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Molt stage and cuticle damage influence white spot syndrome virus immersion infection in penaeid shrimp

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ABSTRACT

Transmission of white spot syndrome virus (WSSV) in shrimp has been reported to occur by feeding and immersion. In the present study, the impact of the molt process and artificial lesions in the cuticle on shrimp susceptibility to WSSV was examined using intramuscular and immersion routes.

For the intramuscular route, *Penaeus* (*Litopenaeus*) *vannamei* shrimp ($n = 450$) were injected with $10^{-2.3}$ up to $10^{2.7}$ shrimp infectious dose 50% end point (SID_{50}) of WSSV in early and late post-molt, inter-molt, early and late pre-molt; resp. A-, B-, C-, D1- and D2-stage. The resulting infection titers demonstrated that no difference ($p > 0.05$) in susceptibility existed between different molt stages when virus was injected.

For the waterborne route, shrimp in different molt stages were immersed in seawater containing 10^4 SID_{50} ml^{-1} of WSSV. In a first study, *P. vannamei* ($n = 125$) incubated in cell culture flasks, became infected with WSSV mostly in post-molt stages. In a second study, 2 groups of *P. vannamei* ($n = 100$) and *P. monodon* ($n = 100$) were transferred into plastic bags to prevent damage to the cuticle; and in 1 group a pleopod was cut off prior to incubation. Induction of damage increased infection significantly ($p < 0.05$) in A-stage from 0–40% to 60–100%, in B-stage from 0–20% to 40–60%, in C-stage from 0–20 to 20–60%, while infection was 0% in D-stages with both immersion methods.

This study proved that shrimp are more susceptible to WSSV infection via immersion after molting than in the period before molting and wounding facilitates infection.

1. Introduction

White spot syndrome virus (WSSV) is one of the most widespread viruses in penaeid shrimp aquaculture and is considered to be responsible for a large portion of crop failures (for reviews on WSSV, see: Sanchez-Martinez et al., 2007; Escobedo-Bonilla et al., 2008). Since the first reports on the virus, it has become generally accepted that transmission between shrimp and other Decapod Crustacea can occur via three routes: (1) oral uptake of tissues from infected hosts; (2) waterborne, when virus is transmitted via the water by immersion or cohabitation and (3) *per ovum* (vertical) and possibly *intra-ovum* from broodstock to offspring. When reviewing literature on WSSV, one finds a high number of experimental studies demonstrated that feeding of WSSV-infected shrimp tissues is an effective way to infect shrimp and other decapods. Especially the early reports on WSSV helped to build the image that the virus is highly contagious, even though many researchers had to administer WSSV-infected tissues more than one feeding, sometimes as long as 7 days. For the waterborne route, many studies

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reported that immersion and even cohabitation exposure readily allowed WSSV to cause infection, although older shrimp were reported to be less susceptible.

It is important to note, however, that most of the studies published so far were performed with non-specific pathogen-free (SPF) animals, without knowing the administered doses of WSSV and without screening the inoculum for the presence of other pathogens. Often, possible secondary transmissions after inoculation were not ruled out, temperature of the rearing water was not under control and most importantly, WSSV infections were rarely confirmed.

These facts make it difficult to reproduce those studies or make reliable conclusions. Probably the best-controlled experimental studies on WSSV transmission so far, were published by Soto et al. (2001), Lotz and Soto (2002), Soto and Lotz (2003) and Prior et al. (2003). Soto and Lotz concluded that ingestion of infected tissues was a far more effective treatment than immersion in infected water. Remarkably however, even when *Penaeus vannamei* were isolated to ensure they had equal chance to consume the infected tissues offered to them, not all shrimp became infected (50–60%). Prior et al. (2003) succeeded in determining the lethal intramuscular dose of a WSSV stock and also tried to develop a controlled bioassay by immersion of *P. vannamei*. Although very large amounts of infectious virus were added to the water (as shown by the injection study), mortality rates stayed below 40%. Recently, another study clearly illustrated the difficulty to infect animals by WSSV immersion challenge (Gitterle et al., 2006), while a study on an ornamental shrimp's susceptibility to WSSV resulted in a discussion of the problems encountered with experimental feeding challenges (Laramore, 2007). Gitterle et al. (2006) showed that merely adding virus inoculum to the water was not sufficient to result in *P. vannamei* infection but needed to place the shrimp in tanks in which orally infected shrimp had previously died to finally obtain successful transmission. Finally, in the PhD thesis by Bayot (2006), less than 17% of *P. vannamei* shrimp became infected upon individual challenge with WSSV via oral route and none or merely 3% by immersion.

