Journal of Marine Science and Technology

Volume 21
Issue 7 *Vol 22, Supplemental*

Article 9

A COMPARATIVE STUDY BETWEEN RANAVIRUS AND MEGALOCYTIVIRUS INFECTIONS IN ORANGE-SPOTTED GROUPER (EPIENPHELUS COIOIDES)

Jiann-Horng Leu
Institute of Marine Biology, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwan, R.O.C. Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, R.O.C., jiholeu@ntou.edu.tw

Ming-Hung Wu
Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwa, R.O.C.

Hsin-Yiu Chou
Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwa, R.O.C. Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.

Recommended Citation

Leu, Jiann-Horng; Wu, Ming-Hung; and Chou, Hsin-Yiu (2013) "A COMPARATIVE STUDY BETWEEN RANAVIRUS AND MEGALOCYTIVIRUS INFECTIONS IN ORANGE-SPOTTED GROUPER (EPIENPHELUS COIOIDES)," *Journal of Marine Science and Technology*: Vol. 21: Iss. 7, Article 9.
DOI: 10.6119/JMST-013-1220-2
Available at: https://jmstt.ntou.edu.tw/journal/vol21/iss7/9

A COMPARATIVE STUDY BETWEEN RANAVIRUS AND MEGALOCYTIVIRUS INFECTIONS IN ORANGE-SPOTTED GROUPER (EPIENPHELUS COIOIDES)

Acknowledgements

This study was financially supported by grants from Center of Excellence for the Ocean, National Taiwan Ocean University. We thank Prof. Chia-Ben Chao for kindly providing TGIV used in this study. Both Jiann-Horng Leu and Hsin-Yiu Chou are corresponding authors for this work.

Journal of Marine Science and Technology, Vol. 21, Suppl., pp. 58-64 (2013)
DOI: 10.6119/JMST-013-1220-2

A COMPARATIVE STUDY BETWEEN RANAVIRUS AND MEGALOCYTIVIRUS INFECTIONS IN ORANGE-SPOTTED GROUPER (*Epienphelus coioides*)

Jiann-Horng Leu[1, 3], Ming-Hung Wu[2], and Hsin-Yiu Chou[2, 3]

Key words: megalocytivirus, ranavirus, Epinephelus coioides, grouper iridovirus.

ABSTRACT

Piscine iridoviruses infect a wide variety of fish and are classified into three genera: *Lymphocystivirus*, *Ranavirus* and *Megalocytivirus*. Lymphocystiviruses cause non-fatal, dermal infections, while ranaviruses and megalocytiviruses produce devastating, systemic infections with mortality reaching up to 100%. Although both ranaviruses and megalocytiviruses cause fatal systemic infections, they induce different pathology. In Taiwan, both ranaviruses and megalocytiviruses have caused serious epidemics in several mariculture fish species, including groupers. In this study, we infected the orange-spotted grouper (*Epinephelus coioides*) with either a megalocytivirus (TGIV, grouper iridovirus of Taiwan) or a ranavirus (GIV, grouper iridovirus), and then the two iridoviruses were investigated and compared in respect of their target organs, virulence, and effects on the expression of several immune-related genes in the spleen and head kidney. By measuring cumulative mortality rate, GIV was shown to have higher virulence than TGIV. By PCR, we found that TGIV mainly infected the spleen, head kidney, kidney, heart and gill, while GIV mainly infected the spleen and intestine. The assayed immune genes were hemoglobin subunit-β-2 (Hb), CC chemokine 19, Toll-like receptor 9 isoform A and B (TLR9-A and B) and Mx (myxovirus resistance). By real-time RT-PCR, we found that of the assayed genes, the expression of CC chemokine 19 was strongly induced in spleen by both viruses, whereas the expression of Mx were strongly induced by both viruses in both organs and the expression of Hb gene was induced only by TGIV in head kidney.

I. INTRODUCTION

In Asia, the orange-spotted grouper *Epinephelus coioides* is a popular seafood fish with high market price, and is an economically important mariculture species not only in Taiwan but also in other Asian countries, e.g. China, Indonesia and Thailand (FAO database; http://www.fao.org/fishery/culturedspecies/Epinephelus_coioides/en). The artificial propagation of *E. coioides* by using hormone-inducing technique was first established in Taiwan in 1979 (FAO database), and since then, the grouper aquaculture industry has expanded rapidly to meet the strong demand for the fish. However, high-density intensive cultivation in cages or tanks has greatly encouraged the spread of the grouper diseases resulting from viral, bacterial and parasitic infections [23]. Among them, the infectious viral diseases are the most serious and have severely affected grouper cultures, causing huge economic losses. The causative agents for viral diseases include a DNA virus, iridovirus, and a RNA virus, nervous necrosis virus (NNV); both are newly emerging viral pathogens, causing mass mortality of grouper especially at larval stage [35].

