test in detecting type-specific antibody is evident in that high antibody titers to one serotype in test sera are frequently observed without any cross-reaction to other SMSV serotypes.<sup>62,66,72</sup> The agar-gel diffusion test has been applied to SMSV, and antibody detection with antigenic cross-reactivity to VESV and other SMSV serotypes is common. A single protein antigen is believed to be responsible for this cross-reactivity.<sup>41</sup> The indirect immunofluorescence test has been useful in calicivirus detection; however, variable antigenic cross-reactivity between serotypes occurs. This assay detects group antigens on cell surfaces, in contrast to the virus neutralization test, which detects type-specific viral antigens. The indirect immunofluorescence test also has been used as an antibody assay and is capable of detecting serum antibody as early as 5–7 days postinoculation and for a period of up to 5 mo.<sup>41,98</sup> The radioimmune precipitation test has been used for calicivirus detection, and, with this test, antigenic cross-reactivity is common between the VESV and the SMSV serotypes.<sup>94</sup>

Exposure to marine caliciviruses, as determined by virus isolation and neutralization testing, is widespread among both marine and terrestrial populations (Tables 3–5). Many species have been exposed to multiple serotypes, and terrestrial animals possess antibody to many of the marine serotypes. Conversely, the marine environment appears to have an ongoing involvement with the VESV's, which were originally

Table 4.

California s  
(*Zalophus*)

Northern f  
(*Callorhinus*)

Stellar sea  
(*Eumetopias*)

Dolphin (7)

Walrus  
(*Odobenus*)  
Bowhead w  
(*Balaena*)  
Gray whale  
(*Eschrichtius*)  
Fin whale  
(*Balaenoptera*)  
Sperm whale  
(*Physeter*)  
Sei whale  
(*Balaenoptera*)  
Northern c  
(*Mirounga*)  
Hawaiian i  
(*Monachus*)  
Opaleye fish  
(*Girella*)

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Table 4. Marine animal exposures to caliciviruses based on serology.

Species	Serotype	Year	Reference
California sea lion ( <i>Zalophus californianus</i> )	SMSV-1	1972-1983	1, 39, 51, 66, 68, 72
	SMSV-2	1972-1976	1, 39, 51, 66, 72
	SMSV-4	1973-1976	39, 66, 72
	SMSV-5	1973-1986	39, 66, 68, 72
	SMSV-6	1975-1986	13, 64
	SMSV-8	1977	68
	SMSV-10	1983-1986	68
	SMSV-13	1977-1986	16
	TCV	1983-1984	15, 61
	VESV	1972-1976	72
Northern fur seal ( <i>Callorhinus ursinus</i> )	SMSV-2	1972-1976	51, 72
	SMSV-5	1973-1984	66, 68, 72
	SMSV-6	1975-1977	64
	SMSV-10	1984	68
Stellar sea lion ( <i>Eumetopias jubatus</i> )	SMSV-1	1976-1985	9, 25, 68
	SMSV-2	1960-1978	1, 25
	SMSV-5	1976-1985	9, 25
	SMSV-6	1976-1986	9, 13, 25, 57
	SMSV-7	1985	9, 13, 25
	SMSV-8	1985	9, 25
	SMSV-10	1976-1985	9, 25
	SMSV-13	1976-1986	9, 25
Dolphin ( <i>Tursiops</i> spp.)	TCV	1976-1985	9, 15, 61
	SMSV-1, SMSV-2,	1988	68
	SMSV-5, SMSV-6,		
	SMSV-12, SMSV-14,		
Walrus ( <i>Odobenus rosmarus</i> )	SMSV-15, WCV, MCV		
	SMSV-5, SMSV-8, WCV	1983	14
Bowhead whale ( <i>Balaena mysticetus</i> )	SMSV-5, SMSV-8,	1980	84
	SMSV-1, VESV		
Gray whale ( <i>Eschrichtius robustus</i> )	SMSV-2, SMSV-5, VESV	1968	1, 72
Fin whale ( <i>Balaenoptera physalus</i> )	SMSV-1, SMSV-5, VESV	1972	72
Sperm whale ( <i>Physeter catodon</i> )	SMSV-5, VESV	1972	72
Sei whale ( <i>Balaenoptera borealis</i> )	SMSV-5, VESV	1972	72
Northern elephant seal ( <i>Mirounga angustirostris</i> )	SMSV-4	1973	1
Hawaiian monk seal ( <i>Monachus schauinslandi</i> )	VESV	1978	38
Opaleye fish ( <i>Girella nigricans</i> )	SMSV-6, SMSV-7	1981	87

thought to be of swine origin. Some marine mammal species that are spacially isolated from one another in the marine environment exhibit exposure to many of the same serotypes. Apparently, the land-ocean interface is not a significant barrier to calicivirus movement.

Because the VESV and SMSV serotypes have been indistinguishable, several attempts have been made to compare them. For example, the disease syndromes produced by marine caliciviruses have been compared in several species. In northern fur seals, SMSV-induced lesions are indistin-



Some significant differences have been observed between SMSV-2 and VESV A<sub>48</sub>, using one serotype to represent each group. For example, VESV A<sub>48</sub> has demonstrated a greater resistance to chemical disinfectants than SMSV-2.<sup>18</sup> Variation between the two serotypes in the *in vitro* growth patterns on feline embryo cell lines has also been noted.<sup>24</sup> In addition, differences in the ability of SMSV-1, -2, -4, and -5 and VESV A<sub>48</sub> to grow on Vero, swine testicle, and other cell lines of diverse species origin have been observed.<sup>41,64,73</sup> Again, because the differences among serotypes within each group were not investigated, it is meaningless to speculate on differences between the two groups.

Antigenic relatedness studies concerning the SMSV's and VESV's have been carried out. Immunoelectron microscopy (IEM) testing was utilized for some of these comparisons and has revealed that most of the VESV's and SMSV's are antigenically related to each other. In addition, two types of VESV were found to cross-react with feline calicivirus (FCV) but not with each other. One of these two VESV types reacted with the SMSV's but not with other VESV's.<sup>92</sup> Another study utilizing IEM techniques revealed that SMSV-6 had some partial antigenic relatedness to FCV.<sup>64</sup> Comparisons using the serum neutralization test revealed that the antigenic relatedness between serotypes of the VESV and SMSV groups was as great as that among serotypes within each group.<sup>18,63,89</sup> From this work, an antigenic basis of distinction between VESV and SMSV groups does not exist. The only known difference between the two groups is that all VESV's were first isolated prior to 1957 and all SMSV's subsequent to 1972.

