



FIRST MOLECULAR DETECTION OF INFECTIOUS SPLEEN AND KIDNEY NECROSIS VIRUS (ISKNV) STRAIN OF *MEGALOCYTIVIRUS* IN ASIAN SEABASS (*Lates calcarifer*) AND WATER SYSTEMS FROM NORTHERN PENINSULAR MALAYSIA

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ARTICLE INFO	ABSTRACT
Article History: Received: 12 March 2025 Revised: 11 July 2026 Accepted: 27 July 2026 Published: 15 September 2026 Keywords: Molecular detection, Megalocytivirus, ISKNV, Lates calcarifer, Malaysia	<i>Lates calcarifer</i> is a commercial species in both aquaculture and capture fisheries in Southeast Asian countries and accounts for 50% to 90% of Malaysia's total annual production. However, the most challenging issue facing <i>L. calcarifer</i> farmers is <i>Megalocytivirus</i> infection, which causes high mortality. Thus, this study aimed to detect the presence of <i>Megalocytivirus</i> and its genotype in fish and water samples from a <i>L. calcarifer</i> farm via PCR analysis. A total of 45 samples of <i>L. calcarifer</i> juveniles, eggs, and water samples from different sites (egg pond, juvenile pond, water outlet, untreated reservoir, and treated reservoir) were collected at two sampling times from the <i>L. calcarifer</i> hatchery, Hang Lekir, Perak. The PCR assay demonstrated that all the samples from both sampling times were positive for <i>Megalocytivirus</i> DNA. External clinical signs in the fish samples included tail erosion, scale loss, haemorrhages (mouth), and body erosion during the first sampling; however, no clinical signs were observed during the second sampling. Sequence analysis of the positive samples showed 97% to 98% nucleotide similarity to the Infectious Spleen and Kidney Necrosis Virus (ISKNV) strain, and samples from similar sampling times showed high nucleotide similarity (> 98%) to each other. Furthermore, the positive samples from this study were grouped within the ISKNV strain.

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Introduction

In the twenty-first century, fisheries and aquaculture have become indispensable sectors for global food security, nutrition, and economic stability, contributing significantly to human consumption and livelihoods worldwide (FAO, 2022). The global aquaculture production reached 88 million metric tonnes in 2022, accounting for 49% of total aquatic animal production and valued at USD 265 billion (FAO, 2022). Southeast Asian countries, particularly Malaysia, play a pivotal role in this expansion,

with Asian seabass (*Lates calcarifer*), commonly known as barramundi, standing out for its rapid growth rate, adaptability, and high market value (FAO, 2020). In Malaysia, *Lates calcarifer* (*L. calcarifer*) contributes between 50% and 90% of cultured marine fish production, peaking at 30,440 tonnes in 2014 (Idris *et al.*, 2022). Production decreased to 17,263.61 tonnes in 2019, largely attributed to seed supply constraints and the severe impact of viral disease outbreaks (Department of Fisheries,

2019). *Megalocytivirus* infections represent a significant threat, leading to severe health complications and elevated mortality rates in affected populations (Qin *et al.*, 2023). Despite the severity of infection, critical gaps in disease management and control remain due to a lack of thorough study of the virus's environmental reservoirs and transmission routes in Malaysian aquaculture.

Megalocytivirus, a member of the Iridoviridae family, is a highly pathogenic virus that causes systemic infections in both marine and freshwater fish species (Subramaniam *et al.*, 2012; Johan *et al.*, 2020, 2023). The virus predominantly targets vital organs such as the liver, kidneys, and spleen, causing symptoms such as fatigue, restricted movement, and tissue necrosis, with fatality rates reaching up to 100% during severe outbreaks (Girisha *et al.*, 2020). Globally, major *Megalocytivirus* strains of concern include the Infectious Spleen and Kidney Necrosis Virus (ISKNV), Scale Drop Disease Virus (SDDV), and Red Sea Bream Iridovirus (RSIV), all of which have caused significant financial losses in the aquaculture sector (Zhu *et al.*, 2021; Sumithra *et al.*, 2022). *Megalocytivirus* infection causes high mortalities of farmed *L. calcarifer*. The strains of the *Megalocytivirus* that have been reported in *L. calcarifer* are Infectious Spleen and Kidney Necrosis Virus (ISKNV) (Kerddee *et al.*, 2021), Red Sea Bream Iridovirus (RSIV) in India (Sumithra *et al.*, 2022), ISKNV in China (Zhu *et al.*, 2021), and Scale Drop Disease Virus (SDDV) in Malaysia (Nurliyana *et al.*, 2020). Clinical signs of *Megalocytivirus* infection in *L. calcarifer* include lethargy, darkened body, anorexia, erratic swimming behaviour, gasping for air, liver haemorrhage, pale gills, and skin haemorrhages (Kerddee *et al.*, 2021; Sumithra *et al.*, 2022; Fu *et al.*, 2023).