Iridoviruses are large, cytoplasmic, double-stranded DNA viruses with icosahedral symmetry; their sizes range from 120 to 350 nm in diameter. The *Iridoviridae* family contains five genera, including *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* [40]. Members of the family are distinguished for their wide variety of hosts, ranging from cold-blooded vertebrates (Lymphocystivirus, Ranavirus, Megalocytivirus), such as bony fish, amphibians, and reptiles, to invertebrates (Chloriridovirus, Iridovirus), including insects, crustaceans and mollusks [40]. Piscine iridoviruses infect a wide range of fish and are classified into three genera: *Lymphocystivirus*, *Ranavirus* and *Megalocytivirus* [38]. Lymphocystiviruses cause non-fatal, superficial dermal infections, while ranaviruses and megalocytiviruses

Paper submitted 11/05/13; revised 12/09/13; accepted 12/20/13. Author for correspondence: Jiann-Horng Leu (e-mail: jiholeu@ntou.edu.tw).

[1] *Institute of Marine Biology, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.*
[2] *Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Keelung, Taiwa, R.O.C.*
[3] *Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.*

are devastating and cause serious systemic diseases with mortality reaching up to 100%. Although both ranaviruses and megalocytiviruses cause fatal systemic infections, they induce different pathology: ranaviruses cause systemic necrotizing lesions, whereas megalocytiviruses induce the formation of hypertrophic cells in various organs [38]. In Taiwan, the iridovirus-like infection was firstly reported in 1997 [5]. Subsequently, the causative agent was isolated and characterized as a member of *Iridoviridae* [7]. Since then, iridoviruses have caused serious epidemics in several mariculture fish species, including groupers [5, 7, 22, 36, 37]. A phylogenetic survey based on the viral major capsid protein genes showed that the iridoviruses from Taiwanese fish could be classified into two groups, the genus *Ranavirus* and the genus *Megalocytivirus* [12].

In Taiwan, both ranaviruses and megalocytiviruses infect the orange-spotted grouper (*E. coioides*), causing serious losses. In this study, we infected *E. coioides* with either a megalocytivirus (TGIV, grouper iridovirus of Taiwan) [7] or a ranavirus (GIV, grouper iridovirus) [22], and then the two iridoviruses were investigated and compared in terms of their target organs, virulence, and effects on the expression of several immune-related genes in the spleen and head kidney.

II. MATERIALS AND METHODS

1. Fish

Specimens of *E. coioides* weighing about 1.3 g were obtained from a fish farm in Tainan, southern Taiwan, and maintained in 26-28°C aquaria with aeration and fed commercially obtained artificial food twice a day. Before experiment, several fish were randomly picked up and checked with PCR to monitor their infectious status of GIV and TGIV.

2. Preparation of Virus Inoculum

The GIV was propagated in the grouper swim bladder SB cell line, which was established from *E. coioides* and maintained in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 25°C [6]. To propagate the GIV, the SB cells were inoculated with GIV at a multiplicity of infection (MOI) of 0.1~0.01. The virus was then allowed to multiply for two weeks at 25°C. The culture medium, together with the adherent cells removed by scraping, was pooled, and then frozen and thawed for three times. After centrifugation, the supernatant was harvested and saved at -80°C for later use. The titer was determined by TCID50 assay and calculated by the method of Reed and Muench [30]. The TGIV was propagated *in vivo*. The tissue lysates prepared from TGIV-infected giant sea perch (*Lates calcarifer*) was obtained from Prof. Chao, and was intraperitoneally injected into the anaesthetized *E. coioides*. After injection for 5 days, the spleens and hearts were pooled and ground with a chilled mortar and pestle on ice in the presence of L-15 medium without FBS in the ratio of 9:1. After centrifugation at 2000 × g for 10 min, the supernatant was filtered through a 0.45 μm membrane filter and saved at -80°C for later use.

Table 1. Primer list used in this study.

