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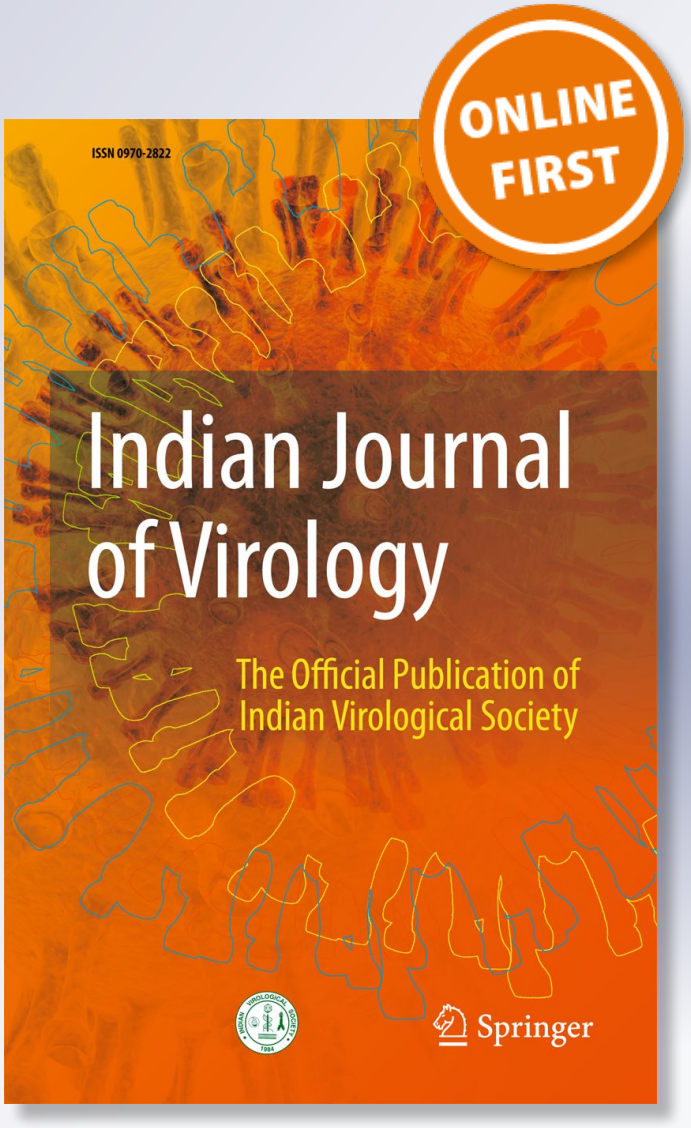

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REVIEW ARTICLE

Biology, Host Range, Pathogenesis and Diagnosis of *White spot syndrome virus*

Balakrishnan Pradeep · Praveen Rai · Seethappa A. Mohan · Mudagandur S. Shekhar · Indrani Karunasagar

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Abstract *White spot syndrome virus* (WSSV) is the most serious viral pathogen of cultured shrimp. It is a highly virulent virus that can spread quickly and can cause up to 100 % mortality in 3–10 days. WSSV is a large enveloped double stranded DNA virus belonging to genus *Whispovirus* of the virus family *Nimaviridae*. It has a wide host range among crustaceans and mainly affects commercially cultivated marine shrimp species. The virus infects all age groups causing large scale mortalities and the foci of infection are tissues of ectodermal and mesodermal origin, such as gills, lymphoid organ and cuticular epithelium. The whole genome sequencing of WSSV from China, Thailand and Taiwan have revealed minor genetic differences among different strains. There are varying reports regarding the factors responsible for WSSV virulence which include the differences in variable number of tandem repeats, the genome size and presence or absence of different proteins. Aim of this review is to give current information on the status, host range, pathogenesis and diagnosis of WSSV infection.

Keywords WSSV · Shrimp · Host range · Pathogenesis · Diagnosis · PCR

B. Pradeep (✉)
Krishi Vigyan Kendra, Indian Institute of Spices Research, Peruvannamuzhi, Kozhikode 673528, Kerala, India
e-mail: nairpradeep2@rediffmail.com; nairpradeep2@gmail.com

P. Rai · S. A. Mohan · I. Karunasagar
Department of Fishery Microbiology, College of Fisheries, Karnataka Veterinary, Animal and Fisheries Sciences University, Mangalore 575 002, India

M. S. Shekhar
Genetics and Biotechnology Unit, Central Institute of Brackishwater Aquaculture, Chennai, India

Introduction

Shrimp culture covers a major portion of the aquaculture industry and its export forms the main source of revenue of many countries. Global aquaculture of penaeid shrimp has grown rapidly during the last two decades and its culture is being undertaken even in saline aquifers, marine, fresh and brackish water areas. The farmed shrimps contributed 42.2 % of total global shrimp production of 6.67 million tones in 2009. This alarming increase was possible due to the intensification of farming practices, expansion of culture and the scientific inputs provided to farmers. Due to the intensification and specialization of shrimp farming, the contribution from farm-raised shrimp to total world supply of shrimp grew by approximately 400 % over one decade during 1985–1995. The shrimp farming sector has grown at an annual average growth rate of over 18.8 % since 1970 [23]. Approximately 75 % of global shrimp production is from aquaculture which is now almost entirely dominated by two species—the black tiger shrimp (*Penaeus monodon*) and the white Pacific shrimp (*Litopenaeus vannamei*) [134].

