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Outbreak investigation attributes *Infectious spleen and kidney necrosis virus* as a necessary cause of a mortality epidemic in farmed grouper (*Epinephelus* spp.) in Bali, Indonesia

Cahya Fusianto [a,b], Paul M. Hick [b], Murwantoko [c], Afri Herlambang [c], Richard J. Whittington [b], Joy A. Becker [a,b,*]

[a] *The University of Sydney, School of Life and Environmental Sciences, Australia*
[b] *The University of Sydney, Sydney School of Veterinary Science, Australia*
[c] *Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Indonesia*

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ABSTRACT

In 2016, famers reported mass mortality events in hybrid grouper within 2–4 weeks following transfer to sea cages on the northern coast of Bali. The objective was to obtain an aetiological diagnosis using a broad range of traditional and emerging diagnostic approaches. A group of 24 and 12 fish with and without clinical signs were sampled from 10 affected populations at 9 farms. Samples for histopathology, ectoparasites evaluation and molecular approaches for microbiology were obtained with a diagnostic post-mortem examination. Fish with clinical signs had a significantly higher likelihood of having pale anterior and posterior kidneys and a liver that was pale and reduced in size compared to fish without clinical signs. There were no differences in the prevalence and quantity of *Megalocytivus* (MCV) or nervous necrosis virus (NNV) in tissues observed from fish with and without clinical signs. Nearly 55 % of fish were infected with NNV irrespective of clinical signs. There were no histopathological lesions consistent with virial nervous necrosis and the NNV infections were considered subclinical. 80 % of grouper were infected with MCV, irrespective of clinical signs. A significant proportion of fish with clinical signs (true prevalence 94.4 %; 95 % CI 79–100) had observed megalocytes and pathology consistent with disease caused by ISKNV compared to those without clinical signs (true prevalence 47.2 %; 95 % CI 27–70). Metagenomic sequences generated using Illumina Miseq and taxonomically labelled using BlastN + revealed that the *Megalocytivirus* was 99.9 % similar to *Infectious spleen and kidney necrosis virus* (ISKNV). The unbiased sequencing did not detect any novel DNA viruses or bacterial pathogens of clinical significance. The monogenean, *Benedenia epinepheli* and a leech, *Zeylanicobdela arugamensis* were detected at 6/9 and 9/9 farms, respectively. Our approach identified several pathogens reported in grouper aquaculture with histopathology showing that ISKNV was a necessary cause for the mass mortality events.

1. Introduction

Grouper aquaculture is a vital and growing source of income in rural communities across in Indonesia (Rimmer and Glamuzina, 2019). This industry is valued at $103 M AUD and supports rural livelihoods through employment and new job opportunities across the value chain (Rimmer et al., 2013). Globally, Indonesia is the third largest producer of grouper and in 2015, exported an estimated 5.6 million fingerlings for grow out in other countries (Rimmer and Glamuzina, 2019). The main market for farmed grouper is live export which can provide considerable revenue with capital payback periods generally less than 1 year for small-scale farms (Pomeroy et al., 2006).

First generation hybrids of grouper species have been widely cultured in South East Asia since first being introduced in 1999 (Kiriyakit et al., 2011; Sun et al., 2016). Superior characteristics include high egg hatching rate and larval survival, rapid growth, tolerance for ocean acidification (Mustafa et al., 2014) and disease resistance (Bunlipatanon and U-taynapun, 2017). The most frequently farmed hybrids are Cantang obtained as a cross from ♀ *Epinephelus fuscoguttatus* and ♂ *E. lanceolatus* and Cantik as a cross from ♀ *Epinephelus fuscoguttatus* x ♂

* Corresponding author at: The University of Sydney, School of Life and Environmental Sciences, Australia.
E-mail address: joy.becker@sydney.edu.au (J.A. Becker).

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E. polyphekadion. Cantang and Cantik hybrids have a better growth rate, food conversion ratio, and survival rate compared to *E. fuscoguttatus* (Sutarmat and Yudha, 2013) and have become more popular for aquaculture in Indonesia compared to non-hybrid species (Fachry et al., 2018).

A constraint on grouper mariculture is inconsistent production from the impact of infectious diseases and repeated significant mortality events (Rimmer and Glamuzina, 2019). Vibriosis is the most commonly reported bacterial disease in grouper culture (Cameron, 2001; Chong et al., 2011). Although *Vibrio* species are commonly isolated during disease events, they are not usually considered the primary cause of disease (Cameron, 2001) and most likely have a pathogenic role in multifactorial disease. Infestations with monogenean ectoparasites (e.g. *Benedenia* sp., *Neobenedenia sp*. and *Pseudorhabdosynochus* sp.) are highly prevalent in grouper aquaculture and cause skin lesions which are the site for secondary bacterial infection (Rimmer et al., 2013). *Vibrio alginolitycus, Vibrio harveyi,* and *Streptococcus iniae* have caused mass mortality of hybrid grouper in sea cages in east Java, Indonesia (Arif et al., 2016), Japan (Kusuda and Salati, 1993) and Taiwan (Lee, 1995).

The predominant viral pathogens in grouper aquaculture are from the Family *Iridoviridae,* genera *Ranavirus*, (Ma et al., 2016) and *Megalocytivirus*, and Family *Nodaviridae*, nervous necrosis virus (NNV) (Ma et al., 2012, 2016). NNV is an RNA virus that can cause the disease viral nervous necrosis (VNN) which is characterised by abnormal swimming and high mortality (e.g. 80–100 %) in larval and young juvenile, predominantly marine fish. Infections are frequently subclinical in adult fish with the exception of a few species, notably adult grouper (Chi et al., 2016). Megalocytiviruses (MCVs) are large double stranded DNA viruses which cause a characteristic histopathological lesion of inclusion body cells in liver, kidneys, and spleen (Subramaniam et al., 2012). Grouper are known to develop persistent sub-clinical infections with both NNV and MCV with disease possible in all age classes (Ma et al., 2012; Jitrakorn et al., 2020; Sah Putra et al., 2020).

