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Cases of White Spot Disease (WSD) in European shrimp farms

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ABSTRACT

White Spot Disease (WSD) caused by White Spot Syndrome Virus (WSSV) is listed as 'non-exotic' to the European Union in EC Directive 2006/88. Two other viral diseases (Taura Syndrome and Yellowhead Disease) are listed as exotic. Despite the listing of WSD as a non-exotic disease, definitive case reports have not been officially reported, or published in the peer-reviewed literature. Here we report on a series of outbreaks of WSD in three European Union (EU) Member States (Greece, Italy and Spain) and in one non-EU European country (Turkey) over the period 1995 to 2001. Samples were submitted by industry representatives over this period and were therefore not officially reported to the Competent Authorities of respective European Member States. At least one of the cases appeared to be associated with the feeding of imported shrimp carcasses from Asia to broodstock while other cases were associated with the importation of post-larvae between hatcheries and on-growing facilities from outside of Europe and further movements within Europe. These case reports demonstrate the ability for WSSV to cause disease and mortality in penaeid shrimp farmed at European ambient temperatures. Furthermore, they demonstrate potential for the introduction of WSSV to new geographic areas via the movement of live crustaceans and their products, both from outside of the EU, and between EU Member and non-member countries within the European region.

1. Introduction

Alongside several diseases of finfish and molluscs, the crustacean diseases White Spot Disease (WSD) caused by White Spot Syndrome Virus (WSSV), Yellowhead Disease (YHD) caused by viruses in the Yellowhead virus complex, and Taura Syndrome (TS) caused by Taura Syndrome Virus (TSV), have recently been listed in European Community Directive 2006/88/EC. The listing of these diseases recognises their global importance in causing significant economic losses and the potential for their international transfer via trans-boundary trade in live crustaceans and their products. An important aspect of the Directive for both exporting nations (3rd countries) and importing nations (European Union Member States) requires recognition of the fact that some of the listed diseases are considered 'exotic' to the European Union (EU) whilst others, with limited or widespread distribution within Member States are deemed 'non-exotic' (Stentiford et al., 2010). In the case of the crustacean diseases, YHD and TS are currently considered exotic to the EU due to their apparent absence and a lack of report of the presence in farmed or wild susceptible species in aquatic habitats of the EU. Conversely, WSD is listed as 'non-exotic' to the EU, despite a lack of published or official reports to confirm the current or historic presence of the causative pathogen (WSSV) in EU aquatic habitats. The listing of WSD as a non-exotic disease appears to be based upon its anecdotal occurrence in penaeid shrimp farms in southern Europe during the late 1990's (see Stentiford et al., 2009 for context). However, to date, these cases descriptions have not been reported in the peer-reviewed literature. In this paper we summarise the available diagnostic data relating to samples of cultured European penaeid shrimp submitted to an OIE Reference Laboratory for WSSV during the 1990's. This period preceded adoption of EC Directive 2006/88/EC. Furthermore, apart from crayfish plague, crustacean diseases were not listed in previous European legislation dealing with finfish and molluscan health (EC Directive 91/67). The description of WSD in penaeid shrimp sampled from European culture facilities confirms the past presence of WSD in susceptible species within the EU. More recent submissions of farmed penaeid shrimp sampled from farms within the EU, to the OIE Reference Laboratory for WSSV (after 2001), have demonstrated an absence of WSSV. Whilst not making assumptions on the current status for WSD of specific EU Member States, or other countries within Europe, this paper provides a definitive statement for historic cases of WSD in Europe.

2. Materials and methods

Samples of marine penaeid shrimp (*Penaeus japonicus*, *Penaeus monodon*, *Penaeus semisulcatus*, *Metapenaeus kerathurus*) and *Artemia* sp., obtained from aquaculture facilities within EU Member States and

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from out-with the EU, but within the European continental area, were submitted by private industry representatives to the OIE Reference Laboratory for WSD at the University of Arizona, USA between 1995 and 2007. Samples for histology and *in situ* hybridization (ISH) were submitted fixed either in Davidson's AFA fixative or in neutral buffered formalin (Bell and Lightner, 1988). Samples for PCR were submitted preserved in absolute ethanol (see Table 1 for sample details).

