Distribution and Detection of *Megalocytivirus* in Ornamental Fish in Thailand

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ABSTRACT

Ornamental fishes were collected from 173 locations in nine provinces of Thailand during 2016-2018. Using real-time PCR analysis, twelve fish species of three families (Poeciliidae, Cichlidae and Osphronemidae) were found positive for *Megalocytivirus*, a pathogenic agent which causes a significantly high mortality in a wide range of aquatic animal hosts. A geographic information system (GIS) displayed the distribution of megalocytivirus to the northern, central and western regions of Thailand. Nevertheless, one sampling province showed megalocytivirus-free status and the overall rate of virus occurrence declined by 10% during the study period. The transport and translocation of ornamental fish could be a key route for megalocytivirus introduction to new geographical areas. Molecular phylogenetic analysis based on the major capsid protein (MCP) confirmed that *Megalocytivius* in the current study belongs to members of an infectious spleen and kidney necrosis virus (ISKNV) cluster. Although most infected fish were asymptomatic, non-specific symptoms such as haemorrhages, loss of coloration on the fish body and pale liver were observed without substantial mortality. Cytomegalic cells with inclusion bodies were examined. Issues associated with host molecular defense mechanism, persistent infection, and mortality risk factors will await further study.

Keywords: ISKNV, MCP gene, *Megalocytivirus*, Ornamental fish,

INTRODUCTION

Double-stranded DNA (dsDNA) viruses of the genus *Megalocytivirusin* the family *Iridoviridae* have caused significant diseases in many Asian countries (Song *et al*., 2008), and also in Australia (Go *et al*., 2006), USA (Fraser *et al*., 1993) and Canada (Waltzek *et al*., 2012). The first outbreak of *Megalocytivirus*, the latest member of *Iridoviridae*, was initially reported from Japanese cultured red sea bream (*Pagrus major*) in 1990. The causative agent was later designated as RSIV (Inouye *et al*., 1992). The disease caused by RSIV is listed in OIE Aquatic Animal Health Code (OIE, 2018). *Megalocytivirus* is classified into three clusters: infectious spleen and kidney necrosis virus (ISKNV), red seabream iridovirus (RSIV) and turbot reddish body iridovirus (TRBIV), based on the major capsid protein (MCP) and the ATPase gene used for phylogenetic analyses (Kim *et al*., 2010; Kurita and Nakajima, 2012; Mohr *et al*., 2015).

Members of the ISKNV cluster presented a high genetic identity with a broad variety of susceptible hosts, from farmed marine finfish to tropical ornamental finfish, namely rock bream (*Oplegnathus fasciatus*) (Jeong *et al*., 2008a), Murray cod (*Maccullochella peelii peelii*) (Go and Whittington, 2006), gourami (Fraser *et al*., 1993; Paperna *et al*., 2001),), angle fish (*Pterophyllum scalare*) (Rodger *et al*., 1997), paradise fish (*Macropodus opercularis*) (Kim *et al*., 2010), as

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Received 5 October 2018 / Accepted 5 February 2019

well as cichlids and poeciliids (Subramaniam *et al*., 2014). Global translocation of live ornamental fish possibly encourages the distribution of megalocytiviruses to new geographic areas (Go *et al*., 2006; Whittington and Chong, 2007; Jeong *et al*., 2008b; Kim *et al*., 2010). Although megalocytivirus causes systemic disease characterized by pale or dark color of visceral organs and splenomegaly, non-specific symptoms or subclinical signs are mostly observed in ornamental fish. Thus, disease mitigation should be made such as post-entry quarantine (Nolan *et al*., 2015). The development of rapid and specific molecular diagnostic techniques (Kurita *et al*., 1998; Go *et al*., 2006; Gias *et al*., 2011; Rimmer *et al*., 2012; Mohr *et al*., 2015) have been widely applied.

Thailand is known as one of the most important ornamental fish producing and exporting countries. Almost 100 countries worldwide import ornamental fish from Thailand. A safe product of high-quality and disease-free fish is demanded by importing countries. In 2014, a quarantine policy was announced by Australia, due to the risk associated with gourami iridovirus and related viruses, for freshwater ornamental finfish from approved exporting countries (DAWR, 2014). Official health certification for gouramis, bettas, paradise fish, cichlids and poeciliids exported to Australia has been requested to confirm megalocytivirus-free status of consignments since 1 March 2016. However, information on disease surveillance in ornamental fish for attesting to megalocytiviruses-free status in Thailand is still lacking. In this study, we investigated freshwater ornamental fish species and geographical distribution of megalocytivirus detection. Clinical signs and histopathological changes due to megalocytivirus infection were examined. Molecular application and phylogenetic tree analysis were employed for megalocytivirus classification.

MATERIALS AND METHODS

Ornamental fish sample and GIS

Sixty fish were collected at each sampling location from the northern, central and western regions of Thailand during January 2016 – August 2018. Ornamental fish in families known to be susceptible to megalocytiviruses were sampled, and geographic coordinates were recorded. Fish were transferred to the Molecular Biological Laboratory at the Aquatic Animal Health Research and Development Division (AAHRDD) Department of Fisheries, Bangkok. External and internal clinical signs of infection were examined. Visceral organs, such as spleen, kidney and liver, were collected for histological and molecular diagnosis. Locations of fish sampling were mapped using geographic information systems (GIS) software (Arc GIS 9.3).