The megalocytivirus, *Infectious spleen and kidney necrosis virus* (ISKNV) was first reported from China following a mass mortality in the freshwater species, mandarin fish (*Siniperca chuatsi*) (He et al., 2000). ISKNV has spread worldwide and caused mortality in grouper aquaculture in Taiwan (Huang et al., 2011), Indonesia (Mahardika et al., 2008), and Malaysia (Razak et al., 2014). Clinical signs of a megalocytivirus infection caused by ISKNV include anorexia, inappropriate regulation of body colour (darker or lighter), and resting at the bottom of the cage with consequent high mortality, in the range 50–90 % (Subramaniam et al., 2012). Red seabream iridovirus (RSIV) is a genotype of the ISKNV species that provides the pathogen name listed by the OIE (OIE, 2019). The ISKNV and RSIV genotypes of ISKNV infect a similar range of marine species used in aquaculture across East and Southeast Asia. Repeated epidemics in barramundi (*Lates calcarifer*) farmed in Vietnam caused by the ISKNV genotype were confused with RSIV infections and an inappropriate vaccine strategy was used (Dong et al., 2017). This was because the PCR assay used was not able to differentiate between ISKNV and RSIV and the typical histopathological lesions for ISKNV infections were not observed (Dong et al., 2017). After the failed vaccine preventative measures, sequence analysis of the major capsid protein confirmed ISKNV infection caused the epidemics rather than RSIV, which was not found (Dong et al., 2017). Grouper sleepy disease is caused by infection with *Singapore grouper iridovirus* (SGIV), a ranavirus. This has been a significant cause of mortality in brown-spotted grouper (*Epinephelus tauvina*) (Chua et al., 1994). Fish infected with SGIV will display similar clinical signs to megalocytivirus infections, including splenomegaly (Qin et al., 2002). Molecular methods can detect persistent infections with viral pathogens in fish with subclinical infection. Further, these viruses have large and overlapping host and geographical ranges which can lead to delays in identifying the primary cause of mortality events and actioning appropriate treatment programs.

Advances in nucleotide sequencing technology have enabled breakthroughs for investigating infectious diseases in which pathogen culture and molecular tests for known pathogens are not available. Metagenomic methods do not require knowledge of the sequence of potential pathogens as they use unbiased approaches to generating sequence information from biological samples that may reveal unknown or novel pathogens (Cheval et al., 2011). For example, a novel *Reovirus* was identified in farmed Atlantic salmon (*Salmo salar*) with heart and skeletal muscle inflammation using a metagenomics approach (Palacios et al., 2010). A veterinary epidemiological framework and complete disease investigation process is needed for this new technology to be useful in attributing a role in disease causation for the detected viruses. Critical to this is the use of histopathology to investigate the disease process and determine the prevalence of diseased animals.

In 2016, a mass mortality disease of hybrid groupers was reported in grow-out farms in north Bali. Farmers reported mortalities began soon after transfer to the sea cages and were associated with the appearance of clinical signs such as inappetence and lethargy. The objective of this study was to obtain an aetiological diagnosis for a disease syndrome considered novel by the farmers that had become prominent in sea-cage grouper production. Specifically, an active targeted surveillance strategy was implemented to source appropriate farm data and specimens with application of a broad range of clinical and laboratory investigation techniques.

2. Materials and methods

2.1. Site description

The disease investigation was undertaken along the northern coast of Bali, Indonesia from August to November 2016. The sampling frame for the study was all grouper sea cages in Pegametan and Sumber Kima Bays during the rainy season (July-December) of 2016. There were 12 farms operating during the time of sampling (Fig. 1). The surface sea temperature data was derived from radiance measurements collected every 8 days by the Moderate Resolution Imaging Spectroradiometer (MODIS) instruments aboard NASA's Terra and Aqua satellites (https://neo.sci.gsfc.nasa.gov/).

2.2. Study design

A prospective strategy for targeted disease detection was used to obtain diagnostic specimens from cases of mass mortality of grouper. Early identification and efficient case detection was encouraged by engaging with farmers during a series of workshops offered to all staff of the sea-cage grouper farms to share information about diagnosis of fish diseases. At the workshops, farmers reported mortality began two to four weeks after transfer to the sea cages and within seven days of showing signs. They reported the final cumulative mortality would be 80–90 % over 14 days. Farmers observed the fish to show signs of inappetence, inactivity with non-responsiveness to stimuli (e.g. being captured in a net) and they reported some fish had a green discoloration on eyes and body surface.

The case definition was the sudden onset of mortality affecting at least 20 % of the population and with signs of inappetence and non-responsiveness. All farms agreed to participate by notifying of any cases in populations of grouper in sea cages and providing access to collect information and specimens. Project staff from Universitas Gadjah Mada were stationed in the area for four months ready to respond immediately to any farmer reports of disease by conducting sampling for the disease investigation.

2.3. Fish collection

The targeted approach to disease detection involved sampling affected populations at the time of disease outbreak under the approval of the University of Sydney Animal Ethics Committee (AEC 2013/6027).

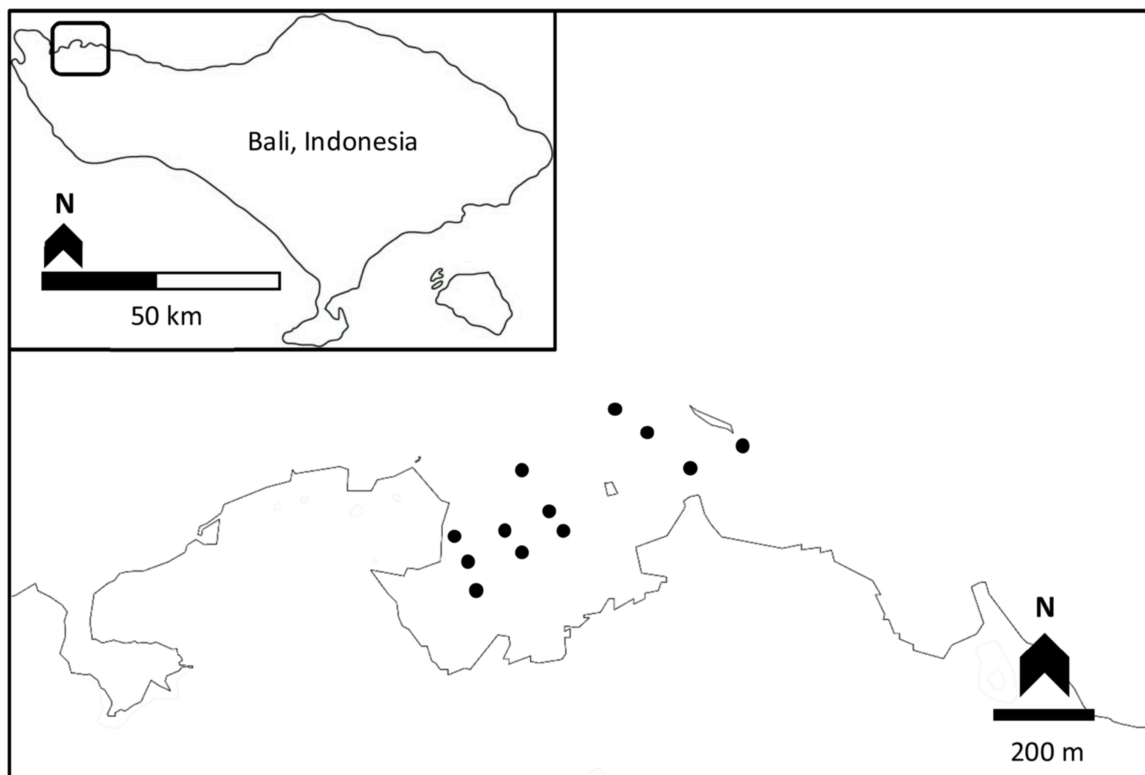

Fig. 1. Sampling locations of the 12 grouper grow-out farms in Bali, Indonesia (Inset) (Map generated using Mapbox ©).

