Journal of Sustainability Science and Management
Volume 17 Number 8, August 2022: 52-61
eISSN: 2672-7226

MOLECULAR DETECTION OF *MEGALOCYTIVIRUS* GENOTYPE: INFECTIOUS SPLEEN AND KIDNEY NECROSIS VIRUS IN MALAYSIAN OVIPAROUS AQUARIUM FISH

SANDRA CATHERINE ZAINATHAN[1,2]*, NURUL ILLIANA NADHIRAH MOHD ZAKI[1], NUR RABIAH JAAFAR[1], AZREEN SYAZANA NAZARUDDIN[1], NOOR ALIA NATASYA OMAR[1], ABDUL HAFIZ ISMAIL[1] AND CHE AZARULZAMAN CHE JOHAN[1,2]

[1]*Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia.*
[2]*Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia.*

**Corresponding author: sandra@umt.edu.my*
Submitted final draft: 17 April 2022 *Accepted: 4 June 2022* http://doi.org/10.46754/jssm.2022.08.003

Abstract: The Malaysian ornamental fish industry's recent production is recorded at RM350 million per annum. An Infectious Spleen and Kidney Necrosis Virus (ISKNV), one of the *Megalocytivirus* strains has been related to significant systemic infections that has ravaged ornamental fish stocks and had a severe financial impact on businesses in the sector. It is vital to identify *Megalocytivirus* host species among Malaysian oviparous ornamental fish as the virus can cripple the ornamental fish trade which is a major contributor to the country's export trade. The purpose of this research is to determine the prevalence of *Megalocytivirus*, ISKNV strain in oviparous aquarium fish, *Betta splendens, Danio rerio, Trichogaster lalius*, *Hyphessobrycon anisitsi* and *Puntigrus tetrazona* in Malaysia. The DNA of the *Megalocytivirus* in the samples was detected using the nested polymerase chain reaction (PCR) method. Out of 239 samples collected in January 2019 and January 2020, six pooled samples (n=30) tested positive for ISKNV infections in *D. rerio and T. lalius*. Positive *P. tetrazona* samples in this study showed a distended abdomen as the only observable clinical sign whereas all other samples were asymptomatic. All the positive samples were similar to each other. In terms of the nucleotide identity of the ISKNV, which stood at between 98% and 100%. In conclusion, the positive samples can be categorised into *Megalocytivirus* genotype I.

Keywords: Genotype I, ISKNV, *Megalocytivirus*, aquarium fish, oviparous, Malaysia.

Introduction

Aquarium/ornamental fish have grown to be one of the most well-known personal interests worldwide (Teletchea, 2019). The ornamental fish industry is estimated at between US$15 billion and US$30 billion per year and the global market includes more than 100 countries. Over a billion wild-caught or captive-bred fish of more than 6,500 species are sold every year (Teletchea, 2019).

Asian countries accounted for 57% of the export market with a value of US$197.7 million (Hans-Georg *et al.,* 2019). In 2014, Malaysia was ranked as the fifth largest producer of aquarium fish valued at approximately US$23 million and Malaysia was the eighth largest world producer in 2017 (Dey, 2016; Department of Fisheries Malaysia, 2017). Currently, he well-known families of freshwater ornamental fish produced in Malaysia are gourami, catfishes, cyprinids, tetras, cichlids, pupfishes/killifishes, guppies, mollies and swordtails (Zainathan *et al*., 2017; Department of Fisheries Malaysia, 2018).

Megalocytivirus is known to affect 40 species of aquarium fish around the world (Jung-Schroers *et al.,* 2016, Zainathan *et al.,* 2017; Lucca-Maganha *et al.,* 2018; Zainathan *et al*., 2019; Baoprasertkul *et al.,* 2019; Johan & Zainathan, 2020). It can cause systemic infections in most fresh and marine fish (Department of Agriculture Australia, 2014).

Plus, the ubiquity of megalocytiviruses in aquarium fish could lead to high mortality rates (Jung-Schroers *et al*., 2016; Cardoso *et al*., 2019) and economic losses (Johan & Zainathan, 2020) for ornamental fish sellers in Malaysia.

The megalocytivirus (Iridoviridae) is a large double-stranded DNA (dsDNA) virus with a diameter of between 120 and 200 nm (Wang *et al.,* 2016). The aquarium fish imported from Sri Lanka, Malaysia and Singapore have been reported to be infected with *Megalocytivirus* at export locations (Nolan *et al.,* 2015; Johan & Zainathan, 2020). In addition, outbreaks of *Megalocytivirus* among Malaysian aquarium fish have been reported, including in aquarium fish exported to quarantine facilities on foreign borders (Subramaniam *et al.,* 2014; Zainathan *et al.*, 2019).