Primer name	Primer sequence (5'-3')	Usage
TGIV-MCP-F1	ATGTCTGCAATCTCAGGTGCG	RT-PCR
TGIV-MCP-R2	CGACACCTCCTCAACTAGATTGTG	RT-PCR
GIV-MCP-F1	AGGTCGGGCGATTACGTGCT	RT-PCR
GIV-MCP-R2	GGCTATGTCGGTAGCAGAGATAGGA	RT-PCR
TGIV-MCP-F2	GCAACGTGCAAAGCAATTACA	Real-time PCR
TGIV-MCP-R2	GCAGATTCACCTTGTTGTTGACA	Real-time PCR
GIV-MCP-F2	TCCCGTTGCCGTTCTTT	Real-time PCR
GIV-MCP-R2	TGAAGCGACCTCAGTTTAATGT	Real-time PCR
EF1-α-F	GGATCTTTTCCTTTCCCATTGTT	Real-time PCR
EF1-α-R	GCAGCTTTGGCCGTGAA	Real-time PCR
Hemoglobin subunit β-F	GTCTTCCCAGGGCGTTCA	Real-time PCR
Hemoglobin subunit β-R	TCCAGGCAGCTTTCCAGAA	Real-time PCR
CC chemokine 19-F	AAGCAGCAGTCCATTGGTATCTC	Real-time PCR
CC chemokine 19-R	ATCCTTTTCATCACCTGCTGCTA	Real-time PCR
TLR9-A-F	TCTCATCCAAAAGAACCAGCATAG	Real-time PCR
TLR9-A-R	TGGTGCAGCAGCGACTTC	Real-time PCR
TLR9-B-F	ATGTGCCAGGAGGTTGTCAGA	Real-time PCR
TLR9-B-R	CAACGGGAATTTTCCAACCTT	Real-time PCR
Mx-F	CATCGACCTCATCCACCGTAT	Real-time PCR
Mx-R	GACGGTAGGCAGCACAAAGTACT	Real-time PCR

3. Determination of TGIV and GIV Copy Number in the Inoculum by Absolute Quantitative Real-Time PCR

The viral genomic DNAs were isolated from the inoculum (prepared as described above) using FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen Biotech Corp.) according to the provided protocol. The GIV and TGIV major capsid protein (MCP) genes were chosen as the target genes for real-time PCR; the used primers are listed in Table 1. Plasmids containing TGIV or GIV MCP genes were used to prepare the standard curve. To generate standard curve, plasmid DNAs were quantified by spectrophotometry, and the gene copy number was determined according to the molar mass derived from the plasmid and MCP gene sizes. The plasmid DNAs were serially diluted 10-fold to generate standard

curves in quantities ranging from 10^3 to 10^9 copies. Real-time PCR was conducted using the Power SYBR® Green Master Mix (Applied Biosystems) and performed using the ABI 7500 Real-Time PCR System (Applied Biosystems). Real-time PCR was carried out as follows: 0.4 µl of each primer (10 µM) and 1 µl of viral genomic DNAs or diluted plasmid DNAs were mixed with 10 µl of Power SYBR® Green Master Mix in a final volume of 20 µl. The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation curve analysis (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec) was included for each sample after PCR to examine the specificity of the PCR products. Three technical replicates were performed for each assay sample and standard dilution. After PCR, the C_t values for the assay samples and each standard dilution were determined. Based on the C_t values, SDS software accompanying with the Real-Time PCR system calculated the standard curve for each standard dilution and then determined the copy number of the two MCP genes for the assay sample by extrapolating values from the standard value.

4. Cumulative Mortality of *E. coioides* after Infection with GIV or TGIV

The fish (1.4 g average weight) were anaesthetized and intraperitoneally injected with 100 µl of 10-fold serially diluted TGIV (1.7×10^8, 1.7×10^7, 1.7×10^6 copies) or GIV inoculum (1.8×10^7, 1.8×10^6, 1.8×10^5 copies); fifteen fish were injected for each dilution. Fish injected with 100 µl of L-15 medium were served as negative control for the infection. The animals were observed twice a day for mortality; the number of deaths recorded and cumulative percentage mortality was calculated. The experiment was conducted in duplicate.

5. Organ Tropism Analyses of TGIV and GIV by PCR

The fish were anaesthetized and infected with 100 µl of TGIV (1.7×10^8 copies) or GIV inoculum (1.8×10^6 copies) by intraperitoneal injection. Five days after injection, the spleen, head kidney, kidney, heart, liver, intestine, gills and muscle were collected. The DNAs were extracted using FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen Biotech Corp.) according to the supplied protocol. The extracted DNAs were subjected to agarose gel electrophoresis for quality check and then quantified using NanoDrop® ND-1000 (Thermo Scientific). For PCR, the concentration of extracted DNAs from each organ was adjusted to 100 ng/µl. PCR was carried out as follows: 0.4 µl of each primer (10 µM) and 1 µl of genomic DNAs were mixed with 10 µl of Taq DNA Polymerase 2 × Master Mix Red (Ampliqon) in a final volume of 20 µl. The thermal cycling conditions were 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min. The PCR products were analyzed by agarose gel electrophoresis. The primers used in this experiment are listed in Table 1.

