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A herpes-like virus in king crabs: Characterization and transmission under laboratory conditions

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ABSTRACT

A herpes-like virus was found infecting the antennal gland and bladder epithelium in the blue king crab *Paralithodes platypus* from the eastern area of the Sea of Okhotsk. Electron microscopic analysis of antennal gland samples from blue king crabs with histologically confirmed signs of disease revealed virus particles, which were mostly hexagonal in shape and located primarily in the nucleus; these particles were rarely observed in the cytoplasm of infected cells. Most virus particles ranged in size from 115 to 125 nm. Hemocytes of the red king crab *Paralithodes camtschaticus* in cell culture could be experimentally infected with virus from thawed antennal gland samples of the blue king crabs with histologically confirmed signs of viral infection. Clear signs of infection were observed in hemocyte cultures at 3–4 days post-inoculation as small foci of highly vacuolated formations. These formations included several nuclei and were surrounded by a halo of small cytoplasmic bubbles containing actin and tubulin. As demonstrated by electron microscopic studies, no virus-like particles were found in the cells 1 day post-inoculation, but particles become abundant at 7 days post-inoculation. We developed a consensus primer PCR method for amplification of a region of the herpesviral DNA-directed DNA polymerase. Primers were designed to target sequences encoding highly conserved amino acid motifs covering a region of approximately 800 bp. Thus, macroscopic, histological and ultra-structural examinations of blue king crabs infected with a virus and the molecular identification of the pathogen revealed the presence of herpesviruses. The frequency of the herpes-like viral infection in natural populations of blue king crabs in the Sea of Okhotsk ranged from 0% to 3% in different years.

1. Introduction

Viral diseases have threatened the aquaculture industry, causing serious economic losses (Takahashi et al., 1994; Hill, 2002; Supungul et al., 2002). Some viral pathogens are highly infectious and easily transmissible via horizontal transmission from animal to animal (Johnson, 1983; Renault and Novoa, 2004). Red king crabs *Paralithodes camtschaticus* and blue king crabs *Paralithodes platypus* are economically important species that are fished in the North Pacific Ocean (Otto and Jamieson, 2001). Crabs infected with viruses typically have mild or no symptoms of disease, but some viruses, particularly the herpes-like virus, are suspected to cause king crab mortality (Sparks and Morado, 1986).

Since the first crustacean virus was described by Vago (1966), more than 30 different viruses have been reported in crabs (Johnson, 1976b, 1978, 1983, 1984, 1986; Payen and Bonami, 1979; Ahne, 1994; Bonami and Zhang, 2011), including those related to the known virus families Reoviridae, Bunyaviridae, Roniviridae and a group of Bacilliform enveloped nuclear viruses (Bonami and Zhang, 2011). By the mid 1970's, TEM observations of infected tissues in marine animals were commonly used as diagnostic tools. Today, more specific and rapid molecular tools, such as PCR, quantitative RT-PCR, *in situ* hybridization and cell culture methods, have been developed for the detection of viruses in tissue samples from infected fish and crustaceans (Lightner and Redman, 1998; Nunan et al., 2000; Assavalapsakul et al., 2003; Li et al., 2006; Li and Shields, 2007; Hope et al., 2010).

The lack of reliable crustacean cell and tissue culture methods that can be used as diagnostic tools has blocked studies of viral

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diseases in commercially valuable species of marine crustaceans (Johnson, 1984; Lightner and Redman, 1998; Rinkevich, 2011). Numerous attempts to develop cell lines from commercially important species such as shrimps, prawns, lobsters and crabs have been reported but almost all of them are unsuccessful (Chen and Kou, 1989; Nadala et al., 1993; Lu et al., 1995; Tapay et al., 1996, 1997; Chen and Wang, 1999; Toullec, 1999; Li and Shields, 2007; Claydon and Owens, 2008; Claydon et al., 2010; Liang et al., 2012). Only recently, a continuous cell line from the testicular tissues of the mud crab *Scylla serrata* was established (Shashikumar and Desai, 2011.

Here, we report the results of macroscopic, histological and ultra-structural examinations of king crabs infected with a virus and the molecular identification of the pathogen. We also report for the first time, that red king crab hemocytes can be experimentally infected in cell culture with the virus from thawed antennal gland samples from infected blue king crabs.

2. Materials and methods

2.1. Materials

Blue king crabs *P. platypus* and red king crabs *P. camtschaticus,* were caught in the eastern area of the Sea of Okhotsk, Russia during two trapping (2006, 2010) and two trawl surveys (2007, 2012). All monitoring was carried out at the site of the West Kamchatka shelf between latitudes 51°18′ and 58°51′N, within depth range 9–322 m (Fig. 1). At each station, all crabs were removed from the catch, sorted by species, sex, size, and molting stage as described in the «Manual for a description of Far Eastern Decapods» (Rodin et al., 1979). Crabs with sigs of disease become lethargic, and fail to demonstrate a defensive reaction to external stimuli (Ryazanova et al., 2013). The abdomen of healthy crabs is tightly pressed to the body, whereas the abdomen of crabs with sigs of disease appears "swollen" and distended. All crabs with these pathological signs of disease and additional individuals that appeared healthy upon gross examination were dissected as described in Johnson (1980). The internal organs of dissected crabs were examined and assessed for disease signs, such as the increase in the volume of connective tissue in the cavity of the cephalothorax, a cream color of the antennal gland and bladder, and the presence of black or brown (presumably melanized) inclusions in the antennal gland, bladder and connective tissue. To calculate prevalence of the disease, we used the ratio of the number of crabs with macroscopic signs of disease (external and internal or only internal) divided by the number of dissected animals.

