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Chapter 32

Nidoviruses of Fish and Crustaceans

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32.1 INTRODUCTION

The order *Nidovirales* comprises four families of viruses containing long RNA genomes (de Groot et al., 2012b; Gorbalenya, 2008; Gorbalenya et al., 2006), the *Coronaviridae* (de Groot et al., 2012a), *Arteriviridae* (Siddell and Snijder, 2008; Faaberg et al., 2012), *Roniviridae* (Mayo, 2002; Cowley et al., 2012) and *Mesoniviridae* (Nga et al., 2011; Zirkel et al., 2011; Lauber et al., 2012). Viruses in the *Coronaviridae* are segregated into two subfamilies. The *Coronavirinae* comprises four genera (*Alpha-*, *Beta-*, *Gamma-* and *Deltacoronavirus*) of viruses infecting diverse mammals or birds and the *Torovirinae* comprises viruses in the *Torovirus* genus that infect horses, cattle and humans and in the *Bafinivirus* genus that infect fish (http://ictvonline.org/virusTaxonomy.asp, de Groot et al., 2012a). Viruses in the single *Arterivirus* genus of the *Arteriviridae* have been isolated from diverse mammalian species such as mice, monkeys, horses and pigs (Balasuriya and Snijder, 2008). Viruses in the single *Alphamesonivirus* 1 genus of the *Mesoniviridae* have only been detected in mosquitoes (Lauber et al., 2012) and viruses in the single *Okavirus* genus of the *Roniviridae* have only been detected in crustaceans (Cowley et al., 2000a, 2012; Sittidilokratna et al., 2008).

Depending on the family and genus, nidoviruses have diverse virion morphologies ranging from spherical to bacilliform, but despite this, they share common genome organization and transcription features (de Groot et al., 2012a). All possess a genome comprised of an infectious linear positive-sense (+) ssRNA, which for coronaviruses (26.4–31.7 kb) is the longest of any known RNA virus (de Groot et al., 2012b). While toroviruses (28.0–28.5 kb) (Snijder et al., 2013), bafiniviruses (26.6–27.3 kb) (Schütze et al., 2006; Batts et al., 2012) and roniviruses (26.2–26.6 kb) (Cowley et al., 2000a; Cowley and Walker, 2002; Sittidilokratna et al., 2002, 2008) possess similarly long genomes, those of mesoniviruses (20.2 kb) (Nga et al., 2011; Zirkel et al., 2011, 2013; Lauber et al., 2012) and arteriviruses (12.7–15.7 kb) (Snijder et al., 2013) are more compact. Despite being diverse in length and gene number, the 5′-genome region of all nidoviruses comprises two long genes encoding overlapping open reading frames (ORF1a and ORF1b) that can constitute up to three-fourths of the genome length (Cowley et al., 2000a; Cowley and Walker, 2002; Sittidilokratna et al., 2002, 2008). At the ORF1a/1b gene overlap, all nidovirus genomes possess a "slippery" heptanucleotide sequence, which in conjunction with a nearby downstream sequence capable of folding into a pseudoknot structure, functions as a translational −1 ribosomal frameshift (RFS) element (Brierley, 1995; Brierley et al., 1989, 1992; Cowley et al., 2002a; ten Dam et al., 1990). This −1 RFS element functions at between 20% and 30% efficiency in pausing polyprotein 1a (pp1a) translation from the ORF1a gene and then slipping the translational machinery back a single nucleotide to be in-frame with the ORF1b gene-coding sequence, thus allowing translation of pp1a to be continued though ORF1b to generate a pp1ab polyprotein. As the ORF1a and ORF1b genes encode nonstructural proteins (nsps) utilized in genome replication and mRNA transcription, the −1 RFS element appears to have been preserved in the evolution of nidoviruses by a need to regulate the abundance of proteins encoded in the ORF1b gene relative to those encoded in the ORF1a gene.

The pp1a polyprotein contains numerous functional motifs, including an ADP-ribose 1-phosphatase, papain-like proteases and a chymotrypsin-like serine protease (3C-like or main proteinase) involved in the site-specific cleavage the pp1a and pp1ab polyproteins into their function units, and the pp1ab polyprotein contains these enzymes in addition to ORF1b gene-encoded motifs, including a RNA-dependent RNA polymerase (RdRp), helicase, ribose methyltransferase, exoribonuclease and endoribonuclease involved in viral RNA transcription and genome replication (de Groot et al., 2012a; Ziebuhr et al., 2000, 2003; Ziebuhr, 2005, 2006; Cowley et al., 2000a; Sittidilokratna et al., 2002). Positioned downstream of the ORF1a/1b gene are as few as 2 genes for roniviruses (Cowley et al., 2002a; Cowley and Walker, 2002; Sittidilokratna et al.,

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2008) to as many as 12 genes for coronaviruses that encode either the virion structural proteins or accessory proteins with various family and genus-specific functions (de Groot et al., 2012a,b).

All nidoviruses also transcribe a nested set of 3′-coterminal capped and polyadenylated subgenomic (sg)mRNAs that encode the virus structural and accessory proteins (Pasternak et al., 2006; Cowley et al., 2002a, 2004a,b; Jitrapakdee et al., 2003; Sittidilokratna et al., 2006, 2008). The mechanism by which these sgmRNAs are transcribed appears to have adopted complexity as nidovirus genomes have acquired greater numbers of genes, presumably to accommodate replication in more highly evolved hosts (Pasternak et al., 2006; Sawicki et al., 2007). In okaviruses, which replicate in crustacean hosts with ancient origins (Siveter et al., 2001; Zhang et al., 2007) and which have a simple genome organization comprising only the ORF1a/1b replicase genes and two genes (ORF2 and ORF3) encoding virion structural proteins, the genomic RNA and sgmRNAs used to translate these ORFs each begin with 5′-AC termini, with these sgmRNA termini mapping to a central position of highly conserved transcriptional regulatory sequences (TRSs) residing within the intergenic regions upstream of ORF2 and ORF3 (Cowley et al., 2002a; Sittidilokratna et al., 2008). Similarly to all but the longest sgmRNA of toroviruses (Snijder et al., 1990; van Vliet et al., 2002; Smits et al., 2005), these TRS elements appear to have roles in terminating negative-strand (−) RNA transcription in a lossy fashion that allows some transcripts to continue through to upstream TRSs or to the (+) RNA genome 5′-terminus. As evidenced by the presence of genomic-length and sgmRNA-length replicative-intermediate double-stranded (ds)RNAs in shrimp cells infected with gill-associated virus (GAV) (Cowley et al., 2002a), the type species okavirus (Cowley et al., 2012), it is speculated that transcription termination of the antisense RNAs might occur at precise positions, resulting in common 3′-termini, and that these then act directly as promoters for transcription initiation of the genomic and sgmRNAs. In all other nidoviruses, however, and for the longest of the sgmRNAs transcribed by toroviruses, the (−) and (+) strand sgmRNAs are transcribed using a far more complex discontinuous process involving the splicing of a common "anti-leader" sequence derived from the genome 5′-terminus to each (−) strand sgmRNA that then acts as a universal promoter for transcribing each (+) strand sgmRNA (Pasternak et al., 2006; Sawicki et al., 2007; Smits et al., 2005; van Vliet et al., 2002).

Nidoviruses of aquatic species include the rod-shaped okaviruses GAV and yellow head virus (YHV) that primarily infect Penaeid shrimp (Longyant et al., 2005; Lightner et al., 1998; Flegel, 2012; Flegel et al., 1997a; Spann et al., 1997; Cowley et al., 2000a, 2002a; Cowley and Walker, 2002; Sittidilokratna et al., 2008) and a morphologically similar virus with a ~22 kb ssRNA genome detected in diseased Chinese mitten crabs (Zhang and Bonami, 2007). They also include the rod-shaped bafiniviruses white bream virus (WBV) detected in white bream in Germany (Granzow et al., 2001; Schütze et al., 2006; Ulferts et al., 2011) and fathead minnow nidovirus (FHMNV) detected in diseased fathead minnows in the United States (Iwanowicz and Goodwin, 2002; Batts et al., 2012). A virus with a spherical coronavirus-like particle morphology has also been detected in color carp in Japan (Miyazaki et al., 2000, 2001), but it has not yet been demonstrated to possess a long (+) ssRNA genome with features characteristic of nidoviruses.

To aid clarity, the fish bafiniviruses and crustacean okaviruses will be described separately together with viruses not yet assigned formally as nidoviruses, but which share common structural features.

32.2 FISH BAFINIVIRUSES

Rod-shaped nidoviruses classified in the genus *Bafinivirus*, subfamily *Torovirinae*, family *Coronaviridae* (de Groot et al., 2012a) have been discovered in wild white bream (*Blicca bjoerkna* L.) in Germany (Granzow et al., 2001) and in diseased fathead minnows (*Pimephales promelas*) cultured as baitfish in the United States (Iwanowicz and Goodwin, 2002; Table 32.1 and Fig. 32.1). White bream support small wild freshwater fisheries in Europe and fathead minnows support a substantial freshwater baitfish culture industry in the United States (Meronek et al., 1997; McCann, 2012). WBV and

TABLE 32.1 Fish Viruses in the Genus *Bafinivirus*, Subfamily *Torovirinae*, Family *Coronaviridae*

Virus Name	Abbreviation	Natural Hosts	References
White bream virus	WBV	White bream (*Blicca bjoerkna L.*)	Granzow et al. (2001)
Fathead minnow nidovirus	FHMNV	Fathead minnow (*Pimephales promelas*)	Iwanowicz and Goodwin (2002)
Ctenopharyngodon idella virus Hungary 33/86[a]	CIVH 33/86	Grass carp (*Ctenopharyngodon idella*)	Ahne et al. (1987)

[a]*Virion morphology consistent with bafiniviruses, but its RNA genome not yet been sequenced to support inclusion in this genus.*

FHMNV possess an infectious 26.6–27.3 kb (+) ssRNA genome organized and transcribed similarly to other nidoviruses, and alignments of deduced amino acid sequences of their pp1ab and structural proteins have identified them to represent different bafinivirus species that appear to have diverged from a common ancestor early in their evolution (Batts et al., 2012; Schütze et al., 2006).

Another rod-shaped fish virus with similar virion morphology to bafiniviruses has been isolated in fish cell lines inoculated with tissue homogenates of healthy grass carp (*Ctenopharyngodon idella*) imported from Hungary into Germany in 1986 (Ahne et al., 1987). Like WBV and FHMNV, *Ctenopharyngodon idella* virus Hungary 33/86 (CIVH 33/86) also contains a ssRNA genome and infection causes syncytia formation in fish cell monolayers (Ahne et al., 1987). However, the genome of CIVH 33/86 must to be sequenced to confirm it is a bafinivirus (Table 32.1). While grass carp is a significant aquaculture species, particularly in China but also throughout Asia, Russia and Europe, with total production estimated to be in the order of 5 million metric tons in 2012 (http://www.fao.org/fishery/culturedspecies/Ctenopharyngodon_idella/en), no evidence has been reported for CIVH 33/86 impacting carp farming.

32.2.1 Virion Morphology and Morphogenesis

Negatively stained WBV particles purified from cell culture supernatants by sucrose density-gradient ultracentrifugation are bacilliform in shape (37–45 nm × 130–160 nm) and possess a lipid envelope decorated with prominent and regularly spaced fuzzy surface projections (20–25 nm in length) (Granzow et al., 2001; Fig. 32.1). Viral nucleocapsids observed in disrupted or partially disrupted virions appear as rigid rod-like structures (19–21 nm × 120–150 nm) with inner and outer sections indicative of their being cylindrical in morphology (Granzow et al., 2001). Negatively stained FHMNV particles semi-purified from cell culture supernatants are also bacilliform and similar in dimensions (31–47 nm × 130–180 nm) (Iwanowicz and Goodwin, 2002). Unlike WBV, however, the FHMNV particles appeared smooth and devoid of surface projections, possibly as a result of how they were purified and stained. Negatively stained rod-shaped virions of CIVH 33/86 are similar in dimensions (50–55 nm × 170–220 nm) and among some particles aligned in close proximity, regularly spaced surface projections like those of WBV have been observed (Ahne et al., 1987; Granzow et al., 2001).

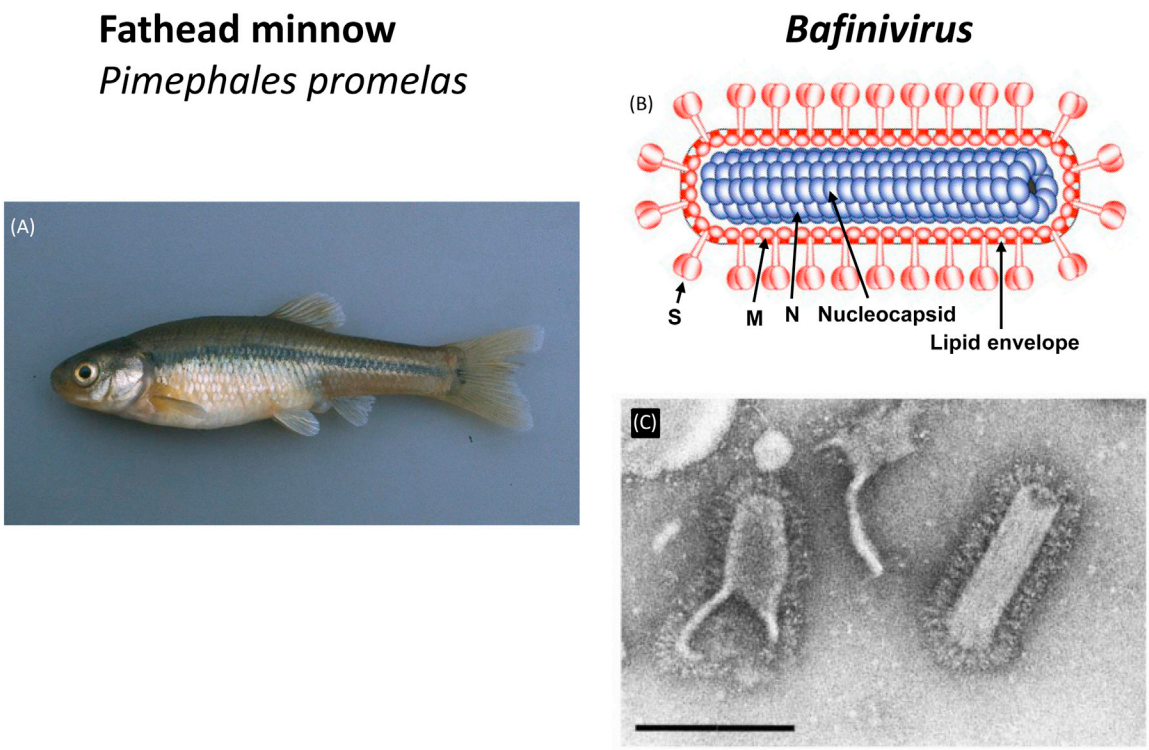

FIGURE 32.1 (A) Photograph of a fathead minnow (*Pimephales promelas*) (Lyons, 2015). (B) Schematic structure of a bafinivirus particle showing the positions of the envelope spike glycoprotein (S), membrane protein (M) and nucleoprotein (N) and (C) electron micrograph of negatively stained rod-shaped virions of WBV (Granzow et al., 2001), showing the presence of regularly spaced club-like spikes protruding from the lipid envelope as well as the underlying helical nucleocapsid (Bar = 150 nm).