In India, commercial shrimp farming started gaining roots only during the mid-eighties. The boom period of commercial-scale shrimp culture in India started in 1990 and the worst came in 1995–1996, with the outbreak of viral disease. The fact that most of the coastal States in India were new to commercial-scale shrimp farming, the general ignorance of good farming practices, and the lack of suitable extension services, led to a series of problems [150]. In India, a variety of farming techniques are practiced like traditional, improved traditional, extensive, semi-intensive and intensive. The scientific shrimp farming has shown phenomenal growth since early nineties, and at present about 150,000 ha is under shrimp farming which is about 15 % of the total potential brackishwater area available in

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the country. Development of shrimp farming in the country has been restricted mainly to east coast, due to availability of shrimp broodstock, seed and location of hatcheries. Shrimp farming in India is mainly restricted to monoculture of black-tiger shrimp, *P. monodon*, which accounts for more than 95 % of the production [39]. Farmed shrimp yield in India has decreased to 75,997 tones in 2009–2010. The decline was around 28.4 % in production and 10.9 % in area compared to previous year as per MPEDA owing to recent disease outbreaks. Despite the decline in production, an increase in shrimp export was recorded, which may be due to high global demand for shrimp and a consequence of lessening of economic crisis. Intensive shrimp cultivation, inadequate sanitation, poor pond management and irresponsible movement of broodstock and larvae have enhanced the disease incidence and its spread [55].

Since 1981, several new viral pathogens have emerged successively, causing mass mortalities and threatening the economic sustainability of the industries in Asia and the Americas [132]. With expansion of the shrimp industry, the number of viral pathogens of penaeid shrimp has also increased, reaching a new high of 22 [94]. Among the known viruses of shrimp, white spot disease caused by *White spot syndrome virus* (WSSV) has had the greatest impact on shrimp culture and continues to be an obstacle to sustainable shrimp farming [135]. The Government of India has recently given approval for culturing *L. vannamei* in India. This shrimp can be cultured only in those farms which have got approval from Coastal Aquaculture Authority (CAA). Many farms in East coast have taken up its culture. The projected production of *L. vannamei* in India is about 50,000 tonnes. Although the farmers got good crops initially there are recent reports of WSSV outbreak in these farms. It should be borne in mind that *L. vannamei* culture crashed in Latin American countries such as Ecuador due to WSSV. This review mainly focuses on current information on the status, host range, pathogenesis and diagnosis of WSSV infection in crustaceans.

Geographical Distribution

Since the discovery of WSSV in China (Fujian) in 1992, the virus spread rapidly to all the major shrimp farming countries of the world due to intensification in shrimp farming and inadequate sanitation [134]. By 1996 the disease had spread to major shrimp farming regions of East Asia, South East Asia, Indonesia and India. In 1992, it was first detected in Taiwan and then spread to all Asian countries [100, 132]. In Japan, white spot disease was observed in *Penaeus japonicus* in 1992 [28, 40]. In 1995 it was first diagnosed in the United States of America (Texas and South-Carolina) [93]. During 1998, shrimp exhibiting gross signs and histopathology of white spot disease (WSD) was reported from India and Korea [45, 46, 49]. In early 1999, WSSV had spread to Latin America and was reported in Central and South-America [94]. In 2002, the virus was detected in Europe (France) and the Middle East (Iran) [95]. In 2004, it was observed in *P. indicus* from Choebdeh area in Iran [116]. Australia is the only shrimp farming country free of WSSV, where shrimp farming is being practiced, which is a result of the strict quarantine measures adopted by the Australian authorities. The original source of virus still remains a mystery [133].

Clinical Signs and Transmission

In cultured shrimp, WSSV infection can result in a cumulative mortality of up to 100 % within 3–10 days [55]. Infected animals show lethargic behavior, reduction in food consumption, reduced preening activities, anorexia, loose cuticle and reddish to pink body discoloration [12]. A characteristic of the syndrome due to the virus include white spots on the exoskeleton especially on the carapace and last abdominal segment (Fig. 1). These spots are the result of calcified deposits that range in size from a few mm to 1 cm or more in diameter [12]. However, in the case of acute (experimental) infections, the only signs of WSSV infection observed are lethargy and lack of appetite. In *P. indicus*, body turns to reddish colour and it is necessary to remove the carapace to confirm WSSV infection [90].