Studies of relatedness among members of the SMSV group have been carried out. Oligonucleotide fingerprinting has shown that serotype relatedness may be associated with host-spectrum characteristics. For example, preliminary studies show that serotypes isolated from similar hosts, such as is the case

with SMSV-6 and SMSV-7, had more oligonucleotides in common than serotypes isolated from dissimilar hosts, such as the reptile and mink caliciviruses.<sup>33</sup>

Summarizing the available information, the marine caliciviruses are capable of producing similar disease in a wide variety of hosts, including many species of domestic livestock and humans. These viruses appear to be widespread in the marine environment and in terrestrial animals. Evidence from serological surveys and experimental transmission studies indicates that the land-ocean interface is not an effective barrier to movement of these viruses. Considering the widespread exposure and disease-causing potential of these viruses, an assessment of the movements and distribution of all animals of ocean origin would be useful to insure that these viruses are not inadvertently spread to susceptible hosts.

#### VIRUS MOVEMENTS

The exposure of domestic terrestrial, wildlife, and zoo species to marine caliciviruses has been clearly established. Questions regarding possible transmission routes and potential disease reservoirs of these viruses should be examined. To address these questions, it is helpful to understand the extent of marine virus exposures in relation to land-ocean margins. By knowing the geographic ranges of marine species exposed to caliciviruses,<sup>59,82</sup> the land masses involved in possible disease transmission routes have been determined. These coastal areas are extensive and include the Northern Hemisphere contiguous with the northern Pacific Ocean.

Considering the marine environment as a direct source of these agents, several transmission routes have been suggested. Diseased marine carcasses washed up on beaches and eaten by scavenging carnivores or contaminated forages eaten by herbivores may lead to exposure in these species. Air bubbles rising through a column of seawater concentrate organic material. When these bubbles burst at the surface, they project

droplets into the air. This may release virus as aerosols into prevailing onshore winds, allowing infectious particles to settle on forages. Haul-out zones for marine mammals could also provide direct contact opportunities for terrestrial species. The movement of coastal feral swine and San Clemente Island goats from these offshore islands into central California and elsewhere across the U.S. for game hunts or pets has been suggested as an avenue for transmission further inland.<sup>63</sup> Inland exposures may also occur from spawning anadromous fish moving into freshwater streams and from the distribution of marine fishery by-products, such as fish meal and fish lysates. Also, the capture and worldwide dissemination of marine animals for display purposes is a likely source of virus spread.<sup>51,58,82</sup>

The use of marine by-products as feed supplements is probably the single most important route of transmission of marine caliciviruses from ocean sources to terrestrial animals.<sup>9,12,54,99</sup> For example, the source of BCV infection in cattle has not been found. However, it is now known that this serotype is of marine origin and that feed supplements could play a role in calicivirus transmission to cattle.<sup>12,15,59</sup> In addition, it now seems certain that the VESV's are all of marine origin and were carried ashore by marine animals at the time the outbreaks of VES occurred.<sup>12</sup> The transmission may have occurred through infected marine products, such as occasional sea lion carcasses or, more likely, raw fish scraps fed in raw garbage. Fish scraps have been implicated as the probable cause of the first three outbreaks of VES because all affected farms fed raw garbage from port city institutions and restaurants.<sup>43,58,63</sup>

The search for marine calicivirus reservoirs has been the object of several studies because it was thought that domestic livestock were aberrant hosts for these viruses.<sup>12,59</sup> It now appears that southern California waters are a central focus of calicivirus activity and that the California sea lion is the marine mammal species most intensely

involved with the marine caliciviruses.<sup>9,12,13,82</sup> More recent evidence shows that opaleye fish, which serve as food sources for California sea lions, are a primary reservoir for marine caliciviruses.<sup>59,82,87</sup> Several characteristics of the opaleye make it especially well suited for this purpose. First, the opaleye population is large enough to maintain the many known calicivirus serotypes on a continuous basis.<sup>63,66</sup> Also, the opaleye habitat lies entirely within the California sea lion breeding range, which is adjacent to the early outbreaks of VES in California and within the area where the greatest activity of the marine caliciviruses has been observed. In addition, opaleyes have a close association with California sea lions and have direct access to the terrestrial environment through the distribution of fish scraps generated by the commercial fishing industry.<sup>10,12,59,66,87</sup> A very high type-specific antibody titer to SMSV-7, the same serotype that was initially isolated from an opaleye, was detected in the serum from a cow sampled in Kansas.<sup>68,87,92</sup>

Disease outbreaks involving the SMSV's in California sea lions appear to be enzootic; SMSV-5 persists in populations of this species for long periods of time.<sup>63</sup> In contrast, disease outbreaks involving the SMSV's in northern fur seal populations have been characterized as epizootic.<sup>12,15,57,61,78</sup> Also, different SMSV serotypes exist in populations of both species at any given time,<sup>16,64,72,78,82</sup> which may be because the food sources for these two species are dissimilar.

The spread of calicivirus to different marine mammal species may be linked by migratory pathways. For instance, Stellar sea lions, northern fur seals, and whales move through the southern California active zone on a seasonal basis, which could account for the spread of calicivirus serotypes to other areas.<sup>13,82</sup> Several disease outbreaks involving specific calicivirus serotypes have occurred in geographically distant northern fur seal populations following initial appearance in California sea lions.<sup>11,66</sup> It is thought

that northern fur seals, while in southern California, may transmit newly acquired viruses during and after their stay at the Pribilof Islands. Several species, such as the harbor seal, and bottlenose dolphin have been isolated. In addition, it is thought that the virus within each species may serve as a

There is evidence in addition to the isolation of marine caliciviruses. An attempt to find a common species of fish in the waters has been fruitless, and the results are inconclusive, and the California sea lion is

Marine caliciviruses (VESV's), appear to be an antigenic shift. Recent types of VESV's have been detected in the terrestrial environment. Of this, it seems that the genetic and antigenic similarity of SMSV's are recent mutations. Serotypes 1 and 2 are both the same. The results (Table 1) show a cycling of the virus. The factor of the marine environment and SMSV serotypes isolated from the initial isolation. Pathotype 1 viruses probably may explain the marine mammals virus serotypes to the isolation.

that northern fur seals acquire infection while in southern California and then transmit newly acquired virus to other fur seals during and after their northward migration to the Pribilof Islands.<sup>88</sup> Exposure in other species, such as the walrus, Hawaiian monk seal, and bowhead whale is difficult to explain because these populations are more isolated. In these cases, it has been suggested that the virus is either maintained entirely within each species or that their food sources may serve as virus reservoirs.<sup>12,14,38,78</sup>

There is speculation that other fish, in addition to the opaleye, may be reservoirs of marine caliciviruses. A preliminary attempt to find calicivirus exposure in 19 species of fish resident to California coastal waters has been made by screening for neutralizing antibodies. These tests were inconclusive, and many other species dwelling in California coastal waters remain untested.<sup>66</sup>

