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Ice as a reservoir for pathogenic human viruses: specifically, caliciviruses, influenza viruses, and enteroviruses

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Summary Hundreds of isolates of viable bacteria and fungi have been recovered from ancient ice and permafrost. Evidence supports the hypothesis that viral pathogens also are preserved in ice repositories, such as glaciers, ice sheets, and lake ice. Proof may depend upon narrowing the search by applying specific criteria, which would target candidate viruses. Such criteria include viral pathogens likely to occur in great abundance, likely to be readily transported into ice, and then participate in ongoing disease cycles suggestive of their having been deposited in and subsequently released from ice. Caliciviruses, influenza A, and some enteroviruses appear to satisfy all three criteria. Environmental ice appears to be an important abiotic reservoir for pathogenic microbes. World health and eradication of specific pathogens could be affected by this huge reservoir.

Introduction

The major events that cause loss of virus infectivity are active during the transit period subsequent to viral release from one host cell and before they infect another host cell. These causes are ultraviolet radiation, drying (especially with cycles of rehydration), contact with inactivating substances (such as enzymes), oxidizing agents, and heat. Retention of virus infectivity is inversely proportional to exposure to these elements. Freezing, especially at very low temperatures without cycles of thawing and refreezing can greatly slow these degradative processes. For the majority of viruses, freezing maintains their integrity and viability. Environmental ice also traps and freezes viruses. It is our hypothesis that it entraps large numbers of pathogenic viruses.

All viruses are intracellular obligate parasites. Castello et al. [1,2] have documented the recovery and identification of viable phage from *B. subtilis*, and also have produced amplicons (PCR-generated

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replicates of genomic segments) of tomato mosaic tobamovirus from ice cores assigned dates of <500 to >100,000 years before present (ybp). Rogers et al. [8] recently postulated that selected pathogenic microbes survive and recycle through ice in a process they call "genome recycling". We now propose the hypothesis that selected viral pathogens of higher animals (mammals including humans) also should recycle from ancient ice repositories. But which animal viruses are likely to be present in ice? Some criteria for candidate viruses include those likely to occur in great abundance, readily transported into ice, and then likely to participate in ongoing disease cycles once released from the ice.

The caliciviruses (specifically those belonging to the genus *Vesivirus* within the family Caliciviridae) have primary cycles in ocean animals, and appear to satisfy all three criteria. Furthermore, they are readily grown and amplified in mammalian cell culture and are available in large numbers representing more than 40 serotypes (each serotype is an independent pathogen and infection with one serotype does not protect against disease from another) [14]. Other candidate viruses include some of the enteroviruses (e.g. polioviruses, echoviruses, and Coxsackie viruses), as well as the influenza viruses (family Orthomyxoviridae). Polioviruses are spread through oral–fecal cycles, and are distributed readily by contaminated water. Also, these viruses remain viable for long periods of time when frozen. Therefore, current disease control efforts should consider ice as a potential reservoir for this virus. Influenza viruses are spread by a number of biotic and abiotic means. Aquatic birds are the primary biotic reservoir for all influenza viruses [4], although influenza A subtypes also have been isolated in various mammals, including swine, horses, seals, whales, and humans. The 1918 H1N1 subtype was responsible for an estimated 20–40 million human deaths. Variants of this subtype have appeared in the human population since 1918, separated in time by decades of absence [10]. It has been detected in ice from Siberian lakes (Rogers et al., unpublished), but has yet to be found in polar ice or temperate glaciers. Surveillance efforts should focus on biotic and abiotic reservoirs of this virus.