6. Expression Analysis of Five Selected Immune-Related Genes in *E. coioides* Infected with TGIV or GIC by Real-Time RT-PCR

The fish were anaesthetized and intraperitoneally injected with 100 µl of TGIV (1.7×10^8 copies) or GIV inoculum (1.8×10^6 copies). Control fish were injected with 100 µl of L-15 medium. At 1, 3 and 5 days post injection, the spleens and head kidneys were collected and pooled from three fish; there were three pooled samples for each time point. TRIzol® reagent was used for RNA extraction according the supplier's protocol. The purified total RNAs were quantified by NanoDrop® ND-1000 (Thermo Scientific). One µg total RNA was used for cDNA synthesis. After treating with DNase I (Invitrogen) to remove contaminated DNA, the total RNAs were primed with oligo-dT and reverse-transcribed with HiScript I Reverse Transcriptase (Bionovas) at 42°C for 1 hr. After reverse transcription, the cDNAs were 10-fold diluted, and then an aliquot of diluted cDNAs were subjected to quantitative real-time PCR analysis. The primers for the five immune-related genes are listed in Table 1. In this experiment, *E. coioides* EF-1α gene was used as a reference gene for internal standardization and the corresponding primers are shown in Table 1. The quantitative real-time PCR was carried out as describe above. The C_t values for the immune-related genes and the C_t value for the internal control EF-1α gene were determined for each sample. The expression levels of immune-related genes in infected fish relative to control fish were then determined using the $2^{-\Delta\Delta C_T}$ method [21].

III. RESULTS

1. Cumulative Mortality of *E. coioides* after Infection with TGIV or GIV

As shown in Fig. 1, for TGIV, the cumulative mortality rate for the fish groups injected with 1.7×10^8, 1.7×10^7 and 1.7×10^6 copies of virus were 93.3%, 73.3% and 36.7%, respectively; for 1.7×10^8 and 1.7×10^7 groups, mortalities were first appeared at 5 and 7 days post injection (dpi). For GIV, the cumulative mortality rate for the 1.8×10^7, 1.8×10^6 and 1.8×10^5 groups were 100%, 100% and 73.3%, respectively. For 1.8×10^7 and 1.8×10^6 groups, mortalities were first appeared at 4 and 5 dpi, and reached 100% mortality at 9 and 10 dpi, respectively. Clearly, GIV had much higher virulence than TGIV. No morbidity or mortality was observed in control fish injected with L-15 medium. For the following comparative studies between the two viruses, the injection dosages of 1.7×10^8 copies of TGIV and 1.8×10^6 copies of GIV per fish were used, because at these dosages, mortalities were first appeared at 5 dpi.

2. Organ Tropisms of TGIV and GIV

Fig. 2 shows the results of organ tropisms of the two viruses. For TGIV, high viral loads were observed in spleen, heart and gills; head kidney and kidney had lower viral loads; intestine

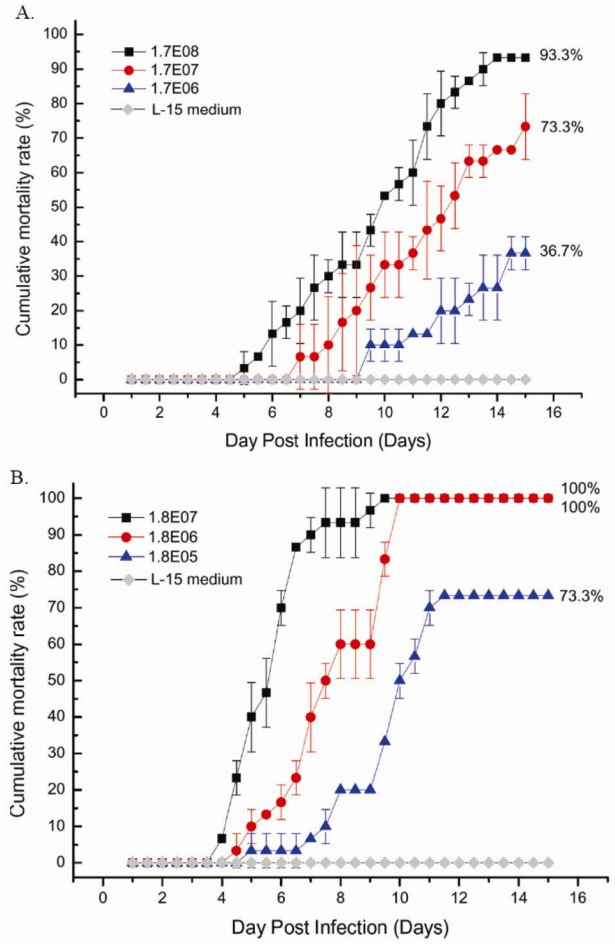

Fig. 1. Cumulative mortality of *E. coioides* infected with TGIV (A) or GIV (B). The fish were intraperitoneally injected with 10-fold serially diluted TGIV (1.7×10^8, 1.7×10^7, 1.7×10^6 copies) or GIV inoculum (1.8×10^7, 1.8×10^6, 1.8×10^5 copies); fish injected with L-15 medium were served as negative control. Fifteen fish were injected for each dilution. The experiment was conducted in duplicate. The cumulative mortality rate for each viral dilution is indicated on the right.