Malaysian freshwater aquarium species have tested positive for the presence of *Megalocytivirus* in *Trichopodus trichopterus* (*T. trichopterus*) with non-specific clinical manifestations such as pale gills, liver enlargements and abdominal distensions (Zainathan *et al.,* 2017). Similarly, Zainathan *et al.* (2019) demonstrated that 110 samples tested positive for ISKNV in *Xiphophorus maculatus* (*X. maculatus)*, *Poecilia reticulata* (*P. reticulata*), *Trichogaster leeri* (*T. leeri*) and *Apistogramma ramirezi* (*A. ramirezi*) from Malaysia.

Other than the darkened body in *X. maculatus,* no clinical symptoms were observed in the positive samples (Zainathan *et al*., 2019). *Trichogaster lalius* (*T. lalius*) and *Puntius. tetrazona* (*P. tetrazona*) sampled by Subramaniam *et al*. (2014) showed negative results whereas only four samples of *Danio rerio* (*D. rerio*) tested positive for *Megalocytivirus*.

In addition, two genotypes within the *Megalocytivirus* had infectious spleen and kidney necrosis virus (ISKNV) as genotype I and genotype II were predominantly found in orange-spotted grouper iridovirus, OSGIV and red snapper iridovirus, RBIV (Oh *et al.*, 2006; Wang *et al.,* 2007). Meanwhile, phylogenetic analysis by Subramaniam *et al*. (2014) revealed three strains of *Megalocytivirus* and all of the ISKNV strains identified in fish in freshwater aquariums on the Malaysian Peninsula were assigned to genotype 1.

Sequencing analysis of the major capsid protein (MCP) of the *Megalocytivirus*, ISKNV strains of various Malaysian aquarium fish species previously showed high nucleotide identities with each other (between 96% and 99%) and reference ISKNV (between 96% and 100%) and could be categorised as *Megalocytivirus* genotype I (Zainathan *et al.,* 2017; 2019). Therefore, this study elucidates the molecular detection of *Megalocytivirus,* ISKNV strains in oviparous aquarium fish in Malaysia: *Betta splendens* (*B. splendens*)*, Danio rerio* (*D. rerio*)*, T. lalius, Hyphessobrycon anisitsi* (*H. anisitsi*) and *P. tetrazona*.

Materials and Methods

Sampling

As a part of its annual screening for research purposes, different aquarium fish species were screened for *Megalocytivirus*. Thus, adult selected aquarium fish (*B. splendens*, n = 50, *H. anisitsi,* n = 49, *T. lalius,* n = 40, *D. rerio*, n = 50) were sampled in January 2019 from an aquarium fish farm in Southern Malaysia. In contrast, *P. tetrazona* (n = 50) was collected in January 2020 from aquarium shops from East Coast Malaysia.

The sample collection was conducted at the farm and aquarium shops, then transported back to the Aquatic Organism Health laboratory in Universiti Malaysia Terengganu (UMT). The samples were observed for any external and internal clinical signs.

Organs such as stomach, gills, kidneys, intestines and spleens were processed accordingly (Zainathan *et al.*, 2017). The selected organs from the pooled samples (5 samples from the same species of fish were pooled into one pool) were cut into small pieces and kept in viral transport media (VTM) in 1.5 ml microcentrifuge tubes. The samples were kept at -80°C until their DNA was extracted.

PCR Analysis

The selected organs (gills, stomach, kidney, spleen and stomach) from each fish sample were approximately 50 mg. The selected organs were pooled in 5 samples into one 1.5 ml microcentrifuge tube after collection.

Next, the GF-1 viral nucleic acid extraction kit (Vivantis Technologies) was used to extract the DNA from the samples. Then, the nested PCR analysis was performed based on Rimmer *et al.* (2012) primers and developed by Whittington *et al.* (2009). At the primary PCR level, forward primer (C1105) and reverse primer (C1106) were used and at the nested PCR level, forward primer (C1073) and reverse primer (C1074) were used.

A total of 25µL of PCR mixture containing: 12.5 µL 2× MyTaq Mix (Bioline), 9.0 µL RNase – free water, 0.5 µL (10 µM) C1105 and 0.5 µL (10 µM) C1106 was added to 2.5 µL extracted DNA. Like the primary PCR mix, the nested PCR was conducted with the following primers (C1073 and C1074) added to the primary PCR products.