Populations of fish within each farm were defined as all fish of the same species (or hybrid) and age that were obtained at the same time from one source.

A sample size of 36 fish was obtained by hand-netting from the affected population and consisted of 24 fish with typical clinical signs and 12 fish without clinical signs. This design aimed to sample fish at different stages of the disease. The sample size was calculated based on an estimated 70 % sensitivity and 90 % specificity for the series of diagnostic steps, to be 95 % certain of detecting a disease process with a minimum expected prevalence of 90 %. The sample size calculation used the Epitools epidemiological calculator for tests with imperfect sensitivity and specificity (https://epitools.ausvet.com.au/freecalctwo) (Cameron and Baldock, 1998).

2.4. Examination for ectoparasites

A skin mucus scrape and a gill and fin biopsy were obtained on farm from each sampled fish for ectoparasite examination following brief anaesthesia with clove oil (0.5 mL/L). Specimens were placed immediately onto glass microscope slides with a drop of filtered sea water and a cover slip. Observations at 40x, 200x and 400x magnification were conducted with a light microscope (Olympus CX31) connected to a laptop computer using an Optilab microscope camera (Miconos). Parasites were counted and representative exemplar specimens from each population were collected in 80 % ethanol for subsequent identification.

Identification was based on morphology and 18 s RNA gene sequence approaches. DNA purification was performed as described in Section 2.6. The PCR reaction was performed in a final volume of 50 μL containing 24 μL FastStart™ PCR Master (Roche), 20 μL nuclease free water, 2 μL DNA sample, 2 μL each of forward and reserve primers (Supplemental Table S1). PCR assays were performed using a T100 Thermocycler (Biorad) with the following profile: one cycle of denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min and one cycle of final extension 72 °C for 5 min. PCR products were visualised by gel electrophoresis on a 1% agarose gel containing 0.003 % FloroSafe DNA staining (1st Base) and DNA Marker XIV (Roche) was used for size comparison. Sequencing of 18 s rRNA was done using BigDye® Terminator v3.1 cycle sequencing kit chemistry on ABI PRISM®3700 DNA Analyzer. Taxonomic labelling of 18 s rRNA sequences was completed by MegaBLAST on the NCBI website (blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Necropsy and tissue collection

Fish were euthanized using clove oil in seawater and then measured and weighed. A necropsy was undertaken while avoiding cross contamination between individual fish. Necropsy instruments and work surfaces were disinfected by immersion in 200 ppm sodium hypochlorite for 10 min, rinsing in water and spraying with 70 % ethanol between each use. New sterile disposable scalpel blades were used and gloves were changed for each fish.

Specimens were preserved for nucleic acid purification from 12 fish with clinical signs and six fish without clinical signs. Samples of a portion of the liver, spleen, anterior and posterior kidney from each individual fish were pooled in a 5 mL tube. Samples of the retina and brain from individual fish were pooled in a separate 5 mL tube containing. A 3:1 volumetric ratio of 80 % ethanol was used for nucleic acid preservation. Brain, eye, gill, heart, liver, spleen, anterior and posterior kidney were collected from the remaining fish (i.e. 12 with, 6 without clinical signs) and preserved in 10 times volume of 10 % neutral buffered formalin for histopathology.

2.6. Nucleic acid purification

Nucleic acid purification was conducted at the Universitas Gadjah Mada, Indonesia. Ethanol was removed by evaporation and preserved tissues were rinsed once in PBS. Nucleic acids for MCV testing were purified from a pool of 0.15 g of tissues with equal parts subsampled from the liver, kidney and spleen of individual fish. The High Pure Viral

Nucleic Acid Kit (Roche®) was used according to the instruction manual. The tissue sample was homogenised by manual grinding using a plastic pestle (Geneaid) in 400 μL binding buffer with addition of 50 μL Proteinase K followed by incubation at 72 °C for 10 min. Nucleic acids for NNV assays were purified from a 0.15 g subsample of equal parts retina and brain tissue from individual fish. The High Pure Viral RNA Kit (Roche®) was used according to the instruction manual. The final elution volume for each procedure and was 50 μL. Purified nucleic acids were stored at −20 °C for approximately one week before being were shipped on dry ice to the University of Sydney and stored at −80 °C until testing.

2.7. qPCR assay for MCV

A previously described qPCR test for ISKNV and RSIV was applied to detect and quantify the MCV genome (Rimmer et al., 2012). This assay was expected to detect multiple genotypes within the genus *Megalocytivirus*. Briefly, individual samples were tested in duplicate 25 μL reactions containing: 12.5 μL of Quantitech SYBR green master mix (Qiagen); 250 nM each of the forward (C1073) and reverse (C1074) primers; 2.5 μL of template DNA; and 7.25 μL molecular biology grade nuclease free water. The controls used were: a tissue homogenate from a fish confirmed to be infected with ISKNV; a negative extraction control; and a no template control (water). A standard curve was prepared for each run by amplification of the standard pDGIV-MCP1 which contained the MCV major capsid protien gene sequence in a preparation of linearised plasmid DNA (Rimmer et al., 2012). For each PCR plate, duplicate reactions were prepared from a 7-step, 10-fold dilution series contianing 10^1 to 10^7 copies of the standard in molecular grade water. The PCR assay was performed using an Mx3000 P Multiplex Quantitative PCR System (Stratagene) under the following conditions: 1 cycle of initial denaturation at 95 °C for 15 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s, with fluorescence acquisition at the end of the annealing step. A dissociation curve was determined after the amplification cycles by: dissociation of the reaction products at 95 °C for 1 min; annealing at 55 °C for 30 s; and then heating to 95 °C at a rate of 1 °C every 30 s. A valid PCR run was defined as amplification of both replicates of the positive control with a cycle threshold (Ct) within the range of the standard curve ($r^2 > 0.95$ and efficiency between 90 and 110 %) and no amplification of negative controls. A fluorescence threshold was determined by the Mx3000p software (Stratagene) based on amplification of the plasmid standard and applied to the experimental samples. A threshold cycle (Ct) was assigned to samples when the ROX normalised baseline corrected SYBR signal exceeded the threshold. A sample was considered positive for MCV when a threshold cycle (Ct) value was obtained for at least one of the duplicate reactions and the melting temperature of the product was within the range +/−0.5 °C of the positive control sample. Quantification of viral DNA in positive samples was determined by interpolation from the plasmid DNA standard curve. A sample was classified as below the level of quantification (BLOQ) when a positive result was obtained but the copy number for the MCV genome was below 100 (Rimmer et al., 2012).

