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Persistent Infectivity of a Disease-Associated Herpesvirus in Green Turtles after Exposure to Seawater

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ABSTRACT: Herpesviruses are associated with several diseases of marine turtles including lung-eye-trachea disease (LETD) and gray patch disease (GPD) of green turtles (*Chelonia mydas*) and fibropapillomatosis (FP) of green, loggerhead (*Caretta caretta*), and olive ridley turtles (*Lepidochelys olivacea*). The stability of chelonian herpesviruses in the marine environment, which may influence transmission, has not been previously studied. In these experiments, LETD-associated herpesvirus (LETV) was used as a model chelonian herpesvirus to test viral infectivity after exposure to seawater. The LETV virus preparations grown in terrapene heart (TH-1) cells were dialyzed for 24 to 120 hr against aerated artificial or natural seawater or Hank's balanced salt solution (HBBS). Fresh TH-1 cells were inoculated with dialyzed LETV, and on day 10 post-infection cells were scored for cytopathic effect. Virus samples dialyzed up to 120 hr were positive for the herpesvirus DNA polymerase gene by polymerase chain reaction. Electron microscopy revealed intact LETV nucleocapsids after exposure of LETV to artificial seawater or HBSS for 24 hr at 23 C. LETV preparations remained infectious as long as 120 hr in natural and artificial seawater at 23 C. Similar results were obtained with a second culturable chelonian herpesvirus, HV2245. LETV infectivity could not be detected after 48 hr exposure to artificial seawater at 30 C. Since LETV and HV2245 remain infectious for extended periods of time in the marine environment, it is possible that FP-associated and GPD-associated herpesviruses also may be stable. These findings are significant both for researchers studying the epidemiological association of herpesviruses with diseases of marine turtles and for individuals who handle turtles in marine turtle conservation efforts.

Key words: Green turtle, *Chelonia mydas*, herpesvirus, infectivity, seawater, disease.

Herpesviruses have been associated with several diseases of chelonians (tortoises and turtles). Tortoise herpesviruses cause conjunctivitis, stomatitis, tracheitis, and pneumonia in Hermann's (*Testudo hermanni*), spur-thighed (*Testudo graeca*), and Central Asian tortoises (*Testudo horsfieldii*) (Biermann and Blahak, 1994; Kabish and Frost, 1994; Marschang et al., 1997). A herpesvirus also has been identified in green turtles (*Chelonia mydas*) with experimentally induced fibropapillomatosis (FP), and has been associated with naturally occurring FP in green, loggerhead (*Caretta caretta*), and olive ridley (*Lepidochelys olivacea*) turtles (Jacobson et al., 1991; Herbst et al., 1998; Quackenbush et al., 1998; Lackovich et al., 1999). FP has been transmitted by scratch inoculation with cell free tumor filtrates (Herbst et al., 1995a). In addition, herpesviruses have been associated with two diseases of mariculture-reared green turtles, gray patch disease (GPD), a necrotizing dermatitis of post-hatching green turtles (Rebel et al., 1975) and lung-eye-trachea disease (LETD), characterized by conjunctivitis, pharyngitis, tracheitis and pneumonia (Jacobson et al., 1986). GPD has been transmitted by scratch inoculation of naïve sea turtles with bacteria-free preparations derived from GPD lesions (Rebel et al., 1975). Many herpesviruses have been successfully cultured from tortoises. However, despite numerous attempts to cultivate GPD-associated and FP-associated herpesviruses, the LETD-

associated herpesvirus (LETV) is the only herpesvirus which has been successfully isolated and maintained in culture from a marine turtle.

The ability of herpesviruses to be transmitted among sea turtles in a marine environment is likely to be influenced by the ability of these viruses to maintain infectivity for extended periods in seawater. While persistence of enteric viruses including hepatitis A virus, poliovirus, echovirus, and rotavirus in the ocean has been well documented (Lo et al., 1976), the ability of herpesviruses to survive in a marine environment has not been previously investigated. This study investigated the ability of LETV to remain infectious after prolonged exposure to seawater.