32.2.2 Genome Organization and Transcription

The capped and polyadenylated (+) ssRNA genomes of WBV (Schütze et al., 2006) and FHMNV (Batts et al., 2012) each possess five genes encoding ORFs (ORF1a, 1b, 2, 3 and 4). The ORF1a/1b gene encodes pp1a and pp1ab polyproteins containing proteinase, polymerase and other replicase enzymes shared across all nidoviruses and ORF2, ORF3 and ORF4 encode a spike (S) glycoprotein, membrane (M) protein and nucleocapsid (N) proteins, respectively. Both viruses utilize −1 RFS elements comprised of an UUUAAAC "slippery" heptanucleotide sequence and a downstream RNA pseudoknot structure similar in predicted structure to facilitate translational read-through of the ORF1a/1b gene overlap, and possess intergenic nonanucleotide TRS elements near identical in sequence (Schütze et al., 2006; Batts et al., 2012). Subgenomic mRNAs transcribed for each gene possess 5′-genome leader sequences upstream of the conserved TRS, indicating the use of a discontinuous transcription strategy comparable to those used by nidoviruses other than roniviruses and for the longest sgmRNA of toroviruses (Pasternak et al., 2006; Sawicki et al., 2007; van Vliet et al., 2002; Smits et al., 2005; Snijder et al., 1988).

32.2.3 Disease and Pathology

WBV (virus isolate DF 24/00) was discovered in 2000 in fish cell cultures inoculated with a mixed tissue homogenate of white bream (*Blicca bjoerkna* L.) as part of a virus monitoring program of wild fish populations in Germany (Granzow et al., 2001). Although highly cytopathic for *Epithelioma papulosum cyprini* (EPC) cell cultures derived from carp (*Cyprinus carpio*) epidermal herpes virus-induced hyperplastic lesions, the health status of the white bream from which WBV was isolated was not reported, and there is no information on its pathogenic potential for this or other fish species.

FHMNV was isolated in 1997 from moribund wild-type and rosy red fathead minnows (*Pimephales promelas*) being reared as baitfish in ponds in central Arkansas (Iwanowicz and Goodwin, 2002). The minnows were a mixture of wild and farmed fish from various unspecified US locations and were experiencing chronic mortality associated with eye and skin hemorrhages. Like WBV, FHMNV is highly cytopathic for EPC cells characterized initially by cell rounding, with multifocal syncytia (fused multinucleated cells) then forming as the cell monolayer degrades. Among several others examined, rainbow trout gonad (RTG-2) and fathead minnow (FHM) cell lines were also highly susceptible to FHMNV-induced cytopathology (Iwanowicz and Goodwin, 2002). In FHMNV-infected EPC cells, histopathology is characterized by pyknotic nuclei displaying marginated chromatin, and in moribund FHMs, by lesions in the anterior kidney, liver and spleen, as well as severe multifocal necrosis of hepatocytes (Iwanowicz and Goodwin, 2002).

In FHMs challenged with FHMNV, morbidity becomes evident by behavioral changes characterized by fish initially swimming erratically in circles before becoming listless either at the tank bottom or with their head orientated upward at the water surface (Iwanowicz and Goodwin, 2002). In 19°C water, mortalities accumulate rapidly from 3 days post-challenge, but 17°C water delays mortality onset to 13 days post-challenge. Clinical signs consistent with the index case develop that include petechial hemorrhages in the eyes and skin (Iwanowicz and Goodwin, 2002). Also evident can be hemorrhaging of muscle, generally localized to the epaxial musculature anterior to the vent, and below the epidermis at the cranium apex. The kidneys in many fish also become visible due to musculature edema and darkening of the trunk kidney. Productive infections do not establish in challenged channel catfish, rainbow trout or goldfish or in golden shiners cohabitated with challenged FHMs, suggesting that FHMNV is highly host-specific (Iwanowicz and Goodwin, 2002).

32.2.4 Diagnostic Methods

FHMNV infection and disease can be diagnosed tentatively by behavioral changes and characteristic gross signs. Development of cytopathic effects in EPC and other fish cell lines that include syncytia formation can also assist diagnosis of FHMNV and WBV, with detection of rod-shaped virions by transmission electron microscopy (TEM) providing additional diagnostic confidence. Opportunities exist to develop specific and sensitive diagnostic reverse transcription-polymerase chain reaction (RT-PCR) tests because complete genome sequences are available for FHMNV (Batts et al., 2012) and WBV (Schütze et al., 2006). For FHMNV, PCR tests have been described that amplify either a relatively conserved region in the ORF1b gene helicase motif or an ORF2 gene sequence (Batts et al., 2012). Sequence analysis of the ORF2 gene region amplified by PCR has provided a means of differentiating FHMNV strains isolated in cell culture from FHMs being reared in Minnesota, Wisconsin and Illinois as well as present in jack pike (*Esox masquinongy*) fed live FHMs in Nebraska and a batch of creek chub (*Semotilus atromaculatus*) (Batts et al., 2012; McCann, 2012). A constant-temperature RT loop-mediated isothermal amplification (RT-LAMP) test with a sensitivity of ~5 RNA copies has also been developed for FHMNV diagnosis and surveillance (Zhang et al., 2014). While no molecular tests have been reported specifically

for WBV diagnosis, immunoblotting using a polyclonal rabbit antiserum to a fusion protein to the pp1a main proteinase (Mpro) motif expressed in bacteria has been used to detect predicted Mpro cleavage products of pp1a and pp1ab in WBV-infected fish cells (Ulferts et al., 2011). Opportunities thus exist to employ this or other WBV protein-specific antibodies in diagnostic immunotests.

32.2.5 Prevention and Control

There has been little impetus to control WBV, presumably because it has only been isolated once and not been identified to be pathogenic, because white bream is not an aquaculture species and as the wild fishery in Europe is small (Granzow et al., 2001). This contrasts to FHMNV, which, due to its pathogenicity for FHMs, poses a significant threat to the multi-million dollar baitfish industry in the midwestern United States (Meronek et al., 1997; McCann, 2012). Due to the industry value to this region and because FHMs are distributed widely in brooks, ponds and small lakes, the potential for virus dispersal via movements of live baitfish is currently regulated by state import policies and health certifications, as well as an overarching US Federal law called the Lacey Act (McCann, 2012). While these regulations rely primarily on observing fish for disease symptoms, a survey of the three primary baitfish species farmed in Wisconsin employing cell culture in conjunction with PCR testing has proved useful in isolating, identifying and determining the prevalence and species distribution of FHMNV (McCann, 2012). In Arkansas, it has been reported that destroying the farmed FHM population affected by FHMNV in 1997 in conjunction with high summer water temperatures might have been effective at eradicating the virus from this state (Batts et al., 2012). As for any pathogen of aquaculture species, FHMNV control will rely on robust surveillance, translocation regulations and farm biosecurity, including stock destruction and water decontamination protocols.

32.3 CRUSTACEAN RONIVIRUSES

The sequence and organization of the 26.2–26.6 kb (+) ssRNA genomes of GAV (Cowley et al., 2000a, 2004a; Cowley and Walker, 2002) and YHV (Sittidilokratna et al., 2002, 2008, 2009; Jitrapakdee et al., 2003), together with the transcription of a nested set of 3′-coterminal sgmRNAs used in protein translation (Cowley et al., 2002a; Sittidilokratna et al., 2008), has identified them to be related distantly to diverse vertebrate and invertebrate viruses classified in the order *Nidovirales* (Cowley et al., 2012; de Groot et al., 2012a). Due to observations of the viruses replicating and causing pathology in the lymphoid or "Oka" organ of shrimp (Oka, 1969; Spann et al., 1995), GAV was assigned as the type species of a genus called *Okavirus* in a family named *Roniviridae* (sigla, *ro*d-shaped *ni*dovirus) based on the evolutionary divergence of GAV and YHV from other nidoviruses (Gorbalenya, 2008; Gorbalenya et al., 2002, 2006) and on their rod-shaped enveloped virions (Spann et al., 1995, 1997; Spann and Lester, 1997; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Nadala et al., 1997b) being distinct from the spherical enveloped virions formed by viruses in either the *Coronaviridae* or the *Arteriviridae* families of the *Nidovirales* known to exist at that time (de Groot et al., 2012a).

In addition to GAV and YHV that have caused widespread disease in shrimp farmed throughout Eastern Hemisphere countries, a few viruses forming okavirus-like rod-shaped virions have been detected in crab species. Of these, *Eriocheir sinensis* ronivirus (*Es*RNV), which causes disease in the Chinese mitten crab, has been shown to possess a ~22 kb ssRNA genome (Zhang and Bonami, 2007) supportive of it being related to okaviruses, but no genome sequence information is yet available to confirm this relationship. The molecular and biological characteristics of GAV and YHV and the management of virus transmission and disease in farmed shrimp have been the subject of several comprehensive reviews (Dhar et al., 2004; Cowley and Walker, 2007; Lightner, 2011; Munro and Owens, 2007b; OIE, 2014; Walker and Sittidilokratna, 2008). For completeness, information available on okaviruses (Table 32.2) has also been reviewed and updated in the next sections, including more recent developments in diagnostic methods and potential antiviral therapies as well as brief descriptions of the crab viruses with virion morphologies suggestive of them being related to okaviruses.

32.3.1 YHV and GAV

32.3.1.1 Discovery, Disease Symptoms and Pathology

YHD was first reported as the cause mass mortality in Giant Tiger shrimp (*Penaeus monodon*) being cultured intensively in Thailand in the early 1990s (Limsuwan, 1991). The disease generally affected juvenile to subadult *P. monodon* 50–70 days into culture, and its pending onset was typically announced by a rapid abnormal increase in feed consumption followed 2–4 days later by an equally rapid cessation in feeding. Within 3–5 days of feeding cessation, moribund shrimp would amass at pond edges and die, often leading to almost complete pond losses. The name YHD was conceived because the bodies of

TABLE 32.2 Crustacean Viruses in the Genus *Okavirus*, Family *Roniviridae*

Virus Name	Abbreviation	Natural Hosts	References
Yellow head virus	YHV	Giant Tiger shrimp (*Penaeus monodon*) Pacific whiteleg shrimp (*Litopenaeus vannamei*)	Boonyaratpalin et al. (1993)
			Chantanachookin et al. (1993)
			Wongteerasupaya et al. (1995a,b)
			Nadala et al. (1997a,b)
			Sittidilokratna et al. (2002, 2008)
			Jitrapakdee et al. (2003)
Gill-associated virus	GAV	*Penaeus monodon*	Spann et al. (1995, 1997)
			Cowley et al. (1999, 2000a, 2002a)
			Cowley and Walker (2002)
Eriocheir sinensis ronivirus[a]	*Es*RNV	Chinese mitten crab (*Eriocheir sinensis*)	Zhang and Bonami (2007)

[a]*Virion morphology and ~22 kb ssRNA genome consistent with okaviruses but its genome not yet sequenced to support inclusion in this genus.*

many diseased shrimp had a bleached appearance often with a yellowed cephalothorax due to the gills developing a brownish color and the hepatopancreas becoming swollen and yellowed rather than dark brown (Limsuwan, 1991; Flegel et al., 1995b; Fig. 32.3). Transmission trials, histology and TEM undertaken to identify the cause of YHD identified a rod-shaped enveloped virus (40–50 nm × 150–200 nm) speculated at the time to be an atypical baculovirus and accordingly given the name yellow-head baculovirus (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). However, when purified virions were identified to contain a genome comprised of a long (>22 kb) ssRNA, its name was revised to YHV (Wongteerasupaya et al., 1995b; Nadala et al., 1997a,b). While thought to have emerged in Thailand, a review of histopathology and the morphology and morphogenesis of a virus cultured in primary shrimp lymphoid organ (LO) cells inoculated with a tissue extract of diseased *P. monodon* investigated at the time when shrimp aquaculture collapsed in Taiwan in the late 1980s suggests that YHV was the likely cause of that collapse (Chen and Kou, 1989).

Soon after YHV was identified in Thailand, virions indistinguishable in morphology were detected in cells with hypertrophied nuclei sequestered within LO spheroids of overtly healthy *P. monodon* being farmed in eastern Australia (Spann et al., 1995). The virus was named lymphoid organ virus (LOV) because it was not associated with disease and virus particles were only evident in LO spheroids; in addition no genome sequence information was available to determine its relationship to YHV (Spann et al., 1995; Spann and Lester, 1997). Around the same time LOV was discovered in 1994, mass mortalities of subadult *P. monodon* were reported at four farms in North Queensland, Australia (Anderson and Owens, 2001; Spann et al., 1995, 1997; Spann and Lester, 1997; Walker et al., 1998; Munro et al., 2011). As with YHD, the disease was accompanied by rapid feeding increases and cessation, shrimp swimming erratically at the water surface and as morbidity became acute, shrimp congregating in large numbers at pond edges before death and mortality rates of up to 80% at pond harvest. As more widespread disease outbreaks occurred during the 1996/97 season generally around 90–120 days into grow-out, the name 'mid-crop mortality syndrome' (MCMS) was adopted to describe the disease (Owens et al., 1998; Anderson and Owens, 2001; Walker, 2000; Walker et al., 2001; Cullen and Owens, 2004). While gills of affected shrimp would typically become pink to brownish in color like YHD, disease gross signs were typified by a generalized reddened of the body and particularly at pereiopod, pleopod and tail fin extremities of experimentally challenged shrimp (Spann et al., 1997).