Both primary and nested reactions were amplified with the same protocol that was programmed as follows: The initial step at 95°C for 10 minutes, followed by 95°C for 30 minutes, 55°C for 30 seconds, extension at 72°C for 1 minute that was run for 35 cycles and the final extension of 72°C for 5 minutes.

Next, approximately 1.8% (w/v) agarose gel powder in Tris-acetate-EDTA buffer stained with SYBR Safe - DNA Gel Stain (Invitrogen) was used in gel electrophoresis analysis. The gel electrophoresis was run for 45 minutes at 70 V for both amplified PCR products. The synthetic positive control was used as a positive control in the study was based on the sequence of *Megalocytivirus* Sabah (GenBank accession number JQ253374.1).

Phylogenetic Tree

Phylogenetic analysis was performed after the results of the DNA sequencing of positive bands were sent by the 1st Based Laboratory Sdn. Bhd. The similarities of the positive bands from the samples were confirmed based on the NCBI BLAST database. Next, Clustal X2.0.12 was used to align the multiple alignments with other *Megalocytivirus*–associated sequences (Larkin *et al.*, 2007).

Lastly, a phylogenetic analysis based on the coat protein genes of the samples and the highly similar published sequences of other viruses was used to construct the phylogenetic tree followed by neighbor-joining method by Molecular Evolutionary Genetics Analysis software (MEGA 7.0.26) (Pennsylvania State University, USA). The maximum composite likelihood method and bootstrap value (100) was also used to compute the evolutionary distances.

Results and Discussion

It was only in *P. tetrazona* (n = 4) (Figure 1) that the distended abdomen was observed as a clinical symptom even though this species was negative for the presence of *Megalocytivirus*. Of the six pooled samples in the secondary reaction: Four pooled samples (n = 20) of showed signs of *D. rerio* and two pooled samples (n=10) of *T. lalius* for *Megalocytivirus* (Table 1) at 167 bp (Figure 2) appeared positive result that demonstrated by conventional PCR assay. In contrast, no bands were visualized for the other species including *B. splendens, H. anisitsi* and *P. tetrazona*.

Table 1: Summary of PCR analyses for the detection of *Megalocytivirus* genotype ISKNV in selected oviparous aquarium fish species from Malaysia

Samples	Number Tested	Number Tested (pooled)	Total Positives (pooled)
P. tetrazona	50	10	0
D. rerio	50	10	4
T. lalius	40	8	2
B. splendens	50	10	0
H. anisitsi	49	10	0
	239	48	6

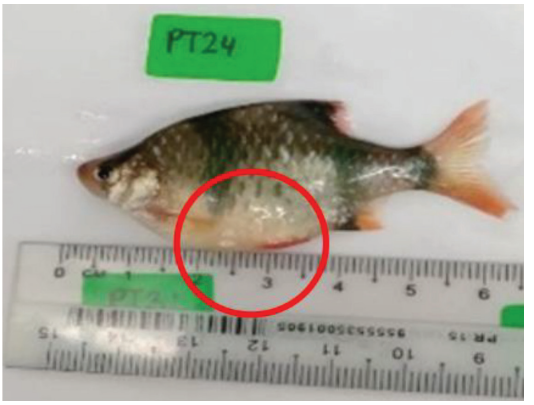

Figure 1: *P. tetrazona* that demonstrated distended body (circle)

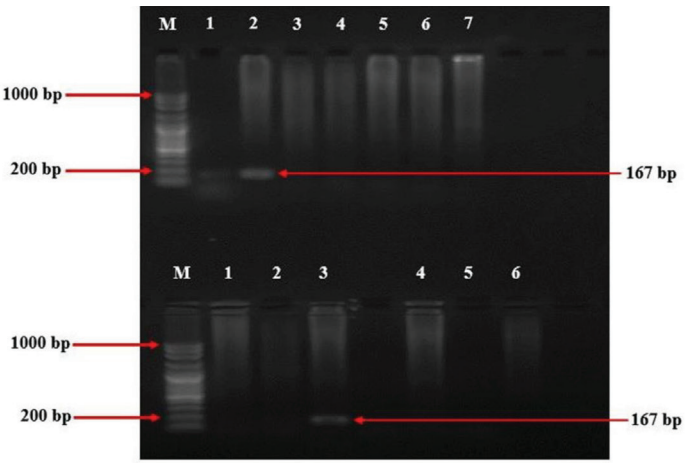

Figure 2: Nested PCR results showed amplification of internal organs of *T. lalius* samples from Malaysia. Above: M: 100 bp ladder. Lane 1 - 5: Samples 1 to 5. Lane 6: Negative control (primary). Lane 7: Positive control (primary). Below: M: 100 bp ladder. Lane 1 - 3: Samples 6 to 8. Lane 4: Negative control (nested). Lane 5: Positive control (primary). Lane 6: Positive control

The positive bands excised from *T. lalius* (sample 2) were examined based on sequencing analysis to confirm the *Megalocytivirus*-genotypes. The samples selected organs were pooled to intensify population-level coverage when ubiquity is low (<10%) and the quantity of the samples was fixed. It is due to the fact pooled samples could intensify the likelihood of including targeted genes from infected fish in a tested pool (Laurin *et al.,* 2019).