The modes of transmission of WSSV in natural environment are mainly by vertical and/or horizontal route. Horizontal transmissions is by ingestion of dead infected shrimp, by contact with water containing infected animals or free virus particles. Infection by the latter is thought to occur primarily through the gills, but may occur via other

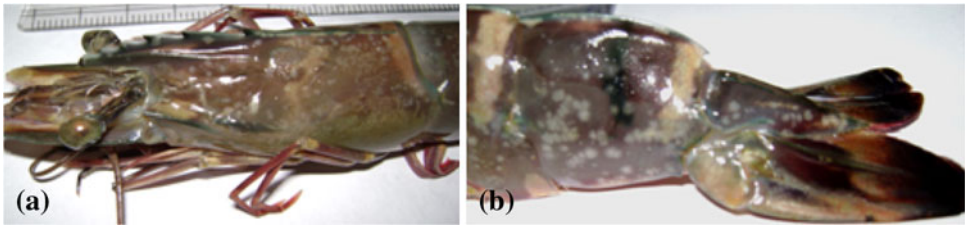

Fig. 1 *White spot syndrome virus* infected *Penaeus monodon* showing white spot symptoms on carapace (**a**) and last abdominal segment (**b**)

body surfaces as well [6, 12, 13]. Lotz and Soto demonstrated that, transmission by cohabitation is of a lower magnitude than ingestion transmission [62]. Vertical transmission occurs from the mother to offspring through viral particles being shed at the time of spawning and then ingested by larvae at first feeding, although it is not clear whether the WSSV virions are present inside the shrimp eggs [35, 58, 84, 117]. By using in situ hybridization and TEM, WSSV viral particles were located in the connective tissue layer surrounding the seminiferous tubules in testes and in the case of ovary, viral particles were detected in follicle cells, oogonia, oocytes and connective tissue cells. In addition, no infection was found in mature eggs, which may imply that infected eggs were killed by the virus before maturation [58]. No penaeid shrimp species are known to be resistant to WSSV infection [55, 61]. Even specific pathogen free (SPF) shrimps were observed to be asymptomatic carriers of WSSV which was attributed to viral latency. The latent infection of WSSV is reported to be associated with three WSSV genes (ORFs 366, 151 and 427) [47].

Taxonomic Affiliation

White spot syndrome virus (WSSV) was earlier known by different names until 2005 when the virus got its internationally approved name [131]. WSSV was originally called a baculovirus based on its cylindrical morphology and on the histological lesions that resembled those of "non-occluded" baculoviruses [146]. The other names by which it was known earlier, includes hypodermal and hematopoietic necrosis baculovirus (HHNBV) [36], rod-shaped nuclear virus of *P. japonicus* (RV-PJ) [113], Chinese baculovirus (CBV), systemic ectodermal and mesodermal baculovirus (SEMBV) [92], penaeid rod-shaped DNA virus (PRDV) [41], white spot baculovirus (WSBV) [57] and now as white spot syndrome virus (WSSV) [55]. The disease caused by WSSV is often indicated as white spot disease (WSD). The taxonomic position of WSSV was assigned by the International Committee of Taxonomy of Viruses (ICTV) to new genus, *Whispovrius*, under the family *Nimaviridae* based on its unique morphology and genomic composition [67, 68, 130]. Since WSSV has a thread-like polar extension at one end of the virus particle, WSSV became the sole species of a new family *Nimaviridae* [53, 100].

Host Range

WSSV has a wide host range which includes all cultured, wild marine shrimps, crabs, lobsters, crayfishes, squilla, copepods and freshwater cultures species of *Macrobrachium rosenbergii* [5, 7, 10, 16, 22, 24, 32, 43, 57, 63, 71, 83, 91, 98, 106, 111, 112, 138, 160]. WSSV is potentially lethal to most of the commercially important species of penaeid shrimps including *P. monodon, P. vannamei, P. indicus, P. japonicus, P. chinensis, P. penicillatus, P. azteus, P. merguiensis, F. duorarum, P. stylirostris* [79]. Table 1 shows the list of host species for WSSV. The susceptibility to WSSV differs significantly between hosts. In some species, WSSV results in a non-lethal or latent infection, making these species potential virus reservoirs and important sources of infection in shrimp ponds. In addition, the occurrence of WSSV in pond soil was detected by nested-PCR [75]. Hence, soil may be an important aspect of WSSV ecology and may have an implication for viral transmissibility. The study conducted in 2006 shows that, rotifer (*Brachionus urceus)* serve as a vector of WSSV to infect the shrimps [159]. By PCR-dot blot hybridization, WSSV was detected in rotifer resting eggs from shrimp culture-pond sediments. Detection of WSSV from disinfected eggs suggests the presence of WSSV within eggs and indicates vertical transmission of WSSV [151]. Studies need to be taken up to know, further on the host range of WSSV as it will be helpful in preventing the spread of disease and also to know the reasons behind the variation in host susceptibility to infection.

Histopathology and Cytopathology

WSSV infects most tissues originating from both ectoderm and mesoderm. Histological signs of WSSV infection include enlarged nuclei in tissues. These include subcuticular epithelium, gills, lymphoid organs, antennal gland, hematopoietic tissues, connective tissue, ovary and the ventral nerve cord [146]. The most convenient tissue for diagnosis is the subcuticular epithelium in tissue sections. The subcuticular epithelium of the stomach usually provides excellent views revealing pathognomonic enlarged nuclei containing basophilic inclusions which are surrounded by vacant cytoplasm. Nuclei at the early stage of infection show marginated chromatin separated from a reddish inclusion by a ring of unstained nucleoplasm. These are called Cowdry A-type inclusions [25]. During early infection stages, the stomach, gills, cuticular epidermis and the connective tissue of the hepatopancreas gets infected. At later stages of infection, the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, hindgut and parts of the midgut also become infected. The nervous system and the compound eyes are infected very late. The stomach, gills, cuticular epidermis, lymphoid organ, hematopoietic tissue and antennal gland are all

