

REVIEW

White Spot Syndrome Virus (WSSV) Concentrations in Crustacean Tissues – A Review of Data Relevant to Assess the Risk Associated with Commodity Trade

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Summary

We have reviewed the available peer reviewed literature on pathogen load for white spot syndrome virus (WSSV) in species susceptible to infection. Data on pathogen load in traded commodities are relevant for undertaking import risk assessments for a specific pathogen. Data were available for several of the major penaeid shrimp species farmed for aquaculture and for one crab and crayfish species. Most data are based on experimental infection, but some data were available for farmed or wild shrimp. Owing to the unavailability of immortal cell lines to determine viral load of viable virus, quantitative PCR was the main method used for quantification. The viral loads measured in shrimp at the onset of mortality events were extremely high (in the order of 10^9 – 10^{10} copy numbers gram^{-1} of tissue). In a farm setting, the onset of increased mortalities will often trigger emergency harvests. Therefore, shrimp obtained from emergency harvests are likely to carry substantial concentrations of viral particles. Viral load did not vary greatly with tissue type. The WSSV load in wild crustaceans, farmed crustaceans not undergoing a mortality event or survivors of a mortality event was significantly lower (usually by multiple logs). Studies have also been undertaken in ‘vaccinated’ shrimp. One of the ‘vaccines’ led to a significant reduction of viral load in WSSV-exposed animals. The data obtained from the literature review are put into context with published information on minimal infectious dose and WSSV survival in frozen commodity shrimp.

Introduction

White spot syndrome is a disease listed by the OIE and in the European Directive 2006/88 (Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals). The causative agent of white spot disease (WSD) is white spot syndrome virus (WSSV); a double-stranded DNA virus assigned by the International Committee on Virus Taxonomy to its own new genus, *Whispovirus*, in the family Nimaviridae (Mayo, 2002a,b). Virions are large (80–120 × 250–380 nm), rod-shaped to elliptical, and with a trilaminar envelope (Inouye et al., 1994; Wang et al.,

1995; Durand et al., 1997; Kanchanaphum et al., 1998; Van Hulten et al., 2001). Negatively stained virions purified from prawn haemolymph show unique, tail-like appendages (Wang et al., 1995). The virus received different names during the first years after it appeared, such as hypodermal and haematopoietic necrosis baculovirus, rod-shaped nuclear virus of *Penaeus japonicus* (RV PJ) (Inouye et al., 1994; Nakano et al., 1994), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya et al., 1995), white spot baculovirus (Chou et al., 1995; Lo et al., 1996) and Chinese baculovirus (Lu et al., 1997). All these isolates are now recognized as one virus: WSSV-1. White spot syndrome virus has a very stable genome. Three isolates have been fully sequenced with minor

differences, except for a single deletion (GenBank Accession No AF332093, GenBank Accession No AF369029 and GenBank Accession No AF440570). The deletion has potentially been related to virulence of strains to penaeid shrimp (Lan et al., 2002).

The disease was initially reported from East, South-East and South Asia. There were some occasional reports of WSSV in North America during the mid 1990s until it created a second pandemic wave that reached North, South and Central America in 1999. It is also present in the Middle East (Nakano et al., 1994; Flegel, 1997; Mohan et al., 1998; Zhan et al., 1998; Wang et al., 2000). Although there are no official reports or publications, outbreaks of WSD have also been observed in South Europe (D. V. Lightner, personal communication, Stentiford et al., 2009).

White spot syndrome virus has been detected in a wide range of decapod and non-decapod crustacean hosts from natural environments. In addition, all decapod crustaceans from marine and brackish or freshwater sources that have been subjected to experimental infection trials have been successfully infected. White spot syndrome virus has also been detected in a number of other species. It should be noted that detection only by PCR does not confirm susceptibility to infection, because the technique only detects the presence of the elements of the viral genome. Whether the viral genome is present intracellularly (infection) or on the surface or in the gut content of the animal (contamination) needs to be confirmed by histology, *in situ* hybridization, immunohistochemistry or transmission electron microscopy. Susceptibility criteria for infection of hosts with WSSV (and with Taura Syndrome and Yellow Head Disease) have recently been reviewed by Stentiford et al. (2009).

All farmed penaeid shrimp species from late post-larvae to juvenile and adult stages are highly susceptible to infection, often resulting in high mortality. Clinical signs appear on-farm after 14–40 days of stocking. The characteristic white spots are rarely present, particularly in *Penaeus vannamei* (and in most other susceptible species in which transmission has been attempted). Mortalities may reach 100% within 5 days after the onset of the disease. Survivors may carry the virus for life and may pass the virus to their progeny (Lo et al., 1996, 1997). Mortality among crabs, crayfish, freshwater prawns, spiny lobster and clawed lobsters is highly variable (Momoyama et al., 1994; Nakano et al., 1994; Takahashi et al., 1994; Cai et al., 1995; Chen et al., 2000; Sahul Hameed et al., 2000; Hossain et al., 2001; Jiravanichpaisal et al., 2001; Rodriguez et al., 2003; Yoganandhan et al., 2003), suggesting that relative susceptibility varies between crustacean taxa. Disease outbreaks may be induced by stressors, such as rapid change in salinity and drop in temperature (Vidal et al., 2001; Granja et al., 2003; Guan et al., 2003).

Egg-associated transmission mechanisms have been confirmed, and true vertical transmission is suspected. Horizontal transmission by consumption of infected tissue (e.g. cannibalism, predation, etc.) and by water-borne routes has been shown (Lo et al., 1997; Chou et al., 1998; Lo and Kou, 1998). Transmission of infection can occur among apparently healthy animals. Dead and moribund animals can be a source of infection (Lo et al., 1997; Chou et al., 1998; Lo and Kou, 1998).