The overall conclusion from these publications is that there are restrictions on the ability of WSSV to gain entry into its host. With feeding of virus-infected tissues to shrimp, this is to be expected as the lack of control on the dose of virus actually reaching the site of entry, inherently creates irreproducible results. The fact that any portion of the animals might not be feeding (due to molting, stress, etc.) for instance, can easily prevent an equal chance to become infected. Another factor which cannot be ignored is that all tissues known to be susceptible to WSSV replication are protected from the outside world by cuticle (Escobedo-Bonilla et al., 2007). This is also true for the gills and the epithelium of stomach and hindgut (Bell and Lightner, 1988).

Although little details are known about the structure and function of the cuticle of penaeid shrimp, it is well known that it changes dramatically in time (Chan et al., 1988; Compère et al., 2004; Promwikorn et al., 2007). During the course of its life, a shrimp passes through consecutive molt cycles. Therefore, in a study examining transmission of pathogens in shrimp, it could be important to take the molt stage into account (Le Moullac et al., 1997; Mugnier et al., 2008).

Considering the inability to reproducibly cause infection in shrimp exposed to WSSV by immersion, the present study was set-up to investigate the factors determining WSSV infection by waterborne route. In a first hypothesis we tested whether the susceptibility of shrimp to WSSV infection changes during the course of their molt cycle. The virus was delivered intramuscularly, thus passing the cuticle in order to compare the internal susceptibility between the different molt stages. In a second approach, the barrier function of the cuticle against natural infection by waterborne virus was tested in a series of immersion inoculation experiments of shrimp in different molt stages. Groups of artificially damaged shrimp were compared with control shrimp to test the hypothesis that the cuticle presents a barrier against WSSV and that wounding can promote infection.

2. Materials and methods

2.1. Experimental animals and conditions

The shrimp used in this study were *Penaeus* (*Litopenaeus*) *vannamei* from Molokai Sea Farms Int., Hawaii, USA and *P. monodon*, from Moana Technologies Nucleus Breeding Centre, Hawaii, USA. The batches of shrimp from Moana Technologies were certified to be SPF by Jim Brock, DVM. Those from Molokai Sea Farms had SPF status according to inspection services by the Aquaculture Development Program, State of Hawaii. Batches of 10,000 PL-10 shrimp were shipped to Belgium and reared in a recirculation system at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. They were fed with *Artemia* nauplii twice daily for a period of 3 weeks and were then weaned onto a commercial pelleted feed (A2 monodon high performance shrimp feed, INVE Aquaculture SA, Belgium), fed twice daily at a total rate of 5% of their mean body weight (MBW). Water temperature was kept at 27 ± 1 °C and salinity at 35 ± 1 g l^{-1}. Regular water changes kept total ammonia-N below 0.5 mg l^{-1} and nitrite-N below 0.15 mg l^{-1}. The room was illuminated 12 h per day by dimmed TL-light. For the viral challenge experiments, shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the experiments were performed under bio-safety conditions.

2.2. Molt stage determination

Molt stages were determined based on the descriptions by Robertson et al. (1987) and Chan et al. (1988). Briefly, shrimp were restrained for a few seconds and their uropods were examined by inverted microscope. At a magnification of 100–200×, the exopodites of uropods were analyzed on the appearance of the cuticle, epidermis and molt processes such as apolysis and the formation of new cuticle. Shrimp were separated into 5 major molt stages: early and late post-molt (A and B), inter-molt (C) and early and late pre-molt (D1 and D2).