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Molecular analysis of RNA1 and RNA2 sequences from a betanodavirus isolated from giant grouper (*Epinephelus lanceolatus*) in Australia

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ABSTRACT

Betanodavirus infections have a significant impact through direct losses and trade restrictions for aquaculture sectors in Australia. The giant grouper, *Epinephelus lanceolatus*, is a high-value, fast-growing species with significant aquaculture potential. With subacute to chronic mortalities reported from a commercial aquaculture facility in northern Queensland, the viral nervous necrosis in the affected fish was confirmed using a RT-qPCR followed by virus isolation using the SSN-1 cell line. The RNA1 and RNA2 segments were sequenced and nucleotide sequences were compared with betanodavirus sequences from GenBank. Phylogenetic analysis revealed that both these sequences clustered with sequences representing red spotted grouper nervous necrosis virus genotype and showed high sequence identity to virus sequences affecting other grouper species. This is the first report confirming infection by betanodavirus in *E. lanceolatus* from Australia with successful isolation of the virus in a cell culture system, and analysis of nearly full length RNA1 and RNA2 sequences.
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1. Introduction

The giant grouper, *Epinephelus lanceolatus*, is a high-value, fast-growing grouper species with significant aquaculture potential. Over the last two decades, commercial aquaculture of marine groupers has been driven by their high market value in Asia (Harikrishnan et al., 2010). Since then the presence of viral nervous necrosis (VNN) has been recorded in different grouper species with disease affecting juvenile and adult fish (Ransangan and Manin, 2012, Tan et al., 2001, Kara et al., 2014, Vendramin et al., 2013). Apart from the groupers, VNN due to betanodavirus infections have been reported in more than 50 fish species in 32 fish families worldwide (Munday et al., 2002, Harikrishnan et al., 2010, Oie-World Organisation for Animal Health, 2013). In Australia, since the presence of betanodaviruses was first reported in 1990 in barramundi (*Lates calcarifer*) hatchery in north Queensland (Glazebrook et al., 1990), several Australian native fish species are known to be infected with betanodaviruses (Moody et al., 2009). In the acute infection, a necrotising vacuolation in the retina and the central nervous system tissue is seen histologically. The virus infection is confirmed in these lesions using polyclonal antibodies raised in sheep against barramundi nervous necrosis virus recombinant coat protein using immunohistochemistry tests (Moody et al., 2009). However, to confirm subclinical infections, isolation of virus using either SSN-1 or E-11 is recommended (Oie-World Organisation for Animal Health, 2013).

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Betanodaviruses belong to the family *Nodaviridae*, are small, non-enveloped, spherical viruses with bipartite positive-sense RNA genomes, which are capped but not polyadenylated (Ball and Johnson, 1999, Schneemann et al., 1998). The RNA1 is approximately 3100 bases in length and encodes for protein A, which is the viral component of the viral RNA-dependent RNA polymerase (RdRp). In infected cells, a sub-genomic RNA3 segment is found, which is approximately 387 bases long transcript of RNA1 and codes for two non-structural proteins (Ball and Johnson, 1999).The RNA2, approximately 1400 bases in length, contains a single open reading frame that encodes the coat protein (Nagai and Nishizawa, 1999). Analysis of the sequences of the variable region of RNA2 from 25 finfish betanodavirus isolate identified four discrete genotypes that were defined as striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). Turbot necrosis virus (TNV) was reported from Norway; the only sequence that segregated from four discrete genotypes could possibly be considered as fifth genotype (Johansen et al., 2004). Australian betanodavirus sequences published so far clustered within the RGNNV group, but most of the information used was from partial RNA2 sequences and very limited RNA1 sequences (Hick and Whittington, 2010, Moody et al., 2009). Moreover there are no reports till date about betanodavirus infections in giant groupers from Australia. While the grouper industry is expanding in Australia, the industry is heavily relied on their Asian counterparts about grouper diseases.

2. Materials and methods

Giant grouper (*E. lanceolatus*) fingerlings were bred from wild caught broodstock in a hatchery in northern Queensland, Australia. Diseased fingerlings from nursery tanks and grow out ponds exhibiting lethargy, uncoordinated swimming and mortalities were submitted to Biosecurity Queensland in 2013 and 2014 for disease investigation.