Tissue tropism analysis from both experimentally infected shrimp and wild-captured brooders shows that tissues originating from the ectoderm and mesoderm, especially the cuticular epithelium and subcuticular connective tissues, as well as other target tissues (e.g. antennal gland, haematopoietic organ, etc.), are the main target tissues for WSSV. Samples of or from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for diagnostic testing (Lo et al., 1997).

White spot disease (caused by infection with WSSV) is considered perhaps the most economically important disease of farmed warm water shrimp (see Stentiford et al., 2010). In the 1990s, the disease spread rapidly across Asia and reached America in 1999; the disease had a serious economic impact on the shrimp aquaculture industry in affected countries (Lightner and Redman, 1998; Begum and Alam, 2002; Mazid and Banu, 2002; Pornlerd and Phillips, 2002; Flegel, 2006). In addition to the well-documented effects of the disease in farmed shrimp, the pathogen has been detected in wild crustaceans (Flegel, 2006; Jang et al., 2009; Meng et al., 2009); though, the impact of infection and its propensity to progress to disease in wild crustacean populations is not known.

Objective of the Review

Owing to unusually wide host susceptibility range to WSSV infection (Stentiford et al., 2009), the listing of the disease (WSD) in international legislation (such as that of the EU) is in recognition of the potential for the pathogen to traverse international boundaries and to potentially establish within farmed or wild stocks in the recipient country. Countries that are currently free from WSSV may therefore identify the introduction of the virus and the establishment of WSD in their territory as a potential hazard and may wish to investigate the likelihood of this to occur. Risk analysis is a method investigating the likelihood and consequences of undesirable events. In the field of animal health, the method is often used to assess the risks of disease introduction via international trade, known as import risk analysis. Guidelines stipulate that a risk assessment is transparent and evidence based (Murray, 2002; Murray et al., 2004; World Organisation for Animal Health OIE, 2010).

The objective of this review is to summarize and assess the literature on WSSV pathogen load in products of aquatic animal origin. The review addresses an important aspect to underpin import risk assessments on WSSV associated with commodity trade. We put the information into context of potential survival of the pathogen in frozen product and the minimal infectious dose (MID) of WSSV for other crustaceans; furthermore, we identify information gaps in these areas relevant for import risk analysis.

Material and Methods

The literature on pathogen load for WSSV was identified by initially creating an endnote library on WSSV by searching two literature databases: The database Scopus was searched for the terms 'White spot syndrome', 'WSD' and WSSV. Furthermore, the database Aquatic Sciences and Fisheries Abstracts was searched for references containing the terms 'White spot syndrome', 'White spot syndrome virus' and WSSV. The library constructed by this method (in November 2010) contained in excess of 1300 references. Within this Endnote library, references were searched containing the words 'Real time', 'real-time', 'quant*' or 'Viral load' in either the title, abstract or keywords. This returned about 220 references. The search was then further narrowed down to references containing the term 'viral load', which returned 14 references. These references formed the core of the literature review on viral load in crustacean tissues. In addition, we included references that were referred to in those 14 references and appeared to include information on pathogen load. The titles of the remaining approximately 200 references were scanned to assess whether these were likely to contain further information on pathogen load. Where the title did not indicate that virus quantification was a part of the study, the references were not assessed. The information is presented by briefly summarizing the approach taken in the respective publication and wherever possible by translating any viral load data into a summary table. For presentation in the table, the data sometimes required transformation; this involved either deriving numeric values from data presented in the original references in format of diagrams, or adjusting units so that the same metric was used.

Review of available literature on pathogen load

One of the first papers to look into relative pathogen load was presented by Soto et al. (2001). The authors investigated differences in transmission potential of WSSV, depending on whether non-infected *Litopenaeus* (= *Penaeus*) *vannamei* (1–3 g) were exposed to cephalo-

thorax, abdomen or whole shrimp cadavers from WSSV-infected shrimp. For the study, the authors placed *L. vannamei* in tanks with the respective tissue type using a variety of experimental set ups (exposed animals kept individually from the start, or separation 24 h post-exposure to limit or exclude possible secondary transmission). The authors observed no difference in mortality, when susceptible shrimp consumed the same quantity of either infected cephalothorax or abdomen. However, when exposing non-starved shrimp, the animals consumed more cephalothorax tissues compared to abdomen, resulting in higher mortality that the authors attribute to the higher amount of tissue consumed. The authors therefore conclude that there was no difference in viral load in cephalothorax tissues compared to abdomen.

In the last 10 years, substantial progress has been made for the quantification of WSSV in infected animals. This is largely because of the development of quantitative PCR assays. Cell lines that would allow quantification of live virus, and are widely used in other sub-disciplines of virology, are still not routinely available.

Dhar et al. (2001) presented a SYBR green assay for the quantification of WSSV. The focus of the paper was on description of the new assay. However, the authors also provided viral copy numbers determined in the hepatopancreas of experimentally challenged (via injection) *Penaeus stylirostris* (1 g). Animals were sampled 40 h post-injection. From the 10 animals tested, the authors reported a viral load from 8091 to 272 270 copies ng⁻¹ of DNA, with a mean of 92 578 and a median of 66 840 copies (mean and median were calculated from data presented in the paper, Table 1).

