Short communication

CpSBV, a systemic virus of the edible crab, Cancer pagurus (L.)

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Following the description of the first crustacean virus by Vago (1966), reports of crab viral diseases were numerous during the 1970s and early 1980s (Bonami 1980; Johnson 1984; Mari & Bonami 1986; Adams & Bonami 1991). During this period, when shrimp aquaculture was beginning, pathological problems were scarce and crabs were used as biological models for crustacean virus investigations. More recently, little information was reported on crab viruses, although several shrimp viral diseases have been described (Lightner 1996). To date more than 50 viruses closely related to the known viral families (van Regenmortel, Fauquet, Bishop, Cartens, Estes, Lemon, Maniloff, Mayo, McGeoch, Pringle & Wickner 2000) have been recognized in aquatic crustaceans, with more than half of those from crabs (Shi 2000).

During experimental investigations (Corbel, Zuprizal, Shi, Huang, Sumartono, Arcier & Bonami 2001) on the possible hosts of white spot syndrome virus (WSSV) – a highly pathogenic shrimp virus (Takahashi, Itami, Kondo, Maeda, Fujii, Tomonaga, Supamattaya & Boonyaratpalin 1994; Huang, Cai, Song, Wang, Yu & Yang 1995; Wongteerasupaya, Vickers, Sriurairatana, Nash, Akarajamorn, Boonsaeng, Panyim, Tassanakajon, Withyachumnarnkul & Flegel 1995) – a new viral

disease was seen in the crab, *Cancer pagurus* (L.). Initially abnormal mortalities were recorded in a batch of negative control animals during WSSV transmission experiments and unknown virus-like particles were seen in the haemolymph of both WSSV-infected and negative control crabs. Because this agent was found free in large amounts in the haemolymph, and its characteristics were quickly recognized as close to those of the Bunyaviridae family (Bouloy 1991; van Regenmortel *et al.* 2000), it was designated *Cancer pagurus* systemic bunya-like virus (*Cp*SBV).

We report here, our investigations on this new virus, its isolation and purification, its structure and size, its development in infected cells and the nature, size and structure of its genome.

Cancer pagurus, locally called 'tourteau' or 'dormeur' (sleeper), were caught in Brittany (France) and send alive to Montpellier. They were maintained in aerated sea water between 11 and 15 °C, which was renewed every 3 days and they were fed three times a week with fresh mussels. After transportation, they were left to recover for 3 days before use in experimentation. After showing signs of disease, animals for further investigations were quarantined for 2 weeks, mortalities recorded, and the presence of virus particles in the blood checked by direct observation in transmission electron microscopy (TEM) (Jeol Europe, Croissy, France) of a drop of haemolymph.

For virus purification Tris-sodium (TN) buffer was used (0.02 M Tris–HCl, 0.4 M NaCl, pH 7.4). Low-speed centrifugations were performed in a JS13.1 rotor using a Beckman J2.21 preparative-centrifuge (Beckman Coulter, Roissy, France). For

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high-speed centrifugation, an SW28.1 rotor in a Beckman L5.50 ultra-centrifuge was used. Sucrose gradients (14–45%, w/w in TN buffer) were prepared and extracted using an automatic gradient maker/extractor Auto-Densiflow (Büchler, Labconco, Kansas City, MO, USA). The optical density (OD) from the fractions was recorded using an ISCO UA6 UV monitor (Isco Inc., Lincoln, NE, USA) at 254 nm wavelength. Fractions were separated using a Gilson FC203 fraction collector (Gilson-France, Villier le Bel, France).

All steps of the purification were checked by TEM after negative staining with 2% phosphotungstic acid (PTA), pH 7.0, on carbon–collodium-coated grids using a Jeol 200X electron microscope (Jeol Europe, Croissy, France).

Tissues of infected animals were fixed in 4% glutaraldehyde, post-fixed in 2% osmium tetroxide in cacodylate buffer (0.2 M cacodylate, pH 7, 4% NaCl) and embedded in Spurr resin. Semi-thin and ultra-thin sections were prepared on an LKB Bromma 2088 ultra-microtome (Bromma, The Netherlands). Sections were contrasted with uranyl acetate and lead citrate according to Reynolds (1963). Semi-thin sections were stained with 2% toluidine blue.

