EVALUATION OF WHITE SPOT SYNDROME VIRUS (WSSV) IN WILD SHRIMP AFTER A MAJOR OUTBREAK IN SHRIMP FARMS AT LAGUNA, SOUTHERN BRAZIL

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RESUMO

Avaliação do vírus da mancha branca em camarões nativos após ocorrência da doença em fazendas de cultivo em Laguna, sul do Brasil

O vírus da síndrome da mancha branca (WSSV) infectou camarões da espécie *Litopenaeus vannamei* cultivados em fazendas na região de Laguna, sul do Brasil, causando enormes perdas econômicas. O objetivo deste estudo foi verificar a presença, ou não, do vírus no camarão selvagem presente no sistema estuarino de Laguna, que recebe efluentes das fazendas de camarão. As amostras foram coletadas no verão de 2006 (fevereiro – 48 camarões juvenis) e no inverno (agosto – 66 juvenis). A presença do vírus foi testada por PCR "single-step" e "nested" nos 114 animais selvagens de três espécies diferentes (*Farfantepenaeus paulensis, Farfantepenaeus brasiliensis* e *Litopenaeus schmitti*). Todas as amostras foram negativas para os métodos de PCR empregados, indicando que a prevalência desta doença em animais selvagens deve ser menor de 5% por período de amostragem, ou menor que 3%, se considerarmos o número total de camarões amostrados. Os resultados sugerem que WSSV não deve estar distribuído extensamente entre os camarões nativos, mesmo após a grande dispersão da doença nas fazendas. Entretanto, mais esforços são necessários a fim de confirmar esses resultados.

PALAVRAS-CHAVE: Vírus da Síndrome da Mancha Branca, WSSV, Reação em cadeia da Polimerase, camarão, Brasil.

ABSTRACT

White spot syndrome virus (WSSV) infected *Litopenaeus vannamei* cultivated in ponds at Laguna, Southern Brazil, and caused huge economical losses. The objective of this study was to determine if WSSV infected wild shrimp were present in the Laguna estuarine system, which receives effluents of the shrimp farms. Samples were collected in the 2006 austral summer (February – 48 juvenile shrimp) and winter (August – 66 juvenile shrimp). Virus presence was tested by one-step and nested PCR in the 114 wild shrimp of three different species (*Farfantepenaeus paulensis, Farfantepenaeus brasiliensis* and *Litopenaeus schmitti*). All samples were negative for PCR method employed, indicating that prevalence of this disease in wild animals must be smaller than 5% per sampling period, or smaller than 3% when considering total number of tested shrimp (114 shrimp). So far, we may say that WSSV is not widely spread among native shrimp, even after strong disease outbreak in farms. However, more efforts need to be made in order to confirm these results.

KEYWORDS: White spot syndrome virus, WSSV, Polymerase Chain Reaction, shrimp, Southern Brazil.

1 – INTRODUCTION

White spot syndrome virus (WSSV) is the cause of a viral disease which affects mainly cultured shrimp (Lightner 1996). It is a double-stranded rod-shaped DNA virus, and belongs to the Nimaviridae family (Wang *et al*. 1995, Mayo 2002, Yi *et al.* 2003). The virus targets various tissues of ectodermal and mesodermal origin (Momoyama *et al*. 1995, Chang *et al*. 1996, Lightner 1996) and clinical signs include lethargy, anorexia, presence of white spots on the cuticle and reddish to pink discoloration (Durand *et al*. 1997). However, WSSV can also infect asymptomatically (Peneido-Guevara & López-Meyer 2006). Very high mortality rates have been reported for this disease, with 100% death of raised shrimp within 3–10 days after the onset of clinical signs (Nakano *et al*. 1994, Wang *et al*. 1995).