In the same year, Tan et al. (2001) also published work on the quantification of WSSV in tissues, using *Penaeus monodon* as the study species. The authors developed a competitive PCR assay to quantify virus copy numbers. *Penaeus monodon* (7.3–10.2 cm) were offered WSSV-infected shrimp tissue as food for 1 h. Shrimp were removed from the exposure tanks after predefined time intervals. The DNA was extracted from samples of gill, integument and muscle and analysed by PCR. The study revealed that viral copy numbers were below detectable levels during the first 12 h post-exposure, and that viral DNA was first detected 14 h post-exposure in gills and integument. In muscle tissue, first detection was 22 h post-exposure in one out of three animals. Following the eclipse phase (first 24 h), viral number exponentially increases between >24 and 48 h post-exposure and eventually reaches a plateau phase (>48–120 h post-exposure) (Table 1a).

Durand and Lightner (2002) developed a new real-time PCR assay for the detection of WSSV. In their paper, the authors also presented data on samples they analysed

Table 1. Synopsis of results of published studies on mean copy numbers of White Spot Syndrome Virus (WSSV) DNA from various tissues of decapod crustacea following injection, *per os* challenge or natural infection

Species	Tissue sampled	Pathway of infection	Sampling	Mean copy number μg^{-1} of total extracted DNA ($\pm\text{SD}$, where provided)	Mean copy number gram^{-1} of tissue or ml haemolymph	Study
(a)						
Pacific blue shrimp, <i>Penaeus stylirostris</i> (1 g)	Hepatopancreas	Injection	40 h post-injection	9.3×10^7 ^a	Not provided	Dhar et al. (2001)
Giant tiger prawn, <i>Penaeus monodon</i> (7.3–10.2 cm)	Gills	<i>Per os</i>	($n = 3$) ^b 14 h post-exposure 24 h 48 72 96 120	Not provided	3.6×10^4 ^c 4×10^5 ^c 1.1×10^8 ^c 7.9×10^9 ^c 1.5×10^{10} ^c 2.0×10^{10} ^c	Tan et al. (2001)
	Integument		14 h post-exposure 24 h 48 72 96 120		2×10^3 ^c 1.0×10^4 ^c 1.4×10^8 ^c 8.9×10^8 ^c 1.6×10^9 ^c 5.0×10^9 ^c	
	Muscle		14h post-exposure 24 h 48 72 96 120		0 ^c 5×10^3 1.0×10^7 ^c 1.6×10^8 ^c 7.9×10^8 ^c 1.5×10^9 ^c	
<i>Cambarus clarkii</i> (20–25 g)	Haemolymph	Injection	3–5 days post-injection		3.6×10^8	Huang et al. (2001)
<i>Penaeus japonicus</i> (150 g)	Haemolymph	Injection	3 days post-injection, moribund animals ($n = 3$)		1.5×10^{10}	Wu et al. (2002)
<i>P. stylirostris</i> (juveniles)	Pleopods	<i>Per os</i>	Moribund ($n = 8$)	3.0×10^{10} ($\pm 3.13 \times 10^{10}$)	Not provided	Durand and Lightner (2002)
<i>P. monodon</i> (juveniles)	Pleopods	From naturally infected farm population	Moribund ($n = 10$)	2.2×10^6 ($\pm 4.56 \times 10^6$)	Not provided	
<i>P. stylirostris</i> (PL; 0.02 – 0.03 g)	Whole animal	<i>Per os</i>	Moribund ($n = 3$)	4.3×10^9	5.7×10^{11}	
<i>Penaeus vannamei</i> (juvenile) Whiteleg shrimp; Pacific white shrimp	Pleopods	Injection	Clinical stage not reported; $n = 5$	1.6×10^9 ($\pm 6.24 \times 10^8$)	Not provided	
	Gill		$n = 5$	1.2×10^9 ($\pm 8.5 \times 10^8$)		
	Hepatopancreas		$n = 5$	9.0×10^7 ($\pm 6.0 \times 10^7$)		
	Muscle		$n = 5$	1.9×10^8 ($\pm 1.3 \times 10^8$)		
	Haemolymph		$n = 3$	2.6×10^9 ($\pm 3.5 \times 10^8$)		

Table 1. (Continued)