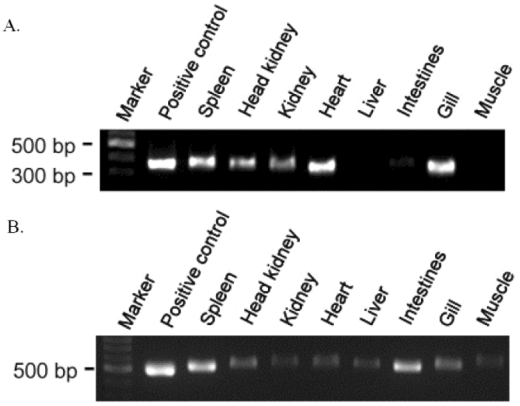

Fig. 2. Organ tropism of TGIV (A) and GIV (B) in *E. coioides*. After infecting the *E. coioides* with intraperitoneal injection for 5 days, the indicated organs were collected and their DNAs were extracted and subjected to PCR analysis.

had lowest viral loads; TGIV loads were undetected in liver and muscle (Fig. 2A). For GIV, high viral loads were detected in both spleen and intestine, while lower viral loads were identified in head kidney and gill. The lowest viral loads were detected in kidney, heart, liver and muscle (Fig. 2B).

3. The Expression Levels of Five Immune-Related Genes in *E. coioides* Infected with TGIV or GIV

The effects of the two idiroviruses on the expression of several immune-related genes in both spleens and head kidneys were investigated. These two organs were chosen because they are the main immune organs in fish. The five immune-related genes selected for qRT-PCR analysis were hemoglobin subunit-β-2 (Hb), CC chemokine 19, Toll-like receptor 9 isoform A and B (TLR9-A and B) and Mx. As shown in Fig. 3, the expression of Hb gene was induced in head kidney at 3 days after TGIV injection. The expression levels of CC chemokine 19 were strongly induced by both viruses in spleen at 1 dpi, and then gradually decreased thereafter; similar expression pattern was observed in head kidney, although the expression levels were much lower. For TLR9-A, the expression level in spleen was suppressed by both viruses at 3 and 5 dpi; in head kidney, although not statistically significant, the expression levels were slightly induced. For TLR9-B, the expression was slightly induced at 3 dpi by GIV in head kidney. Lastly, for Mx gene, as shown in Fig. 3, both TGIV and GIV infections induced strong induction of Mx gene at 1 dpi (the mean fold change was greater than 10), but the expression levels were highly variable, leading to statistical insignificance compared to control fish.

IV. DISCUSSION

For the past two decades, megalocytiviruses and ranaviruses have emerged to become important pathogens for cultured and wild fish species, causing severe systemic infections associated with high mortality (100%). These viruses are known for their world-wide distribution and infect a variety of finfish hosts living in marine and freshwater environment [38]. Both viruses, however, induce distinguished pathological characteristics. All members of ranaviruses, except Santee-Cooper ranavirus that infects largemouth bass, induce systemic necrotizing disease, i.e., necrosis in multiple organs, especially in the hematopoieitic tissues. For example, epizootic haematopoietic necrosis virus (EHNV) induces fatal systemic diseases in rainbow trout and redfin perch, causing multifocal necrosis of the liver, spleen and renal haematopoietic tissue [14, 15, 29, 39]. The prominent feature for megalocytivirus infection is the induction of hypertrophied cells, which contain large granular basophilic inclusion bodies in the cytoplasm. In most cases, large amounts of hypertrophied cells are found throughout various organs, especially the spleen, kidney, gastrointestinal tract and gills [4, 9, 25, 32, 37]. These hypertrophied cells are virally infected monocytes [4, 18].