DNA isolation

DNA isolation was performed using DNeasy Blood and Tissue Kit (Qiagen) as described in the manufacturer's instruction manual. Briefly, 25 mg of fish tissue was cut into small pieces and placed in 1.5-ml microcentrifuge tubes which contained 180 µl of ATL (tissue lysis) buffer. Then, 20 µl proteinase K was added, thoroughly mixed by vortexing the tubes and incubated at 56°C until tissues were completely lysed. Sample tubes were vortexed for 15 seconds and 200 µl AL (lysis) buffer was added into each sample tube and mixed thoroughly by vortexing. Next, 200 µl ethanol (96-100%) was added into each tube and then mixed by vortexing. The mixture from the sample tube was transferred to a DNeasy Mini Spin column and centrifuged for 1 minute at ≥ 6,000 x g. The Mini Spin column tube was then placed into a new collection tube, followed by adding 500 µl AW2 (wash buffer2) buffer. The tube was centrifuged for 3 minutes at ≥ 20,000 x g, and the DNeasy Mini Spin column was placed into a sterile 1.5 ml microcentrifuge tube. Finally, 200 µl AE (elution) buffer was pipetted directly onto the DNeasy membrane, then incubated for 1 minute at room temperature. DNA was eluted by centrifuging for 1 minute at ≥ 6,000 x g. DNA samples were kept at -20°C for further analysis.

Real-time PCR analysis

DNA samples from ornamental fish were examined for megalocytivirus using TaqMan probe. Primers and probes are detailed in Table 1 (Mohr

Table 1. Primer and probe sequences and PCR conditions for molecular assay.

Assay	Primer name: Sequence 5' to 3'	PCR condition
Real-time PCR	F: 5'-TGA CCA GCG AGT TCC TTG ACT T-3' R: 5'-CAT AGT CTG ACC GTT GGT GAT ACC-3' Probe: 5'-FAM AAC GCC TGC ATG ATG CCT GGC TAMRA -3'	pre-incubation for 1 cycle: 50°C 2 min, amplification for 45 cycles: 95°C 2 min, 95°C 15 sec, 60°C 1 min, cooling: 40°C 30 sec (modified from Crane and Davies, 2006)
Nested PCR	F1: 5'-AGACCCACTTGTACGGCG-3'	pre-denature for 1 cycle: 94°C 3 min, amplification for 30 cycles: 94°C 1 min, 52°C 1 min, 72°C 1 min, final extension for 1 cycle: 72°C 5 min (modified from Wang *et al*., 2007)

et al., 2015). Real-time PCR mixture (20 µl) containing 2 µl DNA template, 10 µl Fast Start Essential DNA Probe Master (Roche), and a final concentration of 1,000 nM for each megalocytivirus primer (Forward and Reverse), and 250 nM for the probe were combined. The real-time PCR assays were performed in a LightCycle® 480 Real-Time PCR system (Roche Applied Science) and analyzed with the LightCycle® 480 software. Positive (DNA from infected fish) and negative controls (water) were included in all tests.

Nested PCR, DNA sequencing and phylogenetic tree analysis

Nested PCR was performed in order to amplify the major capsid protein (MCP) gene from different species of ornamental fish. Primers for nested PCR assay are shown in Table 1 (Wang *et al.*, 2007). For primary PCR assays, 1 µl of DNA template was added into 24 µl master mix containing 1x buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 200 nM of each primer, 1 unit of *Taq* polymerase (Invitrogen) and molecular grade water. 1 µl of primary PCR product was used as DNA template for each nested PCR assay which contained 24 µl of master mix (same as primary PCR assay). Positive and negative controls were included in all tests. The PCR product was analyzed in 1% agarose gel stained with GelStar™ (Lonza). Amplicon at 562 bp was cut from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were sequenced (SolGent, Korea). Sequencing results were analyzed by BLAST searches, while protein translation and multiple alignments were conducted using Cluster Omega. The relevant sequences of megalocytiviruses were retrieved from GenBank for comparative analysis. A phylogenetic tree was drawn by neighbor-joining method with 10,000 bootstrapping replications using the MEGA5 program (Tamura *et al*., 2011).

Histopathology

Gill and visceral organs such as liver, spleen, kidney, heart and testes were dissected and fixed in 10% formalin for 24-48 hours. Samples were processed and stained with hematoxylin and eosin using a modified method from Humason (1979). Histological slides were examined under the light microscope for a confirmatory diagnosis.

Association analysis between fish translocation and megalocytivirus occurrence

The risk associated with disease due to fish translocation was analyzed in the current study using chi-square test statistics.

RESULTS AND DISCUSSION

Seventeen freshwater ornamental fish species were collected from nine provinces located in the northern (Chiang Rai), central (Bangkok, Nakhon Pathom, Nonthaburi, Pathum Thani,

Ratchaburi, Samut Prakarn and Samut Sakhon), and western (Kanchanaburi) regions of Thailand, at a total of 173 sampling locations during January 2016–August 2018. The GIS map for 2016 (Figure 1) showed megalocytivirus was mainly distributed in six provinces located in the central region, but not in Nonthaburi Province. The virus was detected in Chiang Rai, a northern province, but not in the western province, Kanchanaburi. The distribution of megalocytivirus in 2017 (Figure 2) and 2018 (Figure 3) was similar to the first year of study with the positive locations (sites where virus was detected) mostly in the central region, but the number of positive locationschanged. Furthermore, the sampling location in Kanchanaburi showed a positive result for megalocytivirus 2018. Our results showed a trend of decreased number of positive (infected) locations from 51.56% in 2016 to 41.67% in 2017 and 42.86% in 2018. In addition, the GIS maps show that Nonthaburi Province maintained megalocytivirus-free status during these consecutive three years.