Species	Tissue sampled	Pathway of infection	Sampling	Mean copy number μg^{-1} of total extracted DNA ($\pm\text{SD}$, where provided)	Mean copy number gram^{-1} of tissue or ml haemolymph	Study		
<i>P. vannamei</i> (Juvenile, 3 g)	Whole heads	<i>Per os</i>	After first occurrence of mortalities ($n = 8$ for each mean)	2.5×10^7	2.0×10^{10}	Durand et al. (2003)		
	Whole tails			1.2×10^7	1.53×10^{10}			
	Tail muscle			3.4×10^7	1.86×10^{10}			
	Tail shells			4.8×10^8	7.32×10^{10}			
<i>P. vannamei</i> (PL55)	Whole bodies and cephalothoraxes	<i>Per os</i> WSSV challenge after bath treatment with:	Sampled 7 days (moribund) or 10 days (survivors) post-challenge with WSSV			Melena et al. (2006)		
				IHHNV at PL22	Moribund ($n = 5$)		2.4×10^9 ($\pm 1.1 \times 10^9$)	
				Inactivated WSSV at PL35	Surviving ($n = 5$)		4.4×10^2 ($\pm 9.8 \times 10^2$)	
				Inactivated WSSV at PL35	Moribund ($n = 5$)		1.9×10^9 ($\pm 2.0 \times 10^8$)	
				IHHNV at N5/Z1/PL22 (group a)	Surviving ($n = 3$)		Undetectable	Not provided
				IHHNV at N5/Z1/PL22 (group b)	Surviving ($n = 5$)		4.4×10^2 ($\pm 6.0 \times 10^2$)	
Blue crab, <i>Portunus trituberculatus</i>	Adults (average 186–365 g)	Pereiopod	Natural infection (sampled from the wild)			Meng et al. (2009)		
				Normal animals, ($n = 222$; 4 locations; April, June, August, December)	5.2×10^3 ($\pm 6.1 \times 10^3$)		2.1×10^6 ($\pm 2.3 \times 10^6$)	
	Zoea (larval stage)	Whole body	Natural infection (sampled from hatcheries)	Normal animals ($n = 140$; 20 animals each from 7 farms), June 2007	6.0×10^3 ($\pm 13.6 \times 10^3$)	Copy number per animal: 3.2×10^6 ($\pm 1.1 \times 10^7$)		
Fleshy shrimp, <i>Fenneropenaeus chinensis</i>	Adults (average 52–55 g)	Pleopod	Natural infection (sampled from the wild)			Jang et al. (2009)		
				Normal animals, ($n = 159$; 3 locations; April–June 2007)	1.5×10^7 ($\pm 1.8 \times 10^8$)		1.8×10^{10} ($\pm 2.2 \times 10^{11}$)	
				Location 1 ($n = 57$)	7.9×10^3 ($\pm 1.2 \times 10^4$)		8.8×10^6 ($\pm 1.6 \times 10^7$)	
				Location 2 ($n = 70$)	3.4×10^7 ($\pm 2.7 \times 10^8$)		4.1×10^{10} ($\pm 3.3 \times 10^{11}$)	
				Location 3 ($n = 32$)	4.8×10^4 ($\pm 2.0 \times 10^5$)		2.8×10^7 ($\pm 1.2 \times 10^8$)	
Post-larvae (PL1-8; mean weight: 5.4 mg)	Whole body	Natural infection (sampled from hatcheries)	Normal animals ($n = 210$; 30 animals each from 7 farms), May–June 2007	2.2×10^5 ($\pm 1 \times 10^6$)	8.5×10^8 ($\pm 6.4 \times 10^9$)			

^aValues estimated from Table 3 of the paper.

^bThe number of samples analysed to generate copy number data is not entirely clear in the paper, but most likely was 3.

^cValues estimated from Figure 5 provided in paper.

IHHNV, infectious hypodermal and haematopoietic necrosis virus.

Table 1. (Continued)

Species	Tissue sampled	Pathway of infection	Sampling	Mean copy number	Mean copy number	Study
(b)						
<i>P. monodon</i>	Gills	Injection	Days post-injection (<i>n</i> not provided)	Mean copy number μg^{-1} of total extracted DNA ^d		Syed Musthaq et al. (2009)
			2	6.3 \times 10 ²	Vaccinated shrimp; 6.3 \times 10 ²	
			5	5.0 \times 10 ⁴	2.0 \times 10 ⁴	
			7	5.0 \times 10 ⁶	1 \times 10 ²	
			10	All animals dead	0.25	
			15		0.016	
			25		0.0025	
Kuruma shrimp; <i>Marsupenaeus japonicus</i> (adults; average weight 11.8 g)	Muscle	Injection	Hour post-exposure; <i>n</i> = 1 animal for each time point	Mean copy number μg^{-1} of total extracted DNA		You et al. (2010)
				Water temp. 27°C	Water temp. 31°C	
			6	1.6 \times 10 ³	1.3 \times 10 ³	
			12	1.6 \times 10 ⁴	7.9 \times 10 ³	
			24	2.0 \times 10 ⁵	2.0 \times 10 ⁴	
			48	2.0 \times 10 ⁶	6.3 \times 10 ⁴	
			72	6.3 \times 10 ⁷	1 \times 10 ⁵	
<i>P. vannamei</i> (4 g)	Haemolymph	<i>Per os</i>	Hour post-exposure; <i>n</i> = 5 for each time point and group	Mean copy number in 1 μl haemolymph		Granja et al. (2006)
				Group 1 (26°C)	Group 2 (32°C)	
			6	1.3 \times 10 ²	4.0 \times 10 ²	
			12	1.6 \times 10 ³	5.0 \times 10 ²	
			18	6.3 \times 10 ³	5.0 \times 10 ⁴	
			24	1 \times 10 ⁵	1 \times 10 ²	
			48	3.2 \times 10 ³	1 \times 10 ²	
			72	1.6 \times 10 ⁷	7.9 \times 10 ⁵	
			96	1.3 \times 10 ⁷	1.6 \times 10 ⁴	
			120	1 \times 10 ⁹	4.0 \times 10 ⁴	
			144	5.0 \times 10 ⁷	4.0 \times 10 ³	
			168	2.0 \times 10 ⁹	1.6 \times 10 ⁴	
			192	6.3 \times 10 ⁶	7.9 \times 10 ²	
			216	No animals surviving	4.0 \times 10 ²	

^dValues estimated from Figure 6 provided in paper.

from selected archived material from moribund juvenile *P. vannamei*, *P. monodon* and *P. stylirostris* (size/weight not provided), and post-larval *P. stylirostris*. For some juvenile *P. vannamei*, which had been infected with WSSV by injection, the authors provided data for viral load by type of tissue (Table 1a).

In a subsequent paper, Durand et al. (2003) presented quantitative data on pathogen load of WSSV in *P. vannamei*, which the authors had exposed to WSSV by oral challenge. Animals were sampled when the first mortalities occurred (only animals alive at the time of sampling were used). The viral loads are shown in Table 1a.