WSSV was first detected in Asia, with occurrences in Taiwan (Chou *et al*. 1995), Japan (Nakano *et al.* 1994), and Korea (Park *et. al.* 1998). Since these first events, the disease has spread among most Asian countries, as well as in the Americas (Lightner 1996, O.I.E. 2003). In Ecuador, WSSV was responsible for the decline of shrimp production, with negative impact on the economy of this country (FAO 2004).

In November 2004, white shrimp *Litopenaeus vannamei* produced in farms (total of 1400 hectares) of Imarui lagoon in Laguna, Southern Brazil, were infected by WSSV. Mortality rates reached 90%, causing losses over US$ 3 million (Seiffert *et al.* 2006). This first WSSV outbreak in Brazil was reported to OIE on January 20, 2005 (available at ftp://ftp.oie.int/infos_san_archives/eng/2005/en_050121v18n03.pdf), and more recently, a new WSSV outbreak was reported in *L. vannamei* farms in Ceará,

Northeastern Brazil, (http://www.oie.int/eng/info/hebdo/AIS_58.HTM#Sec0)

The majority of the 20 viral diseases identified for marine shrimp were first described in culture systems, and prevalence of these viruses in native populations seems to be directly related to the enhancement of shrimp culture activity (Primavera 2006). The spread of viruses in nature occurs despite treatment and monitoring of effluents, mainly because most producers do not conduct proper pond disinfection. Therefore, natural water bodies around ponds may receive contaminated effluents with high loads of pathogenic microorganisms.

To our knowledge, there is no available information regarding possible infection of native shrimp in the Laguna embayment after the major WSSV outbreak in shrimp farms. Therefore, the main goal of this work was to verify the presence of infected wild shrimp in the Laguna estuarine system.

2 – MATERIAL AND METHODS

2.1 – Study Area

The Laguna estuarine system is located in the state of Santa Catarina, Southern Brazil (Lat.: 28ºS, Long.: 48º W). It is a "choked" system composed of three main lagoons (Figure 1): Mirim, at North; Imaruí, the largest central lagoon; and Santo Antonio, in the South, comprising a total area of 220 Km^2. The lagoons are shallow (average 2.0 m depth) with hydrodynamics mainly controlled by wind, since tides play no major role (Fonseca & Netto 2006). Mean air temperatures is 13º C in the winter and 22º C in the summer, and total annual rainfall is 1,260 mm, with no marked differences along the year (Fonseca & Netto 2006).