While up to four virus particle types were detected in MCMS-affected shrimp (Owens et al., 1998), a rod-shaped enveloped virus particle morphologically indistinguishable from LOV and YHV was demonstrated to be the primary disease agent (Spann et al., 1997; Oanh et al., 2011). Due to the lack of genome sequence data to confirm their evolutionary relatedness, and as gill cells of diseased *P. monodon* possessed large numbers of virus particles in addition to LO cells, the virus was named GAV (Spann et al., 1997). However, following subsequent genome sequence comparisons of LOV and GAV (Cowley et al., 2000b) together with shrimp challenge data showing that an inoculum prepared from LO tissue of LOV-infected healthy shrimp could induce disease and mortality typical of GAV and that infection in the absence of disease could be induced using a diluted GAV inoculum (Walker et al., 2001), LOV and GAV were considered the same virus and the name LOV was abandoned.

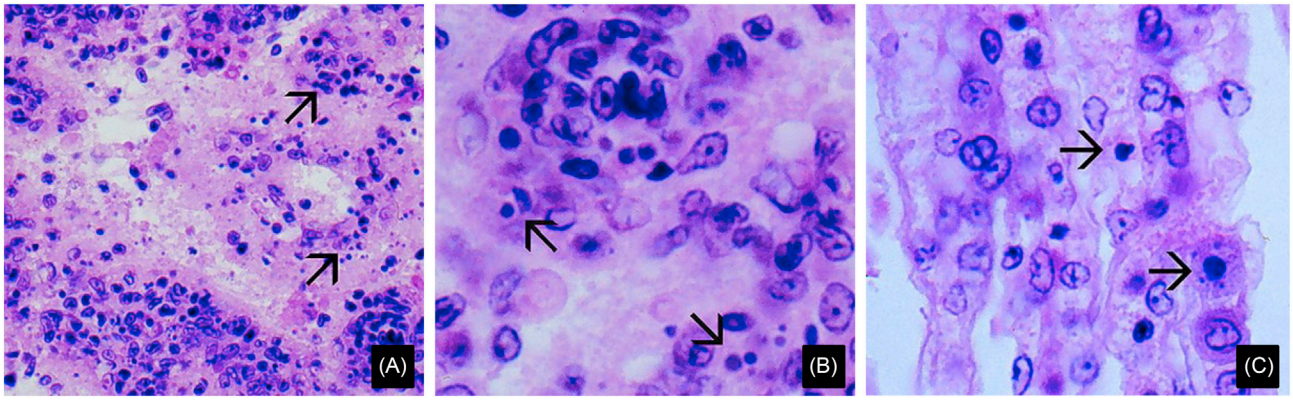

FIGURE 32.2 Histopathology evident in tissue sections stained with H&E. Juvenile *Penaeus monodon* with acute YHD displaying marked cellular necrosis in the lymphoid organ, (A) 525×, (B) 1700× magnification, characterized by affected cells displaying pyknotic and karyorrhectic nuclei, with single or multiple perinuclear inclusion bodies ranging from pale to darkly basophilic (light to dark gray) apparent in some cells (*arrows*) as well as in (C) gill lamellae cells (1000×) displaying similar pyknotic and karyorrhectic nuclei (*arrows*) with some enlarged and generally spherical cells possibly representing immature hemocytes released in response to disease displaying a basophilic cytoplasm (Lightner, 1996).

In acute-stage infections associated with disease, YHV and GAV can invade all tissues of mesodermal and ectodermal origin. These include the LO, circulating hemocytes as well as developing hemocytes in hemopoietic tissues and fixed phagocytes in the heart, gill lamellae and epithelial pillar cells, and spongy connective tissues of various organs including the heart, stomach subcuticulum, antennal gland, gonads and nerve tissues, including neural ganglia, nerve fibers, neurosecretory and glial cells (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lightner, 1996; Spann et al., 1997, 2000; Tang and Lightner, 1999; Soowannayan et al., 2002), as well as the fasciculate zone of eye (Smith, 2000; Callinan et al., 2003). Histopathology evident in hematoxylin and eosin (H&E) stained cephalothorax tissue sections is typified by severe necrosis in which cells display prominent nuclear pyknosis and karyorrhesis, as well as intense basophilic perinuclear inclusions in the cytoplasm resulting from nuclear phagocytosis and virus inclusions (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lightner, 1996; Lu et al., 1994; Nash et al., 1992; OIE, 2014; Spann et al., 1997, 2000; Wang and Chang, 2000). Typical histopathology seen in the LO and gill lamellae of *P. monodon* with acute YHD are shown in Fig. 32.2.

Both YHV and GAV can also persist over the life of wild shrimp in a subclinical or chronic infection state (Spann et al., 1995, 2003; Cowley et al., 2000b; Walker et al., 2001; Pasharawipas et al., 1997). Infection can also persist in *P. monodon* and Pacific whiteleg shrimp (*Litopenaeus vannamei*) that survive experimental YHV challenge (Longyant et al., 2005, 2006; Anantasomboon et al., 2007) and in farmed *L. vannamei* that survive a YHD outbreak (Senapin et al., 2010). The only histopathology observed consistently in asymptomatic shrimp is LO spheroids comprised of partitioned aggregations of cells with hypertrophied nuclei (Spann et al., 1995; Anantasomboon et al., 2007). Cells within these spheroids are often apoptotic and there is evidence for them being granular hemocytes exocytosed due to them having phagocytosed virus-infected cells or cell debris recognized to be foreign (Anggraeni and Owens, 2000; van de Braak et al., 2002; Duangsuwan et al., 2008).

32.3.1.2 Particle Morphology and Morphogenesis

In ultrathin sections of infected shrimp tissue, mature virions of YHV appear bacilliform in shape (40–60 nm × 150–200 nm) and possess a lipid envelope decorated with regularly spaced diffuse surface projections (8 nm dia. × 11 nm long) (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Wang and Chang, 2000; Wongteerasupaya et al., 1995a,b). The morphology of negatively stained virions purified from shrimp hemolymph by sucrose density-gradient ultracentrifugation has been reported to be similar (Wang and Chang, 2000; Wongteerasupaya et al., 1995a,b; Fig. 32.3), except for the presence of narrowed envelope appendages extending from particle ends that can join the ends of curled particles to form donut-like structures (Nadala et al., 1997b). The origin and purpose of the envelope extensions are unknown. However, the donut-shaped YHV particle structures quite closely resemble the virions (120–140 nm dia.) containing curled nucleocapsids formed by viruses classified within the *Torovirus* genus of the *Torovirinae* (de Groot et al., 2012b), with the rod-shaped YHV particles being similar in dimensions and morphology to those of the fish viruses classified within the *Bafinivirus* genus of the *Coronavirinae* (Granzow et al., 2001; Iwanowicz and Goodwin, 2002).

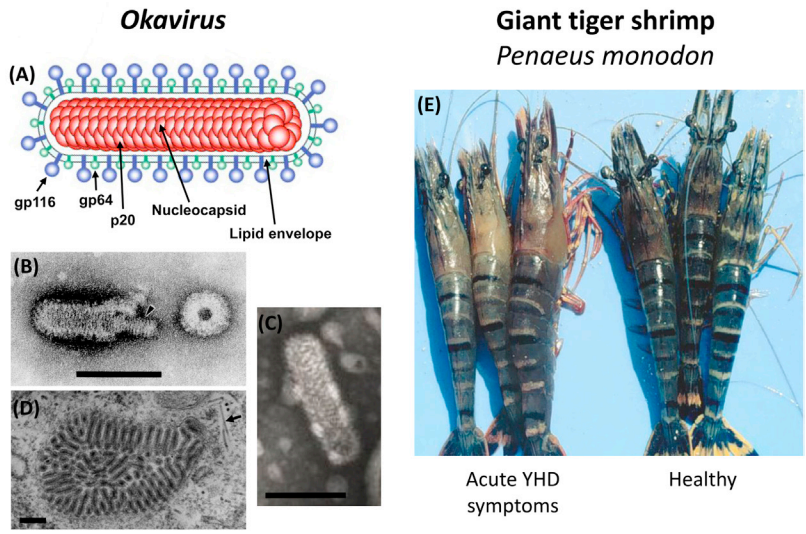

FIGURE 32.3 (A) Schematic structure of an Okavirus particle showing the positions of the envelope glycoproteins (gp116, gp64) and the nucleoprotein (p20) and electron micrographs of negatively stained rod-shaped virions of (B) YHV (Nadala et al., 1997a,b) and (C) GAV (Spann et al., 1995) showing the helical nucleocapsid and diffuse regularly spaced envelope projections as well as (D) GAV particles clustered tightly within a cytoplasmic vesicle surrounded by filamentous nucleocapsid precursors (*arrow*) in a lymphoid organ cell (Spann and Lester, 1997) (Bars = 150 nm). (E) Photograph of Giant tiger shrimp (*Penaeus monodon*) showing yellow-brown (bleached) discoloration of the cephalothorax and gills (left side) as observed in early disease outbreaks in Thailand caused by YHV (Lightner, 1996).

The nucleocapsid component of YHV virions appears as a coiled filament (16–30 nm dia.) with a 5–7 nm periodicity (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Nadala et al., 1997b; Wang and Chang, 2000). Free nucleocapsid precursors (15-nm diameter) that occur abundantly in the cytoplasm of infected cells appear as flexible helical filaments up to 450 nm in length. During virion morphogenesis, nucleocapsids generally bud internally through membranes of the Golgi-endoplasmic reticulum (ER) complex to generate enveloped virions, which sometimes are observed abutted end-to-end, as if formed consecutively from multiple linked nucleocapsids that appear to comprise the long filaments (Chantanachookin et al., 1993; Spann et al., 1997). This consecutive mode of nucleocapsid and virion morphogenesis is also supported by purified virions being identified joined end-to-end (Wongteerasupaya et al., 1995a,b). Virions often occur in the vicinity of nucleocapsid filaments between the outer and inner membranes of the nuclear envelope (Chantanachookin et al., 1993; Wang and Chang, 2000), suggesting that the outer nuclear membrane can serve as an alternative to Golgi-ER membranes for virion maturation. Virions also bud infrequently from the cell plasma membrane (Boonyaratpalin et al., 1993; Spann et al., 1995, 1997). Nucleocapsid precursors and virions accumulate throughout the cytoplasm, with virions commonly seen congregated and sometimes densely packed within cytoplasmic vesicles (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). Exocytosis of these vesicles during cell degeneration appears to be the primary mode of virion release.

32.3.1.3 Genome Organization, RNA Transcription and Viral Proteins

Initial analyses of the YHV genome showed it to be long (~22 kb), sensitive to RNase A, but not DNase 1 digestion (Wongteerasupaya et al., 1995b) and while preliminary in vitro translation analysis suggested it might be negative in polarity (Nadala et al., 1997b), in situ hybridization (ISH) using RNA probes of either polarity confirmed it had positive polarity (Tang and Lightner, 1999). Further characterization of the (+) ssRNA genomes of GAV (Cowley et al., 2000a, 2001; Cowley and Walker, 2002) and YHV (Sittidilokratna et al., 2002, 2008; Jitrapakdee et al., 2003) showed them to be 26,235 and 26,662 nt in length, respectively, to possess a 5′-7-methylguanosine cap structure and a 3′-poly(A) tail and to be organized similarly to coronaviruses and arteriviruses (Fig. 32.4).

Downstream of a short 5′-terminal untranslated sequence, the genome of okaviruses contains a ~20 kb ORF1a/ORF1b replicase gene proceeded by an ORF2 gene that encodes the p20 nucleoprotein, an ORF3 gene that encodes a pp3 polyprotein from which the gp116 and gp64 envelope glycoproteins are derived and, in the case of GAV, a short ORF4 gene encoding a protein of unknown function that is not translated in abundance and that is truncated in YHV (Cowley et al., 2012). Similarly to other nidoviruses, a slippery heptanucleotide (AAAUUUU) in conjunction with a downstream 131-nt sequence predicted to fold into a complex pseudoknot structure resides in the ORF1a/ORF1b gene overlap and functions as −1 RFS

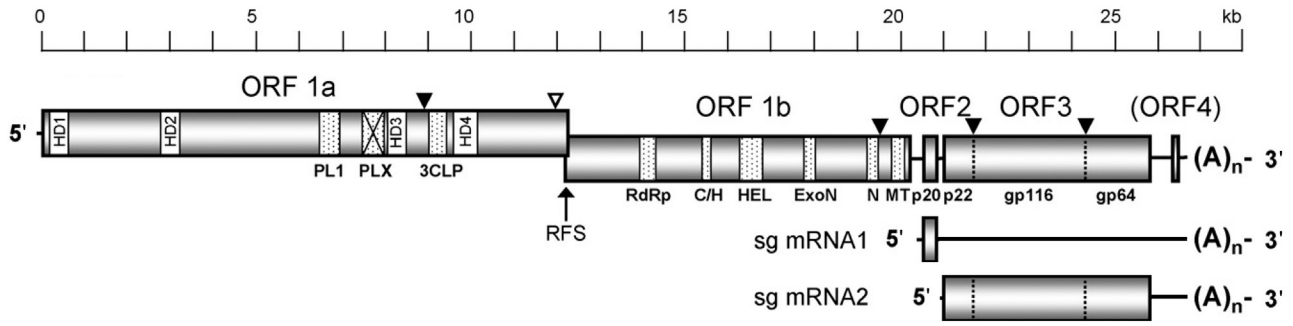

FIGURE 32.4 Schematic representation of the 26,662 nt (+) ssRNA genome of YHV and (subgenomic) sgmRNA1 and sgmRNA2 that translate ORF2 and ORF3, respectively. Each RNA contains a 5′-7-methylguanosine cap and a 3′-poly(A) tail. Functional domains identified in ORF1a include four hydrophobic regions (HD1-HD4), a 3C-like protease (3CLpro), a papain-like protease (PL1) and a domain with homology to PL1, but lacking the canonical α + β fold of papain-like proteases (PLX) and in ORF1b include a RNA-dependent RNA polymerase (RdRp), cysteine- and histidine-rich domain (C/H) Zn fingers, a helicase (HEL), an exoribonuclease (ExoN), a uridylatespecific endoribonuclease (N) and a ribose-*O*-methyl transferase (MT). The −1 ribosomal frame-shift (RFS) element allows translational read-through of ORF1a polyprotein pp1a into ORF1b to generate polyprotein pp1ab. ORF2 encodes the nucleoprotein (p20). ORF3 encodes a polyprotein (pp3) containing six transmembrane (TM) domains that undergoes posttranslational cleavage at the C-terminal side of TM3 and TM5 to generate the envelope glycoproteins gp116 and gp64, respectively, as well as a 22 kDa N-terminal triple-membrane-spanning fragment of unknown function. Known (▼) and likely (▽) sites of proteolytic cleavage in pp1a, pp1ab and pp3 are indicated (Sittidilokratna et al., 2008).

element to facilitate read-through translation of the ORF1a gene pp1a polyprotein into the ORF1b gene at approximately 23% efficiency to generate a pp1ab polyprotein (Cowley et al., 2000a, 2012; de Groot et al., 2012a; Sittidilokratna et al., 2002).