Sathish et al. (2004) developed immunological assays for the detection of a 18-kDa WSSV protein and applied these for the detection of WSSV in tissues of i.m.

injected *Penaeus indicus*. The methodology as well as the results from this study is not sufficiently detailed to clearly interpret the results for the current review. Using an ELISA, the authors tested samples pooled from five shrimp sampled at 12, 24, 36 and 48 h post-injection. In the results section, the authors report that the 18-kDa antigen was detected from 24 h p.i. in head tissue, tail and eyestalk, but took longer before its detection in gills, haemolymph and heart. The authors did not provide data for the detection limit of the ELISA and did not make an attempt to translate the ELISA readings into quantities of viral protein. Therefore, the information is not suitable to estimate viral loads in crustacean tissues. However, what can be derived from the data presented is that virus copy numbers at 48 h post-injection are

highest in head tissues and tail, followed by gills and eyestalk.

Granja et al. (2006) exposed *P. vannamei* via oral challenge to WSSV (via inoculation of purified virus suspension into the oral cavity). Shrimp were maintained at either 26 (Group 1) or 32°C (Group 2), and the authors extracted DNA from haemolymph collected at various times from 6 to 216 h post-infection. The number of viral units was quantified by real-time PCR using SYBR Green. In Group 1, the authors observed a significant increase with time post-infection. In contrast, in Group 2 (32°C), the number of viral units remained largely unchanged, indicating that hyperthermia inhibited, either directly or indirectly, viral replication (Table 1b).

Melena et al. (2006) investigated whether pre-exposure of *P. vannamei* to infectious hypodermal and haematopoietic necrosis virus (IHHNV) or inactivated WSSV had a protective effect against subsequent WSSV infection and disease in post-larvae. As part of their study, the authors analysed WSSV copy numbers in *P. vannamei* that were either moribund following challenge or that were still alive at 10 day post-challenge. White spot syndrome virus challenge was undertaken *per os* at post-larval stage 45 (PL45). By 10 day post-oral challenge, shrimp in all groups challenged with WSSV had died except for those which had been pre-exposed to IHHNV or formalin-inactivated WSSV prior to the WSSV challenge. Viral loads are reported for surviving and moribund shrimp. The authors found high viral loads in moribund shrimp, and relatively low viral load or no detectable WSSV in shrimp surviving 10 day post-challenge (Table 1a).

Peinado-Guevara and López-Meyer (2006) monitored prevalence of WSSV infection in *P. vannamei* in two commercial farms in Mexico. The authors sampled 50 shrimp from each of five ponds (three from farm 1 and two from farm 2) at weekly intervals and analysed the samples by single round and nested PCR. For the PCR analysis, DNA was initially extracted from lamellar tissue (which is not further described; possibly cephalothoracic cuticle or gill lamellae) pooled from 10 animals. If a pooled sample returned a positive PCR result, the authors utilized the appropriate frozen samples and analysed lamellar tissue from individual animals. The authors did not use a quantitative PCR assay. Therefore, the information with regard to viral load is limited as to whether the infection levels were high enough to be detected in a single round or were only detected by nested PCR assay (suggesting lower pathogen load). During the 2- (farm 1) or 3-month (farm 2) observation period, prevalence in ponds in farm 1 increased from low (<10 animals out of 50) or not detectable levels (0%) to 64–88% at the time of harvest, which in farm 1 was undertaken because of mass mortalities (estimated 80%), which occurred following a 3 day period

of rain. For farm 2, several samples from the first weeks of observation had been lost for individual animal analysis. However, based on the pooled samples, the number of PCR positive samples was $\leq 20\%$ in one of the two ponds in the first few weeks and 24% in the second pond. Following a 3-day rain period, prevalence increased markedly in both ponds to a maximum of 58% and 64%, respectively. Farm 2 had also been exposed to the 3-day rain period; however, the farm was not affected by a mass mortality in the way farm 1 had been. The farmer had undertaken a partial harvest immediately following the rain period, which may have prevented mass mortalities. The proportion of individual animals and pools testing positive by single round PCR tended to increase over time. Owing to samples lost for analysis, this observation is mainly based on the proportion of pools testing positive. In summary, the authors show that prevalence of WSSV infection tended to increase over time between seeding and harvest and that prevalence is elevated following a stressful event, such as heavy rain (likely due to a drop in water temperature and salinity). If data were available on the sensitivity of the single round and nested PCR, further information could be gained as to the viral load levels present in the analysed tissue.

Meng et al. (2009) quantified WSSV in hatchery-produced larvae and wild populations of the blue crab *Portunus trituberculatus* in South Korea by TaqMan real-time PCR. Out of 140 *P. trituberculatus* zoea larvae from seven commercial hatcheries, 96.4% were WSSV-positive. The mean WSSV copies were 6.0 ng^{-1} DNA, or 3216.0 per larva. In 222 adult crabs from four wild populations captured in different seasons, WSSV-prevalence was 79.3%, and the WSSV load was 5.2 copies ng^{-1} DNA, or 2116.5 copies mg^{-1} tissue (Table 1). Both the WSSV-prevalence and the viral load of the winter population were significantly lower than those of the other three populations.