For nucleic acid extraction, the last centrifugation pellet of purified virions was re-suspended in 0.01% diethyl pyrocarbonate (DEPC)-treated water. Virions were digested with proteinase K (5% final concentration) for 1 h at 37 °C followed by Sarkosyl treatment (0.5% final concentration) at 60 °C for 2 h. RNA was obtained by two phenol extractions, one phenol/chloroform/iso-amyl alcohol (50/49/1) and two chloroform/iso-amyl alcohol (49/1) extractions. The RNA was then precipitated by two volumes of absolute alcohol after addition of LiCl (0.4 M, final concentration) at −20 °C overnight. It was pelleted by centrifugation for 20 min in an Eppendorf centrifuge, washed with cold 70% ethanol and then dried. The final pellet was re-suspended in DEPC-treated water.

Denaturing (1% formamide) 1% agarose gel electrophoresis was used, according to Sambrook, Fritsh & Maniatis (1989). The size of the viral RNA was estimated using ssRNA markers (GIBCO-BRL, Life Technologies SARL, Cergy Pontoise, France), of 9.46, 7.46, 4.40, 2.37, 1.35 and 0.24 kb. To establish the nature and structure of the genome, the extracted nucleic acid was treated with DNase I, RNase DNase-free and Mung Bean nuclease (MBN) according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

During transmission experiments with WSSV, to determine potential hosts of this virus, mortalities were recorded both in WSSV-injected crabs and in negative control animals injected with sterile TN buffer. These unexpected mortalities in the negative control group (66%) led us to check the WSSV-infected and control crabs for the presence of WSSV, by direct observation using TEM. In WSSV-infected dying animals, WSSV particles were found mixed with another type of rounded particle (Fig. 1). In dying animals from the negative
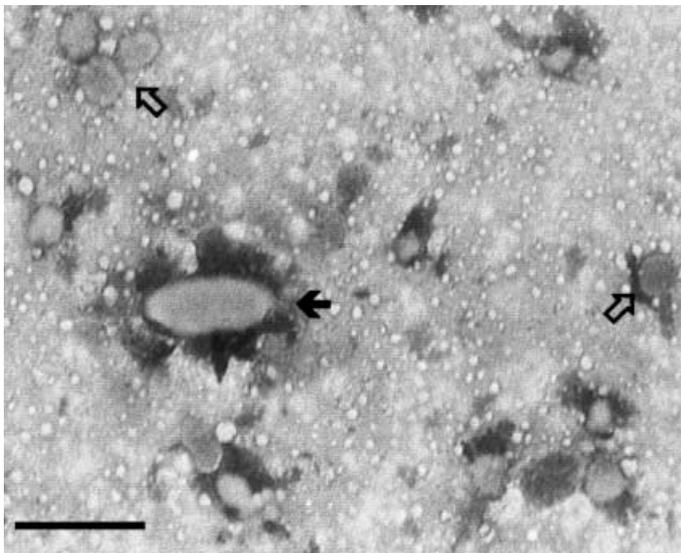

Figure 1 Direct observation by TEM of haemolymph of WSSV-infected crabs. Note enveloped WSS virus (solid arrow) and other rounded particles (open arrows). 2% PTA (bar = 300 nm).

Figure 2 Direct observation by TEM of haemolymph of a moribund negative control crab. Note the numerous enveloped viral particles free in the haemolymph. 2% PTA (bar = 200 nm).
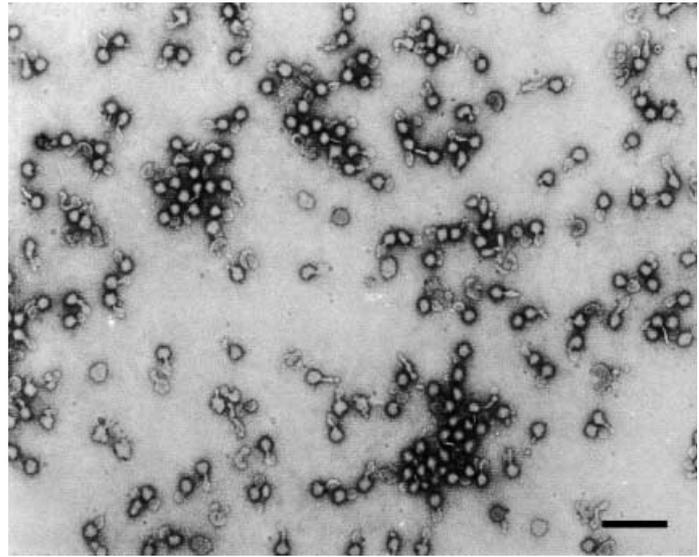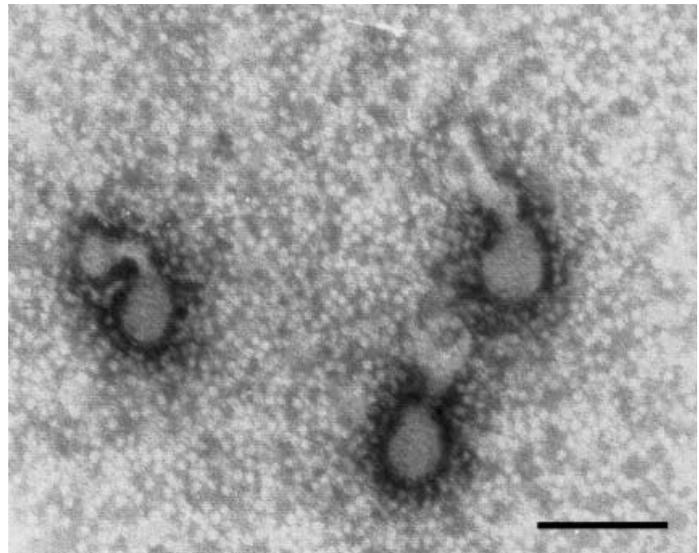