The ORF1a gene coding sequence contains four hydrophobic domains (HD1-HD4), each predicted to comprise multiple transmembrane (TM) sequences (Cowley et al., 2000a, Sittidilokratna et al., 2002). A chymotrypsin-like cysteine proteinase (3CLpro) flanked by HD3 and HD4 and identified tentatively to use a VxHE↓(L,V) consensus cleavage-site motif functions in autocatalytic processing of the pp1a/pp1ab polyproteins into functional nsp units (Ziebuhr et al., 2003). However, the okavirus 3CLpro appears to combine the Cys–His catalytic dyad of the coronavirus 3CLpro with a substrate-binding pocket representative of the 3C-like proteinase of plant potyviruses, thus distinguishing it from the cognate enzymes of other nidoviruses (Ziebuhr et al., 2003). ORF1a also contains two putative papain-like proteinase motifs (PLP1 and PLPx) that lack a Zn^{2+}-binding finger, as in the papain-like proteases (PLpro) of toroviruses, and share only remote structural similarity to PLpro motifs present in similar ORF1a gene positions of other nidoviruses (Sittidilokratna et al., 2002). While shown to cleave consensus sites immediately to the N-terminal side of the 3CLpro domain and near the pp1ab C-terminus (Ziebuhr et al., 2003), the number and nature of okavirus nsp units cleaved from the pp1a/pp1ab polyproteins by the 3CLpro and PLPs have yet to be determined.

The okavirus ORF1b gene-coding sequence component of the pp1ab replicase polyprotein contains homologs of a RdRp polymerase with a "SDD" active site motif characteristic of nidoviruses (Koonin, 1991; Gorbalenya et al., 2002), as well as putative multinuclear Zn^{2+}-binding finger-like domains preceding a 5′→3′ helicase domain (Zn-HEL), a 3′→5′exoribonuclease (ExoN), a *N*idoviral *end*oribonuclease specific for *U*ridylate (NendoU) and a ribose-2′-O-methyltransferase (O-MT) present in the cognate pp1ab polyproteins of other nidoviruses (de Groot et al., 2012a,b; Faaberg et al., 2012; Cowley et al., 2000a, 2012; Lauber et al., 2012; Sittidilokratna et al., 2002; Ziebuhr, 2005, 2006, 2008; Ziebuhr et al., 2000, 2003; Gorbalenya, 2008; Gorbalenya et al., 2006).

Unlike vertebrate nidoviruses in which the viral nucleoprotein gene resides close to the 3′-end of the genome, and more like the mosquito mesoniviruses in which the nucleoprotein appears to be encoded in an alternative reading frame in the N-terminal signal peptidase cleavage product of the ORF2a polyprotein from which the mature spike (S) envelope glycoprotein is also cleaved (Zirkel et al., 2013; Vasilakis et al., 2014), the okavirus p20 nucleoprotein gene (ORF2) resides upstream of the 5 kb ORF3 gene, encoding the gp116 and gp64 envelope glycoproteins (Cowley et al., 2004a; Jitrapakdee et al., 2003; Sittidilokratna et al., 2006). Positioning of the nucleoprotein gene at the 3′-genome end ensures its translation in high abundance because the 3′-coterminal sgmRNA transcription process that is utilized by vertebrate nidoviruses containing larger numbers of genes results in their abundance decreasing relative to their length (de Groot et al., 2012a). However, as ~6 and ~5.5 kb sgmRNAs from which the p20 nucleoprotein and gp116/gp64 envelope glycoproteins are translated, respectively, are transcribed in similar relative abundance (Cowley et al., 2002a; Sittidilokratna et al., 2008), okaviruses appear to have no need to order these genes similarly to coronaviruses or arteriviruses (de Groot et al., 2012a,b;

Faaberg et al., 2012). The mesoniviruses have accommodated this need by having the nucleoprotein and S glycoprotein translated from the same sgmRNA (Lauber et al., 2012). Similar to the mosquito mesoniviruses, the okaviruses also have no gene-encoding a membrane (M) protein integral to the virion structures formed by the vertebrate nidoviruses. Moreover, the presence of only two major genes (ORF2 and ORF3) in addition to the ORF1a/1b gene appears to have allowed okaviruses to preserve use of a sgmRNA transcription mechanism like that used for all but the longest sgmRNA of toroviruses (Snijder et al., 1990; Smits et al., 2005; Van Vliet et al., 2002) and less complex than that used by viruses in the *Coronaviridae* and *Arteriviridae*, and appear to have acquired greater gene numbers to accommodate replication in more highly evolved hosts (de Groot et al., 2012a,b; Faaberg et al., 2012). In addition, YHV particles comprise three structural proteins, a p20 (20–22 kDa) nucleoprotein that binds viral RNA to form the nucleocapsid and two envelope glycoproteins, gp116 (110–135 kDa) and gp64 (63–67 kDa), that form the envelope spikes visible on virions and are utilized in cell attachment and entry (Cowley and Walker, 2002; Cowley et al., 2004a; Nadala et al., 1997b; Jitrapakdee et al., 2003; Sittidilokratna et al., 2006; Soowannayan et al., 2003, 2011; Fig. 32.4). In addition to p20-specific antibodies binding to precursor and virion nucleocapsids (Cowley et al., 2004a; Sittidilokratna et al., 2006), RNA binding analyses using recombinant proteins expressed to various portions of the p20 coding sequence as well as to a synthetic p20 peptide sequence have localized its RNA binding domain to an N-terminal proline/arginine-rich sequence (Met^{11}-Ile^{29}) (Soowannayan et al., 2011). The mature gp116 and gp64 glycoproteins originate from posttranslational cleavage of the pp3 polyprotein encoded by the ORF3 gene (Cowley and Walker, 2002; Jitrapakdee et al., 2003; Soowannayan et al., 2011). Of the six predicted TM domains in pp3, TM3 and TM5 appear to act as signal peptidase type 1-like sites (Fig. 32.4). Cleavage at the C-terminal side of these sites generates the N-terminus of gp116 anchored to the lipid membrane by TM4 and TM5, and the N-terminus of gp64 anchored by TM6. The fate and function of the 25.2 kDa N-terminal pp3 fragment, which contains three TM domains and a N-linked glycosylation site in its predicted ectodomain, have yet to be identified. Of the potential N-linked glycosylation sites in gp116 and gp64, six in gp116 and three in gp64 have been identified to possess mannose-rich glycans, with those in gp116 also possessing *N*-acetylgalactosamine and *N*-acetylglucosamine terminal-type sugars (Soowannayan et al., 2011). Injection of shrimp with tunicamycin to inhibit N-linked glycosylation prior to YHV challenge results in reduced viral RNA and protein levels in hemolymph, numbers of mature virions in cells and mortality rate, confirming that the gp116 and gp64 glycosylation process is critical to YHV replication and thus disease (Soowannayan et al., 2013). In variants of YHV1 (YHV1b) as well as a potential recombinant virus derived from ancestral YHV1b and YHV5 genotypes, a 54 amino acid deletion occurs at the N-terminus of gp116 just downstream of the pp3 TM3 cleavage site, but this deletion appears to have little if any impact on the pathogenic potential of these variants relative to YHV1a (Sittidilokratna et al., 2009; Gangnonngiw et al., 2009; Senapin et al., 2010).

32.3.1.4 Genotypic Variants and Recombinant Viruses

Initial comparisons of three ORF1b gene sequences of GAV from Australia with those reported or determined for YHV samples from Thailand identified 83.0% identity in a 135-nt region (Wongteerasupaya et al., 1997), 80.9% identity in an adjoining 1068-nt region (Tang and Lightner, 1999) and 85.1% identity in a downstream 577-nt region including the C-terminus of the Zn-HEL motif (Cowley et al., 1999, 2000b). These data showed YHV and GAV to be genotypic variants of a YHV complex and were assigned to genotypes YHV1 and YHV2, respectively (Walker et al., 2001; Wijegoonawardane et al., 2008a). Moreover, recent reanalysis of the 577-nt YHV sequence originated from healthy *P. monodon* broodstock sampled from a hatchery in Thailand (Cowley et al., 1999; J.A. Cowley, unpublished) has revealed it to be related mostly closely to YHV genotype 5 (YHV5) strains (Wijegoonawardane et al., 2008a; Gangnonngiw et al., 2009) than the virulent YHV1 genotype that was only type known to exist at the time. Based on a 423-nt ORF1b gene sequence amplified using a commercial YHV/GAV IQ2000 PCR test (Tang and Lightner, 1999; Cowley et al., 2004b) displaying 92.7% and 79.0% nt identity, respectively, to the reference GAV and YHV genome sequences, a third unique YHV genotype (YHV3) was identified in healthy wild *P. monodon* broodstock originating from the Andaman Sea in southern Thailand (Soowannayan et al., 2003).

To identify whether other YHV genotypes might exist in geographically isolated populations of *P. monodon*, a consensus nested (n)PCR was used to amplify an ORF1b gene sequence from large numbers of juveniles, broodstock and postlarvae (PL) sampled during the early 2000s either from YHD outbreaks or from hatcheries in countries distributed widely across the natural Indo-Pacific distribution range of this species from Mozambique in eastern Africa to Fiji in the South Pacific (Wijegoonawardane et al., 2008a). Phylogenetic analysis of the 671-nt sequence determined for 57 strains, including the reference YHV1 and GAV (=YHV2) strains, clustered the viruses into six discrete clades designated as genotypes 1-6 (YHV1-YHV6) (Fig. 32.5). YHV1 strains were associated solely with YHD episodes in Thailand and YHV2, YHV4 and YHV6 were the only genotypes detected in *P. monodon* from eastern Australia, India

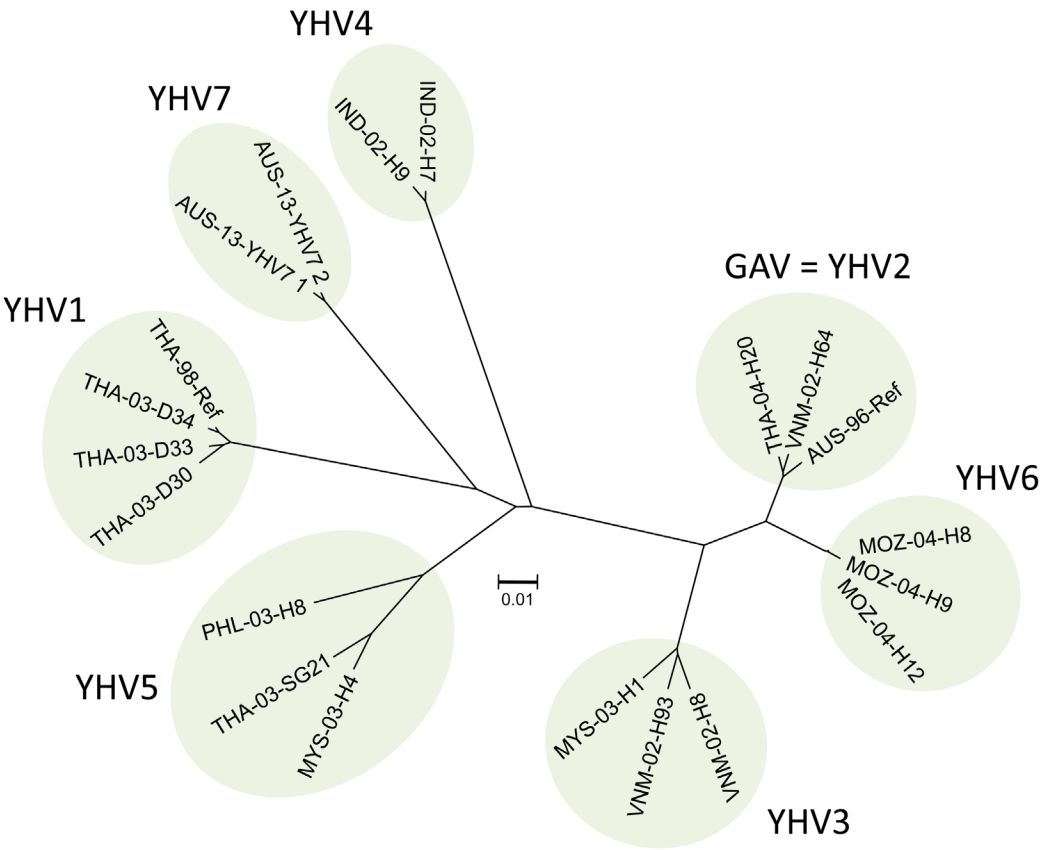

FIGURE 32.5 Phylogenetic analysis of a 646-nt sequence including the 3′ region of the Zn-HEL domain of the ORF1b gene showing clustering and the relative genetic divergence of okaviruses representative of YHV genotypes 1–6 (Wijegoonawardane et al., 2008a) and YHV7 (Mohr et al., 2015). (Bar length = 1% nt difference).

and Mozambique, respectively. Together with some country-specific inter-genotype branches also being evident, the genotype-country associations support the hypothesis of YHV existing as a complex of different genotypes that evolved in isolated shrimp populations, with intensive aquaculture and loosely regulated movements of wild *P. monodon* broodstock in Southeast Asia likely contributing to the mixed genotypes detected in countries like Vietnam (Walker et al., 2001; Wijegoonawardane et al., 2008a).