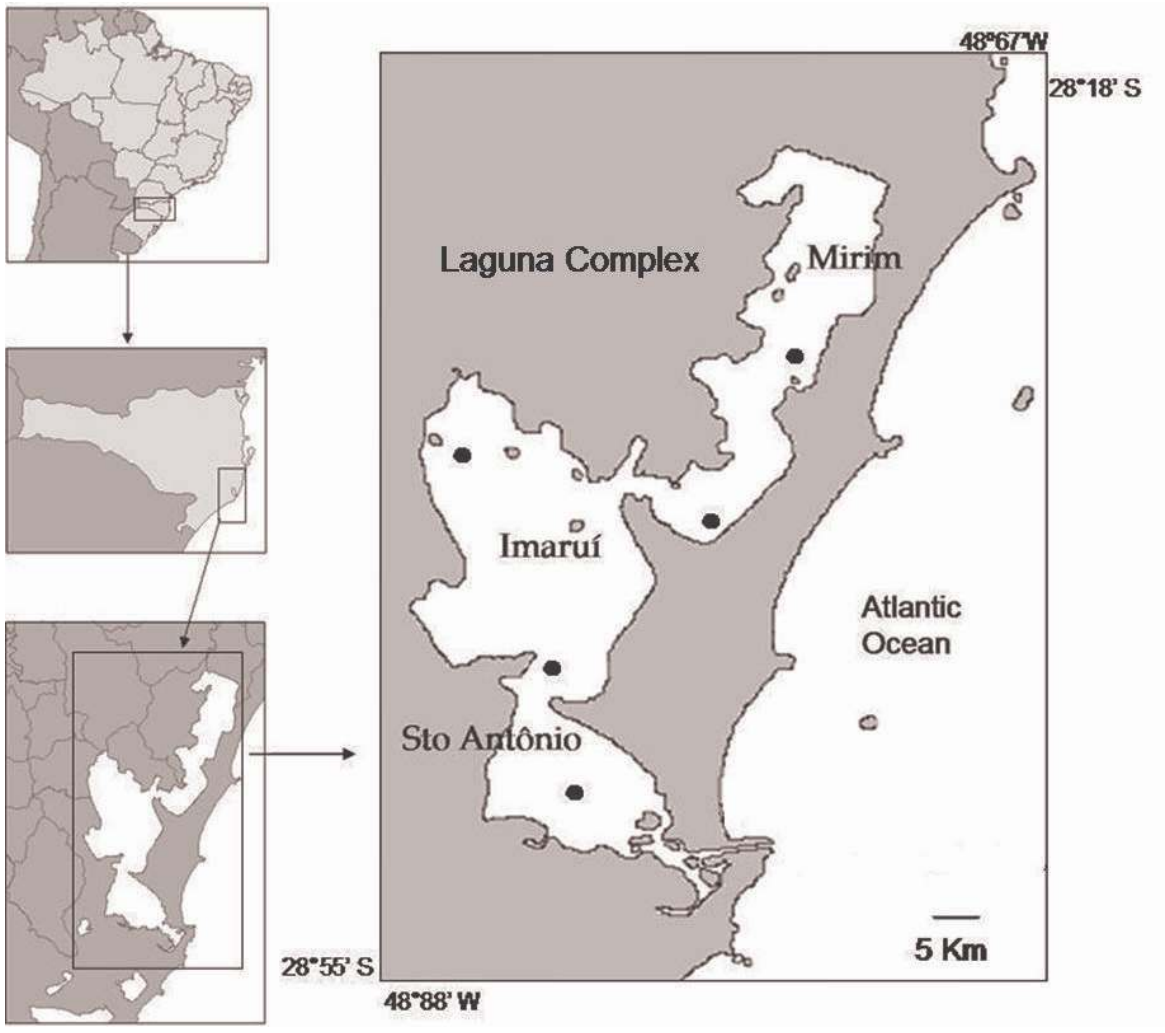


FIGURE 1 – Laguna estuarine system, Southern Brazil (● sampling stations).

2.2 – Shrimp Collection

Juvenile shrimp were collected with a seine net along the Laguna estuarine system (Figure 1). In the austral summer (February 2006), 48 juveniles (6.04 ± 2.72 g) were sampled, while in August 2006 (austral winter) a total of 66 shrimp (5.61 ± 2.01 g) were collected randomly along the main axis of the lagoon. Three penaeid species were found in the lagoon: *Farfantepenaeus paulensis*, *Farfantepenaeus brasiliensis* and *Litopenaeus schmitti*. The shrimp were fixed with ethanol 96 GL immediately after collection and sent to laboratory for DNA analysis. The sampling number of each period was determined considering a large (infinite) population, test sensitivity of 95%, confidence limits of 95% and desired target prevalence of 5%. However, if total number of shrimp collected in both periods is considered, prevalence goes down to 3%.

2.3 – Viral and shrimp DNA extraction

50-100 mg of shrimp gill tissue was used for DNA extraction. Samples were combined in a pool of five specimens. The shrimps were grouped according to species, making 15 pools in summer (10 pools of *F. paulensis*, four of *F. brasiliensis* and one of *L. schmitti*) and 14 pools in winter. In this last case, pools were made of only *F. paulensis*. For DNA extraction DNAzol reagent (Invitrogen) was used, according to manufacturer instructions, with some modification. First, gills were digested with 500 µl extraction buffer (100mM NaCl, 10mM Tris-HCl, 25mM EDTA, 0.2% SDS) and 10 µl Proteinase K overnight. After extraction, DNA pellet was re-suspended in 80 µl of TE-RNAse (10mM Tris, 1mM EDTA). For all analysis, DNA was diluted in distilled water (1:50 µl). DNA concentration in samples was checked with 260 nm readings (Sambrooks *et al*. 1989), using a UV spectrophotometer (Micronal Mod. B382).