Samples were processed for histology following the methods outlined in Bell and Lightner (1988). Samples were processed for ISH using the protocol as outlined in Section 2.5 of Lightner (1996). Samples were processed for nested PCR using the protocol of Lo et al. (1996). According to the OIE manual of diagnostic tests for aquatic animals, histopathology, *in situ* hybridization and PCR are all considered as presumptive and confirmatory tests for use on all life stages for detection of WSSV infection (OIE, 2009).

3. Results

Table 1 summarises the details of samples submitted by anonymous individuals from EU Member States and non-EU European nations during the period 1995 to 2007. Due to the *ad hoc* nature of submissions and the varied format of sample type, submissions from each country are dealt with separately. Specific locations of affected farms within Member States are not provided due to confidentiality agreements between those submitting samples and the testing laboratory.

3.1. Samples submitted from Spain

Six samples of shrimp (*P. japonicus*), one sample of *Artemia* sp., and one sample of pond sediment, collected from Spanish shrimp farms were submitted in 2000, 2001 and 2003 (case numbers 00-251, 00-276, 00-301, 00-333, 01-007, 01-042 and 03-257). One of the samples (00-251) displayed advanced (up to grade 4) WSSV infection by histology (Fig. 1) and ISH (data not shown) and two shrimp samples from 2000 (case numbers 00-301, 00-333) were positive for WSSV using PCR (Fig. 2). The *Artemia* sp. sampled from shrimp ponds in 2001 was negative for WSSV by PCR but WSSV viral DNA was detected in pond sediment collected from the same pond that previously underwent WSSV-associated mortality in 2000. The sample of *P. japonicus* collected during 2003 (case number 03-257) was negative (by histology) for WSSV infection but was positive for 'gut and nerve syndrome' (GNS at grade 3 severity), vibriosis and contained lymphoid organ spheroids (LOS) of unknown cause (Lightner, 1996).

3.2. Samples submitted from Italy

Four samples of *P. japonicus* and *P. semisulcatus* were collected from an Italian shrimp farm stocked with shrimp originating from Turkey in 1997 (case numbers 97-104, 97-110, 97-114 and 97-121). The largest single sample of 100 animals (case number 97-121) revealed a 40% apparent prevalence of WSSV infection based upon histology and ISH (Fig. 3).

3.3. Samples submitted from Greece

One sample of *P. japonicus* (n = 4) collected from a Greek shrimp farm was submitted in 1995 (case number 96-125). Histology revealed grade 3–4 WSSV-associated pathology and grade 2–4 GNS-associated pathology. ISH for WSSV on histological sections of infected shrimp revealed a weak-positive reaction in all 4 cases (data not shown). A second submission of *P. japonicus* collected from Greek shrimp farms in 2005 (case number 05-366) was negative for WSSV (and TSV, YHV and IHHNV) by PCR. A third submission of *M. kerathurus* collected from a Greek shrimp farm in 2007 (case number

Table 1
Summary of cases submitted from Europe to the OIE Reference Laboratory for WSD at the University of Arizona, USA over the period 1995 to 2007.

