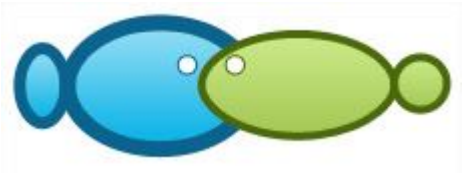

Multiple viral pathogens occurrence in tiger shrimp (*Penaeus monodon*) broodstock from Sulawesi coastal waters

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Abstract. Viral infections are a major obstacle in the propagation of the tiger shrimp, *Penaeus monodon*, in Indonesia. This study investigates the occurrence of viral agents in tiger shrimp broodstock collected along the coast of Sulawesi, Indonesia including Makassar, Pangkep, Takalar, Pinrang and Bombana. Broodstock (n = 137) were examined for white spot syndrome virus (WSSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV). Broodstock showed single viral infections: MBV (3.8-77.8%), IHHNV (3.7-20%), double infections: WSSV/MBV (3.7-15.4%), MBV/IHHNV (6.7-26.9%), MBV/HPV (3.7%), and triple infections: WSSV/MBV/IHHNV (3.3-53.8%). Mixed infection of WSSV with other viruses was found to be the highest in Takalar waters with a percentage of infection of 69.2%. Mixed infection of MBV and mixed infection of IHHNV were also high in Takalar with percentage of 100% and 80.8%, respectively. Single infection with WSSV and HPV were not observed. Furthermore, Bombana broodstock that were spawned, their progeny also showed multiple viral agents that were found in their parents based on specific PCR assays. The incidences of viral infections not vary significantly between male and female broodstock. Our findings demonstrate the persistence of viral infections in shrimp broodstock and their progeny from Sulawesi suggesting the need for screening infectious pathogens to ensure the selection of healthy broodstock, improve biosecurity measures and good practices to produce a domesticated and healthy broodstock, and production of uninfected seed stocks for shrimp cultivation.
Key Words: broodstock, *Penaeus monodon*, viral infections, HPV, IHHNV, MBV, WSSV.

Introduction. Shrimp aquaculture in Indonesia constitutes approximately 2,964,331 ha from which only about 650,509 ha are used for shrimp propagation (Ministry of Marine Affairs and Fisheries of Indonesia 2014). Despite the huge area for shrimp culture, the production of tiger shrimp (*Penaeus monodon*) is low in Indonesia due to viral infections and environmental deterioration (Hariati et al 1998; Sunarto et al 2004). Currently, *P. monodon* is only cultivated with low densities of about 1-2 shrimp m^{-2} due to their susceptibility to disease, compared to intensive systems with a density of about 30-35 shrimp m^{-2} commonly applied in the 1980s through the 1990s. At least 20 species of viral agents have been reported in penaeid shrimp (Lightner & Redman 1998; Walker & Mohan 2009). Among the most serious viral pathogens of penaeid shrimp in Asia are white spot syndrome virus (WSSV), decapod penstyldensovirus-1 (*Pst*DV-1) formerly infectious hypodermal and hematopoietic necrosis virus (IHHNV), *Penaeus monodon* nucleopolyhedrovirus (PemoNPV) previously monodon baculovirus (MBV), yellow-head virus (YHV), *Penaeus monodon* densovirus (PmDNV) formerly hepatopancreatic parvovirus (HPV) and taura syndrome virus (TSV) (Flegel 2006). Among these viruses, WSSV is the most widely distributed causing devastating impacts to the shrimp aquaculture industry worldwide (Lightner & Redman 1998; Lightner et al 2012) including mass mortality of shrimps cultured in ponds (Kasornchandra et al 1995, 1998).

Since the emergence of WSSV in Indonesia, *P. monodon* production has decreased sharply leaving most of the productive ponds in idle condition (Sunarto et al 2004). WSSV is difficult to control because other aquatic organisms such as crustaceans (Hossain et al 2001; Vaseeharan et al 2003; Joseph et al 2015a) and worms (Haryadi et al 2015) may act as natural reservoirs of the virus. As wild broodstock is a potential source of viral infection in shrimp hatcheries (e.g. Uma et al 2005), the shrimp aquaculture industry has shifted to cultured broodstock for seed production of *Litopenaeus vannamei* while wild broodstock remain the main source for *P. monodon* seeds (Lightner et al 2012).

In shrimp hatcheries and grow out ponds, infections with MBV and IHHNV are frequently observed (Umesha et al 2008; Sriwulan & Anshary 2011; Joseph et al 2015b; Sriwulan & Anshary 2016) reducing shrimp growth (Kalagayan et al 1991; Flegel et al 1999; Primavera & Quinitio 2000; Chayaburakul et al 2004; Rai et al 2009). Although non lethal in optimal environmental conditions (Fegan et al 1991), MBV has been implicated in mass mortalities of shrimps cultured at high densities (Fulks & Main 1992) particularly in intensive larviculture systems (Ramasamy et al 1995). While infectious to both wild and cultured shrimps, IHHNV is highly pathogenic to *Penaeus stylirostris* but less devastating to *P. monodon* causing runt deformity syndrome and decreased growth reducing its economic value (Primavera & Quinitio 2000). HPV also caused stunted growth in juvenile shrimp including deaths in larvae and post larvae (Flegel et al 1999; Sukhumsirichart et al 1999; Phromjai et al 2001; Umesha et al 2003; Manjanaik et al 2005).