In support of the YHV complex hypothesis, sequence comparisons of ORF1b gene regions have confirmed the existence of a unique seventh genotype (YHV7) in *P. monodon* originating from the remote Joseph Bonaparte Gulf in northern Australia (Mohr et al., 2015, Fig. 32.5), and based on another ORF1b gene sequence being found to be most closely related (87.6% nt identify) to reference YHV1 sequences (Tang and Lightner, 1999; Sittidilokratna et al., 2002), a unique eighth genotype (YHV8) has been identified in *Penaeus chinensis* farmed in China's Hebei Province (Liu et al., 2014; Mohr et al., 2015).

In addition to YHV existing as diverse genotypes, phylogenetic comparisons of ORF1b and ORF3 gene sequences identified 10 putative recombinant viruses among 28 strains selected to be representative of the YHV-YHV6 genotypes from the different Indo-Pacific regions (Wijegoonawardane et al., 2009). The recombinants originated from Vietnam, Taiwan, Indonesia, Malaysia and the Philippines and represented ORF1b:ORF3 genotype permutations 3:2, 3:5, 5:2 and 5:3, with the 3:2 permutation detected in 6 of the 10 viruses (Fig. 32.6). Maximum-likelihood breakpoint analysis of the ~4.6 kb sequence joining the ORF1b-ORF3 gene regions of representative recombinant viruses from Indonesia (3:2), Vietnam (3:5) and the Philippines (5:2) localized the crossover sites to an identical position just upstream of the ORF1a gene-stop codon in the Indonesian and Vietnamese recombinants and to a position 12-nt further upstream in the recombinant from the Philippines (Wijegoonawardane et al., 2009). The ability of virus recombinants to be generated at high frequency at this crossover hotspot was confirmed by PCR analysis of progeny viruses produced in shrimp co-infected with YHV1 and GAV (Wijegoonawardane et al., 2009). These findings together with reports of other naturally occurring recombinant

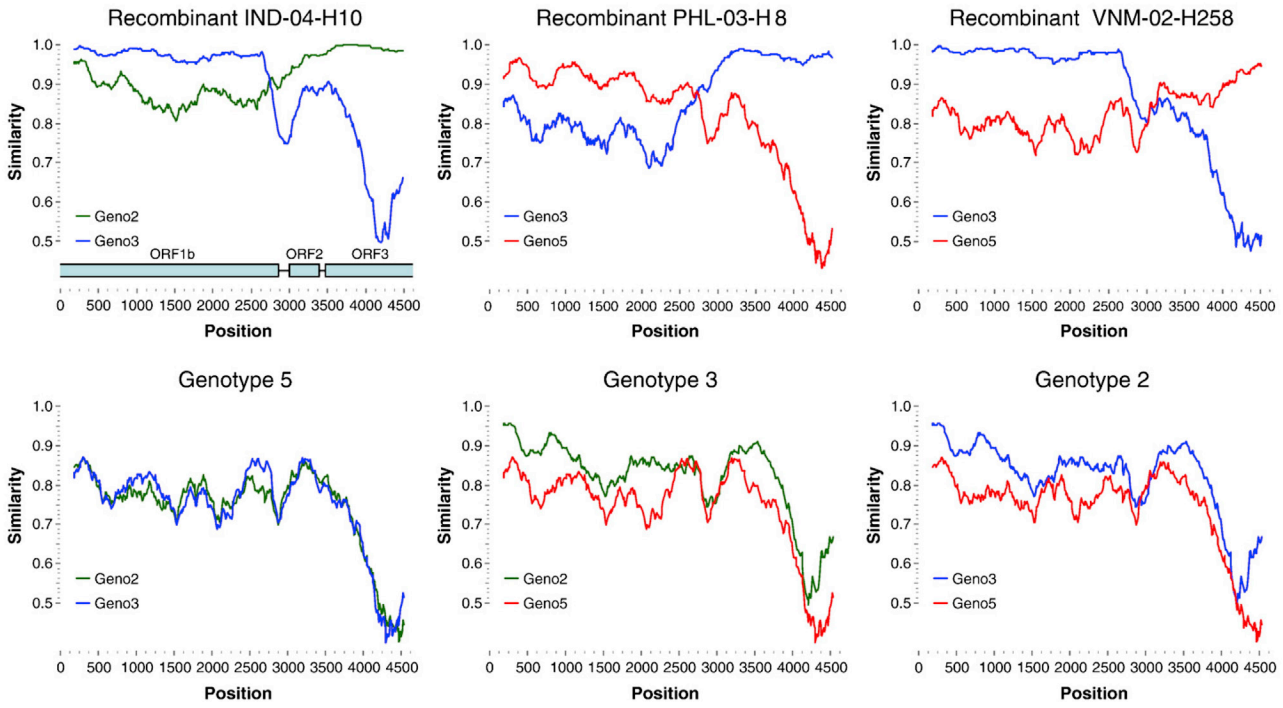

FIGURE 32.6 SimPlot analysis of YHV recombinants IDN-04-H10, VNM-02-H258 and PHL-03-H8 derived from representative parental genotypes 2, 3 and 5 showing the recombination crossover point located near to the ORF1b gene 3′-terminus. Pair-wise divergence between the recombinant and each parental virus or between different genotypes over the ~4.6 kb ORF1b-ORF3 region employed a 350 nt sliding window analyzed at 10 nt intervals and the default Kimura (two-parameter) Distance Model. Percentage identities at each analysis point were plotted on a line chart. The relative positions of the ORF1, ORF2 and ORF3 genes are shown in the first panel (Wijegoonawardane et al., 2009).

YHV strains (Gangnonngiw et al., 2009) indicate that genetic exchange amongst genotypes is occurring commonly in *P. monodon* in southeast Asia, which has profound implications for genotype assignment, disease diagnosis and the potential generation of viruses with increased pathogenic potential.

32.3.1.5 Distribution and Host Range

Within a decade of YHD being discovered in Thailand (Limsuwan, 1991; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993), disease symptoms consistent with acute YHV infection had been reported in most Asian countries with substantial *P. monodon* aquaculture industries, including China, Indonesia, Malaysia, the Philippines, Taiwan, Vietnam, India and Sri Lanka (Albaladejo et al., 1998; Natividad et al., 1999, 2002; Mohan et al., 1998; Wang and Chang, 2000; Walker et al., 2001; Siriwardena, 2000; Subasinghe et al., 2000; Rukyani, 2000; Khoa et al., 2000; Yang et al., 2000). Viruses morphologically indistinguishable from YHV were also detected in Kuruma shrimp (*Penaeus japonicus*) farmed in Taiwan (Wang et al., 1996) and, although described as GAV, in healthy and diseased *P. monodon* farmed in Australia (Spann et al., 1995, 1997; Cowley et al., 1999; Callinan et al., 2003; Callinan and Jiang, 2003; Munro et al., 2011) as well as in wild-caught *P. monodon* broodstock sampled from hatcheries (Cowley et al., 2000b; Walker, 2000; Walker et al., 2001).

In southeast Asia, YHV has been detected in brackish water crustacean species, such as mysid shrimp (*Palaemon styliferus*) and krill (*Ascetes* sp.), as well as shrimp species such as the white banana prawn (*Penaeus merguiensis*) and red endeavor prawn (*Metapenaeus ensis*) that commonly co-inhabit aquaculture ponds growing *P. monodon* (Flegel et al., 1995a, 1997a). Although *P. merguiensis* and *M. ensis* can develop disease and die following experimental challenge (Chantanachookin et al., 1993; Flegel et al., 1995a), these species appear more resilient to developing acute YHV infection in commercial ponds of *P. monodon* dying due to YHD. Similarly in Australia, GAV has been detected at low infection loads in Brown tiger shrimp (*Penaeus esculentus*) inhabiting *P. monodon* ponds affected by GAV disease (Walker et al., 2001). However, when challenged experimentally, *P. esculentus* as well as *P. merguiensis* and *P. japonicus* are susceptible to GAV disease and mortality, although in *P. japonicus*, disease susceptibility was lower in >20 g shrimp compared to 6–13 g

shrimp (Spann et al., 2000, 2003). Following injection challenge, Barred estuarine shrimp (*Palaemon serrifer*), Pacific blue prawn (*Palaemon styliferus*) and the freshwater prawn *Macrobrachium sintangense* are also susceptible to developing mild YHV symptoms that do not necessarily progress to cause mortality, while *Macrobrachium rosenbergii* and *Macrobrachium lanchesteri* are highly refractive to infection, the reasons for which remain to be determined (Longyant et al., 2005). YHV infection in the absence of disease has similarly been induced experimentally in Jinga shrimp (*Metapenaeus affinis*) and the Yellow prawn (*Metapenaeus brevicornis*), but not in any of 16 different crab species challenged by injection (Longyant et al., 2006). Australian red claw crayfish (*Cherax quadricarinatus*) also appear capable of supporting low-level YHV infection following experimental challenge (Soowannayan et al., 2015).

Among shrimp species indigenous to the Americas, such as *L. vannamei* and blue shrimp (*Penaeus stylirostris*) (Lightner et al., 1998; Lu et al., 1994, 1997) as well as white shrimp (*Litopenaeus setiferus*), brown shrimp (*Penaeus aztecus*) and northern pink shrimp (*Penaeus duorarum*) (Lightner et al., 1998), juveniles but not PL stages up to PL20 develop systematic lethal YHV infections when fed carcasses of diseased shrimp (Lightner, 1996). As in these species, *P. monodon* only become susceptible to YHD beyond approximately PL15 (Khongpradit et al., 1995). Injection challenge of Daggerblade grass shrimp (*Palaemonetes pugio*) and the blue crab *Callinectes sapidus* have shown these species to also be susceptible to YHV infection (Ma et al., 2009).

Based on the potential susceptibility of western hemisphere shrimp species, the discovery of infectious YHV together with white-spot syndrome virus (WSSV) in frozen, uncooked commodity shrimp imported into the United States from Asia (Nunan et al., 1998; Durand et al., 2000), and the occurrence of severe LO histopathology thought at the time to be exclusively indicative of YHD in farmed shrimp sampled during outbreaks of white-spot disease (WSD) in the late 1990s, it was speculated that YHV had been introduced into US shrimp farms together with WSSV (Lightner, 1996; Lightner et al., 1998; Lightner and Redman, 1998). However, its presence could not be substantiated by other molecular diagnostic methods, and it was discovered subsequently that in shrimp with severe WSD, LO histopathology can be confused for that caused by acute YHV infection (Pantoja and Lightner, 2003; Lightner, 2011).

Despite the erroneous early reports of YHV occurring in US shrimp farms, a virus consistent in genome sequence to YHV1 was identified subsequently in *L. vannamei* and *P. stylirostris* farmed in northwest Mexico from at least 1999 (de la Rosa-Vélez et al., 2006; Sánchez-Barajas et al., 2009), wild *P. stylirostris* sampled in 2003 from a Gulf of California region nearby to extensive shrimp farms (Castro-Longoria et al., 2008) and *L. vannamei* sampled from low-salinity inland aquaculture enterprises further south in the state of Colima (Sánchez-Barajas et al., 2009). In none of these detections was YHV associated with disease, suggesting it might be a low-virulence strain. Contrary to this assumption, however, *L. vannamei* injected with a high dose of inoculum prepared from healthy YHV-infected *L. vannamei* (YHVLv) developed acute infection resulting in 50% accumulated mortality within 14 days (de la Rosa-Vélez et al., 2006; Cedano-Thomas et al., 2010). Moreover, injection challenge of *L. vannamei* with the Mexican YHV1 strain identified them to be susceptible to disease and mortality and particularly so at seawater salinity extremes (5 and 40 ppt) sometimes occurring in farms along the Mexican Pacific coast (Navarro-Nava et al., 2011).

While the introduction of *P. monodon* into non-native regions might explain how YHV has been introduced into the Gulf of Mexico, it has only been identified and become established as an invasive species in Atlantic coastal regions of the Americas, including the Caribbean (Gómez-Lemos and Campos, 2008), Venezuela (Aguado and Sayegh, 2007) and Brazil (Coelho et al., 2001; Cintra et al., 2011) and in the South Atlantic Bight and Gulf of Mexico regions of the United States (Fuller et al., 2014).

Unlike in Mexico, YHV has been established as a problematic disease of *L. vannamei* since its introduction in the early-2000s and subsequent rapid expansion to become the primary aquaculture species in Thailand and other countries in southeast Asia (Anantasomboon et al., 2007; Senapin et al., 2010). To evaluate the potential for YHV to be translocated and become established in different regions, a comprehensive list of crustacean species capable of carrying infection has been compiled from a meta-analysis of available literature (Stentiford et al., 2009).

32.3.1.6 Transmission

YHV infection can be transmitted horizontally to susceptible crustacean species by several natural challenge routes. These include cannibalization of moribund live shrimp, carcasses or small pieces of flesh from diseased shrimp, cohabitation with infected shrimp and exposure to waterborne virus particles (Flegel et al., 1995a; Lightner, 1996; Lightner et al., 1998; Lu et al., 1997; Walker et al., 2001; Hamano et al., 2015; Soowannayan et al., 2015). Shrimp ingestion of carrier species such as *Acetes* sp. and *Palaemon styliferus* can also transmit YHV infection and disease (Flegel et al., 1995b, 1997a). However, such oral transmission routes only appear to be effective in establishing productive infections and disease in shrimp from late PL life stages (PL17-PL20) onward (Flegel et al., 1995b; Khongpradit et al., 1995; Lightner et al., 1998). Muscle

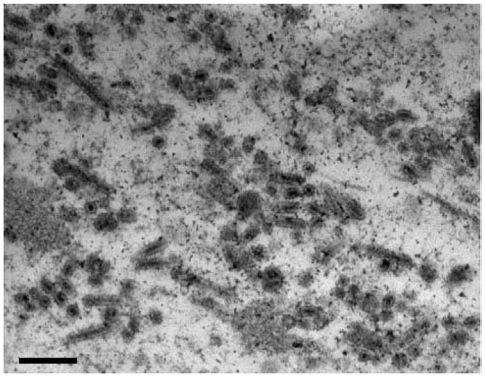

FIGURE 32.7 Transmission electron micrograph showing GAV particles within the seminal fluid of a spermatophore sampled from an adult male *Penaeus monodon* (Cowley et al., 2002b). Bar = 200 nm.

injection of hemolymph or filtered cephalothorax tissue homogenates from diseased shrimp is also very effective at transmitting infection at a defined dose and thus has been used extensively as an experimental challenge method (Boonyaratpalin et al., 1993; Spann et al., 1997; Longyant et al., 2006).