2.8. RT-qPCR assay for nervous necrosis virus

Detection and quantification of NNV by RT-qPCR with hydrolysis probe detection chemistry was completed as described previously (Hick and Whittington, 2010). Briefly, samples were tested in duplicate using 5 μL of purified nucleic acid as template in 25 μL reactions prepared using the QuantiTect Virus OneStep qRT-PCR kit (Qiagen). The primers QR2TF and QR2TR were used at a concentration of 250 nM and QR2T probe at 200 nM. Each plate included a positive control prepared from a fish tissue homogenate confirmed to be infected with NNV, a negative extraction control and a no template RT-PCR control (nuclease free water). Preparation of dilution series and amplification of plasmid standards containing the RNA2 NNV capsid protein gene sequence was as previously described (Hick and Whittington, 2010). The RT-qPCR assay was performed using an Mx3000 P Multiplex Quantitative PCR System (Stratagene) with the following conditions: 1 cycle of reverse transcription at 45 °C for 10 min; 1 cycle of hot start activation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 45 s. The FAM fluorescence threshold determination was described as above. A sample was considered positive for NNV if at least one of the duplicate reactions had a logarithmic increase in fluorescence that exceeded the threshold. A Ct value was assigned and the number of copies of the RNA2 gene was estimated by extrapolation from the standard curve within the range where efficiency was between 90–110 %. A sample was classified as below level of quantification (BLOQ) when the copy number of the virus was below 100 (Hick and Whittington, 2010).

2.9. Histopathology

Histopathological examination was undertaken on 12 fish with clinical signs and 6 fish without clinical signs from each population. At Universitas Gadjah Mada, standard techniques were used to prepare haematoxylin and eosin-stained slides with gill, heart, liver, spleen, anterior and posterior kidney, retina and brain tissues by embedding in paraffin followed by sectioning at 5 μm. To evaluate the relevance of positive PCR tests for MCV and NNV, a systematic observation of all available kidney, liver, spleen and brain and retina sections was undertaken at magnification up to 400 × . Fish with megalocytic inclusion bearing cells in one or more tissues were considered positive for disease associated with MCV infection. Fish with vacuolar lesions in the brain or retina were considered positive for VNN disease associated with NNV infection.

2.10. Unbiased sequencing for potential novel pathogens

An unbiased approach to nucleic acid sequence determination was used to test for a potential novel pathogen associated with this disease syndrome (ie RNA viruses were excluded). It was presumed that sequence from a relevant pathogen would be present at relatively high abundance and high prevalence in samples of pooled internal organs from clinically affected fish. The sample size (n = 8) was calculated to be 95 % confident of identifying a pathogen when the minimum expected prevalence was 90 % with an assumption that the test sensitivity was 90 % and specificity was 100 %. To increase the confidence of identifying a relevant pathogen, 8 samples were selected from clinically affected fish representing a range of affected populations and considered most likely to be diagnostically relevant based on observations at necropsy and histopathology (Supplemental Table S2).

2.10.1. DNA purification and sequencing

A phenol-chloroform precipitation method was used to purify nucleic acids for unbiased sequencing (Green and Sambrook, 2012). A pool of 0.5 g of equal parts of liver, spleen, and kidney were removed from ethanol and washed with PBS. The pooled tissues were homogenised by grinding using plastic pestles in RLT lysis buffer (Qiagen) with addition of 50 μL of 20 mg/mL proteinase K (Sigma-Aldrich) before overnight incubation at 56 °C. After the tissues were completely lysed, the preparation was treated to remove RNA using DNase-free RNase A (2 μg/mL, Qiagen) with incubation at 37 °C for 1 h. Addition of 400 μL phenol:chloroform:isoamyl-alcohol (25:24:1) with incubation at ambient temperature for 15 min was done in a fume hood (Conditionaire International). The aqueous layer was collected after centrifugation at 2000 g for 10 min. DNA precipitation from the aqueous layer was performed by adding 1/20 vol of 3 M Sodium acetate and 3 volumes of absolute ethanol followed by an overnight incubation at −20 °C. The DNA precipitation continued by adding 500 μL of 70 % ethanol (−20 °C), followed by air drying. The DNA pellet was resuspended in

100 μL of Tris-EDTA buffer (pH 8).

The purity and quantity of the DNA was measured using a Qubit® dsDNA BR Assay Kits (Invitrogen) and NanoDrop spectrophotometer 1000 (Thermo Fisher scientific), assessed as an A260/280 ratio between 1.8−2.0. DNA concentration was adjusted to 20 ng/μL by dilution. Nucleic acid samples were submitted to the Australian Genome Research Facility (AGRF) for library preparation using Nextera XT DNA Library Preparation Kit (Illumina). Sequencing used the MiSeq V2 (Illumina) chemistry for 150 bp paired end reads with 4 samples per lane and 300 Cycles to obtain at least 80x depth coverage.

2.10.2. Sequence analysis

Sequences were trimmed to remove Illumina adaptor sequence and exclude low quality base calls using Trimmomatic (Bolger et al., 2014). A minimum Phred score of 20 was applied to provide base call accuracy of 99 % (Ewing et al., 1998). The Fastq files were paired using FASTQ joiner (Blankenberg and Gordon, 2010). As the genome sequence for grouper was not available, sequence presumed to be the host genome was removed from the dataset by mapping the reads to the whole genome of multiple fish species: barramundi (FQ310508.3, FQ310507.3, FQ301506.3), zebra fish (*Danio rerio*) (NC_007112.7-NC_007124.7), large yellow croaker (*Larimichthys crocea*) (NC_040011.1-NC_040034.1 and NC_011710.1) and fugu (*Takifugu rubripes*) (NC_018890.1- NC_018911.1 and NC_004299.1). This process used Bowtie2 without trimming and no mismatch on the read alignment (Langmeat et al., 2009). The unmapped reads were processed to *de novo* assembly using SPAdes with careful correction on mismatch and short indels and K-mers value for reads alignment were set to 21, 33, and 55 (Bankevich et al., 2012). Contigs generated from SPAdes were processed for taxonomic labelling using BLAST+ (Blastn, National Center for Biotechnology Information NCBI) using the nucleotide database through CLC Genomic Workbench 12 (Qiagen). Matches were identified using cut off points for the following parameters: Bit score $\geq$ 50; sequence identity >80 %; sequence length > 500 bp. The results were examined to identify matches that related to fish genomes, manually both using BLAST on NCBI website (blast.ncbi.nlm.nih.gov/Blast.cgi). Additionally, taxonomic classification was performed by submitting non-host reads to Kaiju web-based program (http://kaiju.binf.ku.dk/server) which matched the translated amino acid sequences of each 150 bp paired end read against the NCBI BLAST non-redundant protein database for Bacteria, Archaea and Viruses (Menzel et al., 2016).