Another study applying real-time PCR to assess viral load in a crustacean species was presented by Jang et al. (2009). The authors tested a total of 159 individuals of wild *Fenneropenaeus chinensis* brooders from three locations and 210 post-larvae (PL1–8) from seven commercial hatcheries in South Korea. The authors report a wide range in WSSV load in the wild animals, ranging from 0 to 2.28×10^6 (with a mean of 1.50×10^4) copies ng^{-1} of DNA. Prevalence of infection across all wild animals tested was 75.5%; the majority of all wild animals had low-level infections (96.2% with <100 copies, 69.8% with <10 copies ng^{-1} of DNA). For the 210 post-larvae tested, the range in copy numbers was not provided. However, the authors reported that prevalence within hatcheries was 100% in five of the hatcheries and 77% and 33%, respectively, in the two remaining hatcheries based on 30 animals tested

per hatchery. The overall mean copy numbers per mg tissue were lower by 1.5 \log_{10} s compared to wild animals. Further pathogen load data are presented in Table 1. The mean values in wild animals varied quite substantially depending on location. The means for animals from location 2 were very high as a result of a few (5) heavily infected animals. If those outliers were excluded, mean loads would have been more similar between locations.

Syed Musthaq et al. (2009) constructed a recombinant baculovirus to develop a 'vaccine' against WSSV. Using this baculovirus displaying VP28 as a 'vaccine' against WSSV, the authors observed a significantly higher survival rate of 86.3% and 73.5% of WSSV-infected shrimp (*P. monodon*) at 3 and 15 days post-vaccination respectively compared to non-vaccinated animals. Quantitative real-time PCR also indicated that the WSSV viral load in vaccinated shrimp was significantly reduced at 7 days post-challenge. White spot syndrome virus copy numbers in vaccinated animals versus positive controls are shown in Table 1b. Copy numbers in vaccinated shrimp initially develop in line with copy numbers in non-vaccinated shrimp. However, from 7 days post-challenge, observed copy numbers are significantly lower in vaccinated shrimp and fall to very low levels at 25 days post-challenge. The copy numbers reported by these authors for positive controls are in line with copy numbers previously reported by Tan et al. (2001) for *P. monodon*, if one assumed that virus copy numbers reported per g of tissue are roughly 1000 \times the number of virus copies reported per μ g of DNA, which appeared to be the case based on data reported by Meng et al. (2009) and Durand et al. (2003).

In a further study on possible vaccination methods of *P. monodon*, Krishnan et al. (2009) report reduced mortalities in shrimp that had received DNA constructs expressing long-hairpin RNA (lhRNA). The authors also present data on viral copy numbers in vaccinated shrimp and positive controls using a semi-quantitative assay. They demonstrate that the viral load in vaccinated shrimp is markedly reduced compared to positive control shrimp. Whereas viral copy numbers in non-vaccinated shrimp are $\geq 10^5$ viral particles on day 5 and 7 post-injection, the copy numbers in vaccinated shrimp are in the range of 10^3 – 10^5 copies, or 10–100 copies on those days depending on the DNA construct. Unfortunately, the authors do not describe which tissue was analysed or what amount of DNA or tissue these quantities relate to. Therefore, these data were not included in Table 1.

You et al. (2010) investigated the effect of high water temperature on mortality, immune response and viral replication of WSSV-infected juveniles and adults of Kuruma shrimp, *Marsupenaeus* (= *Penaeus*) *japonicus*. The authors observed that viral load of WSSV (measured by real-time PCR) at 27°C was significantly higher ($P < 0.05$) than that

of shrimp exposed to WSSV at 31°C over 24–72 h post-infection (Table 1b).

Mai and Wang (2010) studied the effects of shrimp lysozyme on cellular and humoral defence mechanisms in blue shrimp [*Litopenaeus* (= *Penaeus*) *stylirostris*]. Amongst other observations, the authors reported a lower viral load (compared to controls) in shrimp that were injected with lysozyme prior to injection with WSSV. Unfortunately, the material and methods applied to quantify virus copy numbers are not described. The authors appear to have determined copy numbers in haemolymph at 0, 4, 8, 12, 24, 48 and 72 h post-injection. Whereas the copy number appears to have increased from 10^7 to 10^9 copies ml^{-1} haemolymph in shrimp positive controls, the increase in viral copy numbers was slightly lower in shrimp injected with lysozyme prior to WSSV challenge. It is unclear how many animals were analysed per time point and what clinical stage the animals were in. Therefore, this report does not add any new useful information in the context of viral loads.

The following two papers were identified to have the words 'viral load' in their abstract, but the information in the paper was not really relevant to the literature review on pathogen load in crustaceans.

Liu et al. (2006) undertook experiments with RNA interference for a specific gene coding for a protein believed to have an influence on WSSV propagation. Using crayfish as an experimental study organism, the authors investigated expression levels at the RNA level rather than copy numbers of viral DNA. Therefore, these data cannot easily be translated into viral loads in crustacean tissues.

Citarasu et al. (2006) investigated the protective effect the addition of herbal extracts to shrimp diets may have against WSSV infection and associated mortalities. The authors observed a delay in progression of the disease and reduced mortalities in the study period. Although the authors describe in the abstract and discussion that the viral load was reduced with increasing amount of herbal extracts, this information is not presented in the results section of the paper.

Two additional publications that were not identified using the method applied for identifying publications to be included in this review but considered relevant by the reviewers are the following:

Huang et al. (2001) studied methods to improve purification of WSSV to allow further characterization of the virus. The authors injected crayfish *Cambarus clarkii* (20–25 g) i.m. with 0.2 ml of virus filtrate obtained from infected *Penaeus chinensis* to amplify WSSV. Crayfish haemolymph was collected 3–5 days post-injection (rearing temperature 20°C). The average yield from approximately 5.0 ml haemolymph is reported as the equivalent of 3.6×10^5 copies μl^{-1} . This is 44 \times to more than 10^3 lower

than average copy numbers reported by Granja et al. (2006) for *P. vannamei* haemolymph following *per os* exposure and 26°C water temperature. It appears that the authors determined the average WSSV yield based on knowledge of the genome size of WSSV, and the DNA concentration determined spectrophotometrically and by comparison with DNA markers of known concentration in agarose gel electrophoresis.