While evidence suggests the presence of *Megalocytivirus* in Malaysian aquaculture systems, particularly affecting *L. calcarifer*, research into its prevalence and transmission routes, especially through water systems and environmental reservoirs, remains insufficient.

This study aims to address these gaps by detecting the presence and genotype of *Megalocytivirus* in *L. calcarifer* and associated water systems in northern Peninsular Malaysia. The findings will contribute to the development of early detection systems, enhance biosecurity practices, and support the long-term sustainability of Malaysia's aquaculture sector, particularly benefiting small-scale farmers and contributing to national food security initiatives.

Materials and Methods

Sampling Location and Sample Collection

The first sampling was conducted on 27 July 2023 at the *L. calcarifer* hatchery in Hang Lekir, Manjung, Perak. The hatchery produces 2 to 3 inches and 4 to 6 inches of *L. calcarifer* juveniles from eggs. The hatchery followed minimum levels of biosecurity protocols. The hatchery experienced unrecorded mortalities from time to time, and no previous detection of any pathogens has been conducted. Sampling times were based on feasibility to record the first detection of ISKNV in *L. calcarifer* from Malaysia. Fish size for both sampling times was based on the availability of the fish samples at the farm. A total of 30 *L. calcarifer* samples (Day 49, 5.5 to 8.5 cm) and a replicate of 500 mL of pond water were collected.

The second sampling was conducted on 31 July 2024 (based on the feasibility of sampling by the farmer) to determine and confirm the presence of *Megalocytivirus* in the water systems (Table 1). The eggs (50 mL centrifuge tube, 50 g) were collected on Day 3 post-fertilisation and originated from the same broodstock as the juvenile samples. A total of 15 samples (2.3 to 2.5 cm) were randomly collected on Day 46 post-hatching and transported to the laboratory in oxygen-filled plastic bags. Water samples were collected in triplicate ($n = 3$) from various locations, including the Egg Pond (EP), Juvenile Ponds (JP), Water Outlet (WO), Untreated Reservoir (UR), and Treated Reservoir (TR), using 500 mL sterile bottles. Water quality parameters, including salinity,

Table 1: The total number of samples collected from the first (2023) and second sampling (2024) of *L. calcarifer* and water samples based on availability of samples and feasibility of sampling

Year	Samples	Total Number of Replicates/Pools	Total Number of Individuals
2023	Juvenile pond water	3	3
	<i>L.calcarifer</i> juveniles	6 pools	30
2024	<i>L.calcarifer</i> eggs	3	3
	<i>L.calcarifer</i> juveniles	3 pools	15
	Egg pond water	3	3
	Juvenile pond water	3	3
	Water outlet	3	3
	Untreated reservoir	3	3
	Treated reservoir	3	3
Total			66

Dissolved Oxygen (DO), and temperature, were measured using a Hydrolab HL7 device. At the same time, pH, nitrate, nitrite, and ammonia levels were assessed using an API® Saltwater Master Test Kit (Zainathan *et al.*, 2019).

Sample Processing

Fish sample length and weight were recorded. Internal and external clinical symptoms were observed and recorded. Selected organs, such as the kidney, liver, and spleen, were collected from the fish and pooled (n = 5) in separate 15 mL centrifuge tubes. Each sample was transported in 3 mL of Viral Transport Media (VTM) according to a modified method by Zainathan *et al.* (2019). The samples were brought back to the Aquatic Organism Laboratory at Universiti Malaysia Terengganu on ice. Isolation was performed in a laminar hood to avoid sample contamination. The fish organs were homogenised in 3 mL of VTM using a tissue homogeniser. The samples were placed in 15 mL centrifuge tubes and then centrifuged for 20 minutes at 7,830 × g and 4°C. Before DNA extraction, the 300 µL supernatant was stored in 1.5 mL microcentrifuge tubes at -20°C. The fish eggs (3 replicates, each replicate weighed approximately 5 g) were cut and minced separately. The samples were homogenised

using sterile scissors in a 50 mL tube containing 5 mL of VTM. The homogenised tissues were centrifuged at 8,000 × g for 5 minutes, and the collected supernatants were stored at -20°C until DNA extraction.

For the analysis of water samples, approximately 300 mL of each sample was divided into six 50 mL sterile centrifuge tubes (Johan *et al.*, 2023). The water samples were centrifuged for 30 minutes at 7,830 × g and 4°C. From each tube, approximately 50 µL of supernatant was transferred into a 1.5 mL microcentrifuge tube, resulting in a final total volume of 300 µL in each tube. The supernatants were stored at -20°C prior to DNA extraction.