2.10.3. Genome assembly and phylogenetic analysis

Assembled contigs included complete MCV genomes from samples with high viral load and partial MCV genomes from other samples. Identification of the assembled genome by Mega BLAST through the NCBI website (blast.ncbi.nlm.nih.gov/Blast.cgi) and annotation was performed using Genome Annotation Transfer Utility (GATU) with the reference genome for Banggai cardinal fish iridovirus (BCIV) (acc number: MN432490.1) (Tcherepanov et al., 2006). The annotated genes and predicted open reading frames (ORF) produced by GATU were assessed using following criteria: (1) larger than 120 nucleotides, (2) not overlapping with another ORF by more than 25 %, and (3) in the case of overlapping ORFs, only the larger ORF was annotated (Koda et al., 2018). Partial MCV genomes were mapped to the reference genome for identification. A progressive multiple sequence alignment of the assembled complete genomes using CLC Genomic workbench 12 (Qiagen) was performed with the published MCV genomes: ISKNV (Acc number: AF371960.1) (He et al., 2001), RSIV (AB104413) (Kurita et al., 2002), Turbot reddish body iridovirus (TRBIV) (GQ273492) (Shi et al., 2010), and SGIV (AY521625). Phylogenetic analyses were conducted in Mega X (Kumar et al., 2018) with the Maximum Likelihood method and General Time Reversible model with 1000 bootstrap value used to generate a maximum likelihood tree.

2.11. Statistical analysis

Given the proximity of the farms to each other, a shared aquatic environment and a common feed source of low value trash fish, the data were collapsed on farms and populations. Thus, fish with and without clinical signs were compared for all observations within the affected populations. Viral loads for MCV and NNV were analysed after log 10 transformation to satisfy the assumption of normality. The viral loads were presented as the back transformed geometric mean with 95 % confidence interval. Univariate associations for the presence of clinical signs, and detection of viruses and ectoparasites were determined using logistic regression and presented with odds ratio and 95 % confidence interval. In all instances, the baseline for the odds ratio was fish without clinical signs. All analyses were conducted using Stata 13 (2013) (StataCorp, USA). The true prevalence for the detection of ISKNV histopathology lesions was calculated using an online tool (Sergeant, 2018). A Bayesian approach was used to estimate the true prevalence of VNN lesions when none were observed (e.g. when the apparent prevalence was 0) (Sergeant, 2018).

3. Results

3.1. Case detection

There were 12 grow out farms in Pegametan and Sumber Kima Bay (Fig. 1). Four farms had more than 30 sea-cages and the rest had 12–15 cages. Generally, one population of fish was kept in two cages that were typically 5 × 5 × 5 m in size. Eight farms had hybrid groupers that were newly transferred to the sea cages from local land-based nurseries and had been at the grow-out farm generally about one month. The other four farms had tiger grouper (*Epinephelus fuscoguttatus*) and or humpback grouper (*Cromileptes altivelis)* which had been in the sea cages for more than 3 months. All farms used locally obtained low-value trash fish as the feed. Leech infestations occurred during the study; previously they were considered rare in this area of Bali. Farmers reported they changed the ectoparasite control from application of a weekly freshwater bath to a treatment with an undisclosed dose of malachite green for 10 min administered once to twice per week.

There was a high incidence of sudden mortality with at least one affected population reported at 9 of the 12 farms from 5 August to 3 November 2016 (Table 1). No sampling was conducted on the three unaffected farms. The presentation of the outbreaks was the same at all farms; affected fish had non-specific clinical signs including inactivity with a preference for the bottom of the net, fin erosion and loss of appetite (Supplemental Table S3). The green colouration reported by farmers on the body surface and eyes was observed on fish with and without clinical signs in all affected populations. The final cumulative mortality reported by farmers at each farm was 80 %–90 % over the course of one month. The surface sea temperature from 4 August to 7 November 2016 was reported to be 29.8 ± 0.84 °C (minimum 28.3 °C; maximum 31.1 °C).

3.2. Ectoparasites and gross pathology

The leech, *Zeylanicobdela arugamensis* was detected at all nine farms with approximately one third of fish affected (Table 2). The monogenean, *Benedenia epinepheli* was detected at six of nine farms with approximately 18–20 % of fish infested (Table 2). The odds of having an ectoparasite infestation was the same for grouper classified as being with and without clinical signs. A broad array of gross external and internal pathology was noted at moderate prevalence on diagnostic post-mortem examination (Table 2). Fish with clinical signs were 2.4 and 4.6 times more like to show fin erosion and green discolouration of the skin and eyes, respectively (Table 2; Supplemental Table S3). Fish with clinical signs had significantly higher odds of pale discoloration of the anterior kidney, posterior kidney, and liver. Fish with clinical signs were 2.3 and

Table 1
Reported cases of acute high mortality disease at grouper farms between August and November 2016 on the northern coast of Bali. Clinical signs in all cases were lethargy, inappetence and non-responsiveness to the stimulus of being captured.

Population ID	Farm ID	Sampling date 2016	Total number of populations on farm at sampling	Species sampled[1]	Days since transferred to sea cage	Number of fish sampled		Weight (g)[2]	Total length (mm)[2]	Condition factor[2,3]
						Without clinical signs	With clinical signs			
1	A	5–6 Aug	5	Cantik	30	12	24	76 ± 25	174 ± 17	1.41 ± 0.32
2	B	1–2 Sep	1	Cantik	30	12	24	54 ± 11	152 ± 10	1.54 ± 0.16
3	C	6 Sep	1	Cantik	30	12	24	47 ± 31	140 ± 27	1.49 ± 0.26
4	D	19 Sep	1	Cantang	30	12	24	71 ± 15	167 ± 11	1.51 ± 0.23
5	E	27 Sep	1	Cantik	45	12	24	82 ± 23	175 ± 15	1.50 ± 0.22
6	F	12 Oct	6	Cantang	60	6	12	108 ± 13	208 ± 13	1.21 ± 0.22
7	G	13 Oct	1	Cantik	30	12	24	50 ± 12	149 ± 12	1.49 ± 0.16
8	H	1 Nov	3	Cantang	60	6	12	139 ± 37	215 ± 12	1.39 ± 0.31
9	I	2 Nov	6	Cantik	210	6	12	467 ± 144	318 ± 38	1.46 ± 0.35
10	H	3 Nov	3	Cantik	30	12	24	47 ± 13	142 ± 13	1.60 ± 0.31

[1] Cantik = Hybrid grouper ♀ *Epinephelus fuscoguttatus* x ♂ *E. polyphekadion*; Cantang = Hybrid grouper ♀ *Epinephelus fuscoguttatus* x ♂ *E. lanceolatus*.
[2] Mean ± standard deviation.
[3] Condition factor = (weight)(L^{-3}).

Table 2
Proportion of individual fish with abnormalities observed at post-mortem and odds ratios for the presence of abnormalities in samples targeted to fish showing behavioural clinical signs of disease at the time of sampling.