YHV transmission from broodstock to progeny in hatcheries was speculated early on to be the source of infection leading to widespread disease in *P. monodon* being farmed in Thailand (Chantanachookin et al., 1993; Pasharawipas et al., 1997). Initially this assumption was poorly supported by data indicating a low prevalence of YHV infection in wild-caught broodstock sampled from Thai hatcheries (Flegel et al., 1997b) and difficulty in detecting YHV in broodstock reproductive organs. However, accumulated subsequent data now strongly supports vertical transmission being a primary route through which infection is perpetuated in wild populations and enters aquaculture ponds. Such data includes the widespread detection of YHV at high prevalence among *P. monodon* broodstock and PL sampled from hatcheries (Phan, 2001; Cowley et al., 2000b, 2002b; Spann et al., 1995; Walker et al., 2001; Wijegoonawardane et al., 2008a) as well as juveniles sampled from farm ponds (Spann et al., 1995; Pasharawipas et al., 1997).

ISH and PCR data have also provided evidence for GAV occurring in male *P. monodon* spermatophores and spermatophore secretions as well as female ovaries (Walker et al., 2001). The presence of mature virions in spermatophore seminal fluid but not in sperm per se has been substantiated by TEM and nested (n)PCR and nPCR has also confirmed the presence of low GAV RNA amounts associated with unwashed eggs, but not newly hatched nauplii (Cowley et al., 2002a; Fig. 32.7). In *P. monodon*, egg activation and fertilization requires a multitude of egg surface alterations that initiate immediately upon contact with seawater (Pongtippatee-Taweepreda et al., 2004). Although the mechanism by which GAV/YHV are transmitted vertically remains unknown, opportunities for virus entry into eggs might present themselves during the extraordinary egg surface ultrastructural changes that occur as a result of the cortical reaction and egg-sperm interaction processes activated within the first few seconds after release from the female (Pongtippatee-Taweepreda et al., 2004).

To directly examine the capacity of either gender of *P. monodon* to transmit GAV infection vertically, female broodstock captured from one location in north Queensland were inseminated artificially with the spermatophores of males captured at a disparate location. GAV strains present as a subclinical infection in each parent as well as in progeny batches reared to an early PL stage could thus be identified clearly by sequence analysis of a more variable region of the ORF3 gene (Cowley et al., 2002a). GAV strain sequences identified in progeny demonstrated that virus could be transmitted from the male or female parent or both, with the predominant strain detected in progeny generally correlating with that existent in either parent at the highest infection load.

32.3.1.7 Diagnostic Methods

32.3.1.7.1 Gross Signs and Light and Electron Microscopy

Gross signs such as erratic swimming and shrimp congregating at pond edges with a yellowed cephalothorax, bleaching/reddening general body appearance and pink discolored gills and appendage extremities can alert to disease caused by YHV. For rapid presumptive diagnosis of YHD, gill squashes or hemocyte smears from moribund shrimp can be observed either directly by phase-contrast microscopy or by normal light microscopy after Wright-Giemsa staining (Nash et al., 1992; Lightner, 1996; FAO Fisheries Technical Paper 402/2 2001). Gill filaments or subcuticular tissue fixed in Davidson's

AFA fixative and stained with H&E can also be observed by light microscopy without wax embedding and tissue sectioning (Lightner, 1996; OIE, 2014). Pathologies predictive of YHD include evidence of nuclear pyknosis and karyorrhexis as well as medium-to-large numbers of evenly stained and deeply basophilic spherical cytoplasmic inclusions ≤2 mm in diameter in cells of mesodermal and ectodermal origins (Lightner, 1996; Flegel et al., 1997a). Histology on wax-embedded cephalothorax tissue sections stained with H&E will typically delineate the nature of the cellular pathologies more precisely as well as assist in determining YHD severity based on the nature and numbers of LO spheroids (Chantanachookin et al., 1993; Boonyaratpalin et al., 1993; Nash et al., 1992; Lightner and Redman, 1998; Spann et al., 1997; Flegel et al., 1995b, 1997a; OIE, 2014; Fig. 32.2).

Despite the diagnostic value of histology, care must be taken when interpreting data because pathology evident in the LO of shrimp with severe WSD can be confused for that caused by YHV (Pantoja and Lightner, 2003; Lightner, 2011). Histology alone is thus insufficient to diagnose YHV infection or disease with confidence unless supported by other methods. TEM on LO or gill tissue can also be used to confirm the presence of variable length (80–450 nm), filamentous nucleocapsid precursors and rod-shaped enveloped particles characteristic of YHV/GAV (Chantanachookin et al., 1993; Boonyaratpalin et al., 1993; Spann et al., 1995, 1997; Fig. 32.3). Like histology, however, TEM is time consuming, requires specialized equipment and, for surveillance and diagnostic purposes, has generally been superseded by more rapid molecular diagnostic methods.

32.3.1.7.2 Shrimp Bioassay, Cell Culture and Hemagglutination Assays

Primary cultures of shrimp LO cells support YHV replication (Chen and Kou, 1989; Chen and Wang, 1999), and assays based on cytopathic effects and cell monolayer disintegration have been used to quantify virus infectivity (Lu et al., 1995a,b; Assavalapsakul et al., 2003). However, because no continuous cell lines are yet available to easily culture YHV, bioassay in naïve shrimp remains the most common means of confirming the presence of infectious YHV (Lightner et al., 1998; Longyant et al., 2006; Lu et al., 1995b; Spann et al., 1997; McColl et al., 2004). While culture methods are useful in amplifying infectious YHV, definitive diagnosis relies on TEM to detect virus nucleocapsids and/or virions or on molecular methods to detect YHV protein or RNA (OIE, 2014).

Hemagglutination (HA) of chicken erythrocytes by YHV (Nadala et al., 1997b) or GAV particles (Munro and Owens, 2005, 2006) in titrations of shrimp hemolymph or gill tissue extracts has also been investigated as a potential pond-side diagnostic method. While the HA method is not particularly sensitive and provides no specificity, there are circumstances in which it could be applied to guide disease management decisions at hatcheries and farms.

32.3.1.7.3 Molecular Diagnostic Methods—Viral Proteins

To diagnose YHV infection unequivocally, various molecular methods have been developed to detect either viral proteins or viral RNA. Methods to detect YHV proteins have employed polyclonal antiserum prepared to purified whole virus (Lu et al., 1996; Munro and Owens, 2007a), monoclonal antibodies (MAbs) generated to purified whole virus and selected for specificity to the gp116 and gp64 envelope glycoproteins or the p20 nucleoprotein (Sithigorngul et al., 2000, 2002; Munro and Owens, 2007a; Sittidilokratna et al., 2006), monospecific antiserum prepared to a p20 peptide sequence or to a p20-fusion protein expressed in *Escherichia coli* (Cowley et al., 2004a) and a recombinant single-chain variable fragment antibody to gp116 expressed in *E. coli* and constructed by PCR from a hybridoma clone generated to purified YHV particles (Intorasoot et al., 2007). The range of antibody-based methods described for detecting YHV or GAV proteins includes dot-blot nitrocellulose enzyme immunoassays (DB-NC-EIA) (Intorasoot et al., 2007; Lu et al., 1996; Loh et al., 1998; Nadala and Loh, 2000; Sithigorngul et al., 2000) and Western blots (Cowley et al., 2004a; Intorasoot et al., 2007; Nadala et al., 1997a; Sithigorngul et al., 2000; Soowannayan et al., 2003; OIE, 2014), a solid-phase noncompetitive enzyme-linked immunosorbent assay (ELISA) (Intorasoot et al., 2007), as well as a sandwich ELISA employing polyclonal chicken antibodies raised against GAV proteins for protein capture and a p20 nucleoprotein-specified MAb for GAV protein detection (Munro and Owens, 2006, 2007a), immunohistochemistry (IHC) for detecting infected cells and tissues in histological sections (Sithigorngul et al., 2000; Soowannayan et al., 2003) and immunoelectron microscopy for detecting viral nucleocapsids and virions in ultrathin tissue sections or protein components of purified and negatively stained virions (Cowley et al., 2004a; Soowannayan et al., 2003).

Of these methods, the DB-NC-EIA protocol, using gill tissue homogenates, provides a rapid and simple potential method to diagnose YHV infection (Nadala and Loh, 2000). However, an even more simple-to-implement lateral-flow immunochromatographic test strip (ICTS) method has been developed for the pond-side diagnosis that employs either shrimp hemolymph or gill/appendage tissue extracts as a protein source, a polyclonal rabbit antiserum prepared to a recombinant YHV p20 protein to capture this viral protein and a p20-specific MAb (Y19) conjugated to colloidal gold as a detector

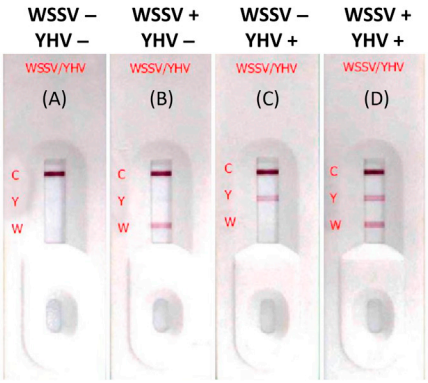

FIGURE 32.8 Dual immunochromatographic strip test showing bands indicating the detection of YHV (Y) and/or WSSV (W) using pleopod tissue homogenates from (A) uninfected shrimp, (B) WSSV-infected shrimp, (C) YHV-infected shrimp and (D) a combination of WSSV- and YHV-infected shrimp, C = control line confirming test performance (Sithigorngul et al., 2011).

antibody (Sithigorngul et al., 2007; Chaivisuthangkura et al., 2014). An ICTS method to co-detect YHV and WSSV in the same strip has also been described (Sithigorngul et al., 2011, Fig. 32.8).

Antibody-based diagnostic methods can offer advantages in speed, simplicity and cost in detecting GAV/YHV compared with molecular methods to detect viral RNA such as PCR (Munro and Owens, 2006; Sithigorngul et al., 2007). However, their analytical sensitivity is generally lower. For example, the lateral-flow ICTS developed to detect YHV is approximately 500-fold less sensitive in detecting YHV p20 protein compared with a one-step PCR test designed to detect YHV RNA (Wongteerasupaya et al., 1997; Sithigorngul et al., 2007). YHV detection sensitivity was reduced further in an ICTS designed to co-detect YHV and WSSV (Sithigorngul et al., 2011). Antibody specificity also needs to be considered. For example, while polyclonal antiserum to recombinant GAV p20 protein cross-detects the YHV p20 protein (Cowley et al., 2004a) and MAbs to the YHV p20 nucleoprotein (Y19) and gp64 glycoprotein (Y18) cross-detect the cognate GAV proteins, MAb V-3-2B specific to the YHV gp116 glycoprotein does not detect gp116 of GAV (Sithigorngul et al., 2000, 2007; Soowannayan et al., 2003). Despite these limitations, protein-based virus detection methods provide practical and relatively cheap diagnostic options for managing YHV infection and disease risks at shrimp hatcheries and farms.

32.3.1.7.4 Molecular Diagnostic Methods—Viral RNA

Molecular methods designed to detect YHV RNA generally provide the highest analytical sensitivity. ISH using digoxigenin (DIG)-labeled DNA or RNA probes is useful, like the IHC method, in associating YHV with specific tissue types and histopathology and thus in diagnosing infection as well as assessing disease severity (Spann et al., 2003; Tang and Lightner, 1999; Tang et al., 2002; OIE, 2014). However, due to its complexity, ISH has been used primarily as a research tool to investigate YHV infection processes and disease progression.

Numerous PCR tests have been reported for either YHV and GAV diagnosis or research (Cowley et al., 2000b, 2004b; Wongteerasupaya et al., 1997; Wijegoonawardane et al., 2008b, 2010; Dhar et al., 2002; Mouillesseaux et al., 2003; de la Vega et al., 2004; Tang and Lightner, 1999; Aranguren et al., 2012; de la Rosa-Vélez et al., 2006). Of these, the one-step RT-PCR first reported for YHV (Wongteerasupaya et al., 1997), a two-step multiplexed nPCR utilizing PCR primers to amplify both YHV and GAV and multiplexed virus-specific nPCR primers to amplify and differentiate YHV from GAV based on amplicon size (Cowley et al., 2004b) and a two-step multiplexed PCR test utilizing combined degenerate primers designed to have broad specificity for YHV genotypes 1–6 and to assist in detecting other genotypic variants that might exist (Wijegoonawardane et al., 2008b) are endorsed for infection/disease diagnosis in the Yellowhead Disease chapter of the OIE Manual of Diagnostic Tests for Aquatic Animals 2014 (OIE, 2014). Each of these tests targets regions in the okavirus ORF1b gene, and are likely to now require revision or caveats to their applicability as new YHV genotypes are discovered such as YHV7 (Mohr et al., 2015) and YHV 8 (Liu et al., 2014) because they were designed to sequences of YHV genotypes known at the time.

The inclusion of a nPCR step can improve YHV/GAV RNA detection efficiency up to 100-fold or more compared with one-step PCR (Cowley et al., 2000b, 2004b). Various real-time RT-PCR tests that provide viral RNA detection sensitivities further improved compared with nPCR as well as improved throughput capacity and linear dynamic range over which viral RNA loads and thus infection severity can be quantified accurately, have been described for detecting

YHV, GAV or YHV genotypes 1–6 that utilize either SYBR-Green chemistry (Dhar et al., 2002; Mouillesseaux et al., 2003) or a TaqMan probe to generate fluorescence (de la Vega et al., 2004; Aranguren et al., 2012; Panichareon et al., 2011; Wijegoonawardane et al., 2010). The detection limits of these real-time PCR tests can approach a single RNA copy. A modified version of the nPCR designed to differentiate YHV from GAV (Cowley et al., 2004b) is also available commercially (IQ2000 YHV/GAV PCR test) together with commercial real-time SYBR-Green PCR and RT-LAMP tests for YHV (GeneReach Biotechnology Corp.).