Marine caliciviruses, including the VESV's, appear to have been maintained in an antigenically unchanged state for many years. Recent exposure to the same serotypes of VESV isolated over 40 yr ago has been detected in both the marine and terrestrial environments (Tables 4, 5). In light of this, it seems unlikely that rapid mutation and genetic instability entirely explain the antigenic variability of caliciviruses.<sup>10,12</sup> The SMSV's are no longer considered to be recent mutants of the VESV's.<sup>53</sup> Many SMSV serotypes have a long standing presence in both the marine and terrestrial environments (Tables 3–5). Evidence for epidemic cycling of the marine caliciviruses has been gathered, using virus isolation as an indicator of levels of SMSV activity in the marine environment. For example, the same SMSV serotype (SMSV-6) has been reisolated from marine mammals 10 yr after its initial isolation from marine samples.<sup>57</sup> Pathotype switching of the marine caliciviruses probably occurs. This phenomenon may explain how asymptomatic marine mammals had been exposed to a specific virus serotype (SMSV-13) several years prior to the isolation of this serotype from mas-

sive vesicular lesions seen during a severe disease outbreak with high morbidity.<sup>61,64</sup>

Although there is strong evidence to support the existence of an ocean-to-land transmission of caliciviruses, a great deal of uncertainty still exists regarding the specific mechanisms involved. However, it now appears that the marine caliciviruses can have terrestrial reservoirs, and for that reason, they could be transmitted to marine species by the natural flow of material from land to ocean, thus giving rise to the concept that the virus may move both ways across the land-ocean interface. The report of persistent BCV shedding in calves provides evidence for the existence of a terrestrial calicivirus reservoir and land-to-ocean movement of these viral agents.<sup>15</sup>

Regardless of calicivirus reservoirs, the land-ocean transmission lines appear to be open and operating. To assume that these viruses are confined to the north Pacific Ocean is not realistic. Marine mammals do not respect geographical boundaries and are capable of spreading these viruses throughout the world.<sup>59,80</sup> Such transmission may have already occurred; disease outbreaks resembling VES were recently reported in swine in Australia, New Zealand, and Tasmania. The affected swine had been fed raw or undercooked feedstuffs from marine sources; however, the causal agent for these disease outbreaks remains unknown.<sup>47</sup> It is also unrealistic to assume that all calicivirus serotypes present in the marine and terrestrial environments have now been isolated and serotyped. New types are continually being isolated, and several important points are beginning to emerge. First, extensive exposure to many serotypes, including the VESV serotypes isolated between 1948 and 1956, is continuing today in the marine and terrestrial environments. For example, a high level of exposure to SMSV-8 was found in selected bovine sera even though this serotype was first isolated from northern fur seals in Alaska in 1975.<sup>68</sup> Recent exposures to viruses of the same serotype have been found in California sea lions, Stellar sea

lions, a bowhead whale, and walrus. These exposures illustrate that the host spectrum can be very broad for many of these serotypes. Also, experimental infections produce significant disease in several host species. These viral agents are relatively resistant to environmental effects, they are antigenically stable, and they appear to have a long-standing presence on land and in the ocean.

There are many aspects of marine calicivirus transmission and activity that are unclear. First, the specific origins, reservoirs, and land-ocean transmission routes are poorly understood. Also, neither the number of calicivirus serotypes nor their worldwide distribution is known. Finally, all susceptible species and the resultant disease syndromes caused by calicivirus infection are not known. SMSV-5 naturally infects four species of pinnipeds, six species of cetaceans, domestic swine, feral fox, musk ox, domestic cattle, and humans. Experimental infectivity studies add fish and monkeys to this list, and clinical disease was noted in seals, swine, monkeys, and humans.

Several recommendations for further action have been formulated, focusing on calicivirus exposures in important marine and terrestrial species. First, the official status of these agents needs to be reevaluated. Because exposure to all marine caliciviruses, including some VESV serotypes, is routinely occurring in a variety of animal species, these viral agents can no longer be considered either eradicated or foreign to the U.S. In addition, the occurrence of widespread exposure resulting in only occasional disease should be actively investigated. It is perplexing that clinical syndromes are not being reported when experimental exposures produce characteristic disease in numerous species and these diseases spread horizontally to pen-contact animals. Presumably, this is because of routine misdiagnosis resulting from the difficulty in definitively diagnosing calicivirus disease. Better diagnostic reagents are needed for the identification of the disease syndromes

caused by natural infection in susceptible species. Such reagents will be useful for assessing and controlling marine calicivirus-induced disease in captive marine mammals, other display or exhibit species, domestic pets, livestock, and humans.

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## **Three-dimensional Structure of Calicivirus**

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## Three-dimensional Structure of Calicivirus

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The Caliciviridae comprise a new family of single-stranded RNA viruses. While human caliciviruses cause gastroenteritis, the animal caliciviruses cause a wide range of diseases. We have determined the three-dimensional structure of a primate calicivirus using electron cryomicroscopy and computer image-processing techniques. Calicivirus is one of the rare animal viruses whose capsid is made of a single structural protein. The three-dimensional structure of the virus is distinct from that of any other animal virus. However, there are several architectural similarities with plant viruses such as tomato bushy stunt virus and turnip crinkle virus. The calicivirions are 405 Å in diameter and exhibit  $T = 3$  icosahedral symmetry. The main features of the three-dimensional structure are the 32 large surface hollows, 50 Å deep and 90 Å wide, at the icosahedral 5-fold and 3-fold axes, and the 90 distinctive arch-like capsomeres surrounding these hollows at the local and strict 2-fold axes. Each capsomere is a dimer of the capsid protein. Despite noticeable differences, the three quasi-equivalent subunits show common structural features: the upper bilobed domain, the central stem domain, and the lower shell domain. The 2-fold related capsid proteins interact through the bilobed domains to form the top of the arch. The structural differences between the connectors of the stem and the shell domain among the three subunits suggest the presence of a hinge region that may facilitate the capsid protein to adapt to the three quasi-equivalent environments of the  $T = 3$  icosahedral structure. The shell domains of the pentavalent and hexavalent capsid proteins associate to form a continuous shell between the radii of 115 and 150 Å. A  $\beta$ -barrel structure has been suggested for the shell domain. The mass density in the inner shell between the radius of 85 and 110 Å may contain a portion of the capsid protein interacting with the RNA. The features between the 45 and 85 Å radius are suggestive of ordered RNA.

**Keywords:** calicivirus; electron cryomicroscopy; 3-D structure; plant viruses

### 1. Introduction

Recent developments in diagnostic techniques have enabled identification of several small round viruses, of which calicivirus is one, as causal agents of acute non-bacterial gastroenteritis in humans (Appleton, 1987; Cubitt, 1987; Kapikian & Channock, 1990). Caliciviruses also infect a number of animal species including calves, pigs, cats, dogs and the pygmy chimpanzee (Hall *et al.*, 1984; Flynn *et al.*, 1988; Evermann *et al.*, 1985; Smith *et al.*, 1983; Kapikian & Channock, 1990). Illness varies depending upon the species. For example, calici-

virus infection is associated with a stunting syndrome in chickens and with a vesicular exanthem in pigs.