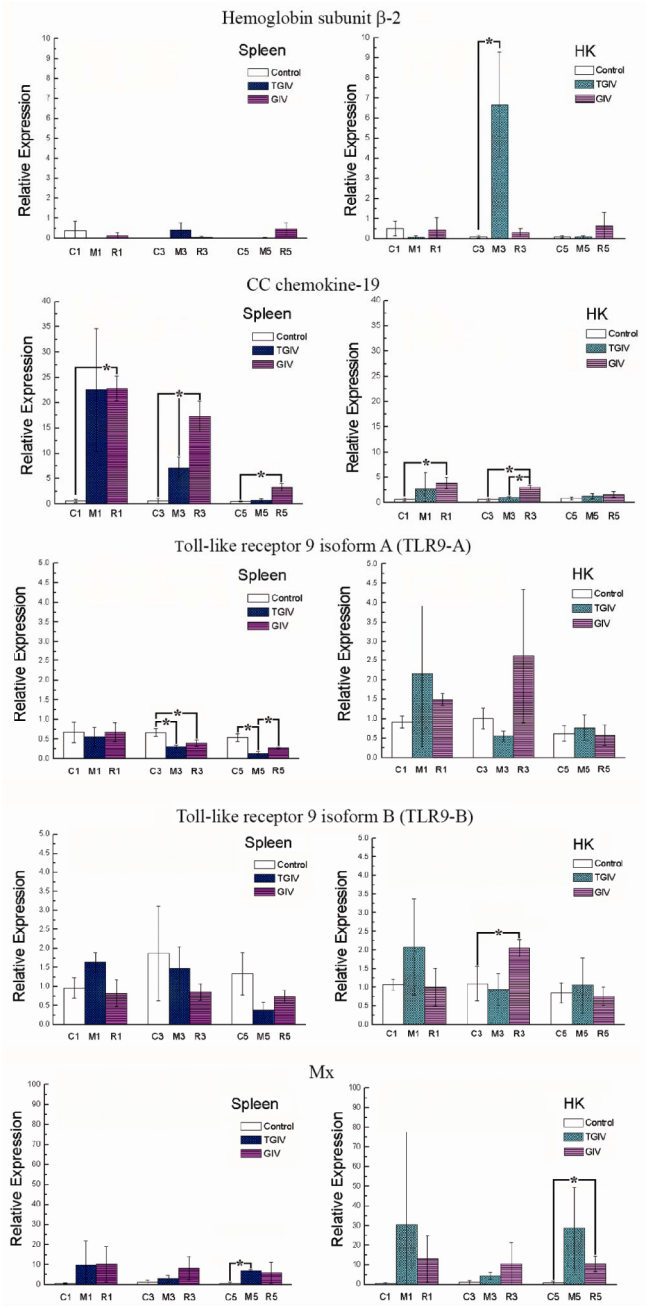

Fig. 3. Expression analysis of the five immune-related genes in *E. coioides* infected with TGIV or GIV. The fish were intraperitoneally injected with TGIV (Megalocytivirus, M), GIV (Ranavirus, R) or L-15 medium (Control, C). At 1, 3 and 5 dpi, the spleens and head kidneys were collected and subjected to quantitative real-time RT-PCR. *E. coioides* EF-1α gene was used as internal control. At each time point, three pooled samples were analyzed, each pooed sample containing three spleens or head kidneys. The expression levels of immune-related genes in infected fish relative to control fish were determined using the $2^{-\Delta\Delta C_T}$ method. Asterisks indicate statistically significant ($p < 0.05$) between the groups by Student's *t*-test.

In Taiwan, the first iridoviral infection was reported in grouper in 1997, and the causative agent was named TGIV (grouper iridovirus in Taiwan) [5]. Based on histological, microscopic and genetic evidence, TGIV was recognized as a member of the genus *Megalocytivirus* [4]. Then, a grouper iridovirus (GIV) was isolated from yellow grouper (*Epinephelus awoara*) in 2000 [13], and subsequent genetic data showed that GIV belongs to the genus *Ranavirus* [22, 34]. In this study, we compared TGIV (megalocytivirus) and GIV (ranavirus), in terms of their virulence, organ tropisms and their effects on the expressions of several immune-related genes. To our knowledge, this is the first report to make a comparative study between the two economically important iridoviruses in *E. coioides*.

For virulence comparison, as shown in Fig. 1, the cumulative mortality rates for the fish injected with either 1.7×10^7 copies of TGIV or 1.8×10^5 copies of GIV were 73.3%. This clearly showed that GIV had much higher virulence than TGIV (approximately 100-fold). However, we noted that the cumulative mortality curve for 1.8×10^5 copies of GIV reached plateau at 12 dpi, whereas the curve for 1.7×10^7 copies of TGIV was still rising at 15 dpi. This suggests that if the experiment period is extended beyond 15 days, TGIV might exhibit higher mortality rate.