Additional data from fish translocation revealed that the movement of ornamental fish from megalocytivirus-infected sources most likely resulted in positive occurrences at their destinations (Figure 4). A similar result was found for the movement of megalocytivirus-negative sources of fish.

The megalocytivirus was detected in eight out of nine sampling provinces. Positive sites were mostly located in Nakhon Pathom, Pathum Thani, Ratchaburi and Bangkok, the producing and collecting areas for megalocytivirus-susceptible fish species. We also discovered megalocytivirus in poeciliids that were moved from Nakorn Pathom for use in aquaria in Chiang Rai. The movement of megalocytivirus-infected fish from producing

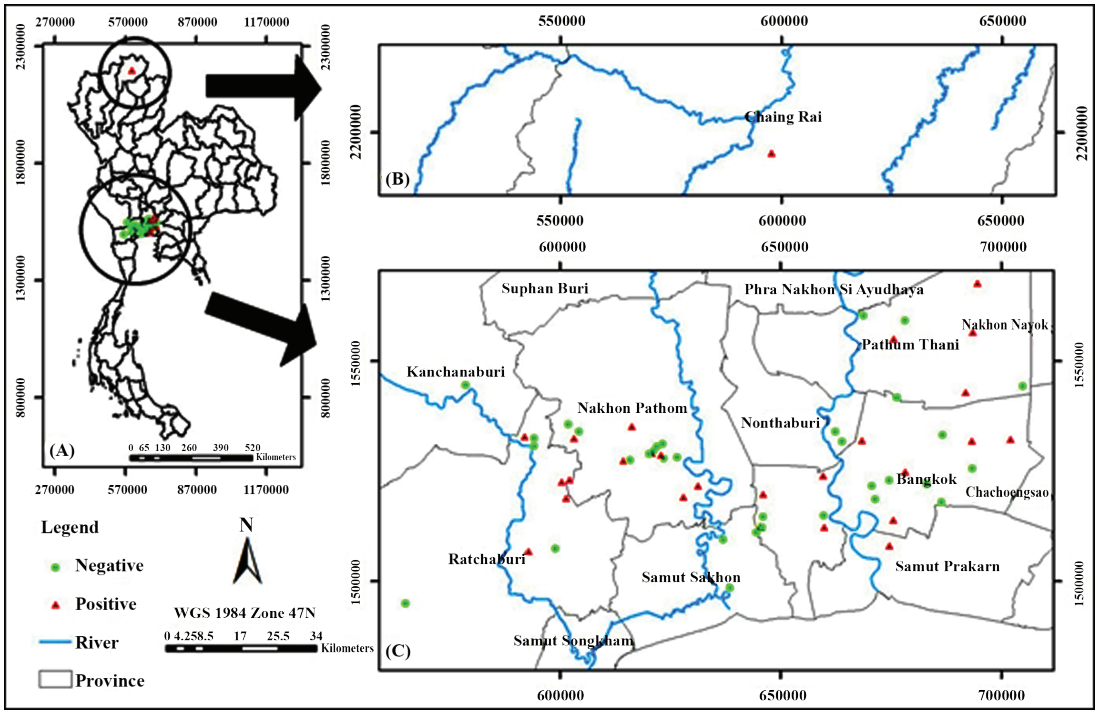

Figure 1. Geographical distribution of ornamental fish samples positive (red triangle) and negative (green circle) for megalocytivirusin 2016: (A) the distribution in the whole country, (B) the distribution in the northern region of Thailand and (C) the distribution in the central region of Thailand.

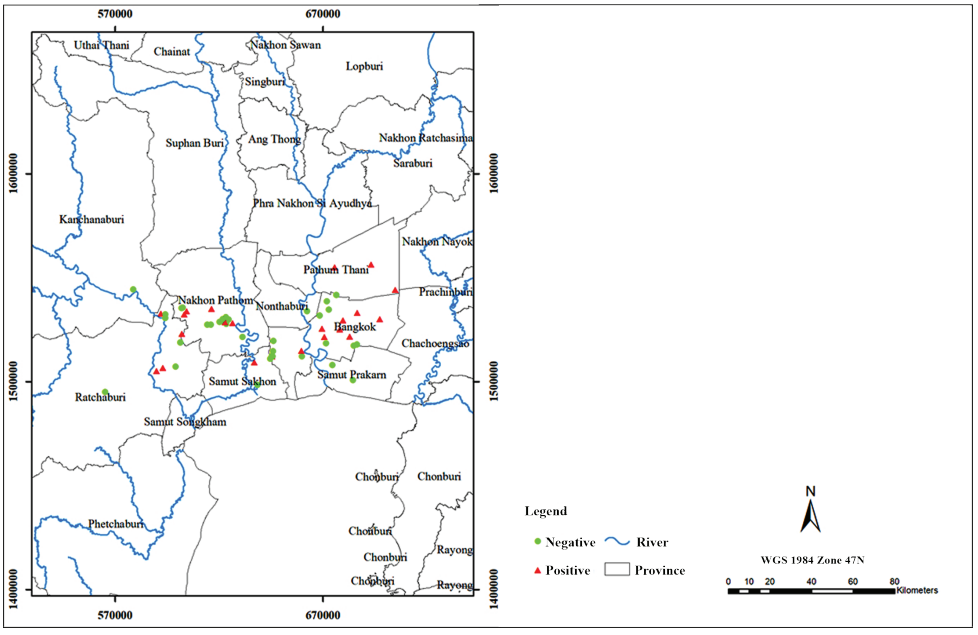

Figure 2. Geographical distribution of ornamental fish samples positive (red triangle) and negative (green circle) for megalocytivirus in 2017 in the central region of Thailand.