The samples did not show any clinical signs except the distended body in *P. tetrazona* (negative sample). Distended body in a negative fish for *Megalocytivirus* infection could be caused by other factors that were not studied. Dropsy is a commonly applied term for coelomic (i.e., abdominal) distention due to ascites or the effusion and collection of fluid freely throughout the coelomic cavity. It is a nonspecific syndrome and a clinical presentation instead of a defined disease (Densmore, 2019).

Nonetheless, a distended body or abdominal cavity is a clinical sign of *Megalocytivirus* (Subramaniam *et al.,* 2014). Ornamental fish infected with *Megalocytivirus* are known to develop non-specific gross symptoms like

other diseases (Johan & Zainathan, 2020). Others signs because of a *Megalocytivirus* infection consist of drowsiness, lack of appetite, darkening, unusual swimming (consisting of rotation), high respiration, swelling of the body (coelomic distension), ulcers, bleeding (injury on the skin and gills), anemic gills, eroded fins, white excreta and excessive mortality (Yanong & Waltzek, 2013).

Same as the Brazilian-affected red piranha, (*Pygocentrus nattereri*) also had non-specific symptoms for instance anorexia, uncoordinated swimming, proximity to the water surface, exhaustion and difficulty in breathing, characteristic of ISKNV infection (Cardoso *et al.,* 2017).

The presence of *Megalocytivirus* in this study showed positive results from *T. lalius* and *D. rerio* was consistent with previous studies. In addition, *Megalocytivirus* was found in zebrafish (*D. rerio*) in the field (Subramaniam *et al.,* 2014; Bermúdez *et al.,* 2018) and experimental challenges (Xu *et al.,* 2008).

A total of four asymptomatic zebrafish out of 15 samples showed positive results of ISKNV in Peninsular Malaysia (Subramaniam *et al.,* 2014). In another study by Xu *et al.* (2008), zebrafish was also defenseless against ISKNV infection during experimental challenges, although there were no antecedent occurrences of ISKNV infection in zebrafish from similar breeding farms.

Jeong *et al.* (2008) recorded positive results for *T. lalius* sampled from wholesalers and exporters. Similarly, high numbers of *T. lalius* (n = 115) were found to be positive for *Megalocytivirus* in samples imported from Indonesia, Singapore and Thailand into Australia (Rimmer *et al.,* 2015) and *T. lalius* (n = 10) sampled from aquarium shops in Australia (Go *et al.,* 2006).

No previous study has shown that *H. anisitsi* was infected by *Megalocytivirus* previously. However, only one sample of *Hyphessobrycon innesi* from Korea showed positive results using the 2-step PCR method (Jeong *et al.,* 2008). Gibson-Kueh *et al.* (2003) demonstrated that *B. splendens* with systemic iridovirus infections showed pathology similar to *Megalocytivirus*.

The Siamese fighting fish (*B. splendens*) was reported to be ISKNV-positive in Thailand (*B. splendens*) (Puttharat *et al.,* 2017), contrary to this study. Furthermore, the existence of *Megalocytivirus*-like viruses was certified by PCR in a barb fish species, cherry barb *Puntius titteye* in India and it was reported initiatory in the world (Girisha *et al.,* 2020). These results indicate that the incidence of ISKNV in healthy Malaysian aquarium fish is high and may exist as a vector due to the omission of clinical symptoms.

Sequencing and Phylogenetic Analysis

The sequences of the samples based on nested PCR analysis covering 167 bp region of *Megalocytivirus*, ISKNV strain was determined for sample 2 from *T. lalius* (TL2). However, positive samples of *D. rerio* were unable to be sent for sequencing analysis.

The strains detected in this study have been aligned by Clustal X2.0.12 (Larkin *et al.,* 2007) and confirmed to have equal to every difference with nucleotide sequences identification with an expected value (e-value) of 100% and with discovered sequences (98%-100%) (Table 2) from the NCBI BLAST database. The e-value represents the expectation of finding that sequence by random chance.