LETV was established in cell culture from an infected green turtle (Jacobson et al., 1986). Virus infected cell suspensions were diluted 1:10 into Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 5% fetal bovine serum and antibiotics, and then incubated on 80 to 90% confluent terrapene heart cells (TH-1; American Type Culture Collection CCL 50) grown as monolayers in vented flasks in 5% CO_2 at 28 C. For comparison, herpesvirus HV2245, isolated from a Hermann's tortoise (Biermann and Blahak, 1994), was similarly prepared and included in initial experiments. Both LETV- and HV2245-infected cells showed 75 to 80% cytopathic effect (CPE) in approximately four days. Virus was harvested by removing the majority of the medium, freezing and thawing the flask, and scraping the infected cell monolayer culture into suspension. Each virus preparation was titered at time zero and after dialysis. Virus preparations were serially diluted 10-fold in DMEM/F12 out to 10^{-8} and inoculated on TH-1 cells grown in 96-well flat-bottom plates (Costar, Corning Incorporated, Corning, New York, USA). An aliquot of each serial dilution was plated into five wells (150 μl per well). On day 10 post-infection, cells were scored for CPE and tissue culture infectious dose 50 ($\log_{10}$ $TCID_{50}$/ml) was calculated.

Virus preparations were dialyzed against artificial seawater (Aquarium Systems, Mentor, Ohio, USA), Hank's balanced salt solution (HBSS, Life Technologies, Gaithersburg, Maryland, USA) or natural seawater. HBSS was used instead of DMEM/F12 as medium control because HBSS maintains neutral pH without CO_2 buffering. Artificial seawater salinity was adjusted to 35 parts per thousand and specific gravity to 1.026. Osmolality was measured with a vapor pressure osmometer (Wescor, Logan, Utah, USA). Osmolality of artificial seawater was 1,000 mmol/kg which was equal to the osmolality of natural seawater collected from the Whitney Marine Laboratory (Marineland, Florida, USA). The osmolality of HBSS was 280 mmol/kg, and the osmolality of natural seawater collected from Sebastian Inlet, Indian River Lagoon was 550 mmol/kg (Melbourne, Florida, USA). Virus preparations (0.5–1.0 ml) were transferred to 10 kD molecular weight cutoff dialysis cassettes (Pierce, Rockford, Illinois, USA) and dialyzed against 1 L aerated artificial seawater, HBSS, or natural seawater maintained in incubators at 15, 23, or 30 C with continuous stirring. Incubation temperatures were based on seawater temperatures measured during FP transmission studies conducted with green turtles (Herbst et al., 1995a). Osmolality equal to dialysis medium was achieved inside the dialysis cassette within 30 min. After 0, 24, 48, 72, 96, and 120 hr, virus preparations were removed from dialysis cassettes and tested by polymerase chain reaction (PCR) for the presence of the herpesvirus DNA polymerase gene (VanDevanter et al., 1996). All samples were positive. PCR positive samples exposed to artificial seawater and HBSS for 24 hr at 23 C were visualized by electron microscopy. Particles with icosahedral nucleocapsids, characteristic morphology of herpesviruses, were evident in those samples (Fig. 1). Preparations containing viral particles were then