Large–scale aquaculture of shrimps has been associated with the spread of viral and other microbial infections worldwide. One mode of dissemination is via movement of live shrimp stocks that can transfer viral agents from their initial origin to global spread (Lightner et al 2012) causing mixed infections in hatcheries from broodstocks to offspring and horizontal transmission in grow out ponds. Co-infections with viral agents are commonly observed such as WSSV and MBV in shrimp hatcheries (Otta et al 2003), triple infections with MBV, WSSV, and HPV in ponds and hatcheries in India (Ramasamy et al 2000, Madhavi et al 2002; Manivannan et al 2002; Umesha et al 2006, 2008) including multiple infections with WSSV, MBV, IHHNV and HPV from grow out ponds in Thailand (Flegel et al 2004), and WSSV, TSV and IHHNV in *L. vannamei* in Hainan, China (Tan et al 2009). As shown in these studies, viral infections are prevalent in all stages of shrimp cultivation from larvae to juveniles and broodstock.

In Indonesia, a hatchery technician mainly purchases shrimp broodstock from collectors, who obtained broodstock from various sources from different fishermen. The wild caught broodstocks were mainly maintained collectively in one container, which highly possibly results in cross contamination. In general, selection of broodstocks to be used as spawners is mainly based on their physical appearance and application of viral detection is not a common practice in small scale hatcheries in Sulawesi. In Indonesia, WSSV has been reported from wild *P. monodon* broodstock in Pangandaran (81%) and Banten (20-50%) (Supriyadi et al 2005) while MBV, IHHNV, HPV including WSSV have been detected in post larval cultures in the hatchery (Rahmi 2011; Sriwulan & Anshary 2011, 2016). Based on these previous studies indicating the range of viral infections in shrimp culture in the region, we investigated other viral agents such MBV including HPV and IHHNV to determine the incidence of these viruses across life stages of shrimps in South Sulawesi, Indonesia. To the best of our knowledge, this is the first study to document mixed viral infections in wild shrimp broodstocks in the Sulawesi coastal region. Our objectives in the current study are to describe the occurrence and identity of viral agents in shrimp broodstock collected from Sulawesi coastal waters and to assess whether viral infections are also present among the progeny (post larvae) of infected broodstock.

Material and Method

Collection of wild broodstock and hatchery spawning. Broodstocks were caught by fishermen and divers using trammel net, placed in aerated bags filled with seawater, and

transported in cooled styrofoam boxes to the Laboratory of Fish Parasites and Diseases, Tamalanrea, Hasanuddin University (UNHAS).

A total of 137 broodstock consisting of 50 males and 87 females were examined for the presence of WSSV, MBV, IHHNV and HPV from Makassar, Pangkep and Takalar in May 2014, Pinrang (northern part of South Sulawesi) in June 2014 and Bombana districts in August 2014 (southern part of Southeast Sulawesi) (Figure 1). These sites are the primary source of broodstock for hatchery operations in the Sulawesi region.