Viral DNA Extraction

The GF-1 Viral Nucleic Acid Extraction Kit was used for DNA extraction according to the manufacturer’s guidelines. Briefly, approximately 200 µL of the sample was mixed with 50 µL of proteinase K. Subsequently, 215 µL of Buffer VL, which contains carrier RNA, was added and mixed thoroughly using a pulsed vortex mixer. This mixture was then incubated for 10 minutes at 65°C. Following the incubation, 280 µL of absolute ethanol was

added, and the solution was mixed well. After rinsing the solution with 500 µL of Wash Buffer 1, the column was centrifuged at $5,000 \times g$ for 1 minute, and the flow-through was discarded. Next, the column was washed with 500 µL of Wash Buffer 2, centrifuged at $5,000 \times g$ for 1 minute, and the flow-through was discarded. The column was then washed once more with 500 µL of Wash Buffer 2 and centrifuged for 3 minutes at $14,000 \times g$. Afterwards, the column was placed into a new microcentrifuge tube. To elute the DNA, 50 µL of Elution Buffer was added to the column membrane and allowed to sit for 2 minutes. Finally, the tube was centrifuged at $5,000 \times g$ for 1 minute to release the DNA from the column.

PCR Analysis and Gel Electrophoresis

Nested Polymerase Chain Reaction (PCR) was conducted using a modified protocol from Zainathan *et al.* (2019) and PCR primers as described by Rimmer *et al.* (2012). For the primary and secondary reactions, we used primers C1105 and C1106 for the primary reaction and C1073 and C1074 for the secondary reaction. Each 25 µL PCR mixture contained 12.5 µL of 2X MyTaq™ Mix (Bioline), 9.0 µL of RNase-free water, 0.5 µL of each primary primer, and 2.5 µL of extracted DNA. The amplification program included an initial step of 10 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, concluding with a final extension at 72°C for 5 minutes. Nested PCR was performed with a total of 25 µL PCR mixture containing similar mixtures, with C1073 (0.5 µL, 10 µM) and C1074 (0.5 µL, 10 µM) included instead of the primary reaction primers. The expected amplicon size was 430 bp for the primary primers and 167 bp for the nested primers. Positive controls included sequences derived from *Megalocytivirus* Sabah (JQ253374.1) along with a field-positive sample (Zainathan *et al.*, 2017). The PCR products from both reactions were subsequently analysed by

electrophoresis for 45 minutes at 70 V on a 1.7% (w/v) agarose gel in Tris-acetate-EDTA buffer and stained with SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, Massachusetts, United States of America).

Sequencing and Phylogenetic Analysis

Positive samples (SBFS: Seabass Fish Sample and SBWS: Seabass Water Sample from first sampling; SFE: Seabass Fish Eggs and SW: Seabass Water sample from second sampling) were sent for sequencing analysis to Apical Scientific Sdn. Bhd. The DNA sequencing results were used for phylogenetic analysis. To confirm the likely identification, the sequences were compared against data from the NCBI BLAST database. Various alignments were performed with additional *Megalocytivirus*-related sequences using Clustal X 2.0.12 (Larkin *et al.*, 2007). Finally, a phylogenetic tree was generated from the MCP gene of all known *Iridoviridae* using Molecular Evolutionary Genetics Analysis software (MEGA X) (Johan *et al.*, 2023).

Results

The water parameters measured at the sampling site for *L. calcarifer* included temperature, Dissolved Oxygen (DO), nitrite, and nitrate. All water parameters were within the normal range, except for salinity during the first sampling in 2023 and pH, salinity, and ammonia during the second sampling in 2024 (Table 2). Ammonia, nitrite, and nitrate values were zero ppm during the first sampling in 2023. During the second sampling (2024), pH was consistently below 7 at all sites, while ammonia levels were above 1 ppm at the water outlet. The egg pond showed a higher salinity (26 ppt), as eggs are cultured in a marine environment compared with the brackish environment of the other sites.

Clinical signs were observed on samples from the first sampling (2023), however, samples from the second sampling (2024) did not display

Table 2: Water quality parameters of the water system throughout the sampling at *L. calcarifer* farm

Parameters	2023				2024		
	Pond Water	Egg Pond	Juvenile Pond	Water Outlet	Untreated Reservoir	Treated Reservoir	Normal Range
pH	8 ± 0	4.2 ± 0.5	5.3 ± 0	4.0 ± 0.1	3.8 ± 0.1	3.2 ± 0.2	7.0–8.5
Salinity (ppt)	4.89 ± 0.02	26.3 ± 0	13.6 ± 0.1	15.2 ± 0.1	19.9 ± 0.2	19.7 ± 0.2	10–30
DO (mg/L)	7.03 ± 0.02	5.6 ± 0.1	6.9 ± 0.2	6.4 ± 0.1	5.9 ± 0.2	6.5 ± 0.6	> 3
Temperature (°C)	30.06 ± 0.01	33.3 ± 0.1	30.2 ± 0.2	31.3 ± 0	32.5 ± 0	31.9 ± 0	26–32
Ammonia (ppm)	0.0	0.5 ± 0	0.5 ± 0	1 ± 0	0.5 ± 0	0.5 ± 0	< 1
Nitrite (ppm)	0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	> 0.01
Nitrate (ppm)	0.0	5 ± 0	0 ± 0	10 ± 0	0 ± 0	0 ± 0	0–100

Note: All the parameters are presented as mean ± SD

any clinical signs. During the first sampling, the clinical signs observed in sample two included tail erosion. In contrast, a few other samples (n = 12) exhibited scale loss, mouth haemorrhages, and body erosion (Table 3).