Case number	Date	Submitting country	Country of origin (if other)	Species	Test(s) ran	Diagnostic findings
96-125	6/21/95	USA	Greece	*P. japonicus*	Histology ISH	Histology: WSSV G3-4; GNS G2-4 ISH: 4/4 weak positive but on re-test 4/4 negative
97-104	6/4/97	Italy	Turkey	*P. japonicus* *P. semiculcatus*	Histology	WSSV G2-4
97-110	6/10/97	Italy	Turkey	*P. japonicus*	ISH	Poorly fixed tissues. ISH — WSSV positive
97-114	6/16/97	Italy	Turkey	*P. japonicus* *P. semiculcatus*	ISH	Poorly fixed tissues. ISH — WSSV positive.
97-121	6/23/97	Italy	Turkey	*P. japonicus*	Histology ISH	Histology: 27/100 WSSV G3-4; 13/100 Susp. WSSV ISH: 4/40 WSSV positive; 2/40 suspect WSSV
97-138	7/15/97	Turkey	n/a	*P. japonicus* *P. monodon* *P. semiculcatus*	Histology	Histology: GNS G4; WSSV not detected.
97-179	9/3/97	Turkey	n/a	*P. japonicus* *P. monodon* *P. semiculcatus*	Histology	Histology: WSSV G3-4; GNS G1-4; MBV G1-4
98-28	3/30/98	Turkey	n/a	*P. japonicus* *P. semiculcatus*	Histology	Not suitable for diagnosis
98-74	4/29/98	Turkey	n/a	*P. japonicus* *P. semiculcatus*	Histology ISH	Histology: WSSV positive ISH: WSSV positive
98-115	6/18/98	Turkey	n/a	*P. japonicus*	Histology	Histology: WSSV G2-3
00-251	7/28/00	Spain	n/a	*P. japonicus*	Histology	Histology: WSSV G1-4
00-276	8/15/00	Spain	n/a	*P. japonicus*	PCR	PCR: WSSV not detected
00-301	9/14/00	Spain	n/a	*P. japonicus*	PCR	PCR: WSSV positive
00-333	10/19/00	Spain	n/a	*P. japonicus*	PCR	PCR: WSSV positive
01-007	1/12/01	Spain	n/a	*P. japonicus*	PCR	PCR: WSSV, IHHNV, YHV not detected
01-042	2/13/01	Spain	n/a	*Artemia* and sediment	PCR	PCR: *Artemia* — WSSV not detected. Sediment — WSSV positive
03-257	8/7/03	Spain	n/a	*P. japonicus*	Histology	Histology: GNS G3; LOS G1; vibriosis, biofouling
05-366	10/4/05	Greece	n/a	*P. japonicus*	PCR	WSSV, TSV,YHV, IHHNV not detected
07-357	11/8/07	Greece	n/a	*P. kerathurus*	Histology ISH PCR	Histology: bacterial infection ISH: bacteria positive WSSV not detected