Caliciviruses are non-enveloped, single-stranded RNA viruses, formerly classified within the Picornaviridae (Cooper *et al.*, 1978). In 1980, because of the presence of a single structural protein and evidence of subgenomic RNAs during replication, these viruses were classified into a separate family called Caliciviridae (Schaffer *et al.*, 1980a). Sequenced genomes of calicivirus are linear positive-sense RNA molecules of about 7.5 kb (Neill *et al.*, 1991; Myers *et al.*, 1991). The viral RNA encodes a single capsid protein of molecular mass ~70 kDa in addition to non-structural proteins totaling about 200 kDa. The molecular mass of the individual non-structural proteins have not been determined. As in picornaviruses, the genomic RNA is covalently linked to a small protein VPg of 10 to 15 kDa (Black *et al.*,

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1978; Burroughs & Brown, 1978; Schaffer *et al.*, 1980b).

When caliciviruses are examined by negative-stain electron microscopy, they exhibit characteristic cup-shaped depressions on the surface (Schaffer *et al.*, 1980a; Carter & Madley, 1987). The virus family takes its name from the Latin word calyx, meaning cup, which is descriptive of these surface hollows. The reported diameter for these viruses ranges from 300 to 400 Å. Several groups have proposed models for the virus structure based on the electron micrographs of negatively stained specimens and the evidence that 180 copies of the capsid protein are incorporated into the virus structure (Almeida *et al.*, 1968; Peterson & Studdert, 1970; Burroughs *et al.*, 1978). Despite differences in detail, all these models suggested a  $T = 3$  icosahedral structure. While icosahedral capsids made of a single structural protein are common among plant viruses, they are unusual among animal viruses. The only known examples, apart from calicivirus, are nodaviruses (Hosur *et al.*, 1987; Hendry, 1991).

Three-dimensional structures of several ssRNA viruses of both plant and animal origin have been determined, but no three-dimensional structural study has been reported for any member of the Caliciviridae. We undertook three-dimensional structural studies of these distinct viruses to establish structure-function relationships and to understand their architectural principles, particularly in comparison with the structures of other ssRNA viruses. We describe here the three-dimensional structure of a primate calicivirus determined using electron cryomicroscopy and computer image reconstruction.

## 2. Materials and Methods

### (a) Virus

The primate calicivirus was originally isolated from the lip lesion on a pygmy chimpanzee, *Pan paniscus* (Smith *et al.*, 1983). The original isolate was passaged in African green monkey kidney (Vero) cells for the electron cryomicroscopy studies. Six 850 cm<sup>2</sup> roller bottles of Vero cells were grown to confluence, the growth medium was removed, and the cells were infected at a multiplicity of infection of 5 with primate calicivirus in 10 ml of minimum essential medium (MEM). Virus was adsorbed for 2 h at 37°C on a roller apparatus at 1/3 turn/min. The inoculum was removed by pipetting and 30 ml of MEM without serum was added to each roller bottle. After 20 h incubation at 37°C, cells exhibited 4+ cytopathic effect. Roller bottle contents were centrifuged at 100,000 *g* for 2 h at 4°C. The resulting pellets were resuspended in phosphate-buffered saline (PBS) at pH 7.5 and extracted 4 times with 1,1,2-trichloro-1,2,2-trifluoroethane. The resulting supernatant was centrifuged at 100,000 *g* for 2 h at 4°C. The pelleted virus was resuspended in 100 µl of PBS and was layered on the top of a CsCl gradient. After centrifugation at 100,000 *g* for 16 h at 4°C the light-scattering band was collected by side puncture. To remove residual CsCl, the banded virus was resuspended in PBS and centrifuged for 5 h at 100,000 *g* at 4°C. The supernatant was discarded and the virus was resuspended in 100 µl of PBS.

### (b) Electron cryomicroscopy

A high concentration of calicivirus was embedded in a thin layer of vitreous ice and examined in a JEOL 1200 electron microscope at -155°C (Adrian *et al.*, 1984; Dubochet *et al.*, 1988; Prasad *et al.*, 1992). Images were recorded with electron doses of 4 to 6 e/Å<sup>2</sup> per image at a nominal magnification of 30,000×. The magnification was internally calibrated using tobacco mosaic virus (TMV) as a standard. A set of 2 micrographs was recorded from each specimen area: the first was underfocused by 1 µm and the next by 2 µm. The 2 µm underfocused images were used to confirm the orientations of the particles and the 1 µm images were used in the 3-dimensional reconstructions.

### (c) Computer image processing

The 3-dimensional structure was reconstructed from the electron cryomicrographs using procedures described earlier (Crowther, 1971; Fuller, 1987; Baker *et al.*, 1988; Prasad *et al.*, 1990, 1992). The electron micrographs were digitized on a scanning densitometer with a step size corresponding to 5.33 Å in the object. The orientations of the capsids were determined by the "common lines" procedure (Crowther *et al.*, 1970; Fuller, 1987). Once the orientations of the particles were determined, the phase origins of the particles were refined. The refinement of the origin and the orientation were carried out iteratively until no change occurred in either of them. The orientation parameters and the phase origin of each particle were then further refined with respect to the entire dataset using cross-common lines (Fuller, 1987). When particles adequate to sample the asymmetric unit of the icosahedron were available, an initial 3-dimensional reconstruction was computed using cylindrical expansion methods imposing a lower 522 symmetry (Crowther *et al.*, 1970; Crowther, 1971). This 3-dimensional density map was projected along the orientation parameters of the particles and the respective projected maps were cross-correlated with the images to determine the phase centers more precisely. After another cycle of refinement of the orientations of the particles using cross-common lines with these phase centers, the final 3-dimensional map was reconstructed including data out to 22 Å resolution. No corrections due to contrast transfer function were incorporated in the reconstruction. A total of 24 particles with unique orientations, all from the same micrograph, was included in the reconstruction. The radial density plots were computed from the 3-dimensional density maps by averaging the densities in concentric shells of 5.33 Å width about the center of the map.

## 3. Results

### (a) Electron cryomicrographs

Electron cryomicrographs of the calicivirions embedded in ice and recorded at 1 and 2 µm underfocus are shown in Figure 1. The low-resolution features are accentuated in the micrograph recorded with 2 µm underfocus (Figure 1(b)). The virions are spherical and appear to have a rough outer surface. In comparison with the TMV diameter of 180 Å, the calicivirus particles have a diameter of 405 Å. Some particles in the higher defocus image display the "star-of-David" characteristic of caliciviruses seen in negatively stained preparations (Figure 1(b), arrowhead). Other particles appear to be featureless.