Post-mortem observation	Clinical signs (%)		Odds Ratio[1]	95 % CI	p value
	Present	Absent			
n	204	102			
External					
Fin Erosion	15.2	6.9	**2.43**	1.03 - 5.73	0.042
Green discoloration	85.3	55.9	**4.58**	2.64 - 7.94	0.0001
Zeylanicobdela arugamensis	33.8	34.3	0.978	0.593 - 1.62	0.932
Benedenia epinepheli	19.6	17.6	1.14	0.615 - 2.11	0.68
Internal					
Anterior kidney colour pale	12.7	4.9	**2.83**	1.05 - 7.62	0.039
Fluid in body cavity	22.1	25.5	0.827	0.475 - 1.44	0.503
Gall bladder over distended	71.6	78.4	0.692	0.395 - 1.21	0.199
Liver colour pale	32.4	18.6	**2.09**	1.17 - 3.73	0.013
Liver colour two-toned or patchy	65.7	81.4	**0.438**	0.246 - 0.780	0.005
Liver enlarged (hepatomegaly)	4.4	8.8	0.477	0.183 - 1.24	0.129
Liver reduced	12.7	2.0	**7.3**	1.70 - 31.4	0.008
Posterior kidney colour pale	19.6	4.9	**4.73**	1.81 - 12.4	0.002
Posterior kidney enlarged	4.4	0.0	-		
Spleen enlarged (splenomegaly)	16.2	7.8	**2.27**	1.00 - 5.11	0.048
Spleen reduced	15.2	9.8	1.65	0.774 - 3.51	0.195

[1] Baseline for odds ratio are fish without clinical signs and bolding indicates statistical significance.

7.3 times more likely to show an enlarged spleen and a small liver, respectively (Table 2). There was a significantly higher odds of a two-tone or patchy liver coloration in fish without clinical signs.

3.3. Proportion of fish with NNV infection

NNV was detected at all farms and in all affected populations in both fish with and without clinical signs (Supplemental Table S4). Approximately 55–57 % of all fish were positive for NNV by qPCR. There were no differences in the proportion of fish infected with NNV and the mean genome copy number when comparing fish with and without clinical signs (Table 3). Vacuoles consistent with VNN disease were not observed in the brain and retina of any fish (Table 3). The true prevalence (95 % CI) for the presence of VNN lesions when none were observed for fish with and without clinical signs was <0.01−0.4% and <0.1 – 2.4 %, respectively.

3.4. Proportion of fish with MCV infection

MCV was detected at all farms and in all affected populations, in both fish with and without clinical signs (Supplemental Table S4). Approximately 80–83 % of all fish were positive for MCV by qPCR. There were no differences in the proportion of fish infected with MCV and the mean genome copy number when comparing fish with and without clinical signs (Table 3). Two-thirds of fish with clinical signs had histopathological lesions consistent with disease caused by MVC infection, which was a significantly higher proportion than those without clinical signs (38 %) (Table 3). The estimated true prevalence for the presence of ISKNV histopathology lesions in fish with and without clinical signs is 94.4 % (95 % CI 79–100) and 47.2 % (95 % CI 27–70), respectively. Megalocytic inclusion bearing cells were observed in tissues of fish from all populations (Supplemental Table S4) and were found in multiple organs including the anterior and posterior kidney, spleen and liver. Multiple stages of megalocyte formation were observed with early stages associated with hypertrophied cells and late stages also associated with necrotic cells (Fig. 2). In the anterior and posterior kidneys, megalocytes were observed in glomeruli, rarely in renal tubules and frequently in haematopoietic cells. In the liver, megalocytes were found in hepatocytes and were observed in blood vessels. Large numbers of megalocytes were observed in the spleen, predominantly in red pulp areas containing haematopoietic cells. Megalocytes were often found in the sub-endothelial cells of blood vessels in all observed organs.

Table 3
Results for the detection of viral pathogens by PCR for fish classified on the presence of clinical signs at the time of sampling. Bolding indicates a statistically significant difference.

Virus[1]	Clinical signs		p value
	Present (n)	Absent (n)	
Megalocytivirus			
Percent positive by qPCR	80.8 (120)	83.3 (60)	0.683
Percent of positive samples above BLOQ	76.3 (97)	84 (50)	0.278
Mean genome copy per mg of tissue	1.21×10^4	3.49×10^3	0.0983
95 % CI of mean genome copy	$4.45 \times 10^3 – 3.27 \times 10^4$	$1.42 \times 10^3 – 8.57 \times 10^3$	
Percent positive by histopathology	**66.7 (120)**	**38.3 (60)**	**0.0003**
NNV			
Percent positive by qPCR	55 (120)	56.7 (60)	0.832
Percent of positive samples above BLOQ	45.5 (66)	44.1 (34)	0.895
Mean genome copy per mg of tissue	2.47×10^3	1.74×10^3	0.670
95 % CI of mean genome copy	$7.91 \times 10^2 – 7.74 \times 10^3$	$4.69 \times 10^2 – 6.48 \times 10^3$	
Percent positive by histopathology	0 (120)	0 (60)	0.999

[1] BLOQ: below the level of quantification.

3.5. Metagenomic sequencing for unknown pathogens

The sequencing data provided 5.5–6.2 million 150 bp paired end reads per sample. Host genome depletion using genomic data from other fish species only reduced the number of reads by 1–3 % (data not shown). Comparing two taxonomic labelling methods, BLAST + method gave better accuracy after manual checking using BLASTN. Kaiju gave more diverse result compare to BLAST+ (Supplemental Table S6). BLASTN identified the virus species ISKNV (genus *Megalocytivirus*) as the only virus present and it was detected in all samples (Supplemental Table S5). There were matches for a small number of potentially pathogenic bacteria in some of the samples including *Escherichia coli, Staphylococcus* spp.*, Pseudomonas* spp., *Enterococcus* spp., and *Clostridium*

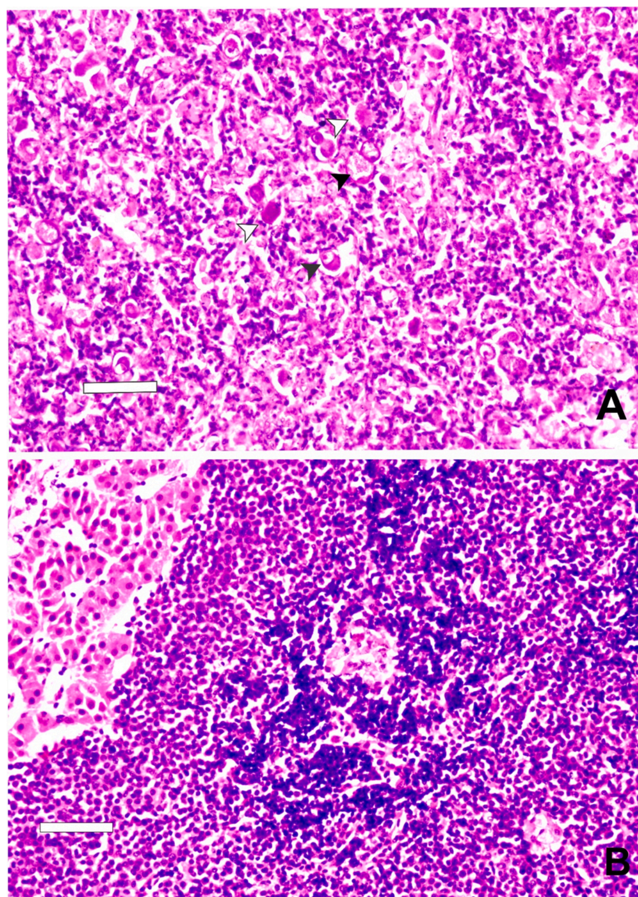

Fig. 2. Megalocytic inclusion bearing cells in (A) infected anterior kidney (from Population 7). Multiple stages of megalocytes (white arrows: early stage, black arrows: late stage of megalocytes) were observed in anterior kidney associated with necrosis. (B) normal anterior kidney of grouper without clinical signs (Population 10). Scale bars =50 μm.