In addition to the PCR designed to detect YHV and/or GAV (Cowley et al., 2004b), a test employing multiplexed PCR primer sets to detect any of six different shrimp viruses and a TaqMan real-time PCR employing multiplexed PCR primers and probes labeled with different fluorophores to co-detect and quantify YHV and Taura syndrome virus (TSV) have been described (Khawsak et al., 2008; Aranguren et al., 2012). As an alternative to gel electrophoresis to detect and confirm the expected size of PCR amplicons, hybridization to biotin-labeled virus-specific oligonucleotides followed by avidin capture and detection using anti-DIG-peroxidase has been used for ELISA-based visualization of DNA amplified in the presence of DIG-dUTP (Khawsak et al., 2008). Hemolymph spotted onto specialized filter papers such as IsoCode (Schleicher and Schuell Inc.) has proved useful for field collection and preservation of YHV RNA for subsequent RT-PCR analysis (Kiatpathomchai et al., 2004). The method avoids the complexities of tissue dissection and collection into liquid-based preservatives, and/or the need to refrigerate or freeze tissues, and can maintain RNA integrity in samples stored for periods of up to 9 months at ambient temperature (Kiatpathomchai et al., 2004). Hemolymph stored as a liquid at either 4°C of 25°C for up to 5 days has also been found show little sensitivity drop-off in real-time PCR detection of YHV RNA (Ma et al., 2008).

While RNA detection by PCR methods can provide exquisite sensitivity, most tests employ primers designed to genome sequences determined for a single strain/genotype and with no regard for them targeting gene regions constrained by their requirement to encode more highly conserved functional domains within YHV proteins. Based on these shortcomings in combination with YHCVs existing as a complex of genotypes that appear to have evolved in geographically isolated populations of *P. monodon* (Walker et al., 2001; Soowannayan et al., 2003; Wijegoonawardane et al., 2008a), extreme care is needed in interpreting data generated by any one particular PCR test. For example, the original RT-PCR test described to detect YHV (Wongteerasupaya et al., 1997) has retained good specificity for the YHV1 genotype due to the 3′-terminus of 10R PCR primer serendipitously targeting a sequence that has diverged in other identified genotypes (Wijegoonawardane et al., 2008b). However, other tests, including the RT-nPCR designed to differentiate YHV1 from GAV (Cowley et al., 2004b) and the TaqMan real-time PCR to detect GAV (de la Vega et al., 2004), have been identified to cross-detect other genotypes at variable efficiency depending on their sequence relatedness to GAV in the genome regions targeted by the PCR primers (Wijegoonawardane et al., 2008b; J.A. Cowley et al., unpublished).

To detect YHV genotypes 1–6 as well as other genotypes that might exist, consensus RT-nPCR and TaqMan real-time RT-PCR tests have been described that use degenerate PCR primers designed to accommodate genotype sequence variations identified in an ORF1b gene region (Wijegoonawardane et al., 2008a,b, 2010). Phylogenetic analyses of sequences amplified from this and another ORF1b gene region have proved useful in assigning YHV genotypes and in identifying new genotypes such as YHV7 found recently in *P. monodon* originating from the previously unstudied Joseph Bonaparte Gulf region of northern Australia (Mohr et al., 2015).

While phylogenetic comparisons of ORF1b gene sequences have proved useful in classifying YHV genotypes, PCR tests designed to amplify sequences within the ORF2/ORF3 genes encoding the virus structural proteins have proved useful in identifying YHV1 subtypes YHV1a and YHV1b (Sittidilokratna et al., 2008, 2009; Wijegoonawardane et al., 2008a) as well as viruses representing recombinants (Wijegoonawardane et al., 2009; Gangnonngiw et al., 2009). Based on the high frequency at which recombinants comprising various permutations of the 5′-ORF1a/1b and the ORF2-3 3′-genome regions of parental YHV genotype 2, 3 and 5 viruses have been detected in *P. monodon* collected from various countries in southeast Asia (Wijegoonawardane et al., 2009), genotype assignments based on ORF1a/1b gene sequences alone need to be viewed with caution, particularly in regions where hatcheries are densely colocated and/or commonly source wild broodstock from diverse locations. In cases when a YHV genotype needs to be defined definitively, it is recommended that regions in both the ORF1a/1b and ORF2/ORF3 genes be amplified, sequenced and compared, as was done to identify the nature of a YHV genotype when first detected in shrimp farmed in Mexico (de la Rosa-Vélez et al., 2006).

As an alternative to PCR, RT-LAMP methods have been described to detect YHV (Mekata et al., 2006, 2009; Jaroenram et al., 2012; Khunthong et al., 2013). A standard RT-LAMP test employing agarose gel electrophoresis to detect the amplified DNA ladder was found to be about 10-fold less sensitive than a commercial IQ2000 YHV/GAV nPCR test (Mekata et al., 2006). In contrast, a real-time RT-LAMP test employing a photometer to quantify turbidity increases in the magnesium pyrophosphate by-product of DNA amplification was found to be about 10-fold more sensitive than the same commercial nPCR test (Mekata et al., 2009). To avoid the need for either gel analysis or a photometer to detect amplified

DNA, a RT-LAMP method modified to amplify biotin-labeled DNA ladder products has also been developed to detect YHV (Khunthong et al., 2013). By hybridizing amplified DNA products to a fluorescein isothiocyanate-labeled DNA oligonucleotide, a simple lateral-flow dip (LFD) stick system was employed to trap and visualize the DNA duplexes as a dark-colored band (Khunthong et al., 2013). A similarly simple tube-based colorimetric method has been described that utilizes gold nanoparticles (AuNP) to bind and detect YHV DNA ladder products amplified by RT-LAMP (Jaroenram et al., 2012). These rapid, specific and highly sensitive methods that only require a constant temperature apparatus and simple LFD stick or tube-based colorimetric detection systems, such as LAMP, offer substantial potential for field diagnosis of YHV. However, due to the complexity of primers required for LAMP, the specificity they provide is likely to limit the applicability of any test to a single genotype.

32.3.1.8 Prevention and Control

32.3.1.8.1 Hatchery and Farm Biosecurity and Management Practices

No commercial treatments are yet available to protect farmed shrimp against YHD. Disease prevention thus relies on the regulatory oversight and surveillance by government quarantine and agricultural agencies (AQIS, 2000; Stentiford et al., 2009; Lightner et al., 1997; McColl et al., 2004), on hatcheries and farms employing robust biosecurity systems that exclude virus entry via water, carrier species, equipment or personnel, as well as infection surveillance in ponds, pond bottom and pond water disinfection systems and farm management practices that avoid exposing shrimp to adverse stressors, such as rapid changes in water pH, pH levels >9 or prolonged periods of low (<2 ppm) dissolved oxygen (Limsuwan, 1996; Lightner, 1999, 2003, 2005; Walker and Mohan, 2009).

32.3.1.8.2 Broodstock Screening and Use of Specific Pathogen-Free or Specific Pathogen-Resistant Broodstock

Since wild shrimp often carry a subclinical YHV or GAV infection (Spann et al., 1995; Pasharawipas et al., 1997; Cowley et al., 2000b; Anantasomboon et al., 2007; Castro-Longoria et al., 2008) and the virus can be transmitted vertically from both male and female broodstock (Cowley et al., 2002b), infected progeny represent a major risk factor in farm disease. PCR screening of hemolymph, gill or pleopod biopsies collected nonsacrificially can provide a means of selecting broodstock that are either free of infection or infected at low levels, posing a lowered risk of virus transmission (Nadala et al., 1997a,b; Cowley et al., 2002b; Pasharawipas et al., 1997). While less ideal, PL batches can also be screened and discarded if deemed to pose an unacceptable disease risk.

Progress made in domesticating and selectively breeding shrimp species of aquaculture importance that are specific pathogen-free (SPF) for YHV and other problematic viruses (Wyban, 1992; Wyban et al., 1992; Lotz, 1992, 1997; Lightner, 2003, 2005; Lightner et al., 2009). However, unlike for infectious hypodermal and hematopoietic necrosis virus (IHHNV) and TSV, in which genetic lines of shrimp have been repeatedly challenged and selected to be specific pathogen-resistant (SPR) of disease caused by these viruses (Tang et al., 2000), as yet only shrimp breeding stocks SPF for YHV are available commercially (Lightner, 2005, 2011). While the farming of virus-free seed stock spawned from SPF *L. vannamei* has assisted in reducing YHV disease impacts in Asian regions that previously farmed *P. monodon* or *P. chinensis* (Lightner, 2011; Lightner et al., 2009), it has not provided a panacea because shrimp remain highly susceptible to YHD and other diseases originating from exogenous sources (Senapin et al., 2010).

In Australia, rearing of domesticated *P. monodon* broodstock over several generations has resulted in profound improvements in production yields due to increased growth rates and survival (Preston et al., 2007, 2010). A strategy employing early rearing of *P. monodon* broodstock in dedicated farm ponds, rather than solely in biosecure facilities, and PCR screening to remove individuals infected with GAV at high loads prior to each breeding cycle has proved useful in establishing breeding populations in which subclinical GAV infection is prevalent, but in which tolerance against GAV-induced disease has developed (J.A. Cowley and M.J. Sellars, unpublished). Disease tolerance among *P. monodon* that have developed a capacity to accommodate YHV/GAV infection in a subclinical state is similar to that observed to have occurred within a few years of YHD emerging in Thailand (Pasharawipas et al., 1997), with some "viral accommodation" process associated with the suppression of cell apoptosis (Flegel and Pasharawipas, 1998; Flegel, 2007; Khanobdee et al., 2002) and/or suppression of YHV gp116 envelope glycoprotein synthesis (Chaivisuthangkura et al., 2008) speculated to be involved. As mortalities in *L. vannamei* exposed *per os* to TSV before challenge with YHV also accumulate more slowly, preexisting infection by some viruses appears able to interfere or compete with YHV infection (Aranguren et al., 2012). In the Americas where subclinical TSV infection occurs commonly in farmed *L. vannamei*, it has been speculated that such infections might have helped prevent YHV infection and disease from becoming established in this region (Aranguren et al., 2012).

32.3.1.8.3 Therapeutic Feed Ingredients

Extracts of various plants, herbs and seaweeds with immunostimulant or antimicrobial activities have been investigated as feed supplements to protect shrimp against viruses including WSSV and YHV (Sivasankar et al., 2015). For example, a feed produced to contain a leaf ethanol extract of the Thai medicinal plant *Clinacanthus nutans* decreased mortalities in shrimp challenged with YHV by water immersion (Direkbusarakom et al., 1998). Leaf extracts of the medicinal herbs *Phyllanthus amarus* and *Phyllanthus urinaria* also neutralize YHV infectivity, but their protective efficacy as a feed supplement was not assessed (Direkbusarakom et al., 1995). An ethanol leaf extract of Guava (*Psidillm guajava*) also neutralizes YHV infectivity, but its addition to feed provides no protection against YHV challenge (Direkbusarakom et al., 1997). While the feeding of such medicinal plant extracts has thus shown promise in protecting shrimp against YHD and other virus diseases, there appears to have been little interest from shrimp feed manufacturers in including such extracts in commercial feed pellets.

When processed and added to shrimp feed pellets, an ingredient produced from flocks of microalgae and bacteria grown in seawater ponds seeded with low-value carbon by-products, such as sugarcane bagasse or rice husks, and patented as Novacq can improve the growth performance of farmed *P. monodon* and *L. vannamei* by 30% and 50%, respectively (Glencross et al., 2012, 2014). Juvenile *P. monodon* fed on pellets containing Novacq have also displayed lowered mortality rates following GAV challenge (Sellars et al., 2015). Thus ingredients like Novacq that will be included in commercial feeds for the primary reason of improving shrimp growth rates and general robustness might provide secondary benefits in reducing farm disease impacts by enhancing shrimp resilience against disease caused by GAV/YHV and other viruses.

32.3.1.8.4 RNA Interference

RNA interference (RNAi) strategies based on the use of short interfering (si)RNA or either long dsRNA or hairpin dsRNA are being explored as a means of preventing diseases caused by most viruses problematic to shrimp aquaculture (Shekhar and Lu, 2009; Sagi et al., 2013). Long dsRNA was identified early to direct a RNAi response more specific than siRNA (Robalino et al., 2004, 2005; Westenberg et al., 2005), presumably through mobilizing the Dicer RNase III dsRNA cleavage enzyme (Chen et al., 2011; Li et al., 2013; Su et al., 2008; Yao et al., 2010) as well as the Argonaut enzyme and other minor components of the RNA-induced silencing complex that mediates RNAi-based gene silencing in shrimp and other organisms (Unajak et al., 2006; Dechklar et al., 2008; Chen et al., 2011). Gene silencing in shrimp mediated by long dsRNA is thus being investigated intensively as a potential therapeutic to combat YHD.

In an initial study using cultures of shrimp LO cells, transfection of in vitro transcribed dsRNA targeted to the YHV ORF1b gene and thus the YHV genomic and antigenomic length RNAs inhibited YHV replication more effectively than dsRNAs targeted to ORF3 gene sequences encoding the gp116 or gp64 glycoproteins (Tirasophon et al., 2005). As okaviruses transcribe 3′-coterminal ORF2 and ORF3 sgmRNAs in greater abundance than genomic-length RNA (Cowley et al., 2002a; Cowley and Walker, 2002; Sittidilokratna et al., 2006), dsRNA targeted to the ORF3 gene would interact with all three RNAs. It is not unexpected, therefore, that dsRNAs targeting the ORF1a/1b gene region of the low-abundance genomic and antigenomic RNAs are more effective in inhibiting YHV replication, and particularly because the genomic-length RNA is responsible for translating the pp1a and pp1ab polyproteins that contain the various enzymes critical for viral RNA transcription and replication.

In *P. monodon* challenged with YHV, an ORF1a/1b gene dsRNA inhibited virus replication specifically when injected up to 12 h post-challenge, and also reduced shrimp mortality when injected earliest at 3 h post-challenge (Tirasophon et al., 2007). Using different methodology, the same ORF1a/1b gene dsRNA injected into hemolymph of *P. monodon* just prior to YHV challenge completed protected the shrimp for 10 days, at which time 90% of noninjected shrimp had died (Yodmuang et al., 2006). Similarly, injection of a hairpin YHV ORF1a/1b gene dsRNA expressed and purified from bacteria into the hemolymph of *L. vannamei* as a therapeutic agent prior to YHV challenge inhibited mortalities over a 16-day period, with no evidence of YHV replication evident by one-step PCR analysis (Assavalapsakul et al., 2009). Consistent with earlier findings, when dsRNA was injected as a prophylactic agent at different times post-challenge, YHV replication levels were suppressed most significantly at the earliest dsRNA injection time points (Assavalapsakul et al., 2009). Injection of an ORF1a/1b gene hairpin dsRNA expressed in bacteria and purified using a cheap and simple process involving phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by RNase A and DNase digestion also suppresses YHV replication and mortalities after *L. vannamei* challenge with YHV (Saksmerprome et al., 2009). Injection of *P. monodon* with a pool of five dsRNAs targeted to different ORF1a/1b gene regions and expressed similarly in bacteria but purified differently, has also proved highly protective against subsequent GAV challenge (Sellars et al., 2011, Fig. 32.9). Moreover, delayed and slowed mortality rates in *P. monodon* challenged by GAV injection has also been provided by injection immediately after challenge of either of two in vitro-transcribed dsRNAs targeted to different ORF1b gene sequences (Oanh et al., 2011).