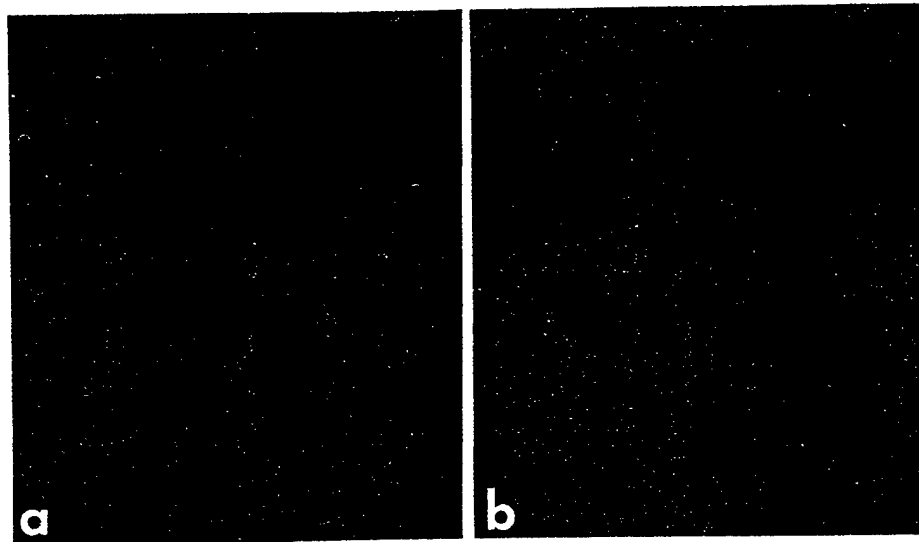


Figure 1. Electron cryo-micrographs of calicivirions embedded in a thin layer of vitreous ice recorded at (a) 1  $\mu\text{m}$  and (b) 2  $\mu\text{m}$  underfocus. The arrowhead shows a particle displaying the "star-of-David" shape. A few TMV particles are also seen in this field of view. The scale bar represents 1000  $\text{\AA}$ .

Occasional incomplete or disrupted particles were seen in other fields.

#### (b) Three-dimensional reconstruction

Three-dimensional reconstruction was carried out using 24 particles chosen from a micrograph, recorded with an underfocus of 1  $\mu\text{m}$ . A portion of the micrograph, along with its focal pair, from which the particles were chosen is shown (Figure 1). The extent to which the data from these particles obey the expected icosahedral symmetry is evaluated by computing the mean phase difference (phase residual) for all pairwise particle comparisons at regular intervals along the cross-common lines in the Fourier transforms of the respective particles (Crowther, 1971; Fuller, 1987). Phase residuals greater than  $90^\circ$ , a value for randomized phases, indicate no correlation with icosahedral symmetry. The phase residual plot, as a function of resolution, for our data is shown (Figure 2). The overall phase residual for the entire data out to 22  $\text{\AA}$  resolution is around  $45^\circ$ . The phase residual is significantly less than  $90^\circ$  for most of the data and approaches  $90^\circ$  for data around  $\sim 22$   $\text{\AA}$  resolution. The resolution limit,  $\sim 22$   $\text{\AA}$ , of the icosahedral correlation is consistent with the defocus level of the micrograph. The point at which the phase residual reaches  $90^\circ$  has been used to estimate the nominal resolution of icosahedral reconstructions. Stewart *et al.* (1991) have validated this criterion by comparing the X-ray structure of adenovirus hexon with that determined by electron cryomicroscopy and computer image processing techniques. However, as we go higher in resolution, the signal to noise ratio decreases and the phase residuals tend to go higher, as seen in Figure 2. Though we have included the data out to a resolution of 22  $\text{\AA}$ , based on defocus level and ico-

hedral correlation of the data, the actual resolution of the reconstruction may, indeed, be lower than 22  $\text{\AA}$ , between 25 and 30  $\text{\AA}$ .

Surface representations of the three-dimensional structure along the icosahedral 5-fold and 3-fold axes are shown in Figure 3. Although a lower 522 symmetry was used in the reconstruction procedure, the reconstruction showed excellent 532 symmetry. As a further check on the reliability of reconstruction, the input images were compared with the projections obtained from the three-dimensional density map in orientations corresponding to the images.

#### (c) Arches and hollows

In the three-dimensional structure of the calicivirus, 90 arch-like capsomeres are arranged on a  $T=3$  icosahedral lattice (Caspar & Klug, 1962) such that there are prominent hollows at all the ico-

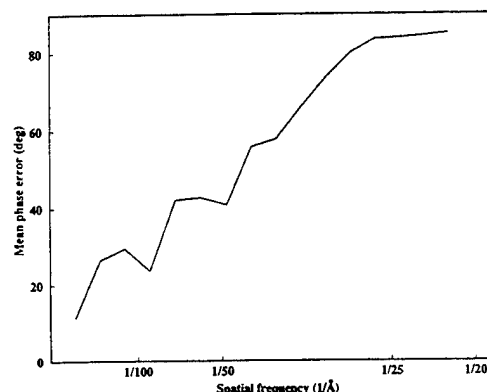


Figure 2. Plot of the average phase residual as a function of resolution.

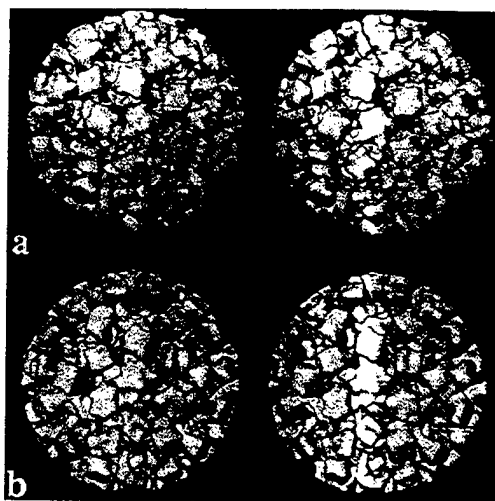


Figure 3. Stereo pairs of the 3-dimensional structure of the calicivirion viewed along the icosahedral (a) 5-fold and (b) 3-fold axes.

hedral 5-fold and 3-fold axes (Figure 3). The arches begin at a radius of 150 Å and extend to a radius of about 202 Å. They have a squarish platform at the top, of dimensions  $\sim 50 \text{ Å} \times 50 \text{ Å}$ . At the center of the platform there is a small dimple. The hole between two sides of the arch is about 30 Å in diameter. The hollows surrounded by these arches are about 50 Å deep and 90 Å wide. Each virus particle has 32 such hollows. The hollows at the 5-fold axes are slightly smaller in width than those at the 3-fold axes.