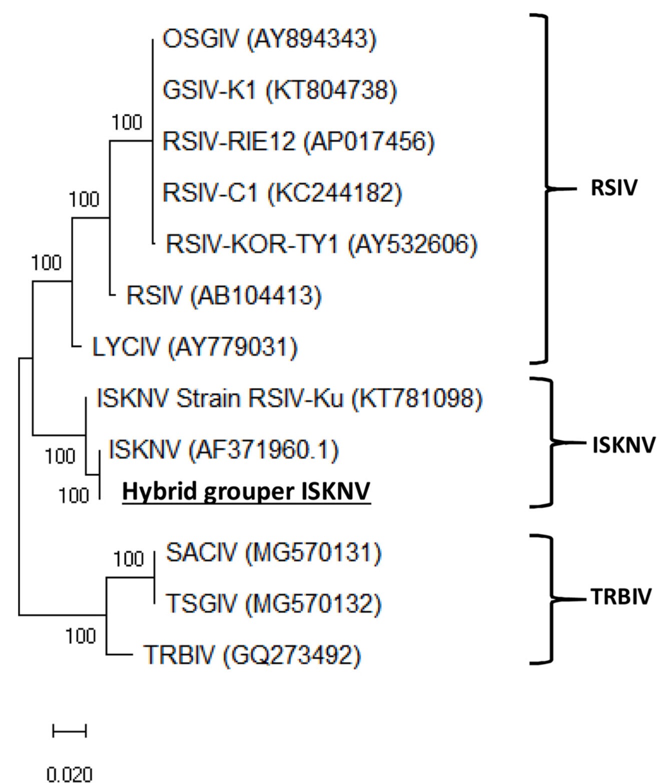

Fig. 3. Phylogenetic tree for representative genotypes of the ISKNV species of megalocytiviruses and the hybrid grouper ISKNV isolate (this study, ID # 3-9 and 7-10 Supplemental Table S5), calculated by the maximum likelihood method with the general time reversible model and 1000 bootstraps. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +*G*, parameter = 0.2099). The scale bar indicates substitution rate per site. Whole genome sequences were used for the analysis excluding gaps. There were a total of 66,660 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

sp. that were not considered to be relevant to the disease process. No internal parasites were identified (Supplemental Table S5).

3.6. Characterisation of the MCV genome

A full length genome sequence of MCV was obtained as a single contig from two samples, ID# 3–9 (Genbank accession number MW557381) and ID# 7–10 (accession number MW464172) (Supplemental Table S2). Partial MCV genomes were obtained from two samples (Supplemental Table S2.; ID # 6−1 and 12−12) which covered 25 % (fully and partially covered 1 and 81 ORFs, respectively) and 49 % (fully and partially covered 5 and 106 ORFs, respectively) of the MCV genome. The other four samples had sequence matches to MCV with less than 10 % genome coverage. The size of the full length genomes was 110,919 bp and 110,961 bp with the average depth of coverage being 164 and 307 times, respectively. The full genome samples had 99.9 % nucleotide simillarity and BLASTN showed that both full length genomes had 99.9 % nucleotide similarity with ISKNV (AF371960.1) and 99.5 % with ISKNV strain RSIV-Ku (KT781098.1). The partial genomes had MCP genes similarity of 100 % (ID# 12−12) and 99.3 % (ID# 6−1) to ISKNV (AF371960.1) and ISKNV strain RSIV-Ku (KT781098.1). Phylogenetic analysis confirmed that the fish were infected with ISKNV rather than RSIV (Fig. 3). Genomes from both samples had 122 predicted genes and the sequence identity of predicted amino acid sequence was 100 % for all genes.

4. Discussion

The main pathogen responsible for the acute mass mortality events in hybrid groupers was ISKNV. A high proportion of fish (80–83 %) were infected with ISKNV regardless of the presence of clinical signs within targeted samples from affected populations. There was histopathological confirmation of disease caused by ISKNV infection in 67 % and 38 % of fish with and without clinical signs, respectively. The true prevalence was estimated to be 94 % (95 % CI 79–100) for the presence of ISKNV lesions in fish with clinical signs. A range of clinical and pathological presentations represented the varying stages of pathogenesis in each population. This is consistent with the sensitivity of histopathology and the proportion of fish that demonstrate lesions with acute infection (Rimmer et al., 2017; Go and Whittington, 2019). This study demonstrated the importance of a sampling framework that accessed a sufficient sample size and included fish from different stages of disease. This together with a multi-modal diagnostic approach, identified relevant lesions and infections considering that the course of infection and pathology is asynchronous in individual fish. Fish identified with clinical signs were likely to be in the late stages of pathogenesis and had evidence of necrosis associated with megalocytes. Fish with clinical signs had a significantly higher likelihood of having fin erosion, green discolouration, pale haematopoietic organs, small liver, and splenomegaly. Fish without clinical signs had a statistically higher likelihood of having a two-toned or patchy discoloured liver and were considered to be in the early stages of the disease. NNV was detected by qPCR at all farms and in all populations with just over half of the fish infected. However, there was no histopathological evidence of disease caused by this virus and the infection was considered subclinical. The ectoparasites, *Z. arugamensis* and *B. epinepheli* were consistently found during the study and these coinfections may have been a contributing factor to the mortality events due to ISKNV. High throughput sequencing contributed to the diagnostic process by identification and genotyping of ISKNV. No other DNA viruses were identified using this method. Potentially pathogenic bacteria were identified, however, in the absence of relevant histopathological lesions, the findings were considered least likely to have caused the high mortality disease.

Farmers reported the mortality events began two to four weeks after the fish were transferred to sea cages. Fish may have arrived at the farm infected with ISKNV or it was newly acquired upon transfer to the sea cage. Either scenario is equally likely as these pathogens are endemic to the region and have persistent infections (Jitrakorn et al., 2020; Sah Putra et al., 2020). ISKNV and NNV were both detected in juvenile grouper at nurseries in Indonesia in a case where NNV was the cause of disease and ISKNV was subclinical (Sah Putra et al., 2020). The source of ISKNV infection at the sea cages could have been from horizontal transmission from infected grouper or wild fish (Wang et al., 2007) or from the feeding of low value trash fish (Lajimin et al., 2015).