The nested PCR results showed that six pools of *L. calcarifer* samples and water samples (n = 3) from the first sampling (2024) were positive for *Megalocytivirus* (Figure 1 and Table 4). Similarly, three pooled samples of *L. calcarifer* (n = 15), three pooled *L. calcarifer* egg samples (SFE), and culture pond water from various sites (SW) (n = 18) were all positive for *Megalocytivirus* DNA. Thereby, the presence of *Megalocytivirus* from both sampling times was 100%. In this study, all water samples (juvenile pond water from the first and second sampling, egg pond, water outlet, untreated reservoir, and treated reservoir) tested positive for *Megalocytivirus* (Table 4 and Figure 1).

Based on the sequencing analysis, the *Megalocytivirus*-positive samples (SBFS and SBWS) from the first sampling demonstrated 97% to 98% nucleotide similarities to the Infectious Spleen and Kidney Necrosis Virus (ISKNV) strain from *L. calcarifer* and ornamental fish (Figure 2 and Table 5). The positive samples (SFE and SW) from the second sampling showed 97% nucleotide similarity to the complete genome of ISKNV’s major capsid protein from Ghana, China, and Vietnam (Figure 3 and Table 6). Pairwise comparisons of the positive samples in this study showed high nucleotide similarity (97.3% to 98.8%) between samples from the first and second sampling (Table 7). It also indicated that samples from similar sampling times showed high nucleotide similarity (> 98%) to each other.

Table 3: Weight, length, and clinical symptoms observed on *L. calcarifer* samples during the study

Year	Sample	Length (cm)	Weight (g)	Clinical Signs
2023	1	7.3	5	
	2	6.3	3	Scale loss, haemorrhages (operculum), tail erosion
	3	6.9	3	
	4	7	4	
	5	6.5	3	
	6	6.2	3	Tail erosion
	7	7.5	4	
	8	6.5	3	Tail erosion
	9	8.5	5	
	10	5.5	2	Tail erosion
	11	5.7	3	Tail erosion
	12	7.2	5	
	13	6.6	4	Tail erosion, body erosion
	14	5.2	2	Haemorrhages (mouth area), tail erosion
	15	6.9	3	Tail erosion, scale loss
	16	7.5	5	
	17	7	4	
	18	7.1	2	
	19	5.7	2	Tail erosion
	20	6.9	3	
	21	6.8	3	
	22	6	3	Tail erosion
	23	5.5	2	Tail erosion
	24	7.5	4	
	25	8	5	
	26	6.4	3	
	27	6.8	2	
	28	7.5	5	
	29	6.6	3	
	30	6.5	3	Tail erosion
2024	31	2.5	0.22	
	32	2.6	0.36	
	33	2.5	0.25	
	34	2.4	0.22	
	35	2.5	0.26	
	36	2.4	0.2	
	37	2.4	0.3	
	38	2.7	0.31	
	39	2.4	0.22	
	40	2.4	0.25	
	41	2.4	0.21	
	42	2.5	0.31	
	43	2.3	0.2	
	44	2.4	0.27	
	45	2.3	0.21	

Table 4: The total number of positive samples obtained from the first (2023) and second sampling (2024) of *L. calcarifer* and water samples

Year	Samples	Total Positives for Replicates	Total Number of Individuals
2023	Juvenile pond water	3/3	3
	<i>L.calcarifer</i> juveniles	6/6 pools	30
2024	<i>L.calcarifer</i> eggs	3/3	3
	<i>L.calcarifer</i> juveniles	3/3 pools	15
	Egg pond water	3/3	3
	Juvenile pond water	3/3	3
	Water outlet	3/3	3
	Untreated reservoir	3/3	3
	Treated reservoir	3/3	3
Total			66