The 90 capsomeres can be classified into two types based on their location with respect to the icosahedral symmetry axes (Figure 4). The first type, designated as type A, are the capsomeres surrounding the icosahedral 5-fold axis. These are located at the local 2-fold axes, midway between icosahedral 5-fold and 3-fold positions. The second type, designated as type B, are located at all the strict 2-fold axes of the icosahedral structure (midway between any 2 neighboring 5-fold or 3-fold axes). The plane of a type A arch lies obliquely to the line joining the neighboring 5-fold and 3-fold axes. Similarly, the plane of a type B arch lies

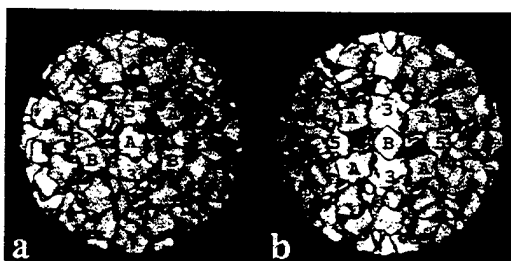


Figure 4. Surface representations of the 3-dimensional structure viewed along (a) the local 2-fold axis and (b) the icosahedral 2-fold axis. Two types of capsomeres, A and B, and a set of icosahedral symmetry axes are indicated.

obliquely to the line joining the neighboring 3-fold axes. There are noticeable differences in the local environments of these capsomere types. Each type A capsomere is surrounded by two type B and two type A capsomeres (Figure 4(a)), while each type B capsomere is surrounded by four type A capsomeres (Figure 4(b)). There is a significant difference between A to A and A to B distances; the A to A distance is about 80 Å and the A to B distance is about 70 Å.

The two sides of a type B arch, each referred to as B1 subunit, are equivalent because they are related by the strict 2-fold axis. However, the two sides of a type A arch, referred to as A1 and A2 subunits, related by a local 2-fold axis, are not strictly equivalent. In the type A arch, the A1 side is closer to the 5-fold axis and the A2 side is closer to the icosahedral 3-fold axis. Each B1 side lies close to an icosahedral 3-fold axis. Despite noticeable differences, A1, A2 and B1 subunits share common structural features: a bilobed distal end (P1) and a single lobed stem region (P2), which merges into a contiguous mass density at a lower radius (S) (Figure 5). One difference between the subunits is that the A2 side has a prominent bulge in the P2 region, which is not seen in A1 and B1 sides. Furthermore, the mass density that connects the P2 domain to the S domain in these subunits appears slightly different. Compared with an A1 side, A2 and B1 have less mass density at this region. In B1, particularly, this mass density is rather thin. Close observation of the capsomeres indicates that the two sides interact with each other with a slight twist.

#### (d) Bases

The lower portions of the A1 sides meet at a radius of about 150 Å to form the base of a hollow at the 5-fold axis. Similarly, the lower portions of three A2 and three B1 sides form the base of a hollow at the icosahedral 3-fold axis (Figure 6(a) and (b)). In Figure 6(a), the mass density beyond the radius of 175 Å has been removed. At this radius, we can still see the lower portions of the P2 domains of the subunits. However, at the radius of 150 Å (Figure 6(b)), we see a relatively smooth surface made by the S domains of the subunits. The base of each of the hollows has a small central protuberance. The mass densities between a 5-fold and 3-fold axis are continuous at the radius of the

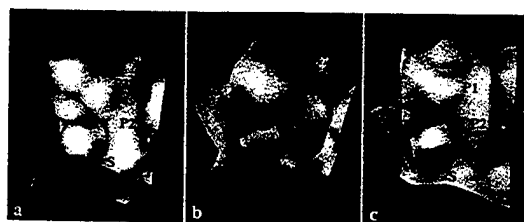


Figure 5. Three quasi-equivalent subunits (a) A1, (b) A2 and (c) B1. The 3 domains, P1, P2 and S in these coat proteins are also indicated.

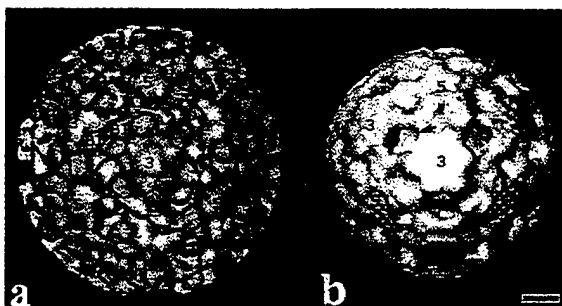


Figure 6. Cutaway of the 3-dimensional structure at a radius of (a) 175 Å and (b) 150 Å along the icosahedral 3-fold axis. Only the upper hemisphere is displayed for clarity. The locations of the lower portions of A1 and A2 of a type A capsomere and 2 B1s of a type B capsomere are indicated. Also marked are icosahedral 5-fold axes and 2 icosahedral 3-fold axes. In (b), the arrow shows the hole at one of the icosahedral 2-fold axes. Refer to Figure 2(b) for the complete particle in the same orientation. The scale bar represents 50 Å.

base across the local 2-fold axis, but not across the icosahedral 2-fold axis (Figure 6(b)). Holes are present at the icosahedral 2-fold axes below the type B arches (Figure 6(b), arrowhead). These holes are about 45 Å in diameter and about 50 Å deep, terminated by the mass density at the radius of 100 Å. The mass density joining the base at a 5-fold axis with that at a 3-fold axis is at a slightly lower radius, forming a small dip.

#### (e) Radial density plot

The radial density plot (Figure 7) provides a convenient way to describe the distribution of mass density as a function of radius. This plot suggests that the mass density in the three-dimensional structure is distributed into four concentric shells: the first between the radii of 155 and 200 Å, the

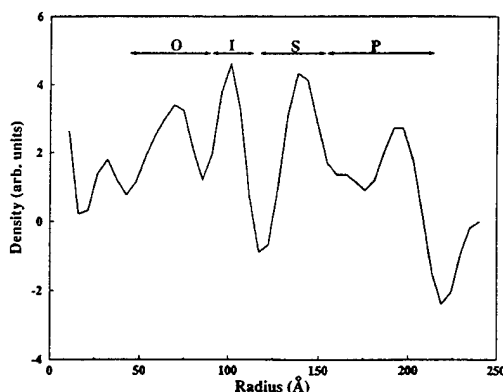


Figure 7. Radial density profile calculated from the 3-dimensional density map. The radial extensions of the proposed P1 and P2 (denoted as P) domains, S domain, the inner shell (I) and putative ordered RNA (O) are indicated by double-headed arrows.