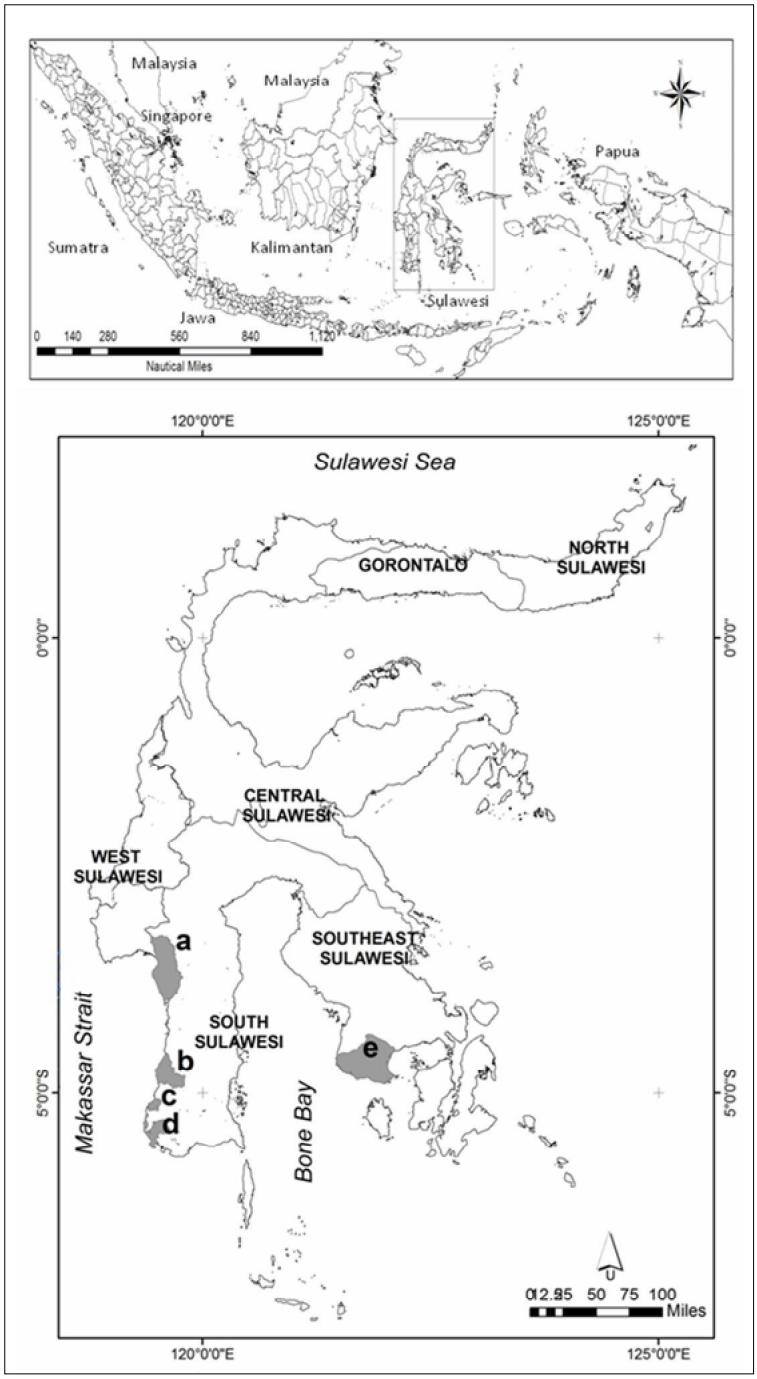

Figure 1. Map of Indonesia showing the five sampling locations of *P. monodon* broodstock in various districts at Sulawesi. Black mark: a = Pinrang, b = Pangkep, c = Makassar city, d = Takalar, and e = Bombana.

Different from the samples mentioned above, other samples of gravid female broodstock, collected from the coast of Bombana in May (n = 22), July (n = 24) and September 2014 (n = 14), were spawned to determine whether broodstock infected with multiple viruses

will produce infected progeny. The broodstocks from Bombana were collected using trawl, placed in aerated bags with seawater, and then transported by air and car to CV Mandiri Hatchery Suppa at Pinrang. The gravid female broodstocks were acclimatized to 30±1°C in round tanks (3 m x 1 m) for 3 to 4 days. A maximum of 30 broodstocks were reared per tank with 70 cm depth seawater level and 50% daily water changes. Mature broodstocks were selected and moved to separate tanks for further maturation and ablation. About 2-3 days after ablation, the broodstocks were transferred to a breeding tank where eggs were released within 1-2 days. The larvae were held and maintained for about one month from eggs to post larvae 12 at 30±1°C, 6.5-6.8 mg L^{-1} dissolved oxygen, pH 8.1-8.3, 200-210 mg L^{-1} $CaCO_3$ alkalinity, 30-32 ppt salinity, and < 0.5 mg L^{-1} ammonia.

Sample preparation and genomic DNA extraction from broodstock and postlarvae. After spawning at the CV Mandiri Hatchery Suppa, the broodstocks from Bombana were tested for presence of WSSV, MBV, IHHNV, and HPV using multiplex PCR. From each broodstock (n = 137), gills, hepatopancreas and pleopods were sampled aseptically, placed in sterile and sealed plastic bag, and transported on ice to the Laboratory of Fish Parasites and Diseases at UNHAS and used for the PCR assays (Tang et al 2007, 2008). The tissues (gills, hepatopancreas, pleopods) from an individual broodstock were pooled and were either directly processed for DNA extraction or stored at -20°C until used. The pooled tissues were homogenized in a mortar and pestle, and 25 mg of the homogenized sample was placed in a 1.5 mL sterile microfuge tube for genomic (g) DNA extraction using QiaAmp DNA mini kit (Qiagen).

The larvae that were produced from the Bombana broodstocks were collected and distributed evenly in 11 larval rearing tanks (10 ton volume) at a stocking density of about 1 larva mL^{-1} seawater, maintained in the tank for about one month until the post larval stage, and then examined for the presence of viruses as found in their parent broodstock by multiplex PCR. Post larvae (n = 25) were randomly sampled from each tank and after removing the compound eyes from the eyestalks, the post larvae were pooled, and homogenized in a sterile microfuge tube and sterile micropestle followed by gDNA extraction using QiaAmp DNA mini kit (Qiagen).

DNA amplification with multiplex PCR. The presence of WSSV, MBV, IHHNV and HPV was determined in broodstock and post larvae with multiplex PCR using published primers as shown in Table 1 and Qiagen multiplex PCR kit.