The following concepts support the hypothesis that caliciviruses would likely be recovered from ancient ice. Both freshwater ice (e.g., glaciers and ice sheets) and sea ice may entrap and preserve enormous numbers of viruses. We estimate that 10^{17}–10^{21} viable microbes (including fungi, bacteria, and viruses) are released from melting ice annually [8]. Oceans interface directly with major planetary ice masses both new and ancient, and contribute to the ice mass content. In turn, oceans are composed partly of new and ancient ice melt water contents, which may be brought into immediate contact with ocean species from protozoa to mammals. Among these would be a heightened probability of viruses contacting the descendant host species of origin, thus increasing the probability of amplifying and recycling old virus. The oceans also provide numerous mechanisms for viral trafficking among marine ecosystems. Ocean currents continuously transport water and its biota to and from ice interfaces. Aquatic mammal species each with an individual viral burden follow ocean migratory routes for sometimes thousands of miles and some birds can migrate from South Africa to Barrow Alaska [6]. Andromadous fish and other migratory poikilotherms have extensive migrations sometimes including estuarine and fresh water routes that can be adjacent to glaciers. Parasites that may be intermediate hosts for viruses can be carried thousands of miles by their primary hosts, and predators can carry infectious viruses from the site of one feeding to numerous others [11]. In addition, the filter feeders (e.g. molluscan shellfish and certain zooplankton) can directly concentrate virus from the water column, which greatly increases the potentially infectious dose of virus delivered to the predator eating them [11]. The oceans also have efficient and massive systems for pumping particulates including viruses out of the water column, and depositing them on ice and land [11,15,20]. A 2 mm bubble rising through the water column will efficiently scavenge minute particulates from that 2 mm cylinder of water then burst at the surface where the surface-tension snap will project a droplet 15 cm into the air to be carried away by prevailing winds and redeposited elsewhere [11]. Storms and surf activities similarly redeposit wind-borne ocean contents, and avian species can deposit fish or other potentially virus-laden materials directly onto ice sheets or shore.

The systems outlined above can result in virus deposition onto ice, while other events assure their return to the ocean thus setting up cycles. These cycles can occur in a time continuum of days to millions of years depending upon the circumstances. Such a slice through time is seen as a spectacular event when eroding glacial cliffs crash into the sea carrying with them viruses recently deposited on their surface and those at all intermediate levels down to the most ancient locked in their deepest layers. Thus, evidence of short, medium, and long cycles for ice-associated pathogenic viruses in ocean animals should be found in present day populations. However, dating the age

of the viruses that infect animals today cannot be determined without first demonstrating the presence of such viruses in ice of known age.

Which viruses?

All viruses contain either DNA or RNA. The general ecology of the RNA viruses is much different than that of the DNA viruses. Their estimated rate of mutation is 10^{-3}—10^{-5} for any given nucleotide in their genome, and they lack the proofreading and repair mechanisms associated with DNA replication [3]. Holland [3] has estimated that substitutions (mutations) could occur in up to 50% of the base sequences without the virion losing viability. For the smaller RNA viruses (those with complete genomes of about 8000 bases) every single replicate of a genome could contain 1—8 errors, thus the estimated number of viable variants possible is 4^{4000} [3]. This does not include genomic recombinants, reassortants, deletions and additions, or gene repeats and relocations. Furthermore, it is not unusual for small RNA viruses to replicate in single cell layers of tissue culture and release up to 10^9 infectious virions (each genetically different) per ml of the surrounding tissue culture media. Thus, RNA viruses may be uniquely suited to survival not by specialization to a specific host or tissue but by using overpowering numbers of variants to adapt to new hosts in a diversity of ecologic settings. The mutagenic characteristics of RNA viruses should make them less useful for tracking mutational changes over time, than the more stable DNA viruses with their slower rates of change. However, the RNA viruses from ancient ice should be more easily found as pathogens active in contemporary populations because of their tremendous adaptive potential. If viable RNA viruses are being released from ice of any age including ancient ice, and if they then come into contact with phylogenetic descendants of previous hosts or perhaps some new host species, they could be expected to establish infection. The oceans and their diverse biota provide an ideal setting for these interactions, but suitable pathogenic viruses active in ocean-animal communities need to be identified to explore and test these possibilities.

There are several animal viral pathogens that are known to occur in the world's oceans, lakes, rivers, and streams. In addition to caliciviruses, enteroviruses [5], and orthomyxoviruses [10], adenoviruses [22], rotaviruses, and herpesviruses [7] are found in water from these sources. Most species within these groups retain viability after freezing and thawing [5]. The first marine mammal virus to be grown in cell culture was isolated from a California gray whale in 1968 in California. The virus was a small RNA virus [25]. Four years later (1972), the first virus isolated from any species of seal was recovered from an aborting California sea lion on San Miguel Island, California. This too was a small RNA virus [12]. Shortly thereafter these viral pathogens of marine mammals, and many more isolated in subsequent years, were found to belong to a newly described viral taxon and were named "caliciviruses" because of their unique cup-like surface features (Fig. 1(a) and (b)) [11,14,16,20]. Enteroviruses also have been isolated from marine mammals [22]. Enteroviruses are small RNA viruses with resistant capsid shells that protect the genome and would be excellent candidates for study except that they have not been shown to occur in abundance in the marine animal populations most extensively evaluated for autochthonous virus populations. Another interesting candidate for study is poliovirus, which is spread in water and retains its viability when frozen. Studies of this virus in ice are non-existent. However, ice has a high potential for being a reservoir for this virus.