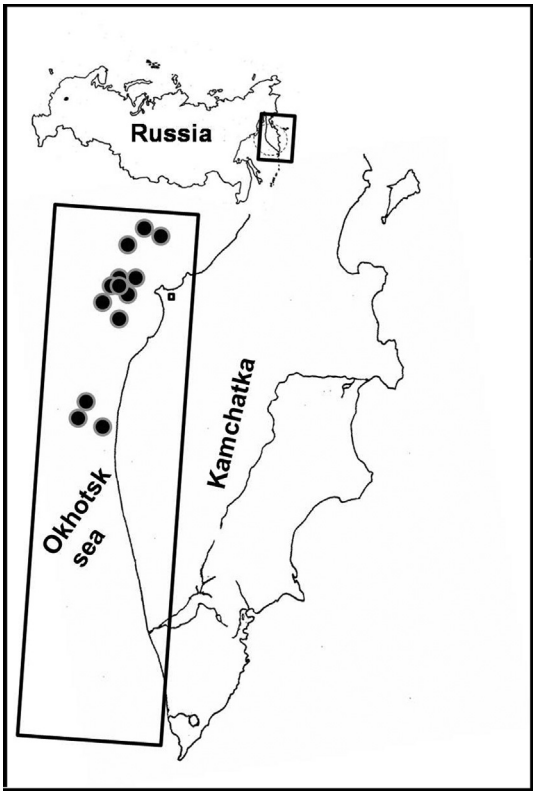

Fig. 1. Map of the study area with sampling stations where infected king crabs *Paralithodes platypus* and *P. camtschaticus* were caught.

Tissue samples were fixed for 24–48 h in Davidson's fixative (Bell and Lightner, 1988) prepared in filtered seawater and processed using standard histological techniques. Five-micron sections were stained with Meyer's hematoxylin–eosin (H&E) and examined using light microscopes (Olympus Al-2 or Leica DM 4500) with digital cameras. Small pieces of antennal glands of the infected crabs with macroscopic signs of viral infection were frozen at −25 °C and then stored for approximately 3 months at -80 °C for later bioassay studies. In addition, the small pieces of the antennal gland of infected blue king crabs were preserved in 96% ethanol at room temperature (RT) for later PCR analysis.

2.2. Raman spectroscopy of infected tissues

Raman spectroscopy, a vibrational spectroscopic technique based on the interaction between monochromatic light and investigated molecules, is a very powerful method to provide a fingerprint by which different molecules can be identified (Hanlon et al., 2000). To identify the main component of pigmented inclusions in crabs with signs of disease, we used Raman spectroscopy. Frozen sections (20–25 microns) of unfixed antennal gland from blue king crabs with histologically confirmed signs of infection were placed onto the aluminum substrate and air dried. The sections were prepared using a HM 560 Cryo-Star cryo-microtome (Thermo Scientific, USA). The micro-Raman spectrometer Renishaw in via Reflex based on a Leica DM 2500 M microscope, with 100x objective Olympus LM Plan FL UIS2, was used to obtain Raman spectra. The spectra were obtained under 532-nm laser excitation; the power of the laser was set to 10 mW; an exposure time for each spectrum was 2 s. Each spectrum was the result of 5 acquisitions. The initial data were smoothed and normalized to the base-line.

2.3. Electron microscopy

For transmission electron microscopy, samples of infected antennal gland tissues from blue king crabs with histologically confirmed signs of infection (12 crabs) were fixed with 2.5% glutaraldehyde in 0.1 M cacodyl buffer (pH 7.2) or in 4% paraformaldehyde (Sigma, USA) in 0.1 M PBS (pH 7.2) for 4 h at 4 °C, rinsed then in sterile seawater several times for 24 h, postfixed with 1% OsO4 in distilled water for 20 min, washed five times in distilled water for 5 h, then processed through an ethanol and acetone series before embedding in resin (Epon-Araldite). Semithin and ultrathin sections were cut using a Leica EM UC6 ultramicrotome (Leica Microsystems, Germany). Semithin sections were stained with 1% of methylene blue. Ultrathin sections were stained with uranyl acetate (0.5% water solution, 15 min) and lead citrate (0.4%, 10 min). For cell culture material, virus-infected and control hemocytes were harvested after 1–7 day-incubation and centrifuged at 3000 × g for 5 min, then the pellets were fixed in 2.5% glutaraldehyde, post-fixed with 1% OsO4, dehydrated as above and embedded in resin (Epon-Araldite). Ultrathin sections were also stained