2.4 – Polimerase Chain Reaction (PCR)

2.4.1 – Decapod PCR assay

Shrimp DNA quality was checked with a primer set that amplifies a decapod gene, carried out before the application of WSSV diagnostic PCR (Lo *et al*. 1996). For this, the primers 143F (5' TGC CTT ATC AGC TNT CGA TTG TAG 3') and 145R (5' TTC AGN TTT GCA ACC ATA CTT CCC 3') were used, with an expected size of 848 bp of amplified fragments.

2.4.2 – WSSV PCR assay

PCR reaction mixture for first-step and nested PCR reactions included 10 mM dNTP, 1.5 mM $MgCL_2$, 1 µM each primer, 0.02 U Taq DNA polymerase (Invitrogen) in a PCR buffer (Tris-HCL, 10 mM; KCl, 50 mM; pH 8.3).

Diagnostic PCR for WSSV was carried out using the following primers: 146F1 (5'-ACT ACT AAC TTC AGC CTA TCT AG-3') and 146R1 (5'-TAA TGC GGG TGT AAT GTT CTT ACG A-3'). For nested-PCR, primers 146F2 (5'-GTA ACT GCC CCT TCC ATC TCC A-3') and 146R2 (5'-TAC GGC AGC TGC TGC ACC TTG T-3') were used, as described by Lo *et al*. (1996). The expected size of the amplified fragment was 1447 bp for the first PCR reaction and 941 bp for the nested PCR reaction. Positive and negative controls were included in each PCR run.

Amplification was performed in a DNA thermocycler (Analitica TC-312) using the following parameters: Initial denaturation at 95 °C for 1 minute, followed by 40 cycles at 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute, with a final extension at 72 °C for 2 minutes, and holding at 10 °C (Crockford 2001).

2.4.3 – Agarose gel electrophoresis

After thermal cycling, 10 µl of the PCR reaction mix were poured onto a 1 % agarose gel for electrophoresis and stained with ethidium bromide (Sigma T-1201) for UV visualization. A 1 Kb DNA ladder (Invitrogen) was used as a marker.

2.4.4 – Polymerase Chain Reaction Sensitivity Test

To evaluate PCR sensitivity, a positive control DNA sample (0.38µg/µL) was diluted in distilled ultra pure water (Invitrogen). The dilution of 1 µL of concentrated shrimp DNA was performed at the following proportions: 1:10; 1:100; 1:1000; 1:10,000; 1:100,000; 1:1,000,000; 1:10,000,000; and 1:100.000.000, resulting in 8 diluted samples. Dilutions were tested for Decapod and White spot primers as described before.

3 – RESULTS

3.1 – Collected shrimp and abiotic factors

Table 1 shows information about the number of collected shrimp per species and period of the year, as well as salinity, temperature and water depth during sampling. During summer, *Farfantepenaeus paulensis*, *Farfantepenaeus brasiliensis* and *Litopenaeus schmitti* were sampled, while in the winter only *Farfantepenaeus paulensis* was found in the lagoon.

TABLE 1 – Month of collection, shrimp species, mean salinity, water temperature and depth during collection. Numbers in parenthesis indicate how many shrimps of each species were obtained in each season.