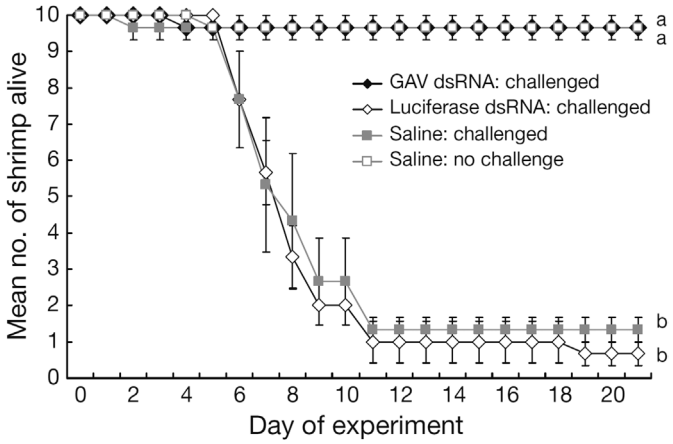

FIGURE 32.9 High survival over time of juvenile *Penaeus monodon* injected with a pool of five dsRNAs targeted to the GAV ORF1b gene, but not with either a nonspecific luciferase dsRNA or saline immediately prior to injection challenge with GAV. Significant differences ($p < 0.001$) are indicated by different letters (Sellars et al., 2011).

In addition to virus-specific dsRNA, dsRNA-mediated RNAi of a *P. monodon* gene (PmYRP65) expressing a protein identified to be a cell-surface receptor for YHV has also been identified to suppress YHV replication in primary LO cell cultures (Assavalapsakul et al., 2006). The same dsRNA injected into shrimp also completely abrogates mortalities following experimental YHV challenge (Assavalapsakul et al., 2014). Muscle injection of shrimp with a dsRNA targeted to another endogenous *P. monodon* gene (PmRab7) encoding a small GTPase has similarly been found to inhibit replication of YHV and WSSV following challenge (Ongvarrasopone et al., 2008). Suppression of its expression might interfere generally with virus entry or the transport of viral proteins into the cytosol because the PmRab7 protein binds to the VP28 envelop glycoprotein of WSSV (Sritunyalucksana et al., 2006) and has been suggested to function in endosomal membrane trafficking (Ongvarrasopone et al., 2008).

The oral delivery of dsRNA expressed in *E. coli* following brief fixation of the bacteria in 0.5% formaldehyde before incorporation into feeds has also been examined for its ability to induce RNAi responses projective against GAV (Sellars et al., 2011) or YHV (Sanitt et al., 2014). When processed into a wet feed, a pool of five dsRNA targeted diverse regions of the GAV ORF1a/1b gene in an attempt to amplify its efficacy did not reduce virus replication or morality rates in shrimp challenged by muscle injection of a minimal lethal dose of GAV (Sellars et al., 2011). In contrast, fixed bacteria containing expressed dsRNA targeted to the Rab7 gene and/or the YHV ORF1a/1b gene and concentrated into small agarose blocks suitable as a feed for juvenile (250–300 mg) *L. vannamei* reduced mortalities following YHV challenge by water immersion, with feeding of the Rab7 dsRNA generating the highest mortality reductions followed by the Rab7 + YHV dsRNA and the YHV dsRNA (Sanitt et al., 2014). Inexpensive and biodegradable polymer nanoparticles derived from chitosan and its quarternized derivative, QCH4, have also been assessed for their ability to bind and deliver YHV ORF1a/1b gene hairpin dsRNA expressed in and purified cheaply from *E. coli* (Saksmerprome et al., 2009; Theerawanitchpan et al., 2012). Based on reduced YHV replication occurring in treated caterpillar (*Spodoptera frugiperda*, Sf9) cells, it was speculated that nanoparticle binding or encapsulation of dsRNA might offer advantages in dsRNA stability and/or delivery efficiency for feed-based dsRNA delivered via the shrimp gut (Theerawanitchpan et al., 2012). Despite these promising data, serious investment in feed-based delivery of dsRNA to prevent YHD via commercial feed pellets seems improbable.

Injection of GAV-infected juvenile *P. monodon* with single or pooled dsRNAs targeted to five different ORF1a/1b gene regions has been identified to reduce GAV infection levels (Sellars et al., 2014). Of the single dsRNAs tested, one targeted to the extreme 5′-end of the GAV genome reduced infection loads most profoundly followed by the dsRNA pool. Injection of the dsRNA pool into GAV-infected female *P. monodon* broodstock has confirmed its ability to reduce virus infection loads without impairing female reproductive capacity (M.J. Sellars et al., unpublished). Thus, if dsRNA injection can be optimized to reduce viral infection loads to levels below which vertical transmission is unlikely to occur, this RNAi strategy might find application in hatcheries reliant on wild *P. monodon* broodstock captured from regions in which GAV or YHV are endemic or in cases when it might be desirable to eliminate viral infection from valuable domesticated breeding populations.

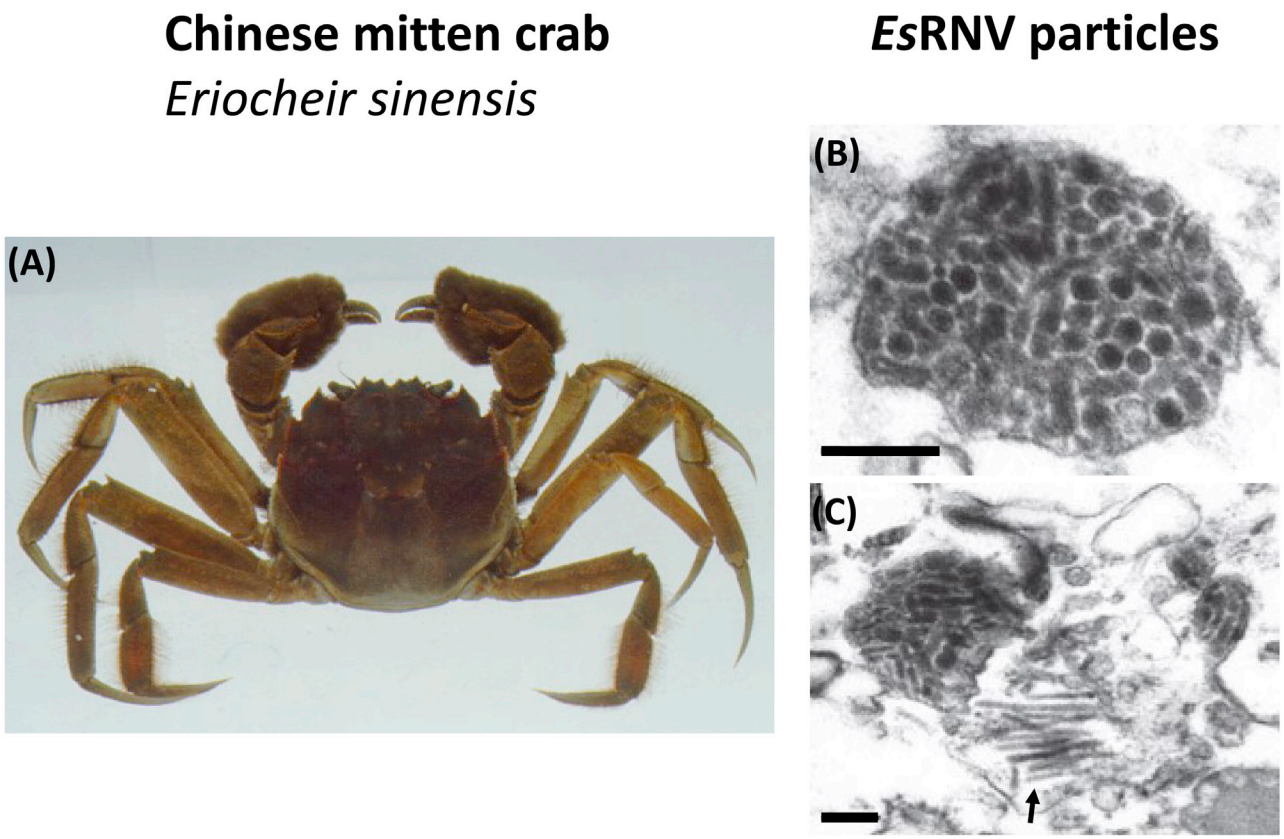

FIGURE 32.10 (A) Photograph of the freshwater Chinese mitten crab (*Eriocheir sinensis*). Transmission electron micrographs of *Eriocheir sinensis* ronivirus (*Es*RNV) particles (B) clustered with cytoplasmic vesicles and (C) filamentous nucleocapsid precursors (*arrow*) in the vicinity of virion clusters in connective tissue cells within the hepatopancreas of diseased *Eriocheir sinensis* (Bars = 120 nm) (Zhang and Bonami, 2007).

32.3.2 *Eriocheir sinensis* Ronivirus

The freshwater Chinese mitten crab (*Eriocheir sinensis*) has become an important aquaculture species in many inland provinces in China (Zeng et al., 2012; Fig. 32.10). With the increasingly intensive aquaculture of *E. sinensis*, several diseases have emerged. Among these, black gill syndrome (BGS) emerged in 1996 as a cause of substantial production losses (Zhang and Bonami, 2007). As other symptoms associated with BGS are generalized, such as sluggishness and anorexia, and as affected crabs display respiratory difficulties manifest by them audibly sounding to "sigh" in the quiet of night as they respire bubbles, the disease is also referred to colloquially as "sighs" disease (SD) (Zhang and Bonami, 2007).

In investigating the cause of SD in crabs collected from a farm in Hubei Province, filamentous viral nucleocapsid precursors (16–18 nm × 150–250 nm) up to 400 nm in length, sometimes closely aligned and amassed adjacent to ER membranes, and enveloped rod-shaped virions (24–42 nm × 60–170 nm) either scattered throughout the cell cytoplasm or packed densely within cytoplasmic vesicles, were identified by TEM in cells of connective tissues (Zhang and Bonami, 2007; Fig. 32.10). Negative staining of virions purified from hemolymph identified regularly spaced surface projections.

In challenge trials, injection of naïve *E. sinensis* with ultra-filtered hemolymph from a disease crab resulted in deaths accumulating rapidly from day 13 to reach 100% by day 17 (Zhang and Bonami, 2007). SD symptoms developed in approximately 30% crabs and none developed BGS suggested that BGS might be a secondary condition caused by crabs becoming moribund in pond culture environments (Zhang and Bonami, 2007). Histopathology in diseased crabs was characterized by clusters (1–5 µm dia.) of pale to deeply basophilic necrotic cells displaying hypertrophied nuclei and nuclear karyorrhesis or pyknosis, and by cytoplasmic inclusion bodies (200–800 nm diameter) filled with enveloped virions. Necrotic cells were prominent in the crab LO and in connective tissues of various organs, including the gills, hepatopancreas, heart, gut and testes. In gills, basophilic inclusion bodies were replaced by eosinophilic foci in necrotic cells and eosinophilic areas in connective tissues (Zhang and Bonami, 2007).

Due to the tissue tropism and histopathology closely resembling those manifest in shrimp infected with okaviruses, filamentous nucleocapsid precursors and enveloped rod-shaped virions sharing striking morphological similarities, except for virions appearing somewhat shorter than those described for YHV (40–50 nm × 150–200 nm, Boonyaratpalin et al., 1993; Chantanachookin et al., 1993) and GAV (34–42 nm × 183–200 nm, Spann et al., 1995, 1997), and a viral genome comprising a long (~22 kb) ssRNA, the virus was named *Es*RNV (Zhang and Bonami, 2007; Bonami and Zhang, 2011). While the properties of *Es*RNV suggest a close evolutionary relationship to okaviruses, genome sequencing is needed to determine whether it should be classified within this or a new genus of the *Roniviridae* (Cowley et al., 2012).

32.3.3 Other Crab Oka-Like Viruses

In the 1970s, viruses forming bacilliform (20–30 nm × 110–170 nm) particles together with long flexible particles up to 600 nm in length, similar in appearance to the virions and filamentous helical nucleocapsid precursors of okaviruses and bafiniviruses, were detected in the Blue crab (*Callinectes sapidus*) (Jahromi, 1977; Yudin and Clark, 1978, 1979; Johnson, 1983). *Callinectes sapidus* is indigenous to the US Atlantic coast and the Gulf of Mexico and while not an aquaculture species, supports an important wild fishery in this region. For the virus particle (20–30 nm × 110–170 nm) named tentatively as *rhabdolike-virus A* (RhVA, Jahromi, 1977; Johnson, 1983) based on morphological similarities to plant rhabdoviruses, virions were detected in Schwann cells in parallel arrays localized at ER and nuclear cistern membranes, and less frequently at plasma membranes. For the virus particle (25–30 nm × 100–150 nm) named *ecdysial gland virus* 2 (EGV-2, Yudin and Clark, 1978, 1979) due to its detection in ecdysial (Y organ or mandibular) glands of eyestalk-ablated blue crabs, virions also matured as arrays aligned perpendicularly to the outer nuclear membrane, as well as at tubular ER membranes and the plasma membrane (Yudin and Clark, 1979). EGV-2 occurred in association with a larger-diameter enveloped bacilliform particle (50–70 nm × 110–170 nm) decorated with surface projections that was named EGV-1 (Yudin and Clark, 1978). EGV-1 and EGV-2 became evident after crab eyestalk ablation, suggesting that the infections were preexisting and escalated in response to stress (Yudin and Clark, 1978). Similarities in virion morphology and morphogenesis suggest that RhVA and EGV-2 are the same virus. However, genome sequence is required to confirm their evolutionary relationships because the diameter of the bacilliform RhVA and EGV-2 virions is somewhat smaller than those described for okaviruses (Cowley et al., 2012) and bafiniviruses (de Groot et al., 2012a).

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