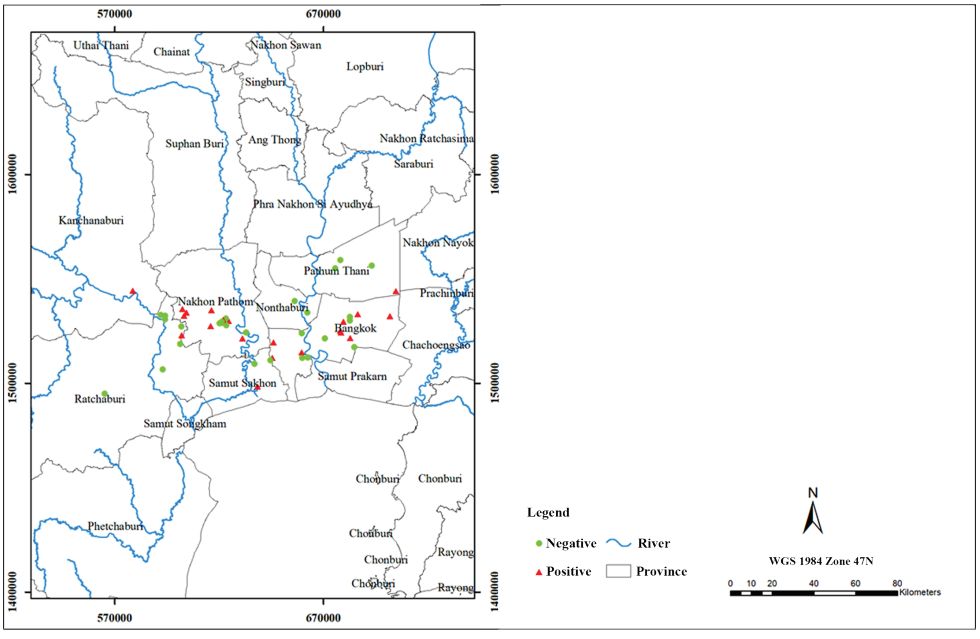

Figure 3. Geographical distribution of ornamental fish samples positive (red triangle) and negative (green circle) for megalocytivirus in 2018 in the central region of Thailand.

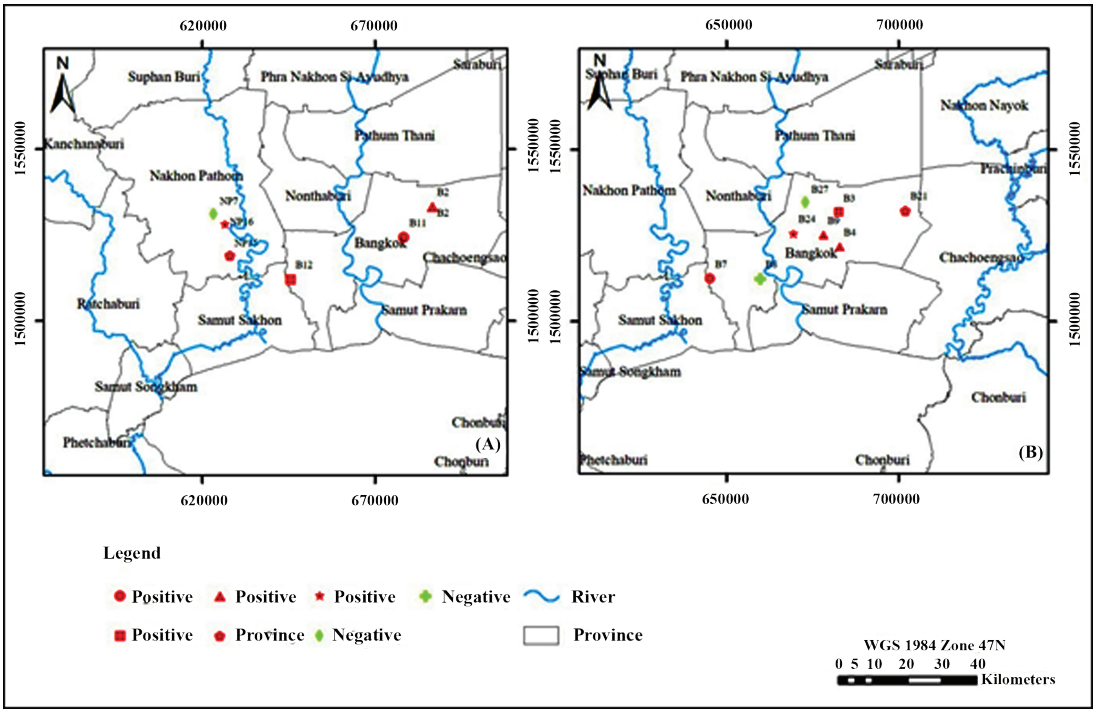

Figure 4. Ornamental fish movement routes in central Thailand. Fish with a common origin (A) and destination (B) are shown with the same symbol, labeled by megalocytivirus status.

sources could introduce the virus into other non-infected areas. The distribution of megalocytivirus possibly arises from translocated ornamental fish, therefore the responsible movement of ornamental fish not only for domestic but also international trade should be seriously taken into consideration.

Real-time PCR results from 17 fish species during the three years revealed that 12 fish species from three families (Poeciliidae, Cichlidae and Osphronemidae) were positive for megalocytivirus (Table 2). Four species of Cichlidae (*Melanochromis auratus*, *Apistogramma* spp., *Amphilophus citrinellus*, *Protomelas taeniolatus*) and one species of Osphronemidae (*Trichopodus leerii*) showednegative results for the virus.