We used PCR to investigate the organ tropisms for TGIV and GIV. TGIV DNAs could be easily detected in spleen, head kidney, kidney, heart and gill, except in liver, intestine and muscle (Fig. 2A). Using *in situ* hybridization, Chao *et al*., [4] found that spleen, head kidney, trunk kidney and gills contained higher number of TGIV-probe labeled cells, whereas heart, muscle, liver and intestine had lower number of labeled cells. Therefore, not considering the heart, our data are largely consistent with the results of Chao *et al*. For GIV, high viral loads were detected in both spleen and intestine; lower viral loads were identified in head kidney and gill; lowest viral loads were detected in kidney, heart, liver and muscle (Fig. 2B). Although no detailed histological and *in situ* hybridization studies have been reported on GIV-infected grouper, a transmission electron microscopic study identified the GIV virions in the cytoplasm of grouper spleen cells [22]. Singapore grouper iridovirus (SGIV) is a ranavirus isolated from *Epinephelus tauvina* [28]. *In situ* hybridization analysis of SGIV showed that in naturally-infected *Epinephelus malabaricus*, strong SGIV-labeled signals were observed in kidney and spleen, intermediate signals were detected in intestine and liver, and the weakest signals were obtained in the stomach and gills [11].

Among the assayed immune-related genes, Mx and CC chemokine 19 genes were highly induced after TGIV or GIV infections (Fig. 3). Chemokines are a large family of chemotactic cytokines that control leukocyte migration and other cellular processes [42]. According to their functional difference, chemokines could be divided into homeostatic chemokines and inflammatory chemokines; the formers mediate leukocyte migration during hematopoiesis and in immune surveillance, and the latters are involved in leukocyte recruitment during infection and inflammation [43]. Chemoki-

nes are defined by the presence of four conserved cysteine residues, and based on the arrangement of the first two cysteine residuese, they are divided into four subfamilies: CXC (α), CC (β), C and CX3C [2]. CC chemokines are the largest subfamily of chemokines with 28 CC chemokines identified from mammalian species [2] and at least 30 from fish [1]. A recent phylogenetic study has classified the teleost CC chemokine into seven groups, i.e., the CCL19/21/25 group, the CCL20 group, the CCL27/28 group, the fish-specific group, the CCL17/22 group, the MIP group, and the MCP group [27]. In this study, we found that the *E. coioides* CC chemokine 19 were highly induced after TGIV or GIV infections. The up-regulation of CC chemokine 19 in other fish has also been reported. The CC chemokine 19 (CsCCK1) in *Cynoglossus semilaevis* was highly induced in spleen, kidney, and liver after *Vibrio anguillarum* infection [19] and the expression of CC chemokine 19 in Atlantic salmon (*Salmo salar*) head kidney was up-regulated after infectious salmon anemia virus (ISAV) [17]. The functions of CsCCK1 have been shown: the recombinant CsCCK1 protein could induce chemotaxis in peripheral blood leukocytes (PBL) of two fish species and enhance resistance of PBL against bacterial infection [19]. Whether *E. coioides* CC chemokine 19 has chemotactic activity or antiviral activity remains to be identified.

Piscine Mx proteins were firstly identified in rainbow trout [33] and since then many piscine Mx proteins have been found and the antiviral activity of some of them has been confirmed [3, 16, 20, 41]. Vertebrate Mx (or myxovirus resistance) proteins are important components of antiviral responses triggered by type I interferon in response to viral infections [31]. They belong to the class of dynamin-like large guanosine triphosphatases (GTPases), which are involved in a wide range of intracellular transport processes [10]. In many cases, Mx proteins exert their antiviral activity by acting on viral nucleocapsids [10]. Although Mx proteins are known for their ability to inhibit the replication of a wide range of RNA viruses, recent studies have shown that Mx proteins inhibit DNA viruses as well [8, 24]. In this study, we found that TGIV and GIV induced Mx protein expression in *E. coioides*, yet this does not necessarily means that the induced Mx protein could inhibit TGIV or GIV. A previous study showed that although TGIV, together with a fish nodavirus and a fish birnavirus, could all induce expression of the barramundi Mx protein in a barramundi brain cell line, the Mx protein only inhibited the two RNA viruses but not TGIV [41]. Therefore, further study is needed to identify whether the induced *E. coioides* Mx protein inhibits TGIV or GIV.

To summarize, in this study, we compared the differences between TGIV (a megalovirus) and GIV (a ranavirus) in *E. coioides*. The results showed that although GIV had higher virulence than TGIV, TGIV exhibited wider organ tropism than GIV. Further, both viruses strongly upregulated the expressions of Mx and CC chemokine 19 genes, and Hb gene was only induced by TGIV.

ACKNOWLEDGMENTS

This study was financially supported by grants from Center of Excellence for the Ocean, National Taiwan Ocean University. We thank Prof. Chia-Ben Chao for kindly providing TGIV used in this study. Both Jiann-Horng Leu and Hsin-Yiu Chou are corresponding authors for this work.