Influenza viruses have been involved in occasional epizootics among seals. They can have widespread and devastating effects on various marine mammal populations. The various subtypes of this virus are spread extensively by migratory waterfowl, and are often asymptomatic in the birds. While the virus particles are enveloped in a membrane, they can survive for days in water (especially true for cold water), and are well preserved when frozen in ice.

The ecology of marine caliciviruses

The marine caliciviruses (Fig. 1) have been extensively reviewed [11,14,20], and fit the proposed criteria as the animal viruses most likely to be preserved in ice. They have numerical density in the ocean [11,14], are resistant to environmental effects [24], have an RNA genome [12], have been extensively studied [11,14,20], and show ecologic evidence of participating in possible ongoing cycles between the ice and the ocean. Caliciviruses (genus *Vesivirus*) are the only viruses known with a primary reservoir in ocean species including fish [21], yet readily infect terrestrial species including humans [13,20]. Approximately 156 calicivirus isolates were recovered from 27 species of animals [11,14]. Ten marine species have yielded 114 of these isolates, and the remaining 42 have been

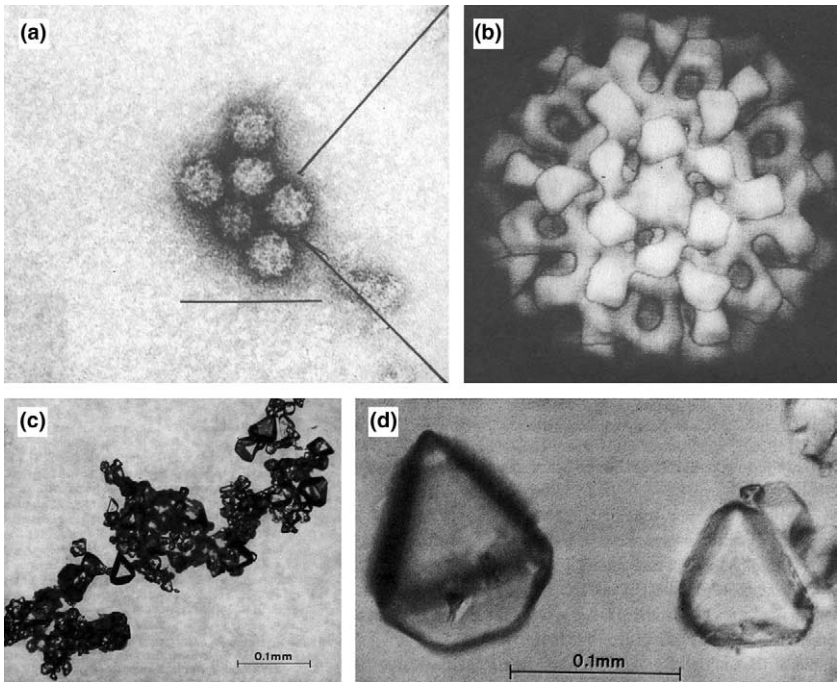

Figure 1 Caliciviruses. (a) Electron photomicrograph (bar equals 100 nm) of Cetacean calicivirus showing typical morphology. The virion in the bracket is oriented along the 5-fold axis. (b) Three dimensional reconstruction of an electron cryo-micrograph of a primate calicivirus shown in the 3-fold orientation at 22 Å resolution. Each of the 90 capsomeres is a dimer made up of capsid protein molecules and is oriented to form arch-like structures. This morphology and structure are not seen in other animal viruses but does occur in several plant viruses. (c) and (d) Crystals of San Miguel sea lion calicivirus type 1. The virus, grown in Vero cells was banded in CsCl from 9 ml of culture media and then pelleted by centrifugation and diluted with 25 volumes of phosphate-buffered saline and centrifuged at 78 g for 2 h. Crystalline samples were transferred to microscope slides for photomicrography (as described in [9]).