All eight species of poeciliids sampled (*Astronotus ocellatus*, *Poecilia lalatipinna*, *Poecilia reticulata*, *Poecilia velifera*, *Pterophyllum* sp., *Xiphophorus hellerii*, *Xiphophorus maculatus* and *Xiphophorus variatus*) were found positive for the virus (Figure 5) at a total of 34, 11 and 20 locations during the years 2016-2018, respectively. Nakhon Pathom, an important area for ornamental fish production, was shown as a positive location for all eight poeciliids species.

As for cichlids, positive results were discovered in two genera: *Cichlasoma* and *Symphysodon* (Figure 6). *Cichlasoma* sp. was found with this viral infection in five provinces. One location in Kanchanaburi, which had negative results during the first two years of this study, was recently detected as positive in 2018. Discus fish (*Symphysodon* sp.) were detected positive only in Bangkok during the whole study period, and the number of positive locations was about 50% compared to positive locations for *Cichlasoma* in the same province.

Table 2. Summary of megalocytivirus diagnoses in different ornamental fish species using real-time PCR:+ = positive result and - = negative result

Family	Common name	Species	Real-time PCR result
Poeciliidae	Oscar	*Astronotus ocellatus*	+
	Ballon	*Poecilia latipinna*	+
	Guppy	*Poecilia reticulata*	+
	Sail-fin molly	*Poecilia velifera*	+
	Angel fish	*Pterophyllum* sp.	+
	Swordtail	*Xiphophorus hellerii*	+
	Platy	*Xiphophorus maculatus*	+
	Variable platy	*Xiphophorus variatus*	+
Cichlidae	-	*Cichlasoma* sp.	+
	Discus	*Symphysodon* sp.	+
	Golden cichlid	*Melanochromis auratus*	-
	-	*Apistogramma* spp.	-
	Midas cichlid	*Amphilophus citrinellus*	-
	Spindle hap	*Protomelas taeniolatus*	-
Osphronemidae	Gourami	*Trichogaster* sp.	+
	Pearl gourami	*Trichopodus leerii*	-
	Siamese fighting fish	*Betta splendens*	+

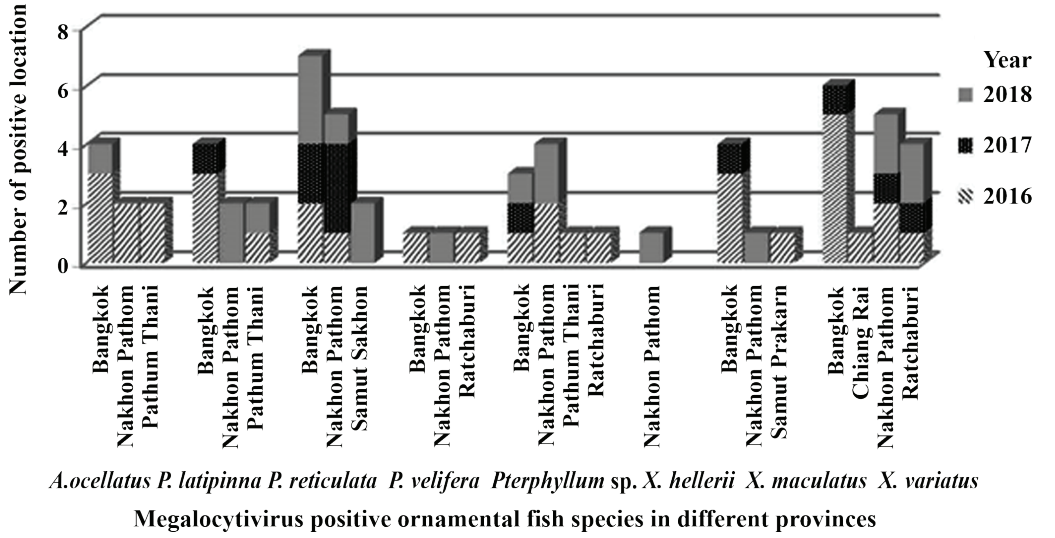

Figure 5. Number of positive locations for megalocytivirus in poeciliids from sampled Thai provinces during 2016-2018

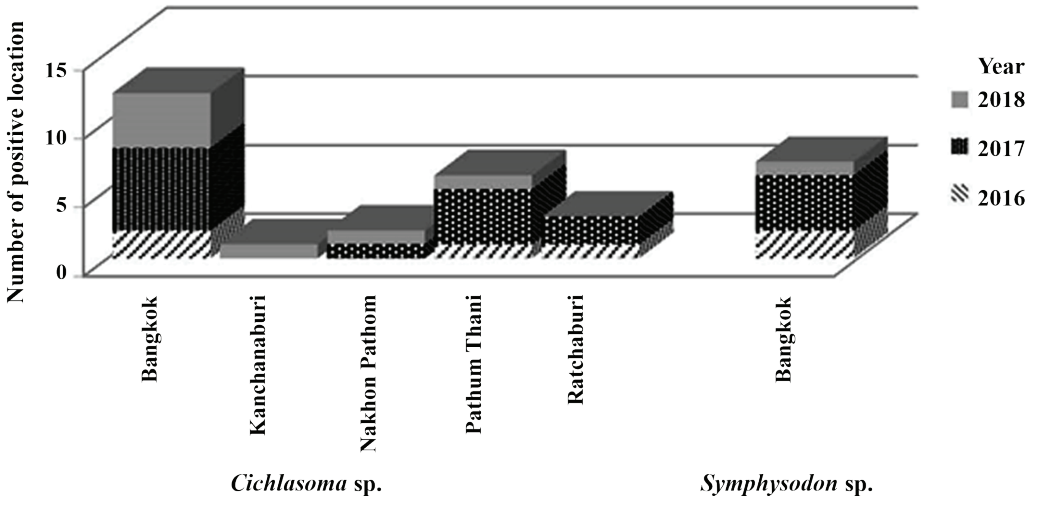

Figure 6. Number of positive locations for megalocytivirus in two cichlids from sampled Thai provinces during 2016-2018

Gouramis (*B. splendens* and *Trichogaster* sp.) tested positive for megalocytivirus in five and three locations, respectively (Figure 7). Bangkok and Nakhon Pathom exhibited the highest number of locations with virus-positive *B. splendens* at 13 locations during three years. As for *Trichogaster* sp., a very low number of positive locations were recorded, in Bangkok, PathumThani and Nakhon Pathom.