Table 1

Nucleotide sequence of primers for multiplex PCR for detection of viral agents in broodstock and post larval shrimps

Virus	*Primer*	*Sequence (5'-3')*	*Size (bp)*	*Reference*
WSSV	WSSV-F	GGT CGT GTC GGC CAT CCT C	436	Khawsak et al
	WSSV-R	GGA GCT ACC GAC AAA GGC CT		(2008)
MBV	MBV261F	AAT CCT AGG CGA TCT TAC CA	261	Surachetpong
	MBV261R	CGT TCG TTG ATG AAC ATC TC		et al (2005)
IHHNV	IHHNV309-F	TCC AAC ACT TAG TCA AAA CCA A	309	Tang et al
	IHHNV309-R	TGT CTG CTA CGA TGA TTA TCC A		(2007)
HPV	HPV-2F	GGA AGC CTG TGT TCC TGA CT	595	Tang et al
	HPV-2R	CGT CTC CGG ATT GCT CTG AT		(2008)

WSSV = White spot syndrome virus; MBV = Monodon baculovirus; IHHNV = Infectious hypodermal and hematopoietic necrosis virus; HPV = Hepatopancreatic parvovirus; F = forward; R = Reverse.

The multiplex PCR master mix comprised of 2 µL of 10x primer mix consisting of the 4 primers for WSSV, MBV, IHHNV and HPV, 2 µL of Q-solution, 10 µL of 2x Qiagen multiplex PCR Master Mix, 2 µL of DNA template, and 4 µL of nuclease free water to achieve a 20 µL of PCR reaction. The final concentration of the multiplex PCR reaction was 0.2 µM of the 10x primer mix, 1x for 2x Qiagen multiplex PCR mastermix and 0.5x for Q-solution, a reagent included in the kit to facilitate DNA amplification. PCR was

performed with initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 7 min. The PCR amplified products were analyzed by electrophoresis using 2.5% agarose gel (110V, 30 min), stained in GelRed Nucleic Acid Gel Stain (Biotin) solution (diluted 3x in distilled water) for 10 min and visualized under UV illumination. The amplified product size was determined using a 100 bp DNA ladder.

Statistical analysis. The prevalence of viral infections between male and female broodstock and across sampling sites was compared and analyzed using chi-square statistical test on Epitools epidemiological calculators available online (Sergeant 2016).

Results. Of the 137 broodstock samples examined, WSSV and HPV were not found in single infections, while MBV and IHHNV occurred in single and co-infections with other viruses (Figure 2). Of the samples examined, MBV infections ranged from 3.8% in Takalar to 77% in Pinrang, whereas the highest single infection of IHHNV ranged from 3.7% in Makassar to 20% in Bombana (Table 2). Although none of the samples were infected with WSSV alone, mixed infections of WSSV with other viruses (Table 2) were observed in other broodstocks. The highest percentage of mixed infection of WSSV with other viruses was found in Takalar (69.2%) and the lowest was in Pangkep (3.7%). These results indicate that mixed infections with viral agents are more common in tiger shrimp compared to single infections.

Infections with MBV were most frequently observed compared to other viral agents. The percentage of co-infection of MBV with any viruses in broodstock was higher in the four locations surveyed ranging from 51.9% in Pangkep to 100% in Takalar (Table 2). Broodstocks positive for IHHNV with any virus ranged from 7.4% in Pinrang to 80.8% in Takalar while broodstocks positive for WSSV with any virus ranged from 3.7% in Pangkep to 69.2% in Takalar. Co-infection of HPV with other viruses was only detected in one broodstock from Pangkep and another from Pinrang. Mixed infections with three viruses (WSSV/MBV/IHHNV) were 13.1%, double infection with WSSV/MBV was 9.5%, and MBV/IHHNV was 10.2%. The highest percentage of broodstock infected with the three viruses (WSSV/MBV/IHHNV) was found in Takalar at 53.8% (Table 3).

Co-infection of WSSV with other viruses was frequently observed particularly with MBV or with MBV/IHHNV (Table 3). Co-infection of WSSV/IHHNV was only found in Bombana district at low prevalence (3.3%). Co–infections with three viruses (WSSV/MBV/IHHNV) were found in broodstock from Makassar, Takalar, Pinrang and Bombana districts at 7.4%, 53.8%, 3.7% and 3.3%, respectively. One broodstock (3.7%) from Pinrang was found simultaneously infected with these four viruses (Table 3). Triple infections with WSSV/MBV/IHHNV were detected in 18 of 137 samples (13.1%) tested. Dual infection with MBV/HPV was positive in 1 (0.7%) sample, while dual infections with WSSV/MBV and MBV/IHHNV were positive in 9.5% and 10.2% samples, respectively. These results indicate that WSSV often co-occurs with other viruses such as MBV or IHHNV as shown in our study.

The samples did not vary ($p > 0.05$) in the percentage of WSSV, MBV, or IHHNV either single or mixed infection between male and female across the four sampling locations (Table 4). From the gravid female broodstock from Bombana that were spawned, mixed infections with MBV/IHHNV were detected in May with 2 broodstocks (9.1%) infected with IHHNV while 4 individuals (18.2%) were infected with MBV (Table 5). A high prevalence of single infections with MBV was seen in July including high incidence of broodstock infected with WSSV (78.6%), MBV (78.6%) and IHHNV (100%) in September (Table 5).

The progeny of the Bombana broodstock were held in 11 tanks and maintained to post larval stages for viral screening. From among the 11 tanks, 18.2% (2 tanks) were infected with MBV, 27.3% (3 tanks) were infected with IHHNV and 18.2% (2 tanks) were infected with both MBV/IHHNV (Table 5). The broodstock collected in July and September showed significantly higher percentage ($p < 0.01$) compared to May in either single or mixed infections with WSSV, MBV, or IHHNV. All of the 11 tanks of post larvae were

positive for WSSV, MBV and IHHNV and eventually all of the post larvae from the infected broodstock from Bombana succumbed to mass mortality.