Month	*Species (n)*	*Salinity*	*Temperature* ℃	*Depth* ***m***
Feb	*Farfantepenaeus paulensis* (38) *Farfantepenaeus brasiliensis*(8) *Litopenaeus schmitti* (2)	7	26.2	1.3
Aug	*Farfantepenaeus paulensis* (66)	14	17.2	1.5

3.2 – Decap Polymerase Chain Reaction

DNA integrity of samples from summer (Fig. 2) and winter (data not shown), was confirmed by the presence of Decapod amplicon (848 bp band) in all electrophoresed agarose gels.

3.3 – WSSV Polymerase Chain Reaction

Negative WSSV results for shrimp collected in summer are illustrated in Figure 2. The 1447 and 941 bp bands indicate positive control. The lack of positive samples indicated that prevalence of WSSV in wild animals is less that 5% in each sampling period or less than 3%, when considering all analyzed shrimp.

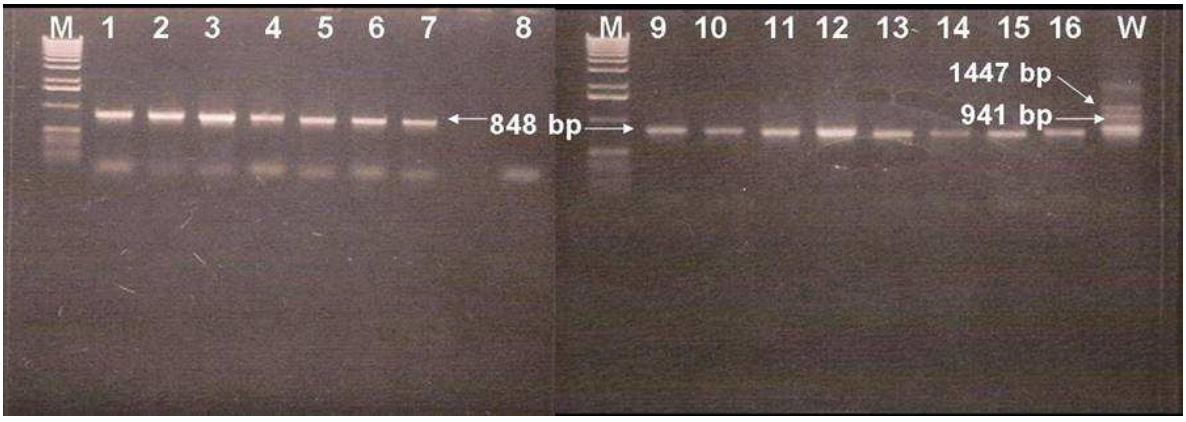


FIGURE 2 – Results of PCR for decap control and nested PCR for WSSV in samples collected in summer: M= DNA ladder; lanes 1-7 and 9 – 16 = summer samples; 8= negative control; W= positive control.

3.4 – Polymerase Chain Reaction Sensitivity Test

Tests showed that shrimp and virus DNA were detected up to a dilution rate of (a) 1:10.000 for Decapod primer, (b) 1:1.000 for WSSV one-step PCR and (c) 1:100.000 for WSSV nested PCR (Figures 3 and 4).



FIGURE 3 – Electrophoresis of Decap template in a 10-fold diluition series from 1:10 to $1:10^9$; 0 = negative control; M = DNA ladder.