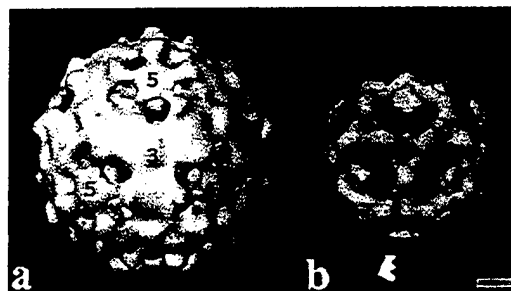


Figure 8. The surface rendering of the mass densities (a) at a radius 115 Å, and (b) between radii 45 and 85 Å, along the icosahedral 3-fold axis. The scale bar represents 50 Å.

second between 115 and 155 Å, the third between 80 and 110 Å, and the fourth inside a radius of 80 Å. The negative peak at the radius of about 210 Å signifies the virion boundary. This negative peak is due to the Fresnel fringes resulting from the relatively high underfocus (1 µm) employed during recording of the electron micrographs. The tops of the arches contribute to the peak at the radius of 185 Å. The stems of the arches, between the radii 150 and 175 Å, contribute relatively less to the average radial density. The mass density between the radii of 115 and 150 Å is due to the continuous shell (Figure 6(b)) that provides the scaffolding for the arches as described earlier. At the radius of about 115 Å there is a significant reduction in the average mass density. The surface-shaded representations of the mass density of the two inner shells, at a radius of 115 Å and at a radius of 85 Å, are shown in Figure 8. While it is quite likely that the mass density between the radii 115 and 202 Å is due to the coat protein molecules, the density below the radius of 115 Å is predominantly due to the genomic RNA, as discussed below.

## 4. Discussion

### (a) Comparison with other ssRNA icosahedral viruses

The three-dimensional structure of the calicivirus has  $T = 3$  icosahedral symmetry. Calciviruses, being non-enveloped and single-stranded RNA viruses, were formerly classified within the Picornaviridae (Cooper *et al.*, 1978). Our studies clearly show that the structure of calicivirus is quite distinct from that of any picornaviruses. Several high-resolution structures of picornaviruses have been determined, including those of human rhinovirus (Rossmann *et al.*, 1985), poliovirus (Hogle *et al.*, 1985), Mengo virus (Luo *et al.*, 1987) and foot-and-mouth disease virus (Acharya *et al.*, 1989). Picornavirus ( $T = 1$ ) structures are much smaller, about 325 Å in diameter, compared with 405 Å for calicivirus. These viruses have a rather smooth outer surface and lack the prominent protuberances and hollows seen in this calicivirus structure. Thus, the three-



dimensional architecture of caliciviruses clearly justifies the classification of caliciviruses into a separate family. Despite the structural differences, some genomic features suggest a common ancestor between picornaviruses and caliciviruses. Gene sequences reported for two animal caliciviruses (Neill *et al.*, 1991; Meyers *et al.*, 1991) indicate that picornaviruses and caliciviruses have some non-structural proteins with the same functions, that these non-structural proteins of the two viruses are aligned on the respective genomes in the same order, and that the primary sequences of the coded proteins share limited sequence similarity.

The  $T=3$  capsid organization is common among ssRNA icosahedral viruses. Notable examples include plant viruses such as tomato bushy stunt virus (TBSV, Harrison *et al.*, 1978), turnip crinkle virus (TCV, Hogle *et al.*, 1986) and southern bean mosaic virus (SBMV, Abad-Zapatero *et al.*, 1980), and insect viruses such as black-beetle virus (BBV, Hosur *et al.*, 1987) and Flock House virus (FHV, Fisher & Johnson, 1993). Like calicivirus, the capsid of these plant viruses is formed by a single protein. While capsid formation by a single protein is common in plant viruses, it is rather rare in animal viruses; apart from caliciviruses, nodaviruses (BBV and FHV) are the only known examples (Hendry, 1991). In its overall architecture, the three-dimensional structure of calicivirus is more similar to TBSV and TCV than to other  $T=3$  viruses. These plant viruses have more prominent projections, unlike SBMV, BBV and FHV, at the local and strict 2-fold axes.

#### (b) Modularity of the coat protein structure

The location and the structural features of the arch-like capsomeres in the calicivirus suggest that they are dimers of the coat protein akin to capsomeres in the  $T=3$  plant viruses. The overall similarity in the structural features between the two sides (A1 and A2) of type A capsomeres and the B1 side of type B capsomeres suggests that each side (A1, A2 or B1) of the arch represents a molecule of the coat protein (Figure 5). The structure of the coat protein in the calicivirus can be described as having three distinct domains. These domains, P1, P2 and S (Figure 5) are so labeled to follow the notation used in the description of the coat protein structure in TBSV (Harrison *et al.*, 1978). The protruding part in calicivirus, 50 Å in height, consists of two domains: the distal head domain (P1) which appears bilobed, and the central stem domain (P2). However, in TBSV and TCV, the protruding portion of the capsid protein is only 25 Å and constitutes a single domain, referred to as P. In contrast to the arch-like formation of the projecting domains in the calicivirus, in TBSV and TCV, the P domains of the 2-fold related subunits oppose each other rather closely to appear more like a cylinder. Such projecting domains, as noted earlier, are absent in SBMV, BBV and FHV. These viruses only have the equivalent of S domains.

The proposed S domains of the individual subunits in the calicivirus structure associate to form a contiguous shell between the radii 115 and 150 Å as shown in Figure 6(b). Despite differences in the overall diameters, it is interesting to note that the S domains, in the  $T=3$  viruses mentioned earlier, form a contiguous shell of similar thickness and radius. It has been shown that, at the S domain level, the tertiary structures of the coat proteins in these viruses share striking similarities not only among themselves, but also with the coat proteins of comoviruses and picornaviruses ( $T=1$ , pseudo  $T=3$ ). In all these viruses the polypeptide chain folds into eight-stranded antiparallel  $\beta$ -barrel (Rossmann & Johnson, 1989; Harrison, 1990). The wedge-like shape of the  $\beta$ -barrel structure seems ideal to form the contiguous shell that is seen in these viruses. We hypothesize that the tertiary structure of the calicivirus coat protein may have a folding pattern, particularly in the S domain, similar to that found in  $T=3$  plant and insect viruses and  $T=1$  picornaviruses.

In the calicivirus structure, the mass density calculations, assuming a protein density of 1.30 g/cm<sup>3</sup> and 180 molecules of the coat protein, indicate that the molecular mass of the S domain is about 15 kDa and that of P1 and P2 domains together is 42 kDa. These molecular masses translate to approximately 150 and 420 amino acid residues in these domains, respectively. Most known  $\beta$ -barrel domains consist of 150 to 200 amino acid residues (Rossmann & Johnson, 1989). Thus, the mass density calculations is consistent with our hypothesis that the S domain may have a  $\beta$ -barrel structure.