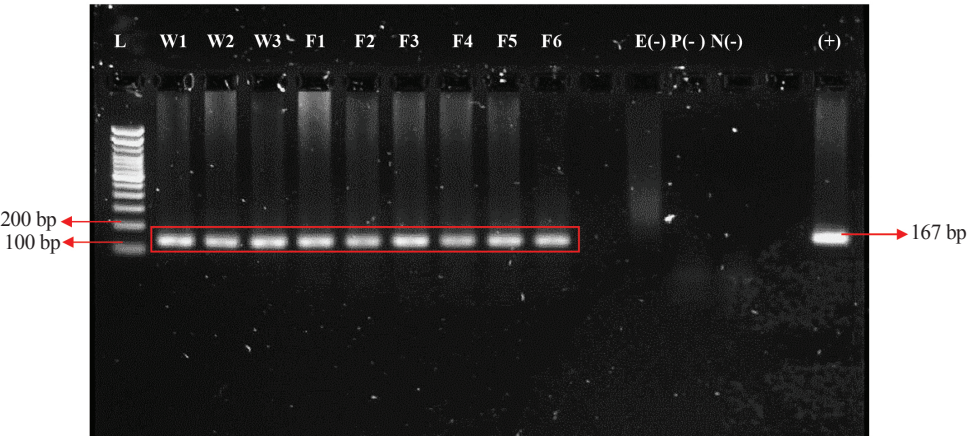


Figure 1: Gel electrophoresis of nested PCR amplification for water samples (n=3) and *L. calcarifer* pooled samples (n = 5) during first sampling

Note: L: 100 bp DNA ladder (Invitrogen) as a molecular weight marker; W1–W3: Water samples; 1 to 3 (Pond water); F1: Fish specimens, 1–5; F2: Fish specimens, 6–10; F3: Fish specimens, 11–15; F4: Fish specimens, 16–20; F5: Fish specimens, 21–25; F6: Fish specimens, 26–30; E(-): Extraction negative control; P(-): Primary PCR negative control; N(-): Nested PCR negative control; (-): Positive control (*Megalocytivirus* Sabah, GenBank accession number JQ253374.1)

Table 5: A comparison between nucleotide sequences of the major capsid protein gene in the positive samples (SBFS and SBWS) from the first sampling and the major capsid protein gene obtained from GenBank using BLAST. The percentage of similarities for a 167 bp segment was computed, uncovering sequence variations within the samples

	1	2	3	4	5	6	7	8	9	10	11	12
1		98	97	97	97	97	97	98	98	98	98	98
2			98	98	98	98	98	98	98	98	98	98
3				100	100	100	100	100	100	100	99	99
4					99	99	99	99	99	99	99	99
5						98	98	98	98	98	98	99
6							100	100	100	100	100	100
7								100	99	99	99	99
8									99	99	99	99
9										99	99	99
10											99	99
11												99
12												

Note: 1 = SBFS (*L. calcarifer* fish sample); 2 = SBWS (pond water sample); 3 = MT178422.1 ISKNV isolate ISKNV/10 MCP gene from Oscar; 4 = MK084827.1 ISKNV isolate M6 MCP gene from molly; 5 = KY440040.1 ISKNV isolate SB04 MCP gene from *L. calcarifer*; 6 = KX354220.1 ISKNV isolate case5 MCP gene from *Pterophyllum scalare*; 7 = OR356116.1 ISKNV isolate NH-2021 from *Siniperca chuatsi*; 8 = OR136939.1 ISKNV isolate KISR_ISKNV2 MCP gene from *L. calcarifer*; 9 = MW883606.1 ISKNV isolate OGIV-PW-2018 from *Osphronemus gourami*; 10 = MW883604.1 ISKNV isolate DGIV_2010 from *Trichogaster lalius*; 11 = MW883603.1 ISKNV isolate HGIV-2015 from *Epinephelus fuscoguttatus* x *Epinephelus polyphemadion*; 12 = MW883602.1 ISKNV isolate XMIV-2015 from *Xiphophorus maculatus*