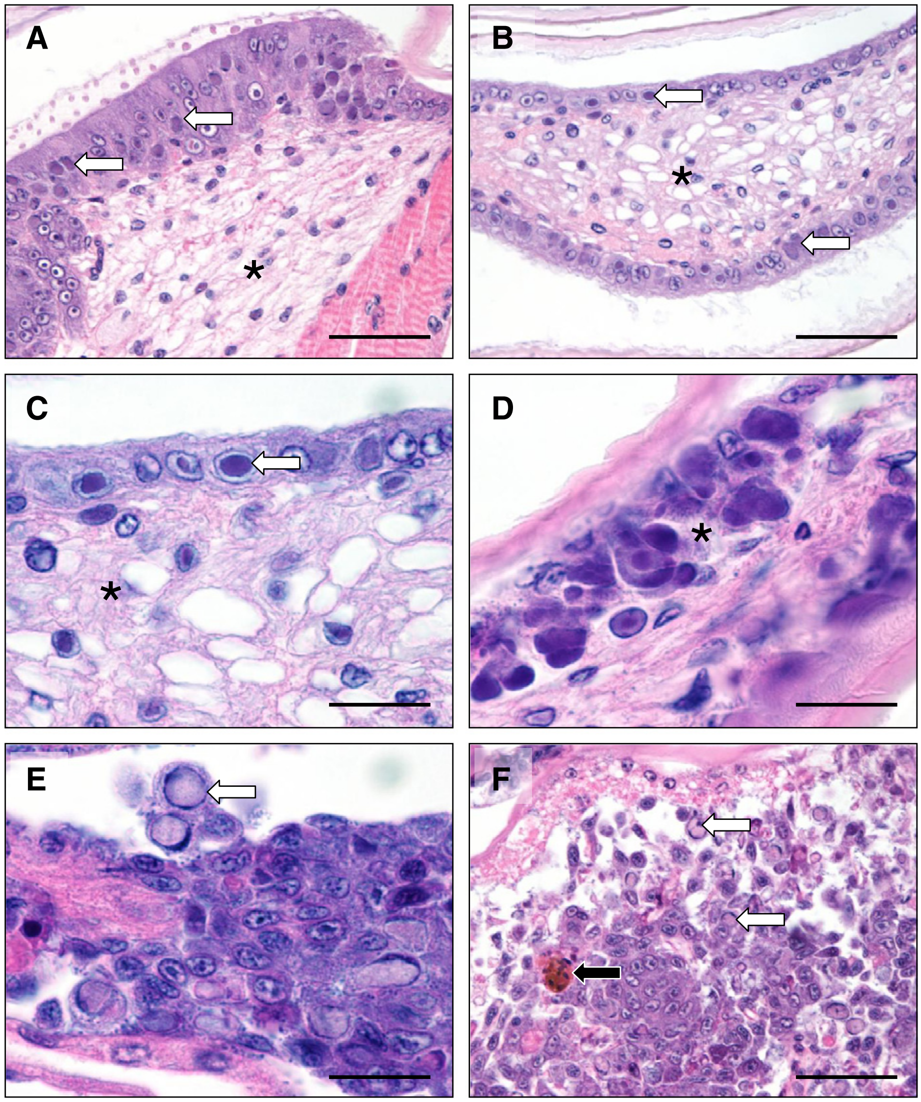

Fig. 1. Histology of White Spot Disease (WSD) caused by White Spot Syndrome Virus (WSSV) in *Penaeus japonicus* collected from a farm undergoing mass mortalities in Spain during 2000. (A) Infected nuclei containing Cowdry-like inclusions in the sub-cuticular epidermis (arrows) and connective tissues (asterisk). Scale = 100 μm. (B) Infected sub-cuticular epidermis (arrows) and connective tissues (asterisk) associated with the stomach. Scale = 100 μm. (C) Classic Cowdry-like inclusions in infected sub-cuticular epidermis (arrow) and connective tissues (asterisk). Scale = 25 μm. (D) Necrosis of heavily infected sub-cuticular epidermal cells within a limb (asterisk). Scale = 25 μm. (E) Infected haemopoietic tissues with formation and liberation of WSSV-infected haemocytes to haemolymph (arrow). Scale = 25 μm. (F) Heavily infected and necrotic lymphoid organ with WSSV-infected phagocytic cells (white arrows) and formation of melanised granulomatous lesions (black arrow). Scale = 25 μm. All images H&E.

07-357) revealed a systemic bacterial infection (by histology and ISH) but was also WSSV negative by PCR.

3.4. Samples submitted from Turkey (non-EU country)

Five samples of shrimp (*P. japonicus*, *P. semisulcatus* and *P. monodon*) collected from Turkish shrimp farms were submitted in 1997 (case numbers 97-138, 97-179) and in 1998 (case numbers 98-28, 98-74, 98-115). Histology revealed advanced grade (3–4) WSSV infection in several cases from 1997 and 1998, with confirmation in most cases by ISH (data not shown). Samples collected during 1997 also revealed the presence (by histology) of GNS in *P. japonicus* and Monodon Baculovirus (MBV) in *P. monodon* (Lightner, 1996 and OIE, 2009).

4. Discussion

This paper summarises the available scientific evidence for outbreaks of White Spot Disease (WSD) associated with infection with White Spot Syndrome Virus (WSSV) in European shrimp farms over the period 1995 to 2001. Samples submitted by private industry representatives from European countries to the OIE Reference Laboratory for WSD (USA) subsequent to 2001 have been negative for WSSV and furthermore, due to the absence of reporting to Competent Authorities by the industry within affected countries over the period covered by these submissions, no official notifications of disease were made by Competent Authorities, either to the European Commission, or to the OIE.