One interesting question is what parts of the amino acid sequence of the capsid protein constitute the S and P domains in the calicivirus structure. The nucleotide sequence of the capsid protein gene of the primate calicivirus (D.O.M. & A.W.S., unpublished results) and of the feline, rabbit, San Miguel sea lion and human hepatitis E caliciviruses have been determined (Neill *et al.*, 1991; Meyers *et al.*, 1991; Tam *et al.*, 1991; Neill, 1992). However, it has not yet been possible to determine the precise molecular mass of the capsid protein in the mature virus. The molecular masses of the capsid precursor proteins in these viruses determined from their respective gene sequences are around 70 kDa. These molecular masses are significantly larger than those of the mature capsid proteins, which are estimated to be around 60 kDa (Schaffer, 1979; Neill, 1992). The amino acid sequences of the capsid proteins in these caliciviruses exhibit fairly good homology among themselves, with percentage similarity ranging from 50 to 70. Comparison of these sequences with the amino acid sequences of either the  $T=3$  plant and insect viruses or  $T=1$  picornaviruses indicate less than 45% similarity. However, there are regions, particularly between the residues 130 and 315, in the calicivirus capsid protein sequences that are homologous to the residues in the VP3 amino acid sequences of the picornaviruses.

Based on these observations, it has been proposed that the residues in this region may fold into a  $\beta$ -barrel structure (Tohya *et al.*, 1991; Neill, 1992). Our preliminary analysis of these sequences using the secondary structure prediction algorithms and three-dimensional profile search method proposed by Bowie *et al.* (1991), has also indicated that the amino acid residues 100 to 300 may fold into a  $\beta$ -barrel structure (A. Liu, S. Adams & B.V.V.P., unpublished results). It is quite possible that these residues in the calicivirus may constitute the S domain. Furthermore, calicivirus sequences show a high degree of homology in this region, upstream from this region they exhibit considerable variability (Neill, 1992). It is plausible that the residues 300 onwards constitute the P1 and P2 domains.

#### (c) Hinge region

One of the important features of the coat proteins in TBSV and TCV is the presence of the hinge peptide located between the P and S domains (Harrison *et al.*, 1978; Hogle *et al.*, 1986). This hinge peptide provides the necessary flexibility for the coat protein to adapt to quasi-equivalent environments. A close look at the regions that connect the P2 and S domains in A1, A2 and B1 suggests that such a hinge may be present in the calicivirus structure as well. The P2 domain of the A2 has a noticeable bend that is not present in the A1 (Figure 5(a) and (b)). In B1, however, the P2 and S domains are connected by only a slender mass density (Figure 5(c)). Furthermore, we can see small, but noticeable, changes in the relative orientation of the P2 domain with respect to the S domain in these subunits. These differences between the regions connecting the P and S domains in A1, A2 and B1 may have been caused by the presumed hinge between P and S domains.

#### (d) Protein-RNA interface

At the present resolution, an unequivocal definition of the boundary that separates the genomic RNA and the protein is not possible. The calculated molecular masses of the P and S domains, 42 kDa and 15 kDa, respectively, based on the mass density calculations, accounts for most of the molecular mass (~60 kDa) of the capsid protein. The S domain extends inward to a radius of 115 Å (Figure 7). Interior to the contiguous capsid shell formed by the association of the S domains, there is another shell of mass density between the radius of 110 and 85 Å (indicated as I in Figure 7). It is not clear whether the mass density in this inner shell is all due to the protein or if it includes some genomic RNA. This uncertainty stems firstly from our inability to choose the exact contour level that represents the protein and, secondly, because we do not know the precise molecular mass of the mature capsid protein. However, because of the contrast present between the solvent and the virus in the electron micrographs, the choice of the contour level

is well restricted, though not exact. The margin of error in the mass density calculations is estimated to be within 10%.

If one assumes that the mass density in this shell represents protein, then each subunit will have a contribution of approximately 8 kDa. It is quite likely that some part, if not all, of the mass in this shell is due to the protein. What portion of the calicivirus capsid protein is likely to be in this shell? It is our conjecture that the amino-terminal portion of the calicivirus capsid protein may be in this shell. It is known that in several of the simple spherical plant viruses and some animal viruses, the basic amino terminus interacts with RNA (Rossmann & Johnson, 1989). In some of these viruses, like TBSV, TCV and SBMV, the region that interacts with RNA is disordered. At the present resolution of our structural analysis it is not possible to address the question whether the protein mass is ordered or disordered. However, the presence of distinct structural features, such as protrusions and holes around the 5-fold axes (Figure 8(a)), in this shell, even prior to imposing the 3-fold symmetry, suggest that the mass density is most likely icosahedrally ordered.

#### (e) Possible ordered RNA

The shell between the radii of 45 Å and 85 Å shows features suggestive of ordered RNA. Tubes of density run between the icosahedral 3-fold axes through the icosahedral 2-fold axis forming a truncated dodecahedral structure (Figure 8(b)). This density at the icosahedral 2-fold axes may represent ordered RNA. The flat bit of mass density seen at the 5-fold axis is a contiguous part of the shell above that extends to this radius. It has been possible to visualize the nucleic acid in the crystal structures of several icosahedral viruses such as beanpod mottle virus (Chen *et al.*, 1989), Flock House virus (Fisher & Johnson, 1993), canine parvovirus (Tsao *et al.*, 1991) and bacteriophage  $\phi$ X174 (McKenna *et al.*, 1992). While the first two are ssRNA viruses, the latter two are dsDNA viruses. It has been also possible to identify the ordered RNA using electron cryomicroscopy and computer image analysis in cowpea mosaic virus, which is a close relative of beanpod mottle virus (Baker *et al.*, 1992).

In the case of the  $T=3$  Flock House virus, the structured RNA is on the icosahedral 2-fold axis forming a helical duplex (Fisher & Johnson, 1993). However, in the case of beanpod mottle virus, the ordered RNA is single-stranded and is found at the icosahedral 3-fold axis (Chen *et al.*, 1989). In both these viruses, it appears that the ordering of the RNA has been imposed by a close interaction between the genomic RNA and the icosahedrally ordered capsid protein. In Flock House virus, it has been suggested that the ordered RNA controls the capsid architecture (Fisher & Johnson, 1993). In this virus, the interaction between the capsid protein and RNA is a necessary element in the capsid assembly, providing a reason as to why the empty particles are not observed. In the calicivirus struc-

ture, it is quite possible that the putative ordered RNA may be in close interaction with the portion of the capsid protein present in the inner shell between the radius of 85 and 110 Å. In our electron microscopic observations, though we have found disrupted particles, we have not found empty particles of calicivirus. It has been found difficult to obtain empty particles of primate calicivirus. Further biochemical and structural studies are required to confirm the suggestion of ordered RNA in the calicivirus structure and to assess its role in the capsid assembly.

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