Table 1 The known host species reported to be naturally or experimentally infected with *White spot syndrome virus*

Scientific Name	Common Name	Type	References
A. Shrimps			
Alpheus brevicristatus	Snapping shrimp	N	[112]
Alpheus lobidens	Apping shrimp	N	[112]
Aristeus sp.	Red shrimp	N	[5]
Callianassa sp.	Mud shrimps	N	[57]
Exopalaemon orientalis	Oriental prawn	N, E	[7, 24, 138]
Farfantepenaeus aztecus	Northern brown shrimp	E	[55]
Farfantepenaeus duorarum	Pink shrimp	E	[55]
Fenneropenaeus penicillatus	Red tail shrimp	N	[12, 57, 138]
Fenneropenaeus chinensis	Fleshy shrimp	N	[24]
Heterocarpus sp.		N	[5]
Litopenaeus vannamei	Whiteleg shrimp	N, E	[55]
Litopenaeus setiferus	Northern white shrimp	E	[55]
Macrobrachium rosenbergii	Giant freshwater shrimp	N, E	[5, 7, 24, 32, 57, 91, 138]
Macrobrachium idella	Sunset shrimp	E	[91]
Macrobrachium lamarrei	Kuncho river prawn	E	[100]
Marsupenaeus japonicus	Kuruma shrimp	N, E	[57, 113, 138]
Metapenaeus ensis	Greasyback shrimp	N, E	[7, 57, 138]
Metapenaeus dobsoni	Kadal shrimp	N, E	[5, 32, 91]
Metapenaeus lysianassa	Bird shrimp	N	[100]
Exopalaemon orientis	Oriental prawn	E	[7]
Metapenaeus monoceros	Speckled shrimp	E	[91]
Metapenaeus elegans	Fine shrimp	N	[5]
Metapenaeus brevicornis	Yellow prawn		[70]
Metapenaeus affinis	Jinga shrimp		[70]
Palaemon adspersus	Baltic prawn	E	[16]
Palaemon styliferus	Grass shrimp	N	[24, 57]
Parapenaeopsis stylifera	Kiddi shrimp	N	[5, 32]
Penaeus monodon	Giant tiger shrimp	N, E	[5, 12, 57, 147]
Penaeus indicus	Indian white prawn	N, E	[24, 91]
Penaeus merguiensis	Banana prawn	N	[24]
Penaeus semiculcatus	Green tiger prawn	N, E	[57, 91, 138]
Penaeus canaliculatus	Striped prawn	N	[70]
Penaeus aztecus	Brown shrimp		[55]
Penaeus schmitti	Southern white shrimp	E	[100]
Penaeus chinensis	Chinese white shrimp	N/E	[2]
Penaeus duorarum	Northern pink shrimp	N/E	[55]
Penaeus setiferus	Northern white shrimp	N/E	[55]
Penaeus stylirostris	Blue shrimp	E	[55]
Solenocera crassicornis	Coastal mud shrimp	N	[32]
Solenocera indica		N	[32]
Seranopsis longipes		N	[70]
Solenocera choprai	Ridgeback shrimp	N	[70]
Squilla sp.	Mantis shrimp	N, E	[5, 32]
Trachypenaeus curvirostris	Southern rough shrimp	E	[7, 138]
B. Crabs			
Atergatis integerrimus	Bashful crab	E	[96]
Cancer pagurus	Edible or rock crab	E	[16]
Calappa lophos	Box crab	N, E	[5, 24]
Calappa philargius	Box crab	E	[98]
Callinectes arcuatus	Swimming crab	N	[26]
Callinectes sapidus	Blue crab	N	[8]
Carcinus maenas	Littoral crab	E	[16]
Charybdis annulata	Swimming crab	N, E	[32, 98]
Charybdis cruciata	Red sea crab	N	[5, 32]
Charybdis feriata	Coral crab	N	[57]
Charybdis granulata	Swimming crab	E	[7]
Charybdis hoplites	Swimming crab	N	[5]
Charybdis lucifera	Swimming crab	N, E	[5, 98]
Charybdis natator	Hairyback crab	N	[24]
Charybdis riversandersoni		N	[70]
Demania splendida		E	[96]