Wu et al. (2002) developed an infection model for Kuruma shrimp *P. japonicus*. The authors determined a suitable injection dose and time point for harvesting haemolymph from exposed Kuruma shrimp in pre-trials. In the main trial, adult shrimp (mean body weight 150 g) were i.m. inoculated and haemolymph collected from moribund shrimp 3 days post-injection. The haemolymph was aliquoted and subsequently analysed by PCR and virulence tests. Based on competitive PCR, the authors estimated the concentration of WSSV in haemolymph to be 1.5×10^7 genome copies μl^{-1} .

Minimal infectious dose

Information regarding the MID is not easily accessible. Owing to the difficulty that to date cell lines for culturing WSSV (or other crustacean viruses) *in vitro* are not routinely available, several transmission trials worked with unknown viral titres using infected tissue for infection trials. Some more recent studies have attempted to establish and apply a standard when undertaking challenge experiments (Prior et al., 2003; Escobedo-Bonilla et al., 2005, 2006; Corteel et al., 2009). Some of the authors used the shrimp infectious dose 50% endpoint ($\text{SID}_{50} \text{ ml}^{-1}$), or the lethal dose 50% endpoint ($\text{LD}_{50} \text{ ml}^{-1}$) to make infection experiments comparable. For example, both Escobedo-Bonilla et al. (2005) and Corteel et al. (2009) prepared a stock of a defined viral concentration, described by the $\text{SID}_{50} \text{ ml}^{-1}$ to undertake challenge experiments via i.m., oral or bath challenge. Escobedo-Bonilla et al. (2005) showed that the absolute dose required to infect *L. vannamei per os* is about 10 times higher compared with the dose per intra muscular injection. The authors observed mortality in *L. vannamei* when they administered an absolute amount of either 10^1 or 10^0 $\text{SID}_{50} \text{ IM}$ orally, but not at a lower dose. Corteel et al. (2009) used a standard concentration of 10^4 $\text{SID}_{50} \text{ ml}^{-1}$ and a volume of 25 ml of this standard solution per g of body weight for bath challenges (3 h exposure). This concentration and challenge conditions appeared to be around the MID per bath challenge, which was indicated by the fact that, depending on moulting cycle stage and whether or not the penaeid shrimp had been injured, the dose was or was not sufficient to induce mortality. Unfortunately, there is insufficient detail provided on the preparation of the standard

solution to trace back how many infected animals were used to generate the virus stock solution. This would be relevant to link virus levels found in the animals, from which the virus stock solution was generated with the SID_{50} . The study does, however, highlight the importance that environmental and host condition may have on calculating absolute MID for a given crustacean species.

In another study, the authors quantified the virus used for either challenge by injection or waterborne challenge by real-time PCR (Durand and Lightner, 2002). The authors found that in waterborne challenge, a concentration of 1×10^4 WSSV copies ml^{-1} of sea water (six animals submerged in a total volume of 5 l) was insufficient to induce mortality, whereas a concentration of 1×10^5 WSSV copies ml^{-1} was sufficient. The authors did not determine the lower infectious dose required to induce infection via the intra muscular route. An absolute dose of 10^4 copies per injection into 5 g *P. vannamei* induced 100% mortality within 52 h.

If we combine the results by Corteel et al. (2009), who found that a concentration of 10^4 $\text{SID}_{50} \text{ IM ml}^{-1}$ was around the MID per bath challenge, with the results by Durand and Lightner (2002), who observed that 1×10^5 WSSV copies ml^{-1} sea water (determined by real-time PCR) were sufficient to induce infection, whereas 1×10^4 WSSV copies ml^{-1} were not, it appears 10 units determined by real-time PCR correspond to approximately 1 $\text{SID}_{50} \text{ IM}$. This may vary to some extent depending on how the virus material has been stored, which may lead to a loss in $\text{SID}_{50} \text{ IM}$, whereas the quantities as determined by real-time PCR may remain largely unchanged.

A study by Wu et al. (2002) further corroborates the link between $\text{SID}_{50} \text{ IM}$ and virus copy quantification by PCR: the authors determined a dose of 9.5×10^2 genome copies g^{-1} shrimp as the LD_{50} of inoculums prepared from haemolymph and stored at -80°C for 40 days. Given that the authors used 0.6 g *P. japonicus* in their challenge trials, the $\text{SID}_{50} \text{ IM}$ in their study would correspond to 5.7×10^2 genome copies shrimp^{-1} . This is approximately 50× more compared to the corresponding values of genome copy numbers and $\text{SID}_{50} \text{ IM}$ determined for bath challenges in *P. vannamei*. However, taking into account that different species were used, and that prior treatment of inoculum may have influenced the available viable virus, these data still provide a valuable indication of virus copy numbers as determined by PCR required to induce infection.

Data presented by Huang et al. (2001) suggest that the infectious dose might be higher for crayfish *C. clarkii*. The authors injected *C. clarkii* (20–25 g) i.m with serial dilutions of WSSV filtrate to investigate the virus quantity sufficient to induce infection. Non-coagulation of

haemolymph, assessed 5–7 days post-injection, was used as the indicator of successful WSSV proliferation. The authors report that $1 \times 10^{4-5}$ virions were sufficient to induce infection if freshly extracted haemolymph was used for injection. If the virus had been frozen prior to injection, the required virion dose was approximately 100× higher (details of storage conditions not provided). However, given that a rather crude method was used to assess whether infection had taken place, the true infectious dose 50 may well have been lower, and the age of the animals has had an impact on the progression of infection.