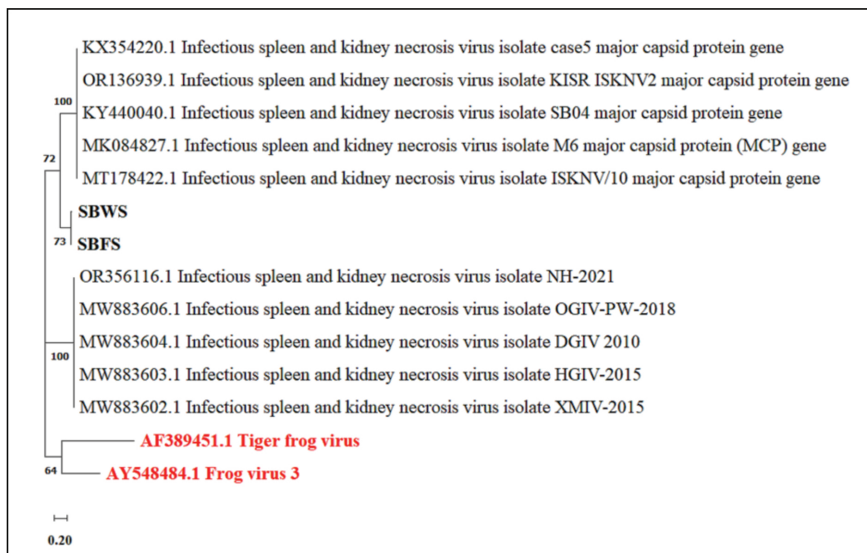


Figure 2: The phylogenetic tree was constructed for 167 bp segments based on major capsid protein gene sequences of ISKNV screened in 10 of the *Megalocytivirus*-positive samples from the first sampling (black bold) and 6 reference viruses from the genus *Megalocytivirus* using the neighbour-joining method with 1,000 bootstrap replicates. Tiger frog virus (AF389451.1) and Frog virus 3 (AY548484.1) (red bold) were included as outgroups. The scale bar indicates distance values, GenBank accession numbers are shown next to the virus species, and bootstrap values (%) are shown next to the clades. The tree is drawn to scale (0.02), with branch lengths measured in the number of substitutions per site

Table 6: A comparison between nucleotide sequences of the MCP gene in the positive samples (SFE and SW) from the second sampling and the MCP gene obtained from GenBank using BLAST. The percentage of similarities for a 167 bp segment was computed, uncovering sequence variations within the samples

	1	2	3	4	5	6	7	8	9	10	11	12
1		98	97	97	97	97	97	97	97	97	97	97
2			97	97	97	97	97	97	97	97	97	97
3				97	97	97	97	97	97	97	97	97
4					97	97	97	97	97	97	97	97
5						97	97	97	97	97	97	97
6							97	97	97	97	97	97
7								97	97	97	97	97
8									97	97	97	97
9										97	97	97
10											97	97
11												97
12												

Note: 1 = SFE (*L. calcarifer* fish eggs); 2 = SW (untreated reservoir water sample); 3 = OP689648.1 ISKNV UG-WACCBIP/BCMB-LV-BC91 complete MCP from Ghana; 4 = PP151097.1 ISKNV-ASB-23 complete genome from China; 5 = OP689623.1 UG-WACCBIP/BCMB-LV-BC58 complete MCP from Ghana; 6 = OP689640.1 UG-WACCBIP/BCMB-LV-BC76 complete MCP from Ghana 7 = OP689621.1UG-WACCBIP/BCMB-LV-BC56 complete MCP from Ghana; 8 = OP689624.1 UG-WACCBIP/BCMB-LV-BC59 complete MCP from Ghana; 9 = OP689633.1 UG-WACCBIP/BCMB-LV-BC68 complete MCP from Ghana; 10 = OP689626.1 UG-WACCBIP/BCMB-LV-BC61 complete MCP from Ghana; 11 = OP689616.1 UG-WACCBIP/BCMB-LV-BC51 from Ghana; 12 = KY440040.1 ISKNV isolate SB04 MCP gene from *L. calcarifer*

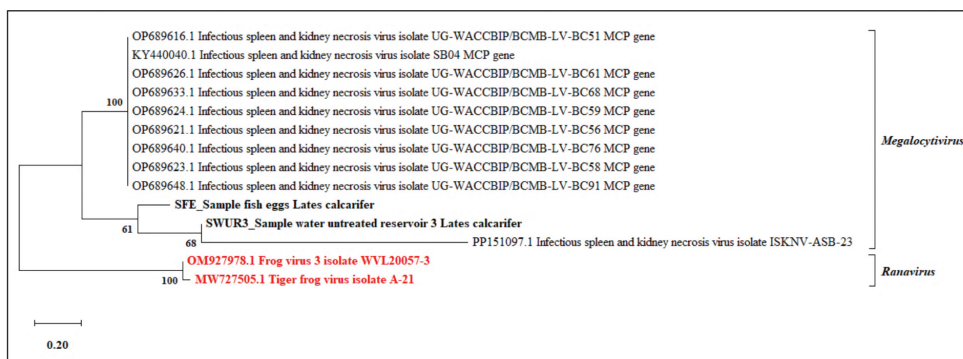


Figure 3: The phylogenetic tree was constructed for 167 bp segments based on MCP gene sequences of ISKNV screened in two of the *Megalocytivirus*-positive samples from the second sampling (black bold) and 10 reference viruses from the genus *Megalocytivirus* using the neighbour-joining method with 1,000 bootstrap replicates

Note: Blast search of the nucleotide sequences from this study showed high similarities and sequence identities to ISKNV/10 which are ISKNV isolate BC51 MCP gene from *Oreochromis* sp. (OP689616.1), ISKNV isolate SB04 MCP gene from *L. calcarifer* (KY440040.1), ISKNV isolate BC61 MCP gene from *Oreochromis* sp. (OP689626.1), ISKNV isolate BC68 MCP gene from *Oreochromis* sp. (OP689633.1), ISKNV isolate BC59 MCP gene from *Oreochromis* sp. (OP689624.1), ISKNV isolate BC56 MCP gene from *Oreochromis* sp. (OP689621.1), ISKNV isolate BC76 MCP gene from *Oreochromis* sp. (OP689640.1), ISKNV isolate BC58 MCP gene from *Oreochromis* sp. (OP689623.1), ISKNV isolate BC91 MCP gene from *Oreochromis* sp. (OP689648.1), ISKNV isolate ASB-23 MCP gene from *L. calcarifer* (PP151097.1). Tiger frog virus (MW727505.1) and Frog virus 3 (OM927978.1) (red bold) were included as outgroups. The scale bar indicates distance values, GenBank accession numbers are presented next to the virus species, and bootstrap values (%) are shown next to the clades. The tree is drawn to scale (0.20), with branches measured in substitutions per site.