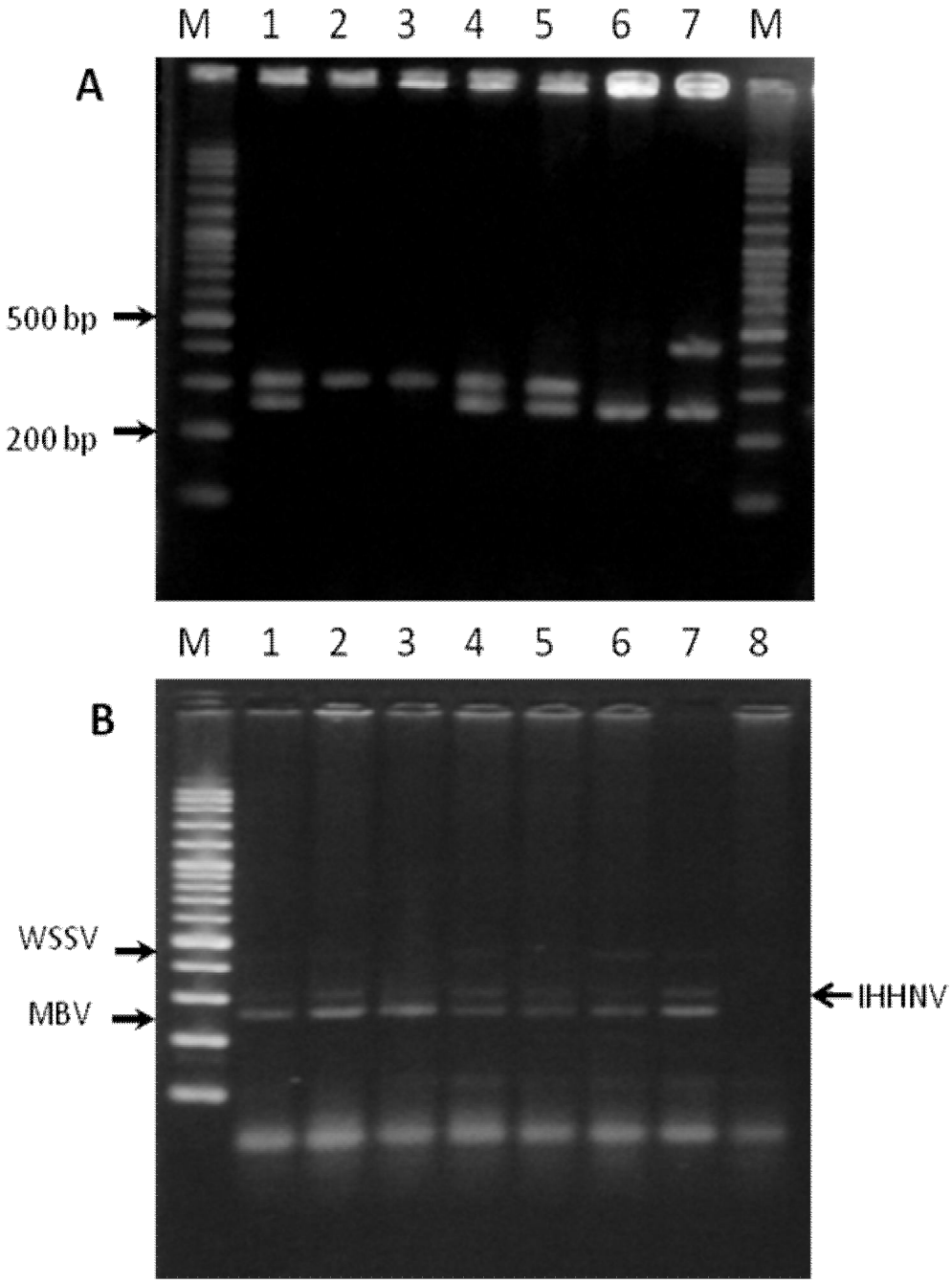

Figure 2. Photo gel showing multiple existences of viral pathogens in shrimp samples. **A**. Lane 1, 4 and 5 infected with MBV and IHHNV, lane 2 and 3 infected with IHHNV, lane 6 infected with MBV, lane 7 positive control (WSSV and MBV positive). **B.** Lane 4 to 7 infected with WSSV, IHHNV and MBV, Lane 8 negative control. M = Marker.