recovered from 17 species of terrestrial animals including humans [20]. Yet all isolates have an ocean presence. Thus, these viruses have unusually wide host ranges. For example, San Miguel sea lion virus type 5 can infect 16 different species as diverse as fish, seals, swine, rabbits, cattle, primates, and humans [20]. In addition to phylogenetically diverse host ranges, caliciviruses also have broad tissue tropisms [20]. Disease manifestations include abortion, agalactia, pneumonia, myocarditis, encephalitis, gastroenteritis, hepatitis and possibly thymic involution [20]. Extensive epidemiological studies have been carried out in some marine species [11]. For example, California grey whales annually migrate from the Sea of Cortez into the Chukchi and eastern Siberian Sea with return routes back through the Bering straits down the coasts of Alaska, Canada, the US mainland and Baja, Mexico into the breeding lagoons in the Sea of Cortez [11]. This species shows extensive exposure to a variety of calicivirus types [14,15]. These whales move through the migratory routes and populations of California sea lions and US and Russian northern fur seals, and Steller sea lions, all of which exhibit repeated exposure to multiple calicivirus types [11]. Extensive movements of caliciviruses into terrestrial species demonstrate active mechanisms for transporting viable viruses out of the sea to the landmass [11,14,20], which for our purposes could include ice. Ocean aerosol-contaminated forage has been suggested as the source of infection for one herbivorous species [15]. Evidence that tremendous numbers of caliciviruses build up in the water columns of the ocean is shown by two different studies. Caliciviruses can replicate $\leqslant 10^7$ infectious viruses per gram of spleen [24] in the opal eye perch, which is an ocean fish known to acquire infections with caliciviruses that cause disease in both marine and domestic mammals [21]. More than 10^6 caliciviruses are present in one gram of feces of a California grey whale sampled in California during its annual migration between the northern ice fields and Mexico, even though this individual had a high level of antibodies [15,20], suggesting that the infection was persistent and that along its migration route as many as $10^{11}-10^{12}$ caliciviruses were being dumped into the water daily by this single whale [20]. If only 10% of the theoretical number of caliciviruses released each

day by this single whale were scrubbed from the ocean and carried ashore on prevailing winds [11], this would deposit one calicivirus on every square centimeter over an area of 10 km^2. Caliciviruses survive more than 14 days when placed directly into artificial seawater and held at 15 °C [22], which greatly exceeds the time needed for ocean sprays and particulates laden with viruses to be deposited on glaciers and preserved by freezing. Perhaps one of the most important features for long term survival of caliciviruses in ice is their ability to form virus crystals (see Fig. 1(c) and (d)) under laboratory conditions involving phosphate buffered saline [9], which may mimic the conditions that occur in the unfrozen component of ancient ice.

Epidemiologic considerations

Calicivirus infections result in type-specific immunity. Therefore, a susceptible host population becomes resistant to that specific virus serotype, which then disappears. Subsequently, new individuals will be recruited into the host population primarily by reproduction, and once again a population that is susceptible will begin to build. If the original virus reappears in this new population, it likely has been reintroduced from some reservoir source outside the host population in question [11,15,17].

The first known occurrence of any calicivirus infection was 1932, when a sudden outbreak of a swine disease in California was thought to be Foot and Mouth disease. It was, however, a "new virus disease" subsequently named vesicular exanthema of swine [11]. After 20 years and numerous cycles of eradication and reintroduction, the disease spread from California throughout the US. Four years later, it disappeared altogether. This new virus was thought to have had a de novo origin or to have come out of some unknown natural reservoir [11,14]. However, in 1972, 40 years after its first appearance, ocean animal reservoirs and fish were recognized as the primary source of infection [12,21]. The sudden appearances, disappearances, and reappearances could not be satisfactorily explained simply on the basis of the known reservoirs and their ecologic relationships; although there were efforts to do so [11,14]. There are other examples of calicivirus pulses that might be explained by releases of virus from ice. Vesicular exanthema of swine calicivirus types J and K were isolated on just one occasion in Secaucus, NJ in swine fed garbage presumed to have contained raw fish [14]. Twenty-four years later bowhead whales in Barrow, Alaska and California sea lions were determined to have antibodies specific to the types J and K caliciviruses [15,19]. This example of active mechanisms for storing and transporting caliciviruses between Atlantic and Pacific oceans has been used often to make the point that calicivirus movements in nature are occurring between oceans but the precise mechanisms have remained a mystery [11]. Now polar ice storage and release in conjunction with east and west seasonal migrations of bowhead whales across the northern Arctic latitudes could explain this mystery. Bowhead whales have an intimate and yearlong association with the Northern ice packs [19], and this could provide a reasonable explanation.