Three different groups of fish (poeciliids, cichlids and gouramis) were found positive for megalocytivirus with different frequencies. The highest proportion of positive-testing species was for poeciliids (66.7%), while the other two fish groups, cichlids and gouramis, each exhibited 16.7% of positive-testing species. Cichlid fish had a high percentage (83.3%) which were negative for this virus. Most infected species found in this study are among those reported in previous publications (Gibson-Kueh *et al*., 2003; Paperna *et al*., 2001; Jeong *et al*., 2008b; Jung-Schroers *et al*., 2016). Megalocytivirus-infected ornamental fish have also been reported to transmit the disease to Murray cod, freshwater fish in Australia (Go and Whittington, 2006) and rock bream, a marine fish species in Korea (Jeong *et al*., 2008a). This information suggests that megalocytivirus can infect a wide host range of ornamental fish and can contaminate and infect other fish species and cross natural environment boundaries.

Gross signs and changes of behavior in infected fish were observed in the current study. Most of the fish showed abnormal swimming behavior and non-specific symptoms. Few infected fishes exhibited clinical signs of hemorrhages on the body (Figure 8A), darkening or loss of coloration (Figure 8B and 8C). The visceral organs showed swollen kidney, splenomegaly, pale liver (Figure 8D) and accumulation of abdominal fluid.

The hemorrhages, loss of coloration and pale liver as non-specific symptoms appeared in few individuals of poeciliids and bettas, while most of the infected fishes in the current study were asymptomatic or appeared to be healthy. This type of healthy condition was also reported by other authors for many megalocytivirus-infected ornamental fish species (Rimmer *et al*., 2012; Subramaniam *et al*., 2014; Mohr *et al.*, 2015) and was described as a persistent infection or a persistent ISKNV infection (Wang *et al*., 2007; Jeong *et al*., 2006; Subramaniam *et al*., 2014). These persistently-infected fish which appear healthy are highly likely

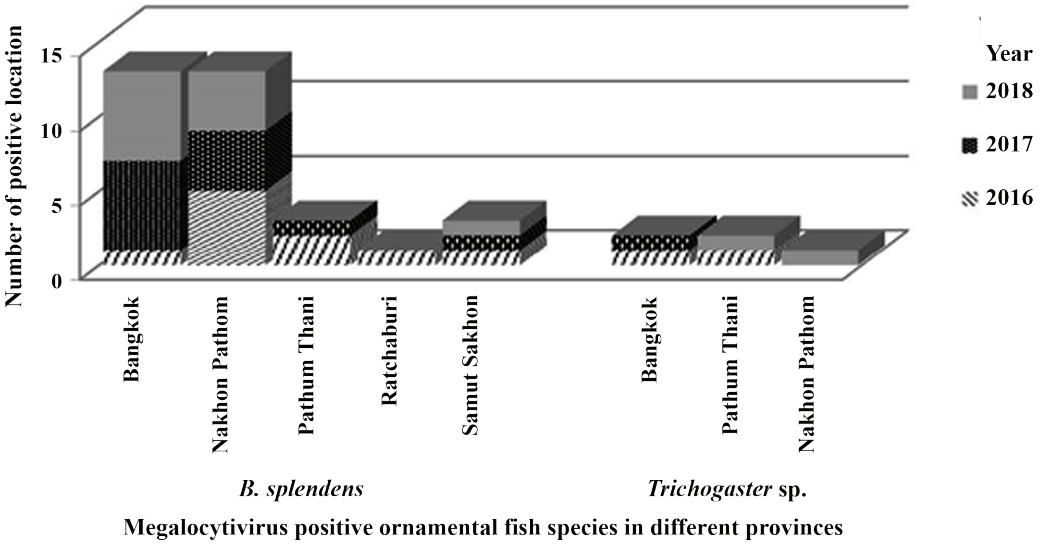

Figure 7. Number of positive locations for megalocytivirus in two gouramisin sampled Thai provinces during 2016-2018