Table 1 continued

Scientific Name	Common Name	Type	References
Doclea hybrida		E	[98]
Gelasimus marionis nitidus		N	[32]
Grapsus albolineatus	Rock crab	E	[98]
Halimede ochtodes	Hairy crab	E	[98]
Helice tridens	Shore crab	N	[57]
Liagore rubromaculata		E	[98]
Liocarcinus depurator	Harbour crab	E	[16]
Liocarcinus puber	Velvet swimming crab	E	[16]
Lithodes maja	Deepsea king crab	E	[98]
Macrophthalmus sulcatus	Ghost/fiddler crab	N	[32]
Mantura sp.		N	[24]
Matuta miersi	Moon crab	E	[98]
Matuta planipes	Moon crab	N	[81]
Metopograpsus messor	Purple climber crab	N, E	[32, 91]
Menippe rumphii	Stone crab	E	[96]
Paradorippe granulata		E	[98]
Parthenope prensor	Elbow crab	E	[98]
Parathelphusa hydrodomous		E	[97]
Parathelphusa pulvinata		E	[97]
Philyra syndactyla	Purse crab	E	[98]
Podophthalmus vigil	Long-eyed swimming crab	E	[98]
Portunus pelagicus	Sand crab	N, E	[5, 57, 111]
Portunus sanguinolentus	Blood spot crab	N, E	[5, 57, 98]
Pseudograpsus intermedius	Mosaic crab	N	[5]
Scylla serrata	Mud crab	N, E	[5, 10, 57, 91, 98, 111]
Scylla tranquebarica	Mangrove crab	E,N	[27, 91]
Scylla olivacea	Orange mud crab	E	[108]
Scylla paramamosain	Green mud crab	E	[108]
Sesarma sp.	Marsh crabs	N, E	[24, 91]
Somannia-thelpusa sp.	Black rice crab	E	[24]
Thalamita danae	Swimming crab	N, E	[24, 98]
C. Lobsters			
Acetes sp.	Krill	E	[111]

Table 1 continued

Scientific Name	Common Name	Type	References
Panulirus homarus	Scalloped spiny lobster	E	[91]
Panulirus longipes	Longlegged spiny lobster	E	[24]
Panulirus ornatus	Ornata spiny lobster	E	[24, 91]
Panulirus penicillatus	Pronghorn spiny lobster	E	[7]
Panulirus polyphagus	Mud spiny lobster	E	[91]
Panulirus versicolor	Painted spiny lobster	E	[7]
Scyllarus arctus	Small European locust lobster	E	[16]
D. Crayfish			
Astacus leptodactylus	Turkish crayfish	E	[16]
Astacus astacus	Broad-fingered crayfish	E	[44]
Cherax destructor albidus	Yabby	E	[22]
Cherax quadricarinatus	Australian redclaw	E	[105]
Orconectes limosus	Spinycheek crayfish	E	[16]
Orconectes punctimanus	Spothanded Crayfish	N	[141]
Pacifastacus leniusculus	Signal crayfish	E	[43]
Procambarus clarkia	Red swamp crayfish	E	[7, 138]
E. Insects			
Ephydridae sp.	Shore fly	N	[57]
Gerries		N	[70]
Notonecta	Backswimmer	N	[70]
Nepa		N	[70]
Ranatra	Water scorpion	N	[70]
Dragon nymphfly		N	[70]
F. Copepod, Polychaeta, Rotifera and Strmatopoda			
Schmackeria dubia	Copepoda	N	[57]
Squilla mantis	Mantis shrimp	N	[97]
Marphysa gravelyi	Polychaeta	N	[129]
Brachionus urceus	Rotifera	NE	[151, 159]
G. Brine shrimps			
Artemia sp.		E	[98]
A. franciscana		E	[54]

heavily infected with WSSV at late stages of infection and become necrotic [6, 58]. Cells of the hepatopancreatocytes and epithelial cells of the midgut have never been shown to be infected with WSSV. A significant reduction in the total

hemocyte count is observed after shrimp are infected with WSSV [30, 43, 121]. This is probably caused by infection of the hemocytes themselves as well as by apoptosis in the WSSV infected hematopoietic tissue, from which the hemocytes derive [145]. WSSV infected hemocytes either undergo apoptosis or are removed from the circulation by attaching to host tissues. As a consequence abnormal low amount of hemocytes weaken the shrimp defenses.

WSSV replication initially occurs in the nucleus and early signs of infection are characterized by the appearance of homogeneous hypertrophied nuclei and marginated chromatin [55, 140, 145]. Virus morphogenesis is initiated by the formation of viral envelopes in the nucleoplasm. The formation of the nucleocapsids begins with extended, empty, long tubules, which break up into fragments of 12–14 rings to form empty nucleocapsid shells. Subsequently, the empty capsids are surrounded by the envelope leaving at one end an open extremity. The nucleoproteins, possibly together with the viral DNA, enter the empty capsid through its open end. Mature virions are obtained after narrowing of the open end and formation of a tail-like extension of the envelope [21, 140]. There are two postulates regarding the transmission of WSSV virions from one cell to another. These are either by budding or by rupture of the nuclear envelope or cell membrane of infected cell.

Morphology and Ultrastructure

Electron microscopy studies of ultra thin sections and WSSV viral suspensions obtained from infected shrimp revealed that, the virion are enveloped, rod shaped, nucleocapsids with a bacilliforms to ovoidal particles of about 275 nm in length and 120 nm in width. These virions have a tail-like appendage at one end, of which neither the function nor the composition is known [21, 130]. Electron microscopic examination of white spot-diseased *P. monodon* and *P. indicus* revealed the occurrence of a non-occluded, rod-shaped, double-enveloped WSSV some of which possessed a tube-like or branched extension and empty capsids. The virions measured 154 ± 2 by 373 ± 5 nm and nucleocapsids 111 ± 1.5 by 293 ± 7 nm [90]. The isolated nucleocapsid have cross hatched appearance and a size about 300×70 nm formed by stacks of rings (about 14 in total), which are in turn formed by regular spaced globular subunits of about 8 nm in diameter, arranged in two parallel rows [21, 72]. The virion contains at least 5 major polypeptides ranging from 15 to 28 kDa [130]. The nucleocapsid contains the viral genome and mainly consists of the WSSV encoded proteins VP664, VP26, VP24 and VP15 [52, 123, 124, 127]. VP664, a remarkable large protein of around 664 kDa, is thought to be the major core protein, responsible for the striated appearance of the nucleocapsids [52]. VP15, a highly basic protein with no hydrophobic regions, is a histon-like, double-stranded DNA-binding protein [144]. The function of VP26 and VP24 in the nucleocapsid are unknown. Furthermore, about 40 WSSV encoded minor virion proteins were identified by protein sequencing of individual bands after applying purified WSSV virions on a SDS-PAGE gel [37, 38, 52, 120, 123, 124, 127, 158]. The various geographic isolates of WSSV, which have been characterized, are very similar if not identical in morphology and proteome [72, 142].