Therefore, the e-value of 100% represents a good sequence match. The nucleotide sequence alignment of the amplified PCR product from the nested reaction confirmed that the sample was sent; TL2 was derived from the same member of the ISKNV strain of the genus *Megalocytivirus* (Figure 2).

Based on the phylogenetic family tree (Figure 3), all ISKNV strains in this study were assigned to genotype I. The length of the branches (horizontal lines) represented the genetic distance. In this study, the positive sample TL2 was more related to the ISKNV strain RSIV-Ku (KT781098.1). However, the

bootstrap value for TL2 (0.24) showed a low confidence level of the node from the topology of the tree due to the increasing numbers of the species (Holman, 2005).

The results of sequencing and phylogenetic analysis in this study were similar to those by Subramaniam *et al.* (2014), Zainathan *et al*. (2017) and Zainathan *et al.* (2019) on several types of Malaysian aquarium fish. Phylogenetic analysis showed that the strains in this study were strongly associated with ISKNV genotype ranging from 96% to 100% based on previous studies (Subramaniam *et al.,* 2014; Zainathan *et al.,* 2017; Zainathan *et al.,* 2019).

The *Megalocytivirus*-MCP sequences in the pet fish samples from 2002 to 2010 were almost entirely identical to each other (99.9% to 100%) and entirely identical to ISKNV and these megalocytiviruses were genetically identical. It provides further evidence that it is different (Go, 2015; Go *et al.,* 2016). Genotype 1 ISKNV virus is omnipresent in numerous fish species in many countries around Asia (Song *et al.*, 2008).

Conclusion

In summation, 30 samples (6 pools) from Malaysian aquarium fish, including *D. rerio* and *T. lalius* were identified as the ISKNV strain, genotype 1. Despite the high occurrence of *Megalocytivirus* in oviparous aquarium fish, the source of infection is unknown.

The epidemiology of *Megalocytivirus* and its associated factors that influence *Megalocytivirus* and treatment or vaccine development for *Megalocytivirus* in oviparous aquarium fish specifically is still limited. Future work should be focused on undertaking such measures as *Megalocytivirus* is persistent in many oviparous aquarium fish.

Acknowledgements

The authors would like to acknowledge the Faculty of Fisheries and Food Science, Institute of Marine Biotechnology, Universiti Malaysia Terengganu and farmers from Malaysia for their support.

Table 2: Nucleotide sequence distinction of *Megalocytivirus*-MCP gene (ISKNV) (%) detected in this study compared to similar reference viruses based on the Genbank database

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1		100	100	100	100	100	100	100	100	100	100
2			100	99.49	99.71	99.78	98.68	100	99.93	100	99.49
3				99.90	100	99.90	98.68	100	100	100	99.90
4					99.34	99.41	98.31	99.41	99.56	99.49	99.41
5						99.49	98.53	99.85	99.78	99.71	99.63
6							98.46	99.85	99.71	99.78	99.71
7								99.12	98.75	98.68	98.60
8									98.75	98.68	98.60
9									99.85	100	99.85
10										99.93	99.85
11											

Notes: 1 = ISKNV (*T.lalius*/Johor/2019/TL2), 2 = ISKNV RSIV-Ku (KT781098.1), 3 = *Anabas testudineus* iridovirus (AB930172.1), 4 = *Megalocytivirus* Sabah (JQ253374.1), 5 = *Megalocytivirus* Sabah (JQ253371.1), 6 = *Megalocytivirus* Sabah (JQ253369.1), 7 = ISKNV (KY440040.1), 8 = ISKNV (KX354220.1), 9 = *Megalocytivirus* Sabah (JQ253368.1), 10 = *Megalocytivirus* Sabah (JQ253372.1), 11 = *Megalocytivirus* Sabah (JQ253373.1)