Figure 3 Higher magnification of *Cp*SBV as observed by negative staining. Virions exhibit a small tail-like structure which appears to be a part of the envelope. 2% PTA (bar = 100 nm).

control group, this new agent was found alone without WSSV (Fig. 2). This indicated that the virus-like particles were not a shrimp virus contaminant but were naturally present in the crabs used for our experiments and may have been activated by the stress of the injection.

Transmission electron microscope observation of the haemolymph from dead crabs by negative staining showed that the *Cp*SBV was about 60–70 nm in size, enveloped, round in shape and exhibited a small tail-like structure (Figs 2 & 3). The infected haemolymph showed large amounts of free viral particles, which persuaded us to consider the disease as true viraemia (Fig. 2).

Transmission electron microscopy of gills, hepatopancreas and heart tissue showed virions in the cytoplasm of connective tissue cells (Fig. 4). The viral particles were free in the plasma, between cells, or accumulated in cytoplasmic vesicles limited by a classic trilaminar membrane. It seems that the *Cp*BSV accumulated in these cytoplasmic vesicles by membrane budding (Fig. 4). No nucleocapsid structure was clearly seen in the vicinity of cytoplasm.
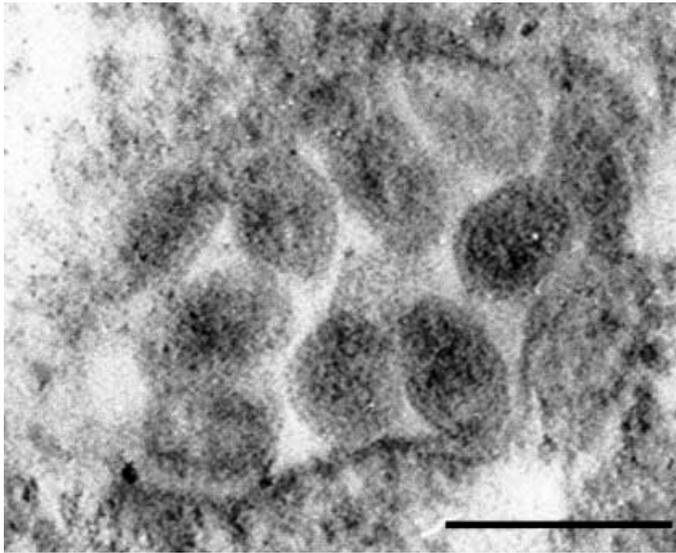
Figure 4 Ultra-thin section of hepatopancreatic tissue of infected crab. Virions accumulate in a cytoplasmic vesicle limited by a unit membrane. Some particles seem to bud through this vesicle membrane in a process of morphogenesis (bar = 100 nm).
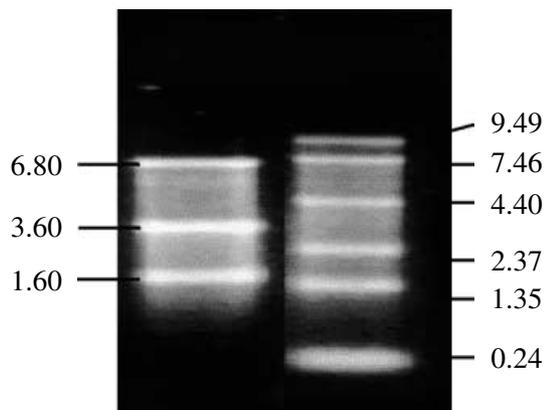

Figure 5 Agarose gel electrophoresis (1%) of viral nucleic acid. In lane A, 3 fragments are seen: a large fragment (L) about 6.80 kb, a medium fragment (M) of about 3.60 kb, and a small fragment (S) of about 1.60 kb. Lane B: ssRNA markers (size expressed in kb).