Table 7: Pairwise sequence comparison of the positive samples

Samples	SBFS	SBWS	SFE	SW
SBFS	-	98.8	96.8	97.3
SBWS	98.8	-	96.9	97.3
SFE	96.8	96.9	-	98.4
SW	97.3	97.3	98.4	-

Note: SBFS: *L. calcarifer* juvenile and SBWS: Pond water from first sampling; SFE: *L. calcarifer* fish egg and SW: Untreated reservoir pond water from second sampling

Discussions

All water parameters were within the normal range, except for salinity during the first sampling in 2023 and pH, salinity, and ammonia levels during the second sampling in 2024. The optimal temperature for *L. calcarifer* culture is between 26°C to 32°C (Islam *et al.*, 2023). Adequate DO above 3 mg/L (Abdullah *et al.*, 2018) is essential for fish growth, as low levels can lead to disease outbreaks (Null *et al.*, 2017; Ali *et al.*, 2022). The optimal salinity range for *L. calcarifer* culture is 10 to 30 ppt (Abdullah *et al.*, 2018). *L. calcarifer* is adaptable to freshwater, brackish, and marine environments (Wijayanto *et al.*, 2021). pH is crucial for *L. calcarifer*’s survival; levels below seven can cause stress, and below five can be fatal (Adeyinka *et al.*, 2020). pH fluctuates with seasons, typically rising in warmer months and decreasing during the rainy season (Adeyinka *et al.*, 2020), with normal ranges between seven and eight and a half (Abdullah *et al.*, 2018). High ammonia levels at the water outlet originate from sediment, where organic matter decomposes and releases ammonia into the water column (Hargreaves *et al.*, 2004). Thus, water quality monitors are essential to improve *L. calcarifer* health on the farm.

Clinical signs associated with *Megalocytivirus* (including tail erosion, scale loss, haemorrhages in the mouth, and body erosion) were observed in fish samples from the first sampling. Similarly, *Megalocytivirus*-positive *L. calcarifer* samples have been reported to demonstrate scale loss, tail erosion,

and ventral haemorrhages (De Groof *et al.*, 2015; Senapin *et al.*, 2019; Nurliyana *et al.*, 2022). Additionally, *Megalocytivirus*-positive *Oreochromis niloticus* samples from Ghana exhibited comparable clinical signs, including tail erosion and scale loss (Ayiku *et al.*, 2024). In India, *Megalocytivirus*-positive *Osphronemus goramy* (*O. goramy*) also showed scale loss as an external clinical sign (Novita *et al.*, 2021). Furthermore, *O. goramy* samples from West Java showed scale loss and body erosion, indicating ISKNV infection (Novita *et al.*, 2021). However, fish samples from the second sampling showed no clinical signs. *Megalocytivirus*-infected fish have been reported to be asymptomatic or apparently healthy due to the asymptomatic carrier stage (reviewed by Johan & Zainathan, 2023). These findings emphasise the importance of early clinical diagnosis in preventing the spread of *Megalocytivirus* infections within aquaculture.

The nested PCR analysis confirmed positive results for all the fish samples. Previously, a Malaysian study showed that 10 samples of *L. calcarifer* were positive for *Megalocytivirus* strain SDDV using PCR (Nurliyana *et al.*, 2020). Zhu *et al.* (2021) confirmed that the causative agent of disease in *Lates calcarifer* from China was ISKNV genotype II (ISKNV-II) of *Megalocytivirus*, using virus isolation and molecular detection. In addition, ISKNV genotype I has been detected via semi-nested PCR in fingerlings and juvenile *L. calcarifer* from Thailand (Kerddee *et al.*, 2021).

Similarly, *Megalocytivirus*-positive ISKNV strain genotype I was detected via nested PCR from cultured *Epinephelus fuscoguttatus* from Malaysia (Zainathan *et al.*, 2022). A semi-nested PCR method was also implemented to detect *Megalocytivirus* in Taiwan, which showed that 35 *L. calcarifer* samples were positive for the TRBIV strain. In 2020, Thanasaksiri *et al.* (2020) reported the first case of ISKNV infection in Asian seabass in Indonesia. PCR analysis of five diseased Asian seabass from East Java, Indonesia, detected the major capsid protein gene of Iridovirus (Thanasaksiri *et al.*, 2020).