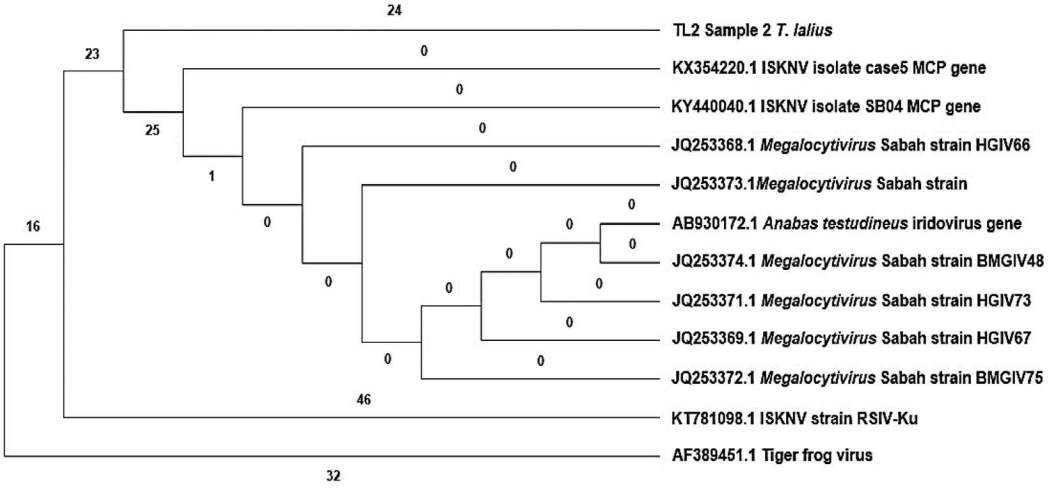

Figure 3: The phylogenetic tree is based on MCP gene sequences of *Megalocytivirus* (ISKNV) detected in aquarium fish from Malaysia and similar reference viruses. The scale bar represents distance values. Note TL2 = *T.lalius* published Genbank sequences: ISKNV strain RSIV-Ku (KT781098.1), *Anabas testudineus* iridovirus gene for MCP (AB930172.1), *Megalocytivirus* Sabah strain BMGIV48 (JQ253374.1), *Megalocytivirus* Sabah strain BMGIV73 (JQ253371.1), *Megalocytivirus* Sabah strain BMGIV67 (JQ253369.1), ISKNV isolate SB04 MCP gene (KY440040.1), ISKNV isolate case5 MCP gene (KX354220.1), *Megalocytivirus* Sabah strain BMGIV66 (JQ253368.1), *Megalocytivirus* Sabah strain

References

Australian Department of Agriculture. (2014). *Importation of freshwater ornamental fish: A review of biosecurity risks associated with gourami iridovirus and related viruses-Final import risk analysis report*. Department of Agriculture, Canberra. Accessed August, 2019, from agriculture.gov.au/biosecurity.

Baoprasertkul, P., & Kaenchan, N. (2019). Distribution and detection of *Megalocytivirus* in ornamental fish in Thailand. *Journal of Fisheries and Environment*, *43*(1), 11-24.

Bermudez, R., Losada, A. P., Azevedo, A. M., Guerra-Varela, J., Perez Fernandez, D., Sanchez, L., Padros, F., Nowak, B., & Quiroga, M. I. (2018). The first description of natural infection with spleen and kidney necrosis virus in zebrafish. *Journal of Fish Diseases, 41*(8), 1283-1294.

Cardoso, P. H. M., Maganha, S. R., Sousa, R. L. M., & Balian, S. C. (2017). First report of *Megalocytivirus* in red piranhas (*Pygocentrus nattereri*) by molecular diagnosis in Brazil. *International Journal of Avian & Wildlife Biology, 2*(3), 82-84.

Chen, F., Sun, J., Han, Z., Yang, X., Xian, J., Lv, A., Hu, X., & Shi1, H. (2019). Isolation, identification and characteristics of *Aeromonas veronii* from diseased Crucian Carp (*Carassius auratus gibelio*). *Frontiers in Microbiology*, *2742*(10).

Citarasu, T., Alfred Dhas, K., Velmurugan, S., Thanga Viji, V., Kumaran, T., Michael Babu, M., & Selvaraj, T. (2011). Isolation of *Aeromonas hydrophila* from infected ornamental fish hatchery during a massive disease outbreak. *International Journal of Current Research*, *2*(1), 37-41.

Department of Fisheries Malaysia. (2016). *Senarai Perangkaan Perikanan Tahunan*. DoF, Malaysia [online]. Updated 21 April 2016. Accessed July, 2016, from http://www.dof.gov.my/perangkaan 2016.

Department of Fisheries Malaysia. (2017). *Senarai Perangkaan Perikanan Tahunan*. DoF, Malaysia [online]. Updated 21 April

2017. Accessed July 20, 2017, from http://www.dof.gov.my/perangkaan 2017.

Department of Fisheries Malaysia. (2018). Annual Fisheries Statistics 2018: Aquaculture Table: https://www.dof.gov.my/dof2/resources/user_29/documents/perangkaan perikanan/2018 jilid 1/table_akua_2018_-new.pdf. Retrieved on 17-10-2020.