Table 2

Percentage of *Penaeus monodon* broodstock with single or mixed viral infections from Sulawesi Coast. Statistical differences across locations for each type of viral infection are presented beneath each column

Sampling location	*No of samples analyzed*	*WSSV*	*MBV*	*IHHNV*	*HPV*	*Mixed WSSV*	*Mixed MBV*	*Mixed IHHNV*	*Mixed HPV*
Makassar	27	0	17 (63%)	1 (3.7%)	0	6 (22.2%)	26 (96.3%)	5 (18.5%)	0
Pangkep	27	0	9 (33.3%)	4 (14.8%)	0	1 (3.7%)	14 (51.9%)	7 (25.9%)	1 (3.7%)
Takalar	26	0	1 (3.8%)	0	0	18 (69.2%)	26 (100%)	21 (80.8%)	0
Pinrang	27	0	21 (77.8%)	0	0	2 (7.4%)	23 (85.2%)	2 (7.4%)	1 (3.7%)
Bombana	30	0	14 (46.7%)	6 (20%)	0	5 (16.7%)	20 (66.7%)	8 (26.7%)	0
Statistical test			$p < 0.01$	NT		$p < 0.01$	$p < 0.01$	$p < 0.01$	NT
Total samples	137	0	62 (45.3%)	11 (8.0%)	0	32 (23.4%)	109 (79.6%)	43 (31.4%)	2 (1.5%)

Mixed = Target virus + other viral agents; NT = Not tested; WSSV = White spot syndrome virus; MBV = Monodon baculovirus; IHHNV = Infectious hypodermal and hematopoietic necrosis virus; HPV = Hepatopancreatic parvovirus; $p < 0.01$ = highly significant.

Table 3

Mixed viral infections in *Penaeus monodon* broodstock from Sulawesi Coast

Sampling location	*WSSV, MBV, HPV*	*MBV, IHHNV, HPV*	*WSSV, MBV, IHHNV*	*WSSV, MBV, IHHNV, HPV*	*WSSV, IHHNV, HPV*	*MBV, HPV*	*WSSV, HPV*	*IHHNV, HPV*	*WSSV, IHHNV*	*WSSV, MBV*	*MBV, IHHNV*
Makassar	0	0	2 (7.4%)	0	0	0	0	0	0	4 (14.8%)	2 (7.4%)
Pangkep	0	0	0	0	0	1 (3.7%)	0	0	0	1 (3.7%)	3 (11.1%)
Takalar	0	0	14 (53.8%)	0	0	0	0	0	0	4 (15.4%)	7 (26.9%)
Pinrang	0	0	1 (3.7%)	1 (3.7%)	0	0	0	0	0	0	0
Bombana	0	0	1 (3.3%)	0	0	0	0	0	1 (3.3%)	4 (13.3%)	2 (6.7%)
N =137	0	0	18 (13.1%)	1 (0.7%)	0	1 (0.7%)	0	0	1 (0.7%)	13 (9.5%)	14 (10.2%)

WSSV = White spot syndrome virus; MBV = Monodon baculovirus; IHHNV = Infectious hypodermal and hematopoietic necrosis virus; HPV = Hepatopancreatic parvovirus.

Table 4

Percentage of male and female broodstock of *Penaeus monodon* with viral infections from Sulawesi coast. Statistical differences between male and female for each type of viral infection are presented beneath each column

District	*Sex*	*No of sample*	*WSSV*	*MBV*	*IHHNV*	*HPV*	*Mixed WSSV*	*Mixed MBV*	*Mixed IHHNV*	*Mixed HPV*
Makassar	Male	12	0	6 (50%)	1 (8.3%)	0	2 (16.7%)	11 (91.7%)	4 (33.3%)	0
	Female	15	0	11 (73.3%)	0	0	4 (26.7)	15 (100%)	1 (6.7%)	0
				p > 0.05			p > 0.05	p > 0.05	p > 0.05	
Pangkep	Male	13	0	4 (30.8%)	3 (23.1%)	0	1 (7.7%)	5 (38.5%)	3 (23.1%)	0
	Female	14	0	5 (35.7%)	1 (7.1%)	0	0	9 (64.3%)	4 (28.6%)	1 (7.1%)
				p > 0.05	p > 0.05			p > 0.05	p > 0.05	
Takalar	Male	11	0	0	0	0	6 (54.5%)	11 (100%)	11 (100%)	0
	Female	15	0	1 (6.7%)	0	0	12 (80%)	15 (100%)	10 (66.7%)	0
							p > 0.05	p > 0.05	p > 0.05	
Pinrang	Male	14	0	10 (71.4%)	0	0	1 (7.1%)	11 (78.6%)	1 (7.1%)	0
	Female	13	0	11 (84.6%)	0	0	1 (7.7%)	12 (92.3%)	1 (7.7%)	1 (7.7%)
				p > 0.05				p > 0.05		

Mixed = Target virus + other viral agents; WSSV = White spot syndrome virus; MBV = Monodon baculovirus; IHHNV = Infectious hypodermal and hematopoietic necrosis virus; HPV = Hepatopancreatic parvovirus; p > 0.05 = no statistical difference.