This report specifically confirms via histology, *in situ* hybridisation and PCR, previous outbreaks of WSD in three EU Member States (Spain, Italy and Greece) and in one non-EU country (Turkey). According to case notes submitted to the OIE reference laboratory during the affected period, all outbreaks were associated with elevated mortalities in marine penaeid shrimp farming operations on the Mediterranean coastline of Southern Europe. Case notes from at least one of the outbreaks (in Spain) appears to highlight that disease followed feeding of wild harvested *P. japonicus* broodstock with carcasses of *P. monodon* imported from Asia. Other cases (e.g. in

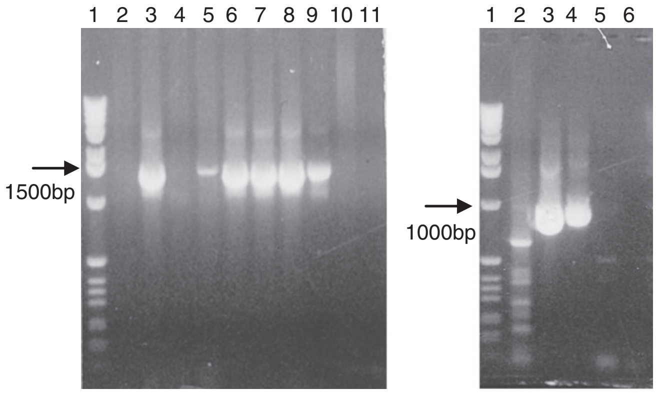

Fig. 2. PCR for detection of WSSV in *P. japonicus* from Spanish shrimp farm undergoing mortality during 2000 (case number 00-301) using the protocol of Lo et al. (1996). (A) 1st round PCR. Lane 1 (1 kb marker), 2 (00-301-A), 3 (00-301-B), 4 (00-301-C), 5 (00-301-D), 6 (00-301-E), 7 (00-301-F), 8 (00-301-G), 9 (WSSV +ve control), 10 (SPF *P. vannamei* −ve control), 11 (no template control). (B) 2nd round PCR. Lane 1 (1 kb marker), 2 (00-301-A), 3 (00-301-C), 4 (WSSV +ve control), 5 (SPF *P. vannamei* −ve control), 6 (no template control). Expected amplicons on 1st round 1447 bp and 2nd round 941 bp. All samples were positive for WSSV apart from 00-301-A.

Italy) were associated with the prior importation of shrimp post-larvae from a European (non-EU) country (Turkey) (Pers. Comm. Professor Giuseppe Bovo, Italy) while the outbreak of WSD in Greece in 1995 was likely due to the importation of WSSV-infected post larval shrimp from Taiwan (Pers. Comm. Dr Thanos Prapas, Greece). The potential for entry of WSSV via live animals (in the case of Italy and Greece) and non-viable animals (in the case of Spain) provides ample demonstration of the potential for WSSV to cause negative impact in countries previously considered free of the pathogen and highlights the role of international legislation (such as that provided in the recently adopted EC Directive 2006/88/EC) in protecting against further outbreaks in susceptible species of farmed and wild crustaceans (Stentiford et al., 2010). These case reports also demonstrate the potential for outbreaks of WSD and other diseases of shrimp (e.g. MBV, GNS) at ambient conditions experienced in European aquatic habitats, particularly in the Mediterranean region. The specific characteristics of WSSV, including a particularly wide susceptible host range which spans across at least the decapod crustaceans (Stentiford et al., 2009) are cause for concern, particularly when its passage to susceptible species does not seem to be hampered by ambient temperatures experienced in the majority of Europe (Corbel et al., 2001; Jiravanichpaisal et al., 2001, 2004). The limited culture industry for penaeid shrimp in Europe (mainly for *P. japonicas*, with a total value of $2 m in 2004) contrasts a large marine coastal and offshore fishery for crustaceans with catches totalling almost 400,000 Mt per annum (source: http://www.fao.org/figis). These wild fisheries are considered as key resources in Europe and in many countries (such as the UK), are amongst the most valuable marine fisheries resources, ranking above most important finfish species in terms of production quantity and value (Stentiford, 2011; Stentiford et al., 2010). The commercial fishery for crustaceans shares an inshore territory with shrimp farming operations in some regions and potential for transfer of pathogens between farm operations and wild stocks cannot be ruled out.