Dey, V. K. (2016). *The Global Trade in Ornamental Fish*. INFOFISH International. Retrieved August, 2017, from http://www.infofish.org.

Gibson-Kueh, S., Netto, P., Ngoh-Lim, G. H., Chang, S. F., Ho, L. L., Qin, Q. W., F. Chua, H. C., Ng, M. L., & Ferguson, H. W. (2003). The pathology of systemic iridoviral disease in fish. *Journal of Comparative Pathology*, *129*(2-3), 111-119.

Girisha, S. K., Kushala, K. B., Nithin, M. S., Puneeth, T. G., Naveen Kumar, B. T., Vinay, T. N., Suresh, T., Ajay, S. K., Venugopal, M. N., & Ramesh, K. S. (2020). First report of the infectious spleen and kidney necrosis virus (ISKNV) infection in ornamental fishes in India. *Transboundary and Emerging Diseases*, *68*(2), 964-972.

Go, J. (2015). *Megalocytiviruses* of freshwater ornamental fish and pathogenicity in marine and euryhaline species. Farm Animal and Veterinary Public Health, University of Sydney, June 2015, pp. 15-18.

Go, J., Lancaster, M., Deece, K., Dhungyel, O., & Whittington, R. (2006). The molecular epidemiology of iridovirus in Muray cod (*Maccullochella peelii peelii)* dwarf gourami (*Colisa Lalia*) from distant biogeographical regions suggest a link between trade in ornamental fish and emerging iridoviral diseases. *Molecular and Cellular Probes*, *20*(3-4), 212-222.

Go, J., Waltzek, T. B., Subramaniam, K., Yun, S. C., Groff, J. M., Anderson, I. G., Chong, R., Shirley, I., Schuh, J. C. L., Handlinger, J. H., Tweedie, A., & Whittington R. J. (2016). Detection of infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) from archival ornamental fish samples. *Diseases of Aquatic Organisms, 122*(2), 105-123.

Hans-Georg, E., John, K. P., & Martin, I. T. (2019). Where are they all from? Sources and sustainability in the ornamental freshwater fish trade. *Journal of Fish Biology, 94*(6), 909-916.

Holman, E. W. (2005). Nodes in phylogenetic trees: The relation between imbalance and number of descendent species. *Systemic Biology*, *54*(6), 895-899.

Johan, C. A. C., & Zainathan, S. C. (2020) *Megalocytiviruses* in ornamental fish: A review. *Veterinary World*, *13*(11), 2565-2577.

Jeong, J. B., Kim, H. Y., Jun, L. J., Lyu, G. H., Park, N. M., Kim, J. K., & Jeong, H. D. (2008). Outbreaks and risks of infectious spleen and kidney necrosis virus disease in freshwater ornamental fishes. *Disease of Aquatic Organisms*, *78*(3), 209-215.

Jung-Schroers, V., Adamek, M., Wohlsein, P., Wolter, J., Wedekind, H., & Steinhagen, D. (2016). First outbreak of an infection with infectious spleen and kidney necrosis virus (ISKNV) in ornamental fish in Germany. *Diseases of Aquatic Organisms*, *119*(3), 239-244.

Laurin, E., Thakur, K., Mohr, P. G., Hick, P., Crane., M. S. J., Gardner, I. A., Moody, N. J. G., Colling, A., & Ernst, I. (2019). To pool or not to pool? Guidelines for pooling samples for use in surveillance testing of infectious diseases in aquatic animals. *Journal of Fish Diseases*, *42*(11), 1471-1491.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23*(21), 2947-2948.

Lucca-Maganha, S. R., Cardoso, P. H. M., de Carvalho Balian, S., de Almeida-Queiroz, S. R., Fernandes, A. M., & de Sousa, R. L. M. (2018). Molecular detection and phylogenetic analysis of *Megalocytivirus* in Brazilian ornamental fish. *Archives of Virology, 163*(8), 1-7.

Nolan, D., Stephens, F., Crockford, M., Jones, J. B., & Snow M. (2015). Detection and characterization of viruses of the genus *Megalocytivirus* in ornamental fish imported into an Australian border quarantine premises: An emerging risk to national biosecurity. *Journal of Fish Diseases*, *38*(2), 187-195.

Oh, M. J., Kitamura, S. I., Kim, W. S., Park, M. K., Jung, S. J., Miyadai, T., & Ohtani, M. (2006). Susceptibility of marine fish species to a *Megalocytivirus*, turbot iridovirus, isolated from turbot, *Psetta maximus* (L.). *Journal of Fish Diseases, 29*(7), 415-421.