Histopathology on specimens including eye and brain from these fingerlings were fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections were cut at 4 μm thickness using a microtome (Microm HM325, Thermo), stained with haematoxylin and eosin (H&E) and examined by light microscopy. Immunohistochemistry test was performed on additional sections cut onto positively charged slides that were dried overnight in a 40 °C incubator using the protocol described earlier (Moody et al., 2009). Briefly, the slides were dewaxed and rehydrated following 10 min incubation in a 60 °C oven. The sections were trypsinised and blocked with bovine serum albumin. The sections were stained with sheep polyclonal antibodies raised against the recombinant coat protein of Barramundi nervous necrosis virus. The slides were counter stained using rabbit α-sheep IgG (H + L) antibody conjugated to horseradish peroxidase and visualised using diaminobenzidine as the substrate. To confirm these results, virus isolation and molecular assays were conducted.

Tissues from the eye and brain of each fingerling were aseptically removed and homogenised in pools of no more than four fish per sample. The tissues were placed in a mortar and pestle with 1200 μL of RLT buffer supplemented with beta-mercapto ethanol and then ground to a homogenous suspension. RNA was extracted using Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Pty Ltd. Australia).

A quantitative reverse transcription PCR (RT-qPCR) for betanodavirus was performed on the extracted samples using 20 μM of each primer and 5 μM of the 6-carboxyfluorescein (6fam) probe. Sequences for primers and probes used for the real time RT-qPCR were NNVqR2T-F: CTT CCT GCC TGA TCC AAC TG; NNVqR2T-R: GTT CTG CTT TCC CAC CAT TTG; NNVqR2T Probe: 6fam-CAA CGA CTG CAC CAC GAG TTG-bhq1 adapted from previously published (Hick and Whittington, 2010) for use with the Rotor-Gene 6000 (Qiagen Pty Ltd. Australia). Reverse transcription was performed at 50 °C for 20 min, prior to an initial denaturation and hot-start Taq activation at 95 °C for 5 min, followed by amplification using 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s, fluorescence was acquired after each cycle. The analysis of the RT-qPCR to determine the threshold cycle (CT) for each sample was performed on Rotor-Gene Q series software with a threshold of 0.05, using slope correct and an outlier removal of 10% to account for background fluorescence.

Virus isolation was attempted using Striped Snakehead (SSN-1) cell line obtained from a commercial supplier (Sigma Aldrich, Australia). The SSN-1 cell line has been shown to be highly permissive to fish betanodaviruses (Iwamoto et al., 1999) and hence was used. SSN-1 cells were grown and maintained using Leibovitz L-15 medium supplemented with 2 mM glutamine, 15–20% foetal bovine serum, 100 IU penicillin/100 μg streptomycin per ml and fungizone at 25 °C temperature, with subculturing on every 7–9th day. Approximately 3×10^5 cells per mL were seeded in 2 mL tissue culture tubes and incubated at 25 °C for at least 24 h. The tissue samples from the eyes and brain of fingerlings were crushed with sterile sand using pestle and mortar. The tissue homogenate was suspended in sterile virus transport medium and centrifuged at 2000 *g* for 10 min. The cell culture medium was removed and 100 μL of the clarified supernatant diluted in 900 μL of cell culture medium was used for inoculation of the monolayer of SSN-1 cells. After 2 h the medium was added back to the tubes without removing the clarified supernatant. The tissue culture tubes were incubated at 25 °C for 14 days, labelled as passage 1. At the end of passage 1, cell supernatant was subcultured on the new monolayer of SSN-1 cells and incubated for another 14 days at 25 °C. Cells were monitored for cytopathic effect (CPE) every third day using light microscopy. The passage 3 cell culture was used for further amplification and sequencing of RNA1 and RNA2 segments.

RT-PCR was carried out using the superscript III/platinum Taq high fidelity one-step RT-PCR kit for endpoint detection (Life technologies, Australia). Nearly full length gene sequences for RNA1 and RNA2 were amplified with combination of three sets of primers. The primer sets were chosen from previously published reports (Tan et al., 2001) and were modified to cover the overlapping regions of both genes. The sequences for the set of primers are: RNA1 RT-PCR primers were NNV1-F1 and NNV1-F6R (approx. 1100 bp); NNV1-F6 and NNV-F10R (approx. 1200 bp); NNV1-F9 and NNV1-R2 (approx. 890 bp) (Fig. 1). The RT-PCR was performed in a 25 μL reaction containing 1x superscript III platinum reaction buffer, 400 nM of each primer selected

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