Infected haemolymph and gills were used for virus purification. Tissues were homogenized in TN buffer using a Potter tissue blender (Wheaton, Polylabo, Strasbourg, France). After a clarification at 2500 *g* for 10 min, the supernatant was pelleted for 1 h at 130 000 *g*. After the pellet was re-suspended in TN buffer, it was layered onto a sucrose gradient (15–45%, w/w) and centrifuged for 1 h at 100 000 *g*. Bands of interest were recovered, diluted in TN buffer and finally pelleted for 1 h at 130 000 *g*.

The sucrose gradient showed two peaks of UV absorbance, each containing a large amount of viral particles. There was no apparent difference in structure and size between the two viral fractions. Purified particles were grossly rounded and about 70 nm in diameter. They all exhibited a characteristic, small tail-like structure, 70–110 nm in length and 25–35 nm in diameter. No other structures, such as nucleocapsids were recognizable.

After nucleic acid extraction, agarose gel electrophoresis revealed three bands, indicating a tri-segmented genome (Fig. 5). The largest fragment (L) is about 6.80 kb, the middle fragment (M) about 3.60 kb, and the smallest (S) about 1.60 kb in size.

The viral nucleic acid was digested with RNase and MBN, and not with DNase. This demonstrated the genome is formed with three ssRNA molecules (L, M and S).

For experimental transmission of the disease, we used crabs previously maintained in quarantine and tested for absence of the agent. Six crabs were injected with 0.2 mL of a *Cp*SBV purified suspension (one in five dilution with TN 1×). The injection was performed at the basal joint of the fifth pereiopod. The animals were maintained in plastic tanks at a water temperature of 14 °C. A control test was carried out with six crabs inoculated with a buffer solution (TN 1×).

Clinical signs and mortality were recorded in both infected and negative control animals for 20 days post-injection. Mortality in the WSSV-infected group reached 100% 2 weeks later. In contrast, no mortality was observed in the negative control group 20 days post-injection. Blood of moribund infected animals exhibited virus particles identical

to those previously injected and ultra-thin sections observed in TEM revealed similar cytological lesions in the target tissues.

The *Cp*SBV is a pathogenic enveloped cytoplasmic virus, developing by budding in connective tissue cells and haemocytes of the marine edible crab, *Cancer pagurus*, collected in Brittany (France). It is grossly spherical, about 60–70 nm in diameter and its genome is formed of three fragments, L, M and S of ssRNA of 6.8, 3.6 and 1.6 kb, respectively. Its characteristics suggest that it is closely related to the Bunyaviridae family (van Regenmortel *et al.* 2000). This agent is not the first crustacean virus related to the Bunyaviridae family (Bang 1971; Hoover & Bang 1978; Johnson 1984), which includes the S virus which infects crabs (*Liocarcinus depurator* L., *Carcinus maenas* L., *Carcinus mediterraneus* Cz.) from French Atlantic and Mediterranean coasts (Bonami, Vago & Duthoit 1971; Bonami, Veyrunes, Cousserans & Vago 1975; Bonami 1980; Mari & Bonami 1986; Mari 1987).

The *Cp*SBV is morphologically different from the S virus of crabs by its general shape (rounded, instead of pleomorphic), size (around 60–70 nm, instead of 80–100 × 180–300 nm), its superficial structure (no spikes associated with a cross-hatched structure of the envelope) and the presence of a characteristic small tail-like structure extruding from the envelope. Both are systemic agents and are found free in very large numbers in circulating haemolymph, indicating a true viraemia. Both appear to develop by budding in cytoplasmic vesicles where they accumulate before release into the circulating blood. Recently, morphologically similar virus particles were observed in diseased mud crab, *Eriocheir sinensis*, in China (Hubei Province) (Z. Shi & S. Zhang, personal communication).

Experimentally, the disease takes 7–12 days to kill injected healthy animals that do not display any gross sign of disease. The occurrence of this virus in wild populations of crabs is unknown, but the fact that the crabs we obtained from storage tanks of a commercial company exhibited a relatively high infection prevalence, suggests that this virus is present in the natural environment and may be associated with the mortalities which occur in storage tanks from time-to-time.

This virus is the first described infecting this species of crab, but records of viral infections in marine crabs are numerous, particularly in the species *Liocarcinus* (*Macropipus*) *depurator* (Bonami 1980), the shore crabs *Carcinus maenas* and *C. mediterraneus* (Mari & Bonami 1986; Mari 1987) and the edible crab *Callinectes sapidus* (Johnson 1984). Viruses constitute the most numerous and most important pathogens of this group of commercially important animals.

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