Table 5

Viral pathogens in *P. monodon* broodstock and their progeny from Bombana

	Broodstock						*Progeny*						
Month	*No of samples analyzed*	*Mixed WSSV*	*Mixed MBV*	*Mixed IHHNV*	*Mixed HPV*	*Infected (p<0.01)*	*No of rearing tanks*	*No of tanks with WSSV pos.*	*No of tanks with MBV pos.*	*No of tanks with IHHNV pos.*	*No of tanks with HPV pos.*	*No of tanks with MBV/IHHNV pos.*	*No of tank with WSSV/ MBV/IHHNV pos.*
May	22	0 (0%)	4 (18.2%)	2 (9.1%)	0 (0%)	5 (22.7%)	11	0 (0%)	2 (18.2%)	3 (27.3%)	0 (0%)	2 (18.2%)	0 (0%)
July	24	0 (0%)	21 (87.5%)	0 (0%)	0 (0%)	21 (87.5%)	11	0 (0%)	11 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Sept	14	11 (78.6%)	11 (78.6%)	14 (100%)	0 (0%)	14 (100%)	11	11 (100%)	11 (100%)	11 (100%)	0 (0%)	11 (100%)	11 (100%)

Mixed = Target virus + other viral agents; WSSV = White spot syndrome virus; MBV = Monodon baculovirus; IHHNV = Infectious hypodermal and hematopoietic necrosis virus; HPV = Hepatopancreatic parvovirus

Discussion. South Sulawesi is the main producer of *P. monodon* in Indonesia providing most of the post larvae for local shrimp farming in the south Sulawesi region, and the main supplier of seeds to shrimp farmers in eastern Indonesia. In the absence of pathogen screening, viral infections are highly likely transmitted upon introduction of shrimp juveniles into grow out ponds. Our studies suggest that the lack of virus testing and quarantine in hatcheries and grow out ponds in Sulawesi may help explain the widespread distribution of shrimp with viral infections in farms that rely on seed stocks potentially generated from infected broodstocks for shrimp cultivation.

Our study showed that although WSSV, MBV and IHHNV were frequently observed, HPV infection was found only in two broodstocks sampled from two of five locations that we examined. Furthermore, HPV was not observed in the Bombana broodstock and in their progeny confirming the low infectivity of this viral agent among broodstock and larval samples examined. Low prevalence of HPV infection (3.8%) in broodstock has also been reported from the South China Sea (Claydon et al 2010). The low prevalence of HPV infection may indicate that broodstock are less susceptible to the virus compared to larval stages as observed among post larvae in India (62.5% HPV positive) (Joseph et al 2015b) and in South Sulawesi, Indonesia (44% HPV positive) (Sriwulan & Anshary 2011). Multiple viral infections are frequently reported in *P. monodon* hatcheries and grow out ponds in Asia (Umesha et al 2008; Joseph et al 2015b). The impact of mixed viral infections compared to single infections is often unclear. Although multiple viral agents of MBV, HPV and WSSV caused morbidity in *P. monodon* post larvae (Manivannan et al 2002), these agents also commonly occur in hatcheries and shrimp ponds (Umesha et al 2008; Joseph et al 2015b). The disparity in viral infectivity suggests that *P. monodon* may be able to tolerate low level of viral infections under optimal environmental conditions as shown in *P. monodon* harboring WSSV that were cultured at low stress environments (Tsai et al 1999). In this study, WSSV infection was mainly found in the form of mixed infection with other viruses and none was found as a single infection, probably due to the small number of samples examined. The results of this study seems to be contradict with the results of previous studies showing that a single infection of WSSV caused mass mortality of shrimp in grow-out ponds. However, in the study conducted by Umesha et al (2008), although most ponds they observed were infected by single infection, they also showed some ponds that were not infected with WSSV alone. The high percentage of viral infections of broodstocks observed from Takalar regency indicating that the broodstocks might be caught adjacent to shrimp ponds in shallow water area.

The most significant viral agent of *P. monodon* mortality in grow out ponds is WSSV due to the difficulties in implementing proper biosecurity in farm levels and in controlling potential vectors (e.g. worms and crustaceans) of viral infection that may be present in the ponds (Hossain et al 2001; Vaseeharan et al 2003; Joseph et al 2015a; Haryadi et al 2015). Furthermore, larval and post larval stages are not routinely screened for WSSV in hatcheries prior to their introduction as juveniles into grow out ponds. A survey on presence of viral infections in post larvae across hatcheries in South Sulawesi showed that the average prevalence of MBV, IHHNV and HPV was 50, 62 and 44%, respectively (Sriwulan & Anshary 2011) while the prevalence of WSSV varied (40-85%) including MBV (10-60%) (Rahmi 2011), suggesting the persistence of these viruses in hatchery facilities and the infected seeds may become the main source of viral infections in grow out ponds. Although the exact mode of transmission of the viral agents in the current study is unknown, MBV (Catap et al 2003), IHHNV (Motte et al 2003) and HPV (Rajendran et al 2012) can be transmitted horizontally or vertically for IHHNV (Lightner et al 1985), MBV (Rajendran et al 2012), and HPV (Safeena et al 2012). In traditional shrimp culture farms, WSSV transmission might be due to persistent infections with the virus within ponds over time in the presence of vectors such as crustaceans or small crabs in the ponds or from neighboring ponds, whereas in semi-intensive farms WSSV is mainly transmitted from neighboring ponds with prior infections with the virus through water exchange (Hoa et al 2011). Moreover, in traditional shrimp aquaculture settings, best management practices and appropriate biosecurity measures are not easy to implement. The main problems are insufficient depth of the ponds, shortage of good

water quality, and aging dikes that can potentially accumulate and release toxic black soils. In such cases, optimum conditions needed for shrimp cultivation are difficult to achieve. These are some of the limiting factors commonly encountered in grow out ponds that predispose the vulnerability of shrimp broodstock to viral infections. Hence, diseases due to viral agents are one of the main risk factors associated with shrimp mortality in Indonesia.

Our studies showed that gender did not affect the prevalence of viral infections in broodstock. Differences in prevalence of MBV infection were not observed in male and female broodstock in the Philippines (de la Pena et al 2008). Stress associated with spawning may alter the development of viral diseases such that mild infections prior to spawning can shift to severe infections at post spawning. Hsu et al (1999) reported that female broodstock that were initially negative or lightly infected with WSSV developed into severe infections after spawning. One potential explanation for the increased susceptibility of female broodstock to WSSV during spawning is stress that could trigger viral replication due to the activation of signal transducer and transcription signaling pathway (Lin et al 2012; Wen et al 2014). The susceptibility of shrimp broodstock to viral infections is also seasonally dependent (Oanh & Phuong 2005; de la Pena et al 2007; Debnath et al 2012). White spot disease was higher in monsoon season (May to August) compared to other months in India (Chakrabarty et al 2014). In our study, the gravid female broodstock from Bombana showed mild double infections with MBV and IHHNV in May, high incidence of single viral infections in July, and severe infections with triple viral agents in September. In Sulawesi, September is the beginning of the rainy season which is characterized by strong winds and high waves that may enhance the flow of contaminated waters. Under these conditions, broodstock collected in shallow contaminated coastal waters may partly explain the higher frequency and severity of viral infections in September. The viruses detected in the broodstock were also detected in the progeny suggesting that viral infections in seed stocks were very likely generated from infected broodstock even though the exact mode of transmission (horizontal vs. vertical) was not confirmed in our study. As infections with MBV or IHHNV can cause only minimal impact particularly under low stress culture conditions (e.g. Tsai et al 1999), viral screening of wild broodstock and post larvae of *P. monodon* can potentially alleviate mortality levels associated with viral infections by selecting relatively healthy breeders. The high percentage of virus–infected broodstock from Bombana and from other coastal areas of South Sulawesi underscores the need for viral screening of broodstock for spawning in captive environments. The impact of seasonal variation and collection sites to health status of tiger shrimp broodstock collected from Indonesian coastal waters need to be studied. Since domestication of *P. monodon* has not been established in Indonesia and globally, viral screening of spawners collected in the wild and their progeny (i.e. juveniles) prior to stocking in grow out ponds will help to reduce the occurrence of viral infections by using disease-free broodstock and seeds thereby contributing to the sustainability of *P. monodon* aquaculture in the Sulawesi region.

Discarding effluents from infected shrimp ponds directly to the ocean without proper treatments or decontamination is a common practice in the Sulawesi region. As a result, coastal waters adjacent to shrimp aquaculture areas could be contaminated with various pathogens from aquaculture farms. Effluents from shrimp ponds were shown to infect wild crustaceans (Sankar et al 2011) that may serve as reservoirs of viral infection by persisting in marine environments (Quang et al 2009). Broodstock from South Sulawesi are mainly caught in polluted coastal waters at a depth of about 30 m, which may explain the high prevalence of infected broodstock in this region. Although data are unavailable to support broodstock quality at different water depths in this region, previous studies in Bay of Bengal in Bangladesh showed that the prevalence of WSSV infection was greater in shallow waters compared to deep waters (Debnath et al 2014).

Our study showed that the prevalence of infected wild broodstock was high suggesting that viral agents may have persisted in wild stocks following culture in hatcheries and ponds. This study indicates that infected broodstock are one of the major reservoirs of viral infections in hatcheries and bottleneck of *P. monodon* aquaculture industry in Indonesia. Shifting from wild stocks of *P. monodon* to cultured *L. vannamei* is

one preventative measure to diminish the consequences of broodstock carrying pathogens from the wild. Domestication of *P. monodon* for broodstock propagation in Indonesia has been unsuccessful so far due to stressful captive conditions in hatchery facilities and water quality. For this reason, implementing good hatchery practices combined with broodstock health management is highly recommended. Managers of hatchery and grow out ponds in research and industry scales must be trained to implement biosecurity, quarantine, and viral screening to support the sustainability of the shrimp industry in Indonesia.

Conclusions. This research demonstrates the persistence of mixed viral infections in shrimp broodstock inhabiting the coastal waters of the south Sulawesi region. Our results are among the first data on shrimp broodstock in Sulawesi harboring mixed viral infections that could potentially serve as reservoir of infections to post larval cultivation in the hatchery. Viral screening using diagnostic PCR assays will identify broodstocks and seeds harboring infectious agents such as WSSV, MBV and IHHNV either as single or mixed infections. Broodstock selection by hatchery managers in the region has been commonly limited to assessments of gross external morphologies thereby precluding any critical information regarding the disease status of spawners and consequences on health of progeny. Our findings suggest the need for pathogen screening and quarantine procedures to help designate healthy broodstock prior to their introduction and spawning for larval production and to ensure effective disease management of shrimp aquaculture in Sulawesi, Indonesia.

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