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Short Communication

Natural occurrence of *White spot syndrome virus* and *Infectious hypodermal and hematopoietic necrosis virus* in *Neohelice granulata* crab

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ABSTRACT

White spot syndrome virus (WSSV) and *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV) are two infectious agents associated to economic losses in shrimp aquaculture. As virus spread occurs through vectors and hosts, this study sought to verify the presence of WSSV and IHHNV in *Neohelice granulata* crab from Lagoa dos Patos estuary in Brazil and nearby shrimp farms. DNA extractions were performed with phenol/chloroform protocol. Molecular diagnosis was carried out by nested PCR for WSSV and one-step PCR for IHHNV. Results showed the presence of WSSV on crabs of both Lagoa dos Patos and farms, while IHHNV was found only on crabs collected in estuary. This is the first study to report IHHNV presence in *N. granulata*. Moreover, as analyzed crabs had no clinical symptoms or showed *in situ* mortality, we suggest its use as a bioindicator for virus occurrence in aquatic environments.

1. Introduction

The economic impact of viral diseases in the world shrimp production has been shown to be a limiting factor for shrimp aquaculture development (Lightner et al., 2012; Walker and Mohan, 2009). Since 1991, losses greater than US$ 6 billion have been estimated worldwide as result of pandemics due to the penaeid viruses (Lightner, 2011). In Brazil, recent problems were caused by *Infectious myonecrosis virus* (IMNV) (Poulos et al., 2006) and by *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV) in northeastern Brazil (Braz et al., 2009), *White spot syndrome virus* (WSSV) in Santa Catarina (Cavalli et al., 2010, 2011; Seiffert et al., 2005), Ceará and Bahia. All these agents are associated to diseases that constrain shrimp production and exportation.

WSSV is a double-stranded rod-shaped DNA virus, which belongs to *Nimaviridae* family (Mayo, 2002). Clinical signs of disease are lethargy, emaciation, presence of white spots on carapace and reddish discoloration of the body (Durand et al., 1997). Since its first appearance in 1992 (Chou et al., 1995), the virus has rapidly spread to different regions of the world. First notifications of WSSV in Brazil occurred in 2005 in the South (Santa Catarina) and Northeast (Ceará). In Santa Catarina, the disease affected more than 1400 ha of shrimp ponds, causing a production decline from 4189 tonnes in 2004 to 480 tonnes in 2006 (Seiffert et al., 2005).

IHHNV is a non-enveloped single-stranded DNA virus, which belongs to *Parvoviridae* family (Bonami et al., 1990). Infected *Litopenaeus vannamei* exhibit clinical signs such as cuticle and rostrum deformities, and reduced growth rate (Kalagayan et al., 1991). Size reduction has a significant impact on production with economic losses that range 50% of market value (Lightner and Redman, 1998). IHHNV infects tissues of ectodermal and mesodermal origins, such as gills, cuticular epithelium, connective tissue, hematopoietic tissue, lymphoid organ, antennal gland and cord nerve. The first occurrence of IHHNV in shrimps was observed in specimens of *Litopenaeus stylirostris* in Hawaii (Lightner et al., 1983). Since then, the virus has been detected on the Pacific coast of South America from Peru to Mexico (Lightner, 2011), in different regions of Asia (Flegel, 1997), Australia (Owens et al., 1992) and Brazil (Braz et al., 2009; Pantoja et al., 1999; Tang and Lightner, 2006).

Both WSSV and IHHNV are spread through vectors, and crustaceans are considered potential hosts for them (Escobedo-Bonilla et al., 2008). Failure to exclude vectors and infected hosts can introduce new virus into the environment (Lightner, 2005). *Neohelice granulata* crab is a semiterrestrial species found in intertidal zones of estuaries, salt marshes and mangroves of Atlantic Ocean (Spivak, 2010), including Lagoa dos Patos, located in Rio Grande do Sul, Brazil. Recently, the presence of WSSV in *N. granulate* was reported in Santa Catarina (Marques et al., 2011), which makes it

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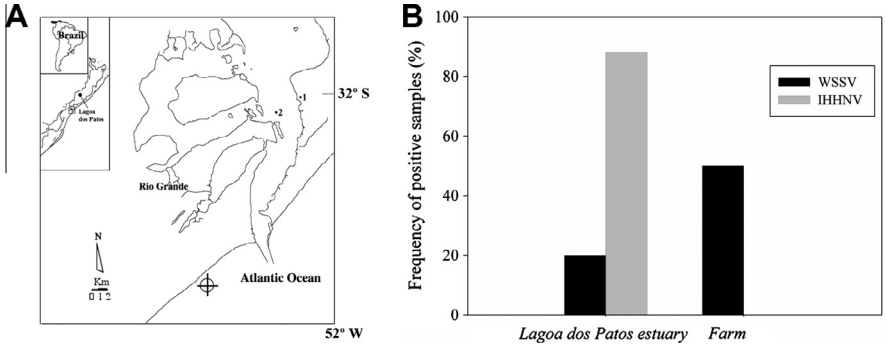

Fig. 1. (A) Map of São José do Norte (1) and Lagoa dos Patos estuary and (2), Brazil, where the samples were collected. (B) Frequency of positive crabs in estuary ($n = 50$) and shrimp farms ($n = 30$). WSSV was detected in 10 crabs from estuary samples (20%) and 15 from farm samples (50%); IHHNV DNA was confirmed in 44 crabs from estuary (88%), but not in farming samples (0%).