Vesicular exanthema virus serotype A_{48} was isolated once from swine in Fontana, California in 1948. It is the "Type Species" for the caliciviruses but was not isolated again until 1968 [11,25]. That isolation was from the California grey whale shedding high numbers of caliciviruses as described in the previous section. If this particular calicivirus serotype was stored in the polar ice, it could have been picked up and carried south by grey whales, which would explain its reappearance after 20 years. In 2002, a Steller sea lion in Alaska was shedding calicivirus, which, based on a 450 base amplicon, has 100% sequence identity with the 54-year old isolate of serotype virus A_{48} [17]. This long-term disappearance and reappearance is remarkably similar to those described for influenza virus [8,10].

Reptilian calicivirus was isolated from five species of poikilotherms at the San Diego Zoo in 1978, but was not isolated from marine mammals on offshore Islands from California to Oregon until 1987. At that time California sea lions and Steller sea lions as well as northern fur seals were all infected [9]. During annual sampling of these marine mammal populations from 1972 through 1994, the Reptilian calicivirus was isolated only during 1987 [11]. This general pattern holds true for nearly all calicivirus types isolated. They tend not to stay in a given population from year to year, but if they do reappear, it is usually years later [14,20]. Once again, this "hiding out" phenomenon could be explained by periodic reinfection of target species with viable viruses released from ice.

Caliciviruses and human health

A calicivirus designated SMSV-5 was first isolated in 1973 from blisters on the flippers of a northern fur

seal in the Pribiloff Island group in the Bering sea [13]. Although the caliciviruses of this class were not known to infect people, the SMSV-5 virus infected a researcher causing blisters on the hands and feet. Shortly thereafter, a new calicivirus type was isolated from the throat of a very sick marine mammal biologist that had been working with Steller sea lions in the Gulf of Alaska [13]. These findings demonstrate that the caliciviruses of ocean origin are human pathogens and represents a paradigm shift in the ecology of human viral diseases [13,20]. Walrus calicivirus was isolated from walruses in 1976 and again in 1986 [11]. It was not associated with overt disease in walrus, but caused hepatitis when inoculated into swine [11], thus suggesting that caliciviruses may account for human cases of unexplainable hepatitis. This theory has been examined [18]. Normal Red Cross blood donors (397 tested) show a 5% incidence of antibodies to the Marine caliciviruses, while blood donors with evidence of hepatitis (200 tested) were 8% positive. Persons (32 tested) who developed hepatitis following transfusion or dialysis, although negative for the known hepatitis viruses, were 22% positive for the Marine caliciviruses [18]. Ten sera from aborting women in Oregon were tested and four (40%) were positive for calicivirus antibody [11,18]. In Sweden, 15% of the sera from 100 aborting women were positive [18]. Caliciviruses having zoonotic potential are also associated with abortion in at least six other mammal species [11,18,23].

The increasingly frequent and mysterious appearance of epidemics involving new and severe human pathogens from unknown reservoirs provides a wake-up call suggesting that traditional disease-monitoring systems lack sensitivity for detecting non-traditional diseases. Ecological studies that address threats to susceptible host populations are complex but usually focus upon host reservoirs rather than ecosystem reservoirs, and have not foretold the emergence of all recent new epidemics. We have identified an ocean reservoir for amplifying and spreading caliciviruses of the genus *Vesivirus*, some of which are newly recognized and emerging human pathogens [18]. Their importance as agents of human disease sequestered in ocean populations and possibly global ice reserves suggests that contemporary disease monitoring systems need to identify and to monitor all potential ecosystem as well as host reservoirs; and that glacial ice is a potentially important ecosystem reservoir for some animal and human viruses.

In summary, the marine caliciviruses described exhibit properties that make them attractive as candidates for viruses surviving in ancient ice. The discovery that viruses are present in ancient ice from polar glaciers [1,2] provides an excellent model that could explain cyclic calicivirus events because their "hiding out" strategies followed by reappearances has not been fully satisfied by other explanations. Similarly, the decades-long disappearances and subsequent reappearances of influenza A subtypes, in addition to their presence in Siberian lake ice lends some circumstantial support to the hypothesis that ice serves as a reservoir for viable pathogenic viruses. Control, surveillance, and eradication efforts for pathogenic viruses should consider potential abiotic reservoirs for these microbes, including the possibility of entrapment in environmental ice.

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