The listing of WSD in EC Directive 2006/88 is in recognition of its classification as perhaps the most serious disease threat facing the global penaeid shrimp farming industry, with cumulative losses exceeding $10 bn since its emergence in the early 1990's (OIE, 2009; Sánchez-Martínez et al., 2007). Recently, Stentiford et al. (2010) have described the implications of EC Directive 2006/88 for EU Member States and for those countries wishing to import live crustaceans and their products into the EU. Based on the listing of WSD as a non-exotic pathogen to the EU, Member States are required to designate their status for the pathogen causing this disease into one of five categories; (1) Disease free, (2) Under surveillance, (3) Undetermined, (4) Under eradication, or (5) Infected. The designated status has implications for the export of live susceptible species (and products associated with them) to other Member States within the EU and possibly, outside of the EU. It also affects the ability of 3rd countries (outside of Europe) to export live crustaceans and their products into the EU, particularly where the disease status of the exporting and importing nations are different.

Improved knowledge of globally significant pathogens such as WSSV and the potential for their transboundary movement in live crustaceans and their products is implied by measures laid down in EC Directive 2006/88. However, significant gaps still exist in defining the risk associated with the movement of these different products across international borders and importantly, on the consequence of their effect should they be released into natural habitats of receiving

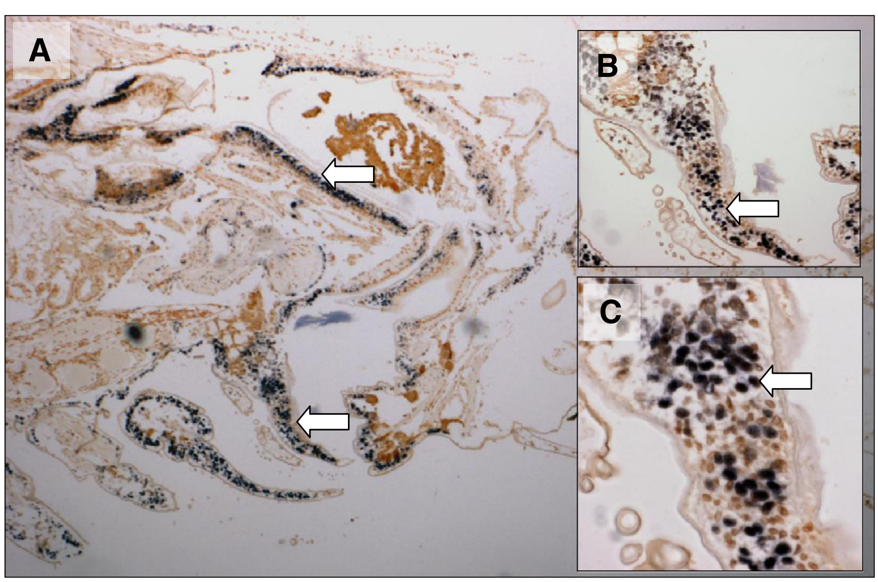

Fig. 3. *In situ* hybridisation of a WSSV-specific gene probe to a section of *P. japonicus* collected from an Italian shrimp farm in 1997 (case number 97-121). (A) Low power image depicting a predominantly sub-cuticular epidermal localisation of the probe (arrows). (B, C) Higher power images of thoracic appendages depicting localisation of the probe to nuclei of sub-cuticular epidermal and connective tissue cells (arrows). ISH protocol according to Lightner (1996).

regions. The responsibility for enhanced biosecurity associated with global movements is therefore a shared one between net producing nations and net consumers.

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