WSSV Genome

The WSSV genome constitutes a circular, supercoiled, double-stranded (ds) DNA of approximately 300 kilobase pairs (kbp) [21, 72, 146]. Sequence analysis of WSSV genome and comparison of sequence data have shown, it to be unique in not showing any homology with any known viruses [58]. Hence, it has been assigned to a new family (*Nimaviridae*) and genus (*Whispovirus*) [122, 130]. It is one of the largest animal virus that has been completely sequenced so far [122, 152]. The various geographical isolates of WSSV identified are very similar in morphology and protein profile [19]. The restriction fragment length polymorphism (RFLP) patterns of isolates show only limited differences, suggesting a high degree of genomic stability [19, 60, 65, 72, 140, 141]. Two complete genome sequences of WSSV isolates were published in 2001, from Thailand (WSSV-TH, AF369029) [126] and China (WSSV-CN, AF332093) [152]. A third complete genome sequence was published in 2002 from Taiwan (WSSV-TW, AF440570) [118]. The complete genome sequences of these three isolates show a considerable difference in genome size of 292,967, 305,107 and 307,287 bp, for the Thailand, China, and Taiwan isolates respectively, but with an overall identity of 99.32 %. The WSSV-CN genome has a total GC content of 41 % [152]. A total of 684, 531 and 507 putative ORFs have been identified by sequence analysis on the WSSV-TH, WSSV-CN, and WSSV-TW isolates, respectively. Among these putative ORFs, only 184 (WSSV-TH) and 181 (WSSV-CN) are likely to encode functional proteins [126, 152]. Several studies have been carried out on WSSV isolates from different parts of Asia on WSSV genome. The variations associated with the three minisatellite ORF 94, ORF 75 and ORF125 were used for genotyping WSSV and for epidemiological purpose [87, 88, 102, 103, 136, 147]. Further studies on genotyping can be carried out based on the microsatellites associated with WSSV genome. Characterization of variable genomic regions of WSSV from different parts of the world need to be done which will be helpful to elucidate the genetic

evolution of WSSV and to know the variations that are taking place in WSSV genome over a period of time and its relevance to virulence.

Virulence

Virulence is the degree of pathogenicity within a group or species. Preliminary studies have shown, slight difference in virulence between different strains of WSSV, but no direct comparisons were made [51, 139]. The first attempt to compare virulence of six geographical origin (China, India, Thailand, Texas, South Carolina, as well as from crayfish maintained at the USA National Zoo) WSSV isolates in *L. vannamei* (PL) and *Farfantepenaeus duorarum* (juveniles) was made by Wang et al. [139]. The study revealed that, all six isolates of WSSV were infectious and highly virulent to *L. vannamei* (PL), while *F. duorarum* (juveniles) showed moderate resistant to WSSV infection. Slight differences of virulence among six geographic isolates of WSSV were detected; with the crayfish WSSV isolate as the least virulent and Texas WSSV isolate as the most virulent forms [139]. Further studies compared the virulence between WSSV-TH-96-II (largest WSSV genome reported to date and considered as the common ancestral to the WSSV isolates described till date) and WSSV-TH (isolate containing the smallest genome) which suggested that the virus with the smallest genome was more virulent and fit than the one with the largest [66]. Furthermore, a comparative study was conducted between six isolates of WSSV from farms experiencing outbreaks (virulentWSSV; vWSSV) and three isolates of WSSV from farms that had infected shrimps but no outbreaks (non-virulentWSSV; nvWSSV). Both vWSSV and nvWSSV isolates resulted in 100 % mortality within 5 days on challenge in shrimps [110].

In 2009, a study was conducted to compare the virulence and fitness between three different Indian genotypes of WSSV by infecting shrimps and crabs. The three different strains of WSSV were isolated from disease outbreak ponds from West and East coast of India. The results showed that, there was no difference in virulence between the three genotypes of WSSV used for infection, as all three strains caused mortality in shrimps and crabs within the same time period and also there were no significant differences between the viral loads ($P < 0.05$) as indicated by real-time PCR. When three strains were mixed and used, only the strains with a smaller genome size (ORF 94, 3 RU and ORF 94, 6 RU) caused disease. This was further supported by the observation that in the subsequent passage only the strain with ORF 94, 3 RU caused disease. In the case of crabs, when a mixture of viruses was used for infection, only the strain with the smallest genome size (ORF 94, 3 RU) caused disease. This result suggests that, although all the strains of WSSV used were equally virulent, a difference in fitness was observed [89]. A recent study by Hoa et al. [31] showed that WSSV mixed-genotype infections correlate with lower outbreak incidence and that disease outbreaks correlate with single-genotype infections. Further studies need to be taken to understand the functions of various WSSV genes, which in turn will explain the reasons for differential virulence of various WSSV strains and viral latency in asymptomatic carriers.