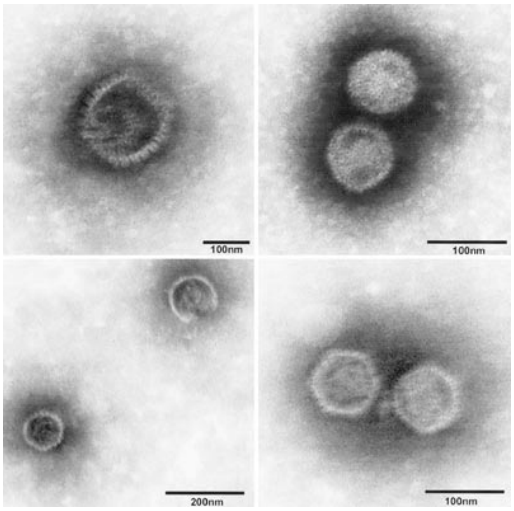

FIGURE 1. Negative stain electron microscopy images of lung-eye-trachea virus (LETV) after 24-hr exposure to artificial seawater or Hank's balanced salt solution (HBSS) at 23 C. The top left and right panels illustrate herpesvirus virions after exposure to HBSS. The icosahedral nucleocapsid structure is clearly visible and is approximately 100 nm in diameter. The bottom left and right panel illustrates herpesvirus virions after exposure to artificial seawater.

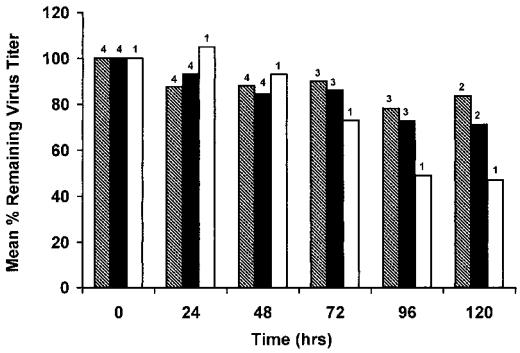

FIGURE 2. Persistent infectivity of lung-eye-trachea virus (LETV) after dialysis for 120 hr at 23 C. Mean % remaining virus titer of LETV exposed to artificial seawater ■, to Hank's balanced salt solution (HBSS) ▨, and natural seawater from Sebastian Inlet □ are shown. Numbers above each bar are equal to number of replicates.

titered for infectivity. Although the viral envelopes were not clearly visible, four independent experiments demonstrated that LETV preparations remained infectious after a 48 hr exposure to artificial seawater or HBSS at 23 C (Fig. 2). The virus preparations used in each experiment had slightly different initial titers (four-six $\log_{10}TCID_{50}$/ml), but the titer consistently decreased approximately one $\log_{10}TCID_{50}$/ml (10%) after 48 hr dialysis. Similar results were obtained for HV2245. In one experiment LETV exposed to natural seawater for up to 120 hr at 23 C remained infectious with a three $\log_{10}TCID_{50}$/ml (50%) decrease in titer. In a separate experiment, LETV infectivity was reduced approximately one $\log_{10}TCID_{50}$/ml after 96 hr when dialyzed against artificial seawater or HBSS at 15 C, and was reduced two $\log_{10}TCID_{50}$/ml after 96 hr at 23 C, but all infectivity (>four $\log_{10}TCID_{50}$/ml) was lost after 48 hr at 30 C.

Many factors contribute to the spread of infectious diseases in animal populations. Stability of a pathogen outside the host is a critical factor in the epidemiology of a disease. The ability of a pathogen to remain infectious in the environment of its host may facilitate disease transmission. These experiments provide the first evidence that disease-associated marine herpesviruses retain their infectivity for extended periods of time outside the host in a marine environment.

These experiments utilized both artificial and natural seawater and virus infected cell lysates that simulated virally infected cells shed or sloughed from a diseased marine turtle into marine habitats. Consistent results obtained in four independent dialysis-exposure experiments supported the conclusion that LETV infectivity persists after exposure to seawater. Additionally, the tortoise herpesvirus HV2245 had similar stability following exposure to seawater, suggesting that other chelonian herpesviruses such as FP-associated and GPD-associated herpesviruses may share attributes necessary for survival under harsh environmental conditions.