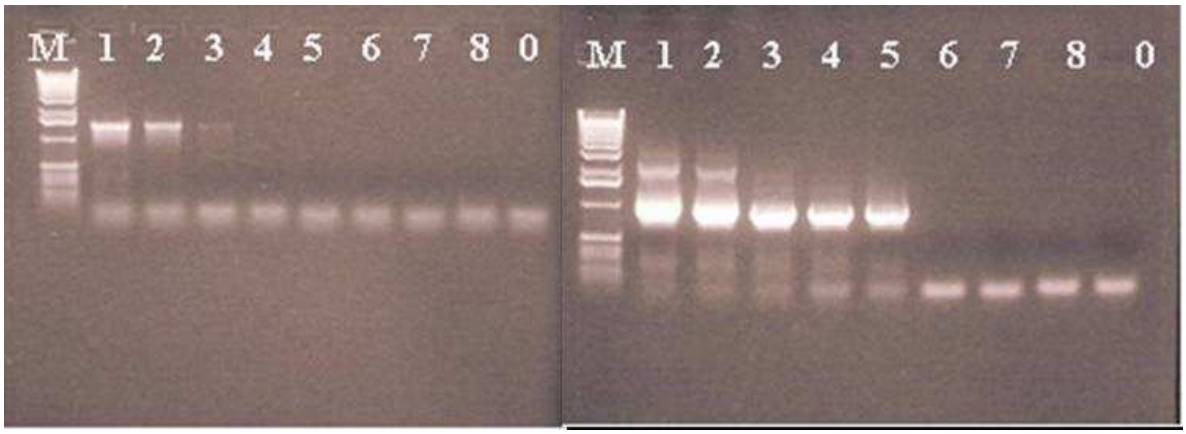


FIGURE 4 – Electrophoresis of positive control WSSV template in a 10-fold dilution series from 1:10 to $1:10^9$; 0 = negative control; M = DNA ladder. Left photo is the first step of the nested assay and the right photo is the second step.

4 – DISCUSSION

Outbreaks caused by White spot syndrome virus are of major concern, considering that this virus produces high mortality rates in infected cultures, generating huge economical losses (Lightner 2003). Therefore, many studies have investigated the presence of this virus in aquaculture systems, as well as in the environment, where other crustacean and aquatic organisms could function as vectors (Flegel 2006, Mijangos-Alquisires *et al*. 2006). However, few studies have considered possible virus infection of wild animals, generated by aquaculture activity (Chakraborty *et al*. 2002, Vaseeharan *et al*. 2003, Chapman *et al*. 2004, East *et al*. 2004). This topic is of great importance since shrimp aquaculture has been identified as an unfriendly environmental activity in several parts of the world (Naylor *et al*. 2000).

The results of this study indicate that, despite the major WSSV outbreaks in aquaculture farms in Laguna, the virus does not seems to be widely spread among native penaeid shrimp. Nevertheless, this result must be carefully considered in the light of: i) methodological approach; ii) environmental conditions, and iii) biology of penaeid species.

Regarding methodological approach, sampling size is, perhaps, the main factor that could lead to underestimation of infected shrimp in Laguna. It is especially important if virus load is low, or if the detection method applied is not sensitive enough. According to the OIE Manual (OIE 2003), tissue samples of few juveniles and sub-adults provide the best samples for WSSV detection. Post-larvae, on the other hand, must be analyzed entirely and in higher number. Thakur *et al*. (2002) suggested the analysis of at least 300 post-larvae shrimp, either pooled or in sub-samples of 50 PL, in order to significantly reduce the probability of false negatives, especially in areas

where infection by WSSV occurs at low prevalence levels.

Undoubtedly, a larger number of sampled organisms provide a better estimation of disease prevalence. However, large sampling numbers are not always possible, due either to shrimp availability, difficulty in sampling or elevated costs of analysis. Therefore, statistical methods are employed for obtaining a representative population sample with the best cost-benefit relationship. Based on the sampling number of this study, we may say with 95% confidence that prevalence of WSSV in natural populations of penaeid shrimp in Laguna was less than 5% per sampling period, or less than 3% when considering the total number of shrimp collected in both periods.

Another point of concern is the sensitivity of analytical methods. There is no doubt that PCR-based analysis are the most sensitive and precise methods for screening or confirmation of WSSV infection (Crockford 2001). However, many studies have demonstrated differences in sensitivity between one-step and nested PCR methods, with the last showing higher sensitivity (Thakhur *et al*. 2002, Flegel 2006, Sritunyalucksana *et al*. 2006). The sensitivity test conducted in this study showed that the nested PCR method was 100 times more efficient than the one-step method and could detect virus in samples containing 3.8 picograms of total DNA. Considering that each microgram of total shrimp+virus DNA contains around 10^7 copies of the virus (Durand *et al*. 2003), at the 1:100,000 dilution rate there would be ca. 38 copies. On the other hand, at the highest dilution rate, where the single-step method presented positive result (1:1000) there would be about 3800 copies in the sample. These values are in the same order of magnitude as those estimated for both methods by real-time PCR (Sritunyalucksana *et al*. 2006).