Techniques for WSSV Detection

It is extremely important to understand that diagnosis for WSSV infection cannot be based only on the gross signs of white inclusions in the cuticle. Studies have shown that bacterial infections of the cuticle can also be associated with the formation of white inclusions, in the absence of WSSV infection [143]. The white spots produced closely resemble those caused by WSSV. They are associated with the presence of rod shaped bacteria revealed by scanning electron microscopy. Thus, white spots in the cuticle are unreliable for diagnosis of WSSV. Histopathology has been one of the pioneer techniques for the detection of WSSV. Other techniques which are developed include conventional PCR, real-time PCR, in situ hybridization, isothermal amplification and monoclonal antibody based detection methods are currently in use. Histology has been the gold coin for detection of WSSV in cases when clinical signs and histological changes are observed but other sensitive diagnostic methods like PCR are helpful even when histopathological changes are not seen. A large number of commercial kits, based on in situ hybridization, PCR and immunodetection, are also available (EBTL, DiagXotics, IQ2000, BIOTEC) for detection of WSSV.

DNA Based Diagnostic Methods

DNA Probe Based Methods

Non-radioactively labeled digoxigenin (DIG) DNA probe has been used in dot blot [22], in situ hybridization [9, 20, 77] and Southern blot hybridization for detection and analysis of WSSV [141]. Shekhar et al. [104] on comparing the sensitivity of DNA dot blot and PCR suggested that although PCR was found more sensitive to detect WSSV, DNA dot blot had an advantage over PCR in allowing use of those tissues which prevented PCR amplification due to inhibiting factors. DNA dot blot was found successful in detecting WSSV from the crude DNA extracted from

infected tissue samples such as eye stalk, eye stalk with eye, gills, cuticle, pleopod, periopods, uropods and telson.

Polymerase Chain Reaction (PCR)

Several PCR methods have been described for WSSV either in single [33, 57, 114, 115, 128] or together with other viruses in a multiplex PCR [48, 119]. For detection of WSSV in carrier animals such as wild caught marine shrimp, two-step nested PCR has been developed [32]. In India, PCR has been used for detection WSSV in cultured and captured crustaceans [128]. In Taiwan, Tsai et al. [119] designed a multiplex RT PCR for detection of WSSV and TSV in penaeids shrimp. A multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed for simultaneously detection of six major shrimp viruses including WSSV, YHV, TSV, HPV, IHHNV and MBV [48]. Recently multiplex PCR was developed for simultaneous detection of four DNA viruses including WSSV, IHHNV, MBV and HPV [69]. The protocol described by Lo et al. [57] is the standard method used by the International Organization for Animal Health, although a recent publication suggests that under some conditions, this test may give false positive results with the Australian crayfish *Cherax quadricarinatus* [15]. A rapid and modified PCR assay has been developed based on the nested PCR procedure described by Lo et al. [57] for detection of WSSV [78].

A rapid and highly sensitive real-time PCR detection and quantification method for dual detection of WSSV and IHHNV infecting penaeid shrimp has been developed using SYBR Green chemistry [18]. A recent comparison using real-time PCR as the gold standard clearly showed that, nested PCR tests were generally superior to one-step PCR tests in that they could reliably detect approximately 10–50 virions per reaction test vial [109]. Recently in South Korea a TaqMan real-time polymerase chain reaction was used to quantify and compare infection of WSSV in cultured *P. (Fenneropenaeus) chinensis* [42]. A real-time multiplex polymerase chain reaction (rtm-PCR) assay was developed for simultaneous detection of WSSV, IHHNV and TSV by using TaqMan probes [149]. A multiplex real-time PCR and high-resolution melting (HRM) analysis has been developed to detect simultaneously three of the major viruses of penaeid shrimp including WSSV, YHV and *Penaeus monodon* densovirus (*Pm*DNV) [82]. Recently real-time PCR assay was developed for detection of WSSV infection in *P. monodon* with sensitivity of 10 copies/µl [69].

Though PCR has been one of the popular detection methods, there have been reports of inter laboratory variations as evident from the ring testing conducted in Thailand [109], India and Indonesia [29]. The main reasons for false positive results could be cross contamination and for false negative could be the primers designed targeting the deletion region. Marks et al. [65] showed that, the probe designed by Nunan and Lightner [77] for in situ hybridization gave false negative results as the sequence of probe was in the deletion region, which is absent in few strains of WSSV. This suggests that, the primers and probe should be designed only targeting conserved region of virus.