Table 1
Primers used in the study. One nested PCR amplified WSSV genome and one simple PCR amplified IHHNV genome.

	Forward primer	Reverse primer	Amplicon	Reference
WSSV 1st reaction	146F1 5′ACTACTAACTTCAGCCTATCTAG 3′	146R1 5′ TAATGCGGGTGTAATGTTCTTACGA 3′	1447 bp	Lo et al. (1996)
WSSV nested PCR	146F2 5′ GTAACTGCCCCTTCCATCTCCA 3′	146R2 5′ TACGGCAGCTGCTGCACCTTGT 3′	941 bp	Lo et al. (1996)
IHHNV	389F 5′CGGAACACAACCCGACTTTA 3′	389R 5′GGCCAAGACCAAAATACGAA 3′	389 bp	Tang et al. (2000)

a potential host of WSSV and IHHNV in cultures and in nearby ponds. In this study, the presence of these viruses was investigated in *N. granulata* crabs from Lagoa dos Patos estuary and nearby shrimp farms.

In summer 2008, *N. granulata* crabs were collected in the Lagoa dos Patos estuary ($n = 50$) and in a shrimp farm (*L. vannamei*) ($n = 30$) from the nearby São José do Norte, Rio Grande do Sul, Brazil (Fig. 1A). DNA extractions from shrimp gill tissue were performed with phenol/chloroform protocol (Sambrook et al., 1989). Shrimp DNA quality was checked with primers 143F 5′ TGC CTT ATC AGC TNT CGA TTG TAG 3′ and 145R 5′ TTC AGN TTT GCA ACC ATA CTT CCC 3′, that amplify 848 bp from decapod gene (Lo et al., 1996).

WSSV and IHHNV DNA amplifications were performed using primers described in Table 1. Amplifications were performed with the following conditions for both steps of WSSV nested-PCR: one cycle of 94 °C for 4 min, 55 °C for 1 min, and 72 °C for 2 min, followed by 39 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min with a final elongation step of 5 min at 72 °C; and for IHHNV one-step PCR: one cycle of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for for 30 s, and 72 °C for 1 min with a final elongation step of 7 min at 72 °C.

Positive and negative controls were included in both reactions. Amplicons were resolved in 1% agarose gel, stained with ethidium bromide and viewed on a UV transilluminator. PCR for all positive cases was repeated at least twice. Partial WSSV and IHHNV DNA of positive samples were sequenced using the automatic sequencer MegaBACE 1000 (GE Healthcare Life Sciences, Brazil). BLASTn tool was used to compare the obtained sequences to equivalent genomic regions of WSSV and IHHNV available at GenBank. For histopathological analysis, gills were gently cut out and fixed in Davidson's fixative for 24 h. Histologic sections were stained with hematoxylin and eosin and Periodic Acid Schiff's (PAS).

The presence of WSSV was detected in 15 crabs from the farm samples and in 10 from the estuary samples. IHHNV DNA was confirmed in 44 crabs collected at the Lagoa dos Patos estuary, but not in farming samples (Fig. 1B). These results were confirmed by genome fragments analysis, whose nucleotide sequences showed high similarity with WSSV and IHHNV sequences available at GenBank (*E value* = 0). Histological examination of WSSV and IHHNV positive individuals showed no significant alterations, indicating that *N. granulate* is an asymptomatic carrier for both viruses.

WSSV is found along the southern Atlantic coast and in Lagoa dos Patos, as well as in shrimp ponds of this region (Cavalli et al., 2010, 2011). As WSSV, IHHNV is also well distributed in coastal and oceanic waters of Brazil, with infected shrimp being reported in northeastern and southern Brazil (Lenoch, 2011). However, infection with this agent in Rio Grande do Sul, the southernmost Brazilian state, had not been previously reported, nor had its presence in *N. granulata* crabs. It is noteworthy that IHHNV was found in crabs living in the natural environment, but not in shrimp farms. This suggests that IHHNV may be a normal constituent within the crabs and raises the possibility that these animals have a role inintroducing the virus into shrimp farms. However, further studies are necessary to confirm this hypothesis.

Viral particles in vectors and reservoirs may represent important sources of contamination, introducing disease and precluding good yields of shrimp. Crustaceans like crabs act as vectors representing important sources of viruses spread. Moreover, infected crabs, like *N. granulata*, with no clinical symptoms or mortality could make them an important tool to monitor the presence of WSSV and/or IHHNV in culture systems or natural waters. An evidence for this role is that WSSV was detected in shrimps collected at Lagoa dos Patos estuary in a close time (Cavalli et al., 2011). Aquatic species have been used as environmental bioindicators to monitoring water quality and presence of pathogenic microorganisms. A recent study suggests *Crassostrea gigas* oyster as a shrimp farm bioindicator of WSSV (Vazquez-Boucard et al., 2012). In this sense, the present work shows that use of crabs as bioindicators in shrimp farms can represent an advance in the control of viral infectious agents.

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