Puttharat, B., Varinee, P., Nudthapol, K., & Paveena, T. (2017). Detection of *Megalocytivirus* from freshwater ornamental fish in Thailand. In Society, A. F. (Ed.), *Diseases in Asian Aquaculture 10* (pp. 71). Asian Fisheries Society, Bali,

Rimmer, A. E., Becker, J. A., Tweedie, A., & Whittington, R. J. (2012). Development of a quantitative polymerase chain reaction (qPCR) for the detection of dwarf gourami iridovirus (DGIV) and other *Megalocytiviruses* and comparison with the Office International des Epizooties reference PCR protocol. *Aquaculture,* 358-359, 155-163.

Rimmer, A. E., Becker, J. A., Tweedie, A., Lintermans, M., Landos, M., Stephens, F., & Whittington, R. J. (2015). Detection of dwarf gourami iridovirus (Infectious spleen and kidney necrosis virus) in populations of ornamental fish prior to and after importation into Australia, with the first evidence of infection in domestically farmed platy (*Xiphophorus maculatus*). *Preventive Veterinary Medicine, 122*(1-2), 181-194.

Song, J. Y., Kitamura, S. I., Jung, S. J., Miyadai, T., Tanaka, S., & Fukuda, Y. (2008). Genetic variation and geographic distribution of *Megalocytiviruses. Journal of Microbiology*, *46*(1), 29-33.

Subramaniam, K., Shariff, M., Omar, A. R., Hair-Bejo, M., & Ong, B. L. (2014). Detection and molecular characterization of infectious spleen and kidney necrosis virus from major ornamental fish breeding states in Peninsular Malaysia. *Journal of Fish Diseases, 37*(7), 609-618.

Teletchea, F. (2019). Ornamental Fish Trade: Overview and emerging questions. *INFOFISH International*, *5*, 8-11.

Wang, Y. Q., Lu, L., Weng, S. P., Huang, J. N., Chan, S. M., & He, J. G. (2007). Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis virus-like (ISKNV-like) virus. *Archives of Virology*, *152*(4), 763-773.

Wang, C. S., & Wen, C. M. (2016). *Megalocytiviruses.* In D. Liu (Ed.), *Molecular detection of animal viral pathogens* (pp. 825-833). Boca Raton, Florida: CRS Press.

Wang, X., 1. King Jordan & Mayer, L. W. (2015). A phylogenetic perspective on molecular epidemiology. In T., Yi-Wei, L., Dongyou, S., Joseph, S., Max & P., Ian (Eds.), *Molecular medical microbiology* (2nd ed., pp. 517-536). Academic Press.

Whittington, R. J., Tweedie, A., Dennis, M. M., Becker, J. A., & Landos, M. (2009). FRDC final report Aquatic Animal Health Subprogram: Optimization of PCR tests for diagnosis of *Megalocytivirus* (gourami iridovirus) and cyprinid herpesvirus 2 (goldfish herpesvirus) FRDC Project No. 2007/007. University of Sydney and Fisheries Research and Development Corporation, pp. 5-33.

Walczak, N., Puk, K., & Guz, L. (2017). Bacterial flora is associated with diseased freshwater ornamental fish. *Journal of Veterinary Research*, *61*, 445-449.

Xu, X., Zhang, L., Weng, S., Huang, Z., Lu, J., Lan, D., Zhong, X., Yu, X., Xu, A., & He, J. (2008). A zebrafish (*Danio rerio*) model of infectious spleen and kidney necrosis virus (ISKNV) infection. *Virology*, *376*, 1-12.

Yanong, R. P. E., & Waltzek, T. B. (2013). *Megalocytivirus* infections in fish, with emphasis on ornamental species. IFAS Cooperative Extension Service, Circular FA – 120 [online]. Updated December 2013. Accessed August, 2016, from http://edis.ifas.ufl.edu/pdffiles/FA/FA18200.pdf.

Zainathan, S. C., Balaraman, D., Ambalavanan, L., Krishna Moorthy, P., Palakrishnan, S. K., & Ariff, N. (2019). Molecular screening of infectious spleen and kidney necrosis virus in four species of Malaysian farmed ornamental fish. *Malaysian Applied Biology*, *48*(1), 131-138.

Zainathan, S. C., Johan, C. A. C., Subramaniam, N., Ahmad, A. A., Halim, N. I. A., & Norizan, N. (2017). Detection and molecular characterization of *Megalocytivirus* strain ISKNV in freshwater ornamental fish from Southern Malaysia. *Aquaculture, Aquarium, Conservation and Legislation Bioflux*, *10*(5), 1098-1109.