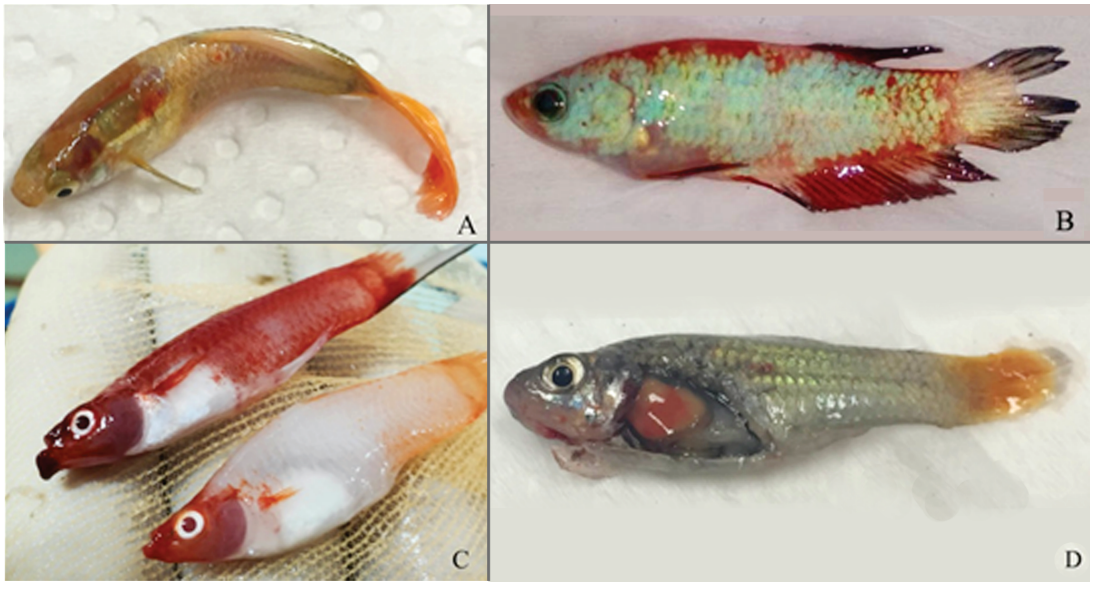

Figure 8. Clinical signs of megalocytivirus in infected fish (A) *Poecilia reticulata* (B) *Betta splendens* (C) *Xiphophorus maculatus* and (D) *Xiphophorus variatus.*

to pose a threat to other ornamental fish facilities by viral transmission. Thus, the validated, rapid, highly sensitive and specific diagnostic technique should be implemented for ISKNV megalocytivirus detection to be able to eliminate vector fish carrying this virus from producing areas and ornamental fish trading consignments.

Because of a lack of validated cell culture techniques (Rimmer *et al*., 2012) and difficulties of isolation and propagation of this virus in cell culture (Gias *et al*., 2011), not only molecular analysis but also histological techniques were applied to detect and confirm megalocytivirus infection in this study. Histopathological changes caused by megalocytivirus included hyperplasia and eosinophilic granular cells in gills. Cytomegalic cells with inclusion bodies which confirmed the infection were observed in several organs such as testes (Figure 9A), kidney (Figure 9B), spleen, liver and gills. All infected samples showed severe necrosis in the kidney, especially in haemopoietic tissue, while damage in the testes was found in the tunica albuginea. However, infected cells with inclusion bodies were less abundant in spleen, liver and gills.

Information from DNA sequencing and molecular phylogenetic analysis indicatedthe megalocytivirus pathogens in ornamental fish from the current study are members of ISKNV (Figure 10). All megalocytivirus MCP genes from six host species (*Trichogaster* sp., *Cichlasoma* sp., *Betta splendens*, *Astronotus ocellatus*, *Xiphophorus variatus* and *Xiphophorus maculatus*) were placed into the same cluster containing other ISKNV members: dwarf gourami iridovirus (DGIV) from Australia, paradise fish from Korea and complete genome of ISKNV obtained from China. The clade containing the MCP gene from Turbot reddish body iridovirus (TRBIV) was separated from ISKNV. This situation is similar to membersof the RSIV cluster, namely red seabream iridovirus (RSIV), rock bream iridovirus (RBIV), orange spotted grouper iridovirus (OSGIV) and large yellow croaker iridovirus (LYCIV). Our finding is consistent with other studies of ornamental fish infected with megalocytivirus (Song *et al*., 2008; Kim *et al*., 2010; Subramaniam *et al*., 2014; Mohr *et al*., 2015).

One interesting finding from the present study is an incidence of megalocytivirus disease in some individuals which was identified through clinical signs and histopathological changes, but with no or fairly low mortality. Virus-positive fish predominantly expressed persistent infection. Significant mortality in susceptible ornamental fish species has never been reported in Thailand. This is different from a previous report on the occurrence of 90% mortality in Australian Murray cod fingerlings due to megalocytivirus, when temperature of water was high at 26-27°C (Lancester

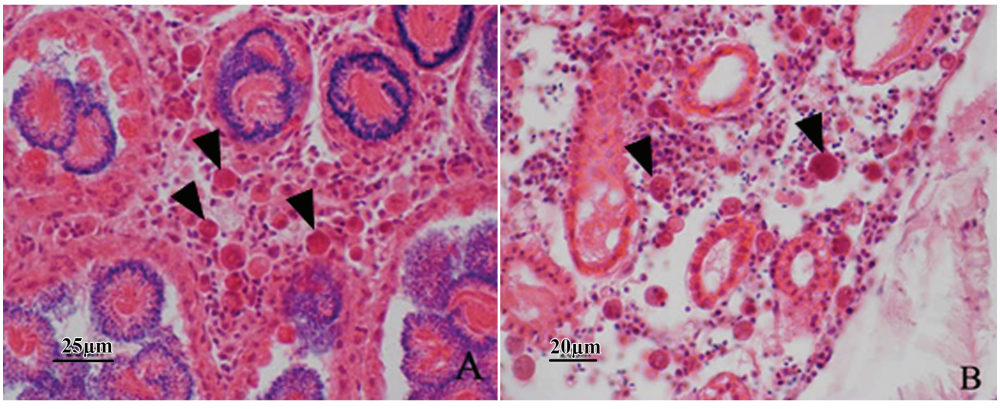

Figure 9. Histopathological results from *Poecilia latipinna* showing cytomegalic cells (arrow head) with inclusion bodies: (A) testes and (B) kidney.