Stressful environmental conditions lead to the decrease of shrimp immunocompetence (Lightner & Redman 1998). It is well know that variation in water salinity and temperature, as well as low oxygen level and high ammonium concentration, contributes to animal stress, generating a situation where viruses can spread rapidly. Among these factors, water temperature is considered to be highly important, since it directly influences the whole biology of the shrimp, directly affecting its metabolism, growth rate and survival rates, among others (Rahman *et al*. 2006). In fact, some challenge experiments indicate that shrimp develop gross signs of the disease when a decrease in temperature occurs, while an increase in temperature lessened WSSV expression and/or appearance of clinical signs (Rahman *et al*. 2006, Reyes *et al*. 2007). It has been considered that increased temperatures may be an important tool for controlling WSSV infections. However, the exact mechanisms that lead to this shrimp response are not well known (Rahman *et al*. 2006). In this study, the collection of samples along two different seasons (austral summer and winter) was intended for comprising maximum seasonal variability of water parameters. For water temperature, a ca. 9ºC difference was observed between sampling periods (February mean = 26.2ºC± 0.83; August mean = 17.2ºC± 0.45). Despite this significant decrease in water temperatures, no positive results were found for shrimp collected in the winter.

Similarly, rapid decrease in salinity, mainly caused by rain, may trigger virus infection, or generate the appearance of gross clinical signs when the virus was already present, but shrimp were asymptomatic (Liu *et al*. 2005, Peinado-Guevara& López-Meyer 2006). Between summer and winter there was an increase in water salinity (February mean = 7.0± 5.4; August mean = 14.0± 5.1) in the Laguna embayment system. Salinity variability is characteristic of every estuarine system like Laguna, and wild shrimp living in such places are supposed to be adapted to such stressful conditions. Therefore, it is very unlikely that this parameter could contribute to the occurrence of WSSV in wild animals.

Finally, it must be considered that the lack of infections in wild shrimp could be related to ecological interactions in the lagoon, or the biology of these organisms. Generally, the natural environment favors a resistance to the illnesses, since it does not generate density-dependent stress, or low water quality. Moreover, the chances of finding infected animals in the environment are smaller than in culture systems. In nature, infected animals are rapidly eliminated mainly due to predation exerted on sick animals (Read 1994). Most diseases cause lethargy,

which facilitates predation, with rapid elimination of infected shrimps. This process diminishes horizontal transmission between individuals in the natural environmental.

Another possibility could be that some wild shrimp species present in Laguna are more resistant to WSSV infection than *L. vannamei*, considering that there are viruses which cause little or no infection in some species (Lightner 1996). Regarding WSSV, Wang *et al*. (1999) demonstrated that *Farfantepenaeus duorarum* did not show any mortality after being infected with this virus in a challenge test, and Chapman *et al*. (2004) observed lower WSSV infection rates in *Litopenaeus setiferus* in comparison to *Farfantepenaeus aztecus* caught in coastal waters of South Carolina (U.S.A.).

In summary, this study showed differences in sensitivity between PCR based methods, with higher sensitivity in nested PCR for WSSV infected shrimp identification. Despite this fact, no positive results were found for wild shrimp captured during austral summer and winter. These results indicate that, so far, white spot syndrome virus – WSSV is not extensively disseminated among wild shrimp in Laguna, Southern Brazil. However, efforts should be directed in order to continue a monitoring program in this ecosystem.

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