In this study, the histopathological examination was critical to assign an aetiological diagnosis when subclinical viral infections were present. Due to the lack of evidence of brain or retinal lesions (true prevalence was <2.4 %), it was concluded that NNV was least likely to be the cause of the high mortality disease. NNV damages nerve cells in the retina and brain leading to abnormal of swimming and vision loss and is often associated with epidemic losses in larval fish (Ma et al., 2012). Mortality up to 100 % was reported in the 1–2 month-old juvenile of *Epinephelus fuscogutatus* and *Epinephelus akaara* (Chi et al., 1997) and generally declines with increasing age as observed in Atlantic halibut (*Hippoglossus hippoglossus*) (Johansen et al., 2004), barramundi (*Lates calcarifer*) (Hick et al., 2011) and Australian bass (*Macquaria novemaculeata*) (Jaramillo et al., 2019). However, NNV was reported to cause mortalities in up to 50 % of market size fish (500−1800 g) in sevenband grouper *Epinephelus septemfasciatus* (Fukuda et al., 1996) and sea bass, *Dicentrarchus labrax* (Le Breton et al., 1997). Further, NNV was found to be the cause of disease outbreaks in juvenile orange-spotted grouper (*E. coioides*) in the nursery stage of production in Aceh, Indonesia (Sah Putra et al., 2020). Given that the duration of NNV persistence is poorly understood, it is essential to pair histopathology from a targeted sampling strategy with the results of molecular testing to identify relevant disease processes. The recent development of a cell culture system for dual propagation of NNV and ISKNV will support studies to investigate host-pathogen interactions and vaccine development (Jitrakorn et al., 2020).

The ectoparasites, *Z. arugamensis* and *Benedenia* sp. were detected during the mortality event. Both are considered common in grouper production in South East Asia (Ruckert et al., 2009; Kua et al., 2010). Weekly to fortnightly freshwater bath treatments are used routinely in grouper aquaculture in Indonesia to reduce the burden of *Benedenia* sp. (Ruckert et al., 2009). *Z. arugamensis* was detected in all populations sampled with the farmers suggesting this is a new parasite in this area of Indonesia. Previous research has shown that the feeding of low value 'trash' fish was the major transmission route for ectoparasites such as *Z. arugamensis* in grouper aquaculture (Ruckert et al., 2009). Leech larvae were found on the gills of *Upeneus vittatus*, a trash fish species in sea cages in Lampung, Indonesia (Ruckert et al., 2009). *U. vittatus* was the main trash fish used in grouper aquaculture in north Bali (data not shown). The use of commercial pellets as food can significantly reduce the risk of parasite transmission in grouper aquaculture (Ruckert et al., 2009). However, there are recognized limitations with the quality and availability of commercial feed for marine fish in Indonesia and the difficulty in adapting grouper to eat pellets (Rimmer and Glamuzina, 2019).

From the workshops with the farmers, they reported that some fish has green discolouration on the body and eyes. During the study, we observed the application of malachite green to the freshwater bath as a treatment for the leech infestation. The use of malachite green in aquaculture has been banned due to human health hazards in Europe (Efsa, 2016), Australia (Aqis, 2008) and the USA (FDA, 2016). Malachite green can be highly toxic to fish and is known to cause pathological changes in the gills including the separation of epithelial cells, leukocyte infiltration, and lamellar cell necrosis (Sudova et al., 2007). Malachite green accumulates and persists in skin, muscle and organs in fish in a coloured and non-coloured form. The coloured form has been detected in rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) muscle for at least 5 and 14 days following a 1 h bath with malachite green, respectively (Sudova et al., 2007). In our study, the green discolouration observed on the skin and eyes was presumed to

have been caused by the repeated weekly to fortnightly treatments with malachite green but the extent to which potential malachite green toxicity contributed to clinical signs was not determined.

Effective disease control is vital for aquaculture to stop the spread of infectious pathogens and reduce the burden of disease on aquatic farming systems. Currently, there are a diverse range of diagnostic tools used to detect aquatic pathogens in fish and the environment including traditional (e.g. culture, microscopy), immunological and molecular methods (Adams and Thompson, 2011). However, a combination of methods is often required for a definitive diagnosis of disease that is complemented with histopathology (Adams and Thompson, 2011). One example is the disease syndrome known as heart and skeletal muscle inflammation in Atlantic salmon that emerged in Norway in 1999 (Palacios et al., 2010). The pathogen responsible, Piscine orthoreovirus was identified a decade later using unbiased sequencing techniques (Palacios et al., 2010), but not before the virus spread to other significant salmon farming industries in Scotland, Chile and Canada (Di Cicco et al., 2017). Unbiased sequencing which was once cost prohibitive is now a complementary tool accessible for pathogen identification during a disease investigation and will assist in timely identification of appropriate biosecurity measures. This method will have the greatest impact for investigating disease events with mixed or coinfections and to identify novel or emerging pathogens for which PCR assays are non-existent or suboptimal. From our study, unbiased sequencing identified that ISKNV was the genotype responsible for the mortality event after using a qPCR assay that did not differentiate between ISKNV and RSIV. Unbiased sequencing for pathogen detection can also be considered for outbreak investigations in remote areas or areas with limited access to veterinary diagnostic laboratories as samples can be easily preserved at the farm.

ISKNV and the other closely related MCV genotypes (i.e. RSIV and TRBIV) are aquatic pathogens with increasing global distribution and a broad host range (Hick et al., 2016). The host range includes fish species from a variety of habitats (Wang et al., 2007; Dong et al., 2017; Rimmer et al., 2017; Tsai et al., 2020) and with a variety of uses including species critical to food security (He et al., 2001; Subramaniam et al., 2016), those used for pets (Rimmer et al., 2015; Koda et al., 2018) and those of high conservation value (Rimmer et al., 2017; Go and Whittington, 2019). It is now known that MCVs were circulating in ornamental fish species in the late 1980s, which precedes the first outbreak in mandarin fish in 1994 ca. The early detection of MCVs from ornamental fish mostly originated from South East Asia (Go et al., 2016). The international spread of MCVs may have been aided by the subclinical nature of infections (Joon et al., 2008; Rimmer et al., 2015) and a lack of effective pre-border disease surveillance polices (Whittington and Chong, 2007; Johnson et al., 2019).

The targeted sampling approach used in our study combined with a thorough disease investigation identified ISKNV as the causative pathogen responsible for the mortality of hybrid groupers along the northern coast of Bali, Indonesia. Our approach identified several known pathogens including NNV and results from the high throughput sequencing did not identify any unexpected or novel DNA viruses. There is a need for greater understanding of the risk factors for disease caused by ISKNV and NNV in grouper aquaculture in Indonesia given the persistent and subclinical nature of these infections and endemicity to the region.

Author statement

All authors have made substantial contributions to the design of the study, acquisition of data and the drafting of the manuscript. We have no conflict of interest to report in relation to the research undertaken in this study. All authors have approved the final submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2021.100723.

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