Serological Based Detection Methods

As an alternative to PCR, the progress in the use of immuno-based detection methods for shrimp viruses has been rather slow. This is primarily because of the difficulties associated with the availability of a purified antigen for immunization and availability of purified antibodies. Immunologically-based diagnosis methods using both polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) have been developed for the detection of WSSV [1, 3, 4, 17, 56, 64, 74, 80, 85, 86, 105, 124, 153, 154, 156, 157] with a diagnostic sensitivity comparable to single step PCR. VP28 is one of the most abundant envelope proteins of WSSV and located on the surface of the viral particles and is crucial for virus entry [125]. This protein has been extensively used for development of MAbs [1, 34, 56]. The monoclonal antibodies specific to VP28 of WSSV could detect 500 pg protein by immunoperoxidase dot blotting [1] and 625 pg by the immunocomb detection method [64] whereas, about 76 % of the nested PCR-positive samples could be detected by immunodot blot using polyclonal antibodies specific to recombinant VP28 protein of WSSV [105]. Major advantage of immuno based detection methods is the ability to detect and grade the severity of viral infections as grading of infections is one of the important criteria required for the proper management of WSSV in culture systems [59].

A simple and rapid dot-blot nitrocellulose enzyme immunoassay (DB-NC-EIA) was developed by using HRP-conjugated virus-specific antibodies for the detection of WSV and YHV in infected shrimp [73]. Another sensitive, specific and simple immunodot assay was developed for the rapid detection of WSSV using polyclonal antiserum specific to the 27.5 kDa envelope protein of WSSV. The immunodot assay detected WSSV in a 1:1,000 dilution of a 10 % gill homogenate and approximately the equivalent of 5 ng total protein [153]. In 2004, a reversed passive latex agglutination (RPLA) assay was developed for detection of WSSV from stomach tissue homogenate of the kuruma shrimp, using high-density latex particles and specific polyclonal antibody in Japan [80]. Enzyme-linked immunosorbent assay (ELISA) and immunodot blot assay was developed for detection of WSSV in shrimp samples using

MAbs against WSSV as primary antibody, and goat anti-mouse Ig serum conjugated with alkaline phosphatase (AP) as secondary antibody [155]. An cost effective, a latex agglutination assay was developed using anti-18 kDa antiserum to detect WSSV in shrimps and crabs and detected the virus as early as 24 h post infection in shrimps [101]. A rapid, single step, sensitive and specific immunochromatographic test (ICT) was described for detecting WSSV using anti-WSSV MAbs, labeled with colloidal gold as a detection reagent and another anti-WSSV MAb as a capture antibody immobilized on nitrocellulose membrane (NCM) [137]. An immunoblot assay using an immunocomb was developed to detect WSSV infection from field samples and had an analytical sensitivity of 625 pg of purified antigen [64]. A sensitive and rapid lateral-flow immunoassay (LFIA) was developed for detection of WSSV using colloidal gold as an indicator with polyclonal antibody raised against recombinant VP (19+8) fusion protein [11]. A study was conducted to compare two chromogenic substrates (3,3′,5,5′-tetramethylbenzidine (TMB) and 3–3′ diaminobenzidine tetrahydrochloride (DAB)) by immunodot blot assay for detection of WSSV. Result suggested that TMB is more sensitive than the DAB method [105]. A immunochromatographic strip test for the simultaneous detection of WSSV and YHV was described using MAbs specific to the WSSV major envelope protein VP28 and the YHV nucleocapsid protein p20 [107]. Recently antibody-based microarray was developed for detection of WSSV in multiple samples [148].

Other Diagnostic Methods

Loop-mediated isothermal amplification (LAMP) method [76] is a novel, sensitive and rapid technique which can be applied for diagnosis of WSSV in aquaculture. It is a highly specific and sensitive diagnostic system using set of four primers, two outer and two inner primers. The detection limit using the LAMP method was found up to 1 fg, when compared to 10 fg by nested PCR [50]. Recently by combining LAMP and amplicon-specific fluorescence energy transfer-based probes, a real-time target amplicon-specific assay was successfully developed for accurate diagnosis of WSSV [14]. A highly sensitive method, capacitive biosensor was developed for the quantitative detection of WSSV in shrimp pond water samples with a linearity that ranged from 1 to 1×10^5 copies/μl [99]. Further studies need to be taken up to know the proportion or ratio of concentration of WSSV DNA and host DNA for getting up a positive reaction for each of the DNA based diagnostics methods. Studies also need to be carried out to know the effect of various preservatives used for storing the tissue samples and period for which the viral DNA is stable in them.

Concluding Remarks

WSSV is now enzootic and widespread in the culture environment, i.e., it is present in many crabs, wild shrimp and plankton crustaceans. Despite the application of numerous strategies, the problems associated with WSSV infection still remains unabated. Fitness and virulence studies on different genotypes of WSSV suggest that, all the strains of WSSV are equally virulent, but the one with smaller genomic size is the fittest. Studies also have shown that, mixed genotype infection of WSSV correlates with lower disease outbreaks. Till date, as there are no treatments available for WSSV infection, good management practices including stocking PCR tested WSSV negative PL may help to prevent the occurrence of WSSV infection in shrimp culture systems. Further research need to be carried out to deploy effective strategies to overcome the spread of WSD by developing different vaccines based on various WSSV proteins and also to develop a cost effective and practical mode for administering it. The changes occurring in WSSV genome due to various environmental stresses which acts as a trigger for WSSV outbreak need studied.

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