The link between virus copy quantification using quantitative PCR assays and the infectious dose is relevant to use data on virus concentrations in tissue determined by quantitative PCR to assess the likelihood that susceptible species exposed to certain quantities of virus may become infected.

Discussion and Conclusions

Data on WSSV load were found for a number of penaeid shrimp species, for one crab species and for one crayfish species. Penaeid shrimp are highly susceptible to infection, and infection in farmed populations is often associated with high mortalities. The species for which data were found are the main warm water shrimp species traded for human consumption.

Amongst the penaeid species, WSSV load was very similar for animals at the respective stages of infection. Furthermore, exposure often leads to similar cumulative mortalities over a given time period. Therefore, even if viral load data are not available for every penaeid species, conclusions can be drawn as to the likely viral load range for a certain clinical stage of infection. In penaeid shrimp, viral load determined as PCR copy numbers was extremely high in moribund shrimp or shrimp without signs sampled at the onset of mortalities within the challenge group (in the order of 10^9 – 10^{10} copy numbers gram^{-1} of tissue).

In a farm setting, the onset of increased mortalities will often trigger emergency harvests. Therefore, shrimp from emergency harvests are likely to carry substantial virus numbers. Viral load did not vary greatly with tissue type. White spot syndrome virus load in wild animals, farmed populations not undergoing a mortality event or survivors of a mortality event was significantly lower (usually lower by multiple logs). Studies have also been undertaken in vaccinated shrimp. One of the vaccines led to a significant reduction of viral load in WSSV-exposed animals.

Cortee et al. (2009) have shown that 10^0 – 10^1 SID_{50} IM is sufficient to induce infection in *L. vannamei* via the oral route. Assuming our derivation of corresponding units measured by real-time PCR or SID_{50} IM is correct;

100 copies detected by real-time PCR would be sufficient to cause infection via the oral route (at least for naïve penaeid shrimp). The literature review has shown that viral load in moribund shrimp and shrimp analysed at a point of infection simulating an emergence harvest is carrying viral copy numbers per mg and even per μg of tissue that are far in excess of 100 copies.

In survivors of experimental studies or infected animals from the wild, copy numbers were lower by several \log_{10} s compared to moribund shrimp or shrimp without signs sampled at the onset of mortalities within the challenge group.

Most viruses are stable at freezing temperatures. Diagnostic and research laboratories, including our own, commonly freeze samples to ensure the preservation of viruses. Under laboratory conditions, maximum preservation of viral infectivity is achieved when samples are held at very low temperatures (-70°C or lower). Experimental studies using commodity shrimp for transmission trials have shown that sufficient viable virus remains in frozen commodity shrimp to induce mortality in exposed *P. vannamei* (Durand et al., 2000; Hasson et al., 2006). The data on WSSV quantities are based on quantitative PCR data and are not necessarily identical with numbers of viable virus. Freezing, storage and thawing are likely to lead to a drop in viable virus numbers. Wu et al. (2002) report that viable WSSV titres present in haemolymph stored at -80°C remained largely unchanged between 40 days and 16 months of storage. They also mention un-published data according to which viable virus titres gradually declined over time if whole shrimp cadavers were stored at -80°C .

Studies by Sritunyalucksana et al. (2010) suggest that commercial processing procedures reduce virus levels of another shrimp virus, Yellow head virus, in shrimp carrying virus levels similar to pre-patent-infected shrimp sufficiently to render the risk of transmission to naïve shrimp negligible. However, the scale of drop in virus level still remains to be shown. Given that freezing is generally used to preserve viable virus, the drop in titre might be limited.

Because the information on available viable virus is relevant to undertake import risk assessments, further studies will need to quantify the loss of viable virus as a result of freezing and thawing. Immortalized cell lines, which are often used to titrate viable virus, are currently not available for use with crustacean viruses. To date, the majority of published studies in this area have attempted to utilize crustacean primary cell cultures derived from different organs for sustaining or growing some crustacean viruses *in vitro* (Jose et al., 2010).

Very few data are available from which a MID may be derived. Studies have only been undertaken in a very

limited number of species and at a certain life stage of these species. Host condition (e.g. sex, moult status, nutritional condition) may further influence the MID. Furthermore, data on susceptible species range and MID of species native to countries where the disease has not yet been detected are very limited or non-existent (Stentiford et al., 2009). Such data are required to assess the likelihood of establishment of WSSV infections in crustacean populations of countries where the disease is currently exotic.

In future studies, it would be useful, if researchers routinely quantified virus concentration in virus preparations for bioassay by qRT-PCR. This would allow to some extent to relate data on viral load in tissues with the likelihood of such viral loads leading to infection – either by oral or waterborne challenge routes.

Based on the information reviewed to date, raw, frozen commodity shrimp imported for human consumption if sourced from emergency harvests is likely to contain relevant virus numbers. If such shrimp were introduced into a country free from the pathogen, and crustaceans in the receiving country were exposed to infected tissues *per os*, there is a considerable risk that such exposed crustaceans may become infected and that the infection may become established in domestic populations of crustaceans. Misdirection of shrimp intended for human consumption as angling bait has previously been identified as a relevant risk for introduction of viral crustacean pathogens (Biosecurity Australia, 2009) and would provide a direct link to the aquatic environment. A pathway identified as a source of introduction of WSSV into the Americas was value added reprocessing of shrimp for the restaurant and retail trade (Lightner, 2011). Several other potential pathways of pathogen release may exist, and countries currently free from the pathogen may need to consider biosecurity measures in place to prevent its introduction.

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