REFERENCES

1. Alejo, A. and Tafalla, C., "Chemokines in teleost fish species," *Developmental & Comparative Immunology*, Vol. 35, pp. 1215-1222 (2011).
2. Bacon, K., Baggiolini, M., Broxmeyer, H., Horuk, R., Lindley, I., Mantovani, A., Maysushima, K., Murphy, P., Nomiyama, H., Oppenheim, J., Rot, A., Schall, T., Tsang, M., Thorpe, R., Van Damme, J., Wadhwa, M., Yoshie, O., Zlotnik, A., and Zoon, K., "Chemokine/chemokine receptor nomenclature," *Journal of Interferon & Cytokine Research*, Vol. 22, pp. 1067-1068 (2002).
3. Caipang, C. M., Hirono, I., and Aoki, T., "*In vitro* inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx," *Virology*, Vol. 317, pp. 373-382 (2003).
4. Chao, C. B., Chen, C. Y., Lai, Y. Y., Lin, C. S., and Huang, H. T., "Histological, ultrastructural, and *in situ* hybridization study on enlarged cells in grouper *Epinephelus* hybrids infected by grouper iridovirus in Taiwan (TGIV)," *Diseases of Aquatic Organisms*, Vol. 58, pp. 127-142 (2004).
5. Chao, C. B. and Pang, V. F., "An outbreak of an iridovirus-like infection in cultured grouper (*Epinephelus* spp.) in Taiwan," *Journal of the Chinese Society of Veterinary Science*, Vol. 23, pp. 411-422 (1997).
6. Chen, H. R., *Establishment of a Continuous Cell Lines from Swim Bladder of Grouper (Epinephelus coioides) and Its Application on Study of TGIV (Grouper Iridovirus of Taiwan)*, Master Thesis, Department of Aquaculture, College of Life Sciences, National Taiwan Ocean University, Taiwan (2001).
7. Chou, H. Y., Hsu, C. C., and Peng, T. Y., "Isolation and characterization of a pathogenic iridovirus from cultured grouper (*Epinephelus* sp.) in Taiwan," *Fish Pathology*, Vol. 33, pp. 201-206 (1998).
8. Fernández-Trujillo, M. A., García-Rosado, E., Alonso, M. C., Castro, D., Álvarez, M. C., and Béjar, J**.**, "Mx1, Mx2 and Mx3 proteins from the gilthead seabream (*Sparus aurata*) show in vitro antiviral activity against RNA and DNA viruses," *Molecular Immunology*, Vol. 56, pp. 630-636 (2013).
9. Gibson-Kueh, S., Netto, P., Ngoh-Lim, G. H., Chang, S. F., Ho, L. L., Qin, Q. W., Chua, F. H. C., Ng, M. L., and Ferguson, H. W., "The pathology of systemic iridoviral disease in fish," *Journal of Comparative Pathology*, Vol. 129, pp. 111-119 (2003).
10. Haller, O., Staeheli, P., and Kochs, G., "Interferon-induced Mx proteins in antiviral host defense," *Biochimie*, Vol. 89, pp. 812-818 (2007).
11. Huang, C., Zhang, X., Gin, K. Y., and Qin, Q. W., "In situ hybridization of a marine fish virus, Singapore grouper iridovirus with a nucleic acid probe of major capsid protein," *Journal of Virological Methods*, Vol. 117, pp. 123-128 (2004).
12. Huang, S. M., Tu, C., Tseng, C. H., Huang, C. C., Chou, C. C., Kuo, H. C., and Chang, S. K., "Genetic analysis of fish iridoviruses isolated in Taiwan during 2001-2009," *Archives of Virology*, Vol. 156, pp. 1505-1515 (2011).
13. Lai, Y. S., Murali, S., Ju, H. Y., Wu, M. F., Guo, I. C., Chen, S. C., Fang, K., and Chang, C. Y., "Two iridovirus-susceptible cell lines established from kidney and liver of grouper, *Epinephelus awoara* (Temminck & Schlegel), and partial characterization of grouper iridovirus," *Journal of Fish Diseases*, Vol. 23, pp. 379-388 (2000).
14. Langdon, J. S. and Humphrey, J. D., "Epizootic haematopoietic necrosis a new viral disease in redfin perch, *Perca fluviatilis* L., in Australia," *Journal of Fish Diseases*, Vol. 10, pp. 289-298 (1987).
15. Langdon, J. S., Humphrey, J. D., and Williams, L. M., "Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia," *Journal of Fish Diseases*, Vol. 11, pp. 93-96 (1988).

16. Larsen, R., Rokenes, T. P., and Robertsen, B., "Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein," *Journal of Virology*, Vol. 78, pp. 7938-7944 (2004).
17. LeBlanc, F., Laflamme, M., and Gagne, N., "Genetic markers of the immune response of Atlantic salmon (*Salmo salar*) to infectious salmon anemia virus (ISAV)