It is likely that the extent of herpesvirus persistence in a natural marine environment will depend upon numerous other environmental factors. The composition of

seawater is in a constant state of flux and virus stability will likely be influenced by changes in temperature, currents, depth, sunlight, and pH. Organic material, especially protein, or cellular debris may stabilize viruses and increase survival (Lipson and Stotzky, 1984). Suspended sediments, such as clay, may also have a similar effect of stabilizing infectious virus (Clark et al., 1998). In addition to the physical and chemical components, the biological composition of a specific site can negatively or positively influence virus survival. Microbes producing antiviral substances have been documented in marine habitats and have been reported to decrease the amount of time infectious virus persists in the environment (Girones et al., 1989). The presence of a higher level of such microbes at one location verses the absence or lower level at another location may account for differences in virus survivability and disease transmissibility.

Because the temperature of seawater in marine turtle habitats fluctuates throughout the year, virus preparations were dialyzed against artificial seawater at temperatures ranging from 15 C to 30 C (Herbst et al., 1995a). LETV lost infectivity after 48 hr exposure to artificial seawater at 30 C, suggesting that at higher temperatures herpesviruses shed into seawater may be inactivated more rapidly. This study simulates the persistence of free virus shed into seawater. It is likely that temperature will affect the persistence properties of herpesviruses within infected lesions differently. In addition, this study does not address the effect temperature may have on the disease process. For instance, lesions in green turtles infected with GPD-associated herpesvirus had faster onset, development, and increased severity at 30 C (Haines and Klesse, 1977). In contrast, green turtles inoculated with filtrates containing FP-associated virus developed tumors during the winter months (low 15 C) and continued to develop as the water temperature increased (high 30 C) (Herbst et al., 1995a). It is difficult to speculate on the true effect of water temperature on virus transmissibility especially since sea turtles traverse numerous marine habitats of varying water temperatures during their life history.

The ability of LETV to retain infectivity in a marine environment is not surprising since herpesviruses are known to be quite stable. Human herpesvirus 1 can persist for eight weeks at ambient humidity and temperature (Mahl and Sadler, 1975). The swine herpesvirus, pseudorabies, remained infectious for at least seven days on fomites such as whole corn, polypropylene, loam soil, and vinyl rubber (Schoenbaum et al., 1991). Marine herpesviruses may also be able to survive out of water for extended periods of time on surfaces of equipment, instruments, boats, or in facilities that contact sea turtles infected with herpesviruses.

The stability of other viruses in marine habitats has been previously assessed (Lo et al., 1976; Fujioka et al., 1980). Most of the research has focused on the potential contamination of marine and estuarine habitats with human enteric viruses as a result of wastewater release. If enteroviruses can survive in marine habitats, coastal waters used for recreation and harvest of shellfish may serve as a reservoir and thus become a public health hazard (Fujioka et al., 1980). Human enteric viruses including hepatitis A virus, poliovirus, echovirus, and rotavirus not only persist after exposure to seawater but have also been documented to be taken up by certain aquatic organisms (Goyal et al., 1979; LaBelle et al., 1980; Hejkal and Gerba, 1981).

Transmission of mammalian herpesviruses is usually by contact of infected cells in saliva, urogenital excretions, or free virus in aerosols (Fields et al., 1996). At this time little is known about the mechanism of transmission of herpesvirus infections in marine environments. Herpesviruses from infected turtles may be transmitted to uninfected individuals by direct contact between turtles, by vectors, by contact with

virus in sediments or suspended in seawater. The data of the present study are evidence that chelonian disease-associated herpesviruses may retain their infectivity for extended periods of time in the marine environment. As a result, common foraging grounds which potentially attract a high density of susceptible hosts may serve as a reservoir for infectious viruses and may facilitate transmission (Herbst and Klein, 1995b).

The results of this study are significant for both researchers studying diseases of marine turtles and various aspects of marine turtle conservation. Caution should be taken when handling marine turtles. Instruments, tools, hands, and work surfaces that have been in contact with turtles that could harbor herpesviruses should be cleaned to minimize risk of transmission. Ongoing attempts are being made to cultivate FP-associated herpesvirus and GPD-associated herpesvirus to assess their ability to maintain infectivity after exposure to seawater.

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