The presence of *Megalocytivirus* was confirmed in all the water samples. Samples were collected in triplicate to increase the chances of detecting *Megalocytivirus*. Treated reservoir water samples tested positive for *Megalocytivirus*. According to the farmer, they apply 30 ppm chlorine for every 20 m³ of juvenile ponds and treated reservoir ponds. However, no chlorine disinfection is applied for egg ponds. While chlorine disinfection can effectively control certain viruses (Ben-Asher *et al.*, 2019), the chlorine application may have been insufficient or incorrect in the treated reservoirs, allowing *Megalocytivirus* to persist. Free chlorine species have been shown to inactivate a range of viruses at C-t (concentration × time) values between 15 and over 2,000 MgCl₂/L·min (Munang'andu *et al.*, 2016). *Megalocytivirus* can be transmitted via contaminated water (Kawato *et al.*, 2021; Johan *et al.*, 2023), and untreated water can facilitate horizontal transmission (Johan & Zainathan, 2020).

In Korea, *Megalocytivirus* RSIV strain has been shown to thrive in seawater at 15°C for up to a week (Kim *et al.*, 2022). Poor biosecurity in fish farming could further contribute to the spread of the virus (Johan *et al.*, 2023) as the farm in this study does not implement biosecurity protocols thoroughly. Based on PCR analysis, this study showed 100% presence of *Megalocytivirus* DNA in all samples. Therefore, it is vital to further analyse water samples with

a larger sample size and longer sampling time to confirm horizontal transmission. This will strengthen the water treatment protocols to minimise *Megalocytivirus* in *L. calcarifer* farms.

Sequencing and phylogenetic analyses showed that the positive samples shared 97% to 98% nucleotide similarity with ISKNV. ISKNV infection has never been reported in *L. calcarifer* from Malaysia. However, Nurliyana *et al.* (2020) reported the first evidence of SDDV infection (sharing 68% genome identity with *Megalocytivirus*) in marine cage-cultured *L. calcarifer* in Malaysia. SDDV occurrences were initially confirmed in Singapore, Indonesia, and Thailand (Nurliyana *et al.*, 2020). SDDV isolates from Malaysia shared 100% nucleotide similarities to Singaporean and Indonesian isolates (GenBank accession numbers MH152405, MH152404, and KR139659) (Nurliyana *et al.*, 2020). In 2018, 40% to 50% cumulative mortalities were reported in adult Asian seabass (*Lates calcarifer*) cultured in an inland freshwater system in Thailand due to ISKNV infection (Kerddee *et al.*, 2021). Out of the 26 fish sampled in this study, 20 samples were positive for ISKNV from six farms. Sequencing analysis revealed 100% similarity to ISKNV clade I (GenBank accession numbers MW269579 and MW269580) (Kerddee *et al.*, 2021). Similarly, Thanasaksiri *et al.* (2020) found that virus isolates from diseased Asian seabass in East Java, Indonesia, were closely related to ISKNV and were classified within genotype I. Based on the sequencing and phylogenetic tree results in this study, the regional distribution pattern of ISKNV suggests that cross-border fish commerce and waterborne transmission may contribute to the virus's widespread prevalence.

Conclusion

This study reports the recent detection of the *Megalocytivirus* ISKNV strain in *L. calcarifer* (eggs and juveniles) and water samples (egg pond water, juvenile pond water, water outlet, treated and untreated reservoir pond water) from a small-scale *L. calcarifer* culture farm in Malaysia. The virus was detected using a nested

PCR assay. In total, 66 sample replicates of *L. calcarifer* and water samples from all sites tested positive for *Megalocytivirus*. Clinical signs in *Megalocytivirus*-positive *L. calcarifer* samples included scale loss, haemorrhages at the operculum, haemorrhage at the mouth, tail erosion, and body erosion. Sequencing analysis showed that all positive samples exhibited 97% and 98% nucleotide similarities to the ISKNV strain found in Asian countries. Further confirmatory studies on horizontal transmission via water and the vertical transmission of *Megalocytivirus* are crucial for identifying the source of infection in farmed fish, as this directly affects the overall production of *L. calcarifer*. In addition, it is recommended that best aquaculture practices be adopted across all farms. Enhancing biosecurity measures is vital; this may include treating water with appropriate chemicals that do not harm the aquatic environment, as well as sanitising equipment prior to use.

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Conflict of Interest Statement

The authors agree that this research was conducted in the absence of any self-benefits, commercial, or financial conflicts and declare absence of conflicting interests with the funders.

Author Contributions Statement

ECZM and NANA carried out the research. SCZ and YKF conceptualised the central research idea and provided the theoretical framework. SCZ and YKF designed the research, SCZ supervised research progress; CACJ assisted

ECZM and NANA in data analysis, manuscript was written by ECZM, NANA, and CACJ; SCZ reviewed the manuscript and approved the article submission.

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