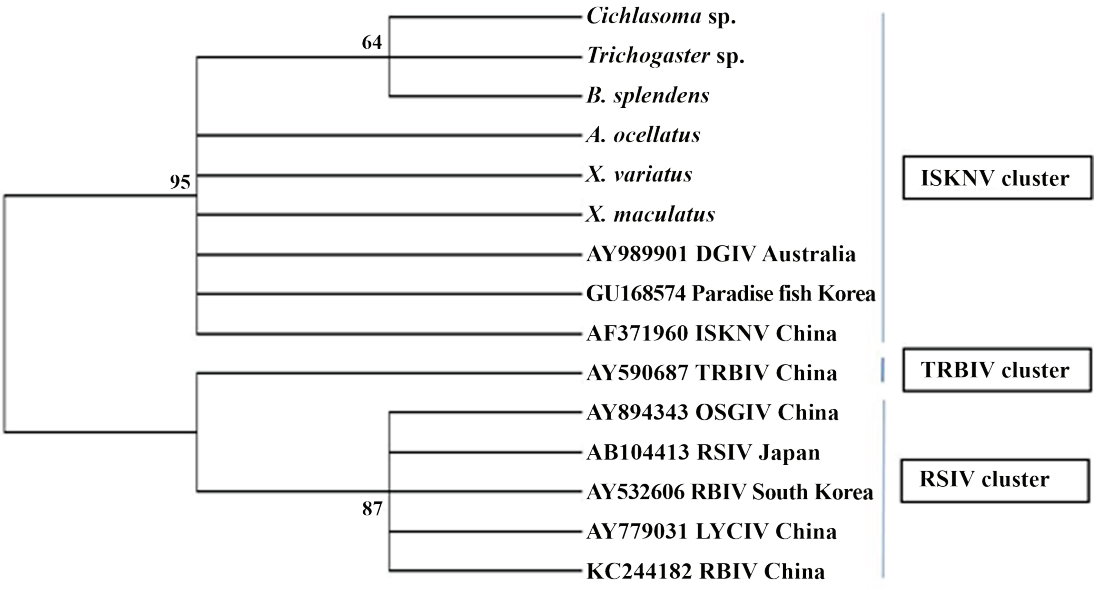

Figure 10. Phylogenetic tree analysis based on megalocytivirus major capsid protein (MCP) gene sequences. The number at the branches represents a confidence level of 10,000 bootstrapping replications. MCP nucleotide sequence genes of six fish species from the current study were used to compare with other reference megalocytivirus sequences obtained from GenBank with the following accession numbers: AY989901, GU168574, AF371960, AY590687, AY894343, AB104413, AY532606, AY779031 and KC244182.

et al., 2003). Go and Whittington (2006) studied transmission pattern of this virus through intraperitoneal injection and found that using viral inoculum from only one strain of gourami ISKNV, they could produce high mortality (> 90%) in Murray cod fingerlings.

Persistent infection is not yet well studied in megalocytiviruses, however this infection has been widely investigated in infectious pancreatic necrosis virus (IPNV), an etiological agent of acute contagious systemic disease, which infects various aquatic species including molluscs and crustaceans (Hill and Way, 1995). Substantial mortality frequently occurs in salmonid fry and fingerlings after an outbreak, while surviving fish exhibit persistent infection (Imajoh *et al*., 2005; Reyes-Cerpa *et al*., 2014; Julin *et al*., 2015). The persistent infection and inhibition of IPNV were associated with antiviral cytokine expression such as Mx1 protein (Larsen *et al*., 2004), interferon gamma (IFNγ) gene and apoptosis (Imajoh *et al*., 2005; Reyes-Cerpa *et al*., 2014). Environmental conditions were discovered as related factors on fish mortality, for example water temperature and exposure period were reported to contribute different mortality levels in rock bream after infection with red seabream iridovirus (RSIV) and rock bream iridovirus (RBIV) (Oh *et al*., 2016; Jung *et al*., 2017). Therefore, factors contributing to disease outbreak, host molecular defense mechanism, viral infection and pathogenesis, persistent infection and mortality risk factors in megalocytivirus infected ornamental fish should be further explored.

The movement of fry or fish could possibly be a cause of the spread of this disease. The authors further analyzed an association between ornamental fish translocation and disease occurrence in five major ornamental fish-producing provinces: Bangkok, Nakorn Pathom, Pathum Thani, Ratchaburi and Samut Sakhon. The statistical analysis showed that an improper management through translocated fish from unknown or megalocytivirus-positive

origin was associated with disease occurrence in Bangkok (χ^2= 28.231, p= 7.4x10^{-7}), Nakorn Pathom (χ^2= 24.149, p = 6x10^{-6}) and Ratchaburi (χ^2= 13.449, p = 0.001). However, there was no association between these two factors in Pathum Thani (χ^2= 2.286, p = 0.131) and Samut Sakhon (χ^2= 2.000, p = 0.157).

CONCLUSION

This is the first report which attempts to shed the light on megalocytivirus in Thailand. The locations in which this virus was found in ornamental fish in 2017 and 2018 were fewer than in 2016. Responsible movement of fish could mitigate the viral distribution. However, the question still remains why ISKNV present in ornamental fish does not cause high mortality as in other finfish such as Murray cod and rock bream, thus further studies are needed to elucidate more valuable information governing the infection process to facilitate proper disease control and prevention.

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Fisheries for funding support of this study. The authors would like to thank the staff at Molecular Biological Laboratory, Aquatic Animal Health Research and Development Division (AAHRDD), Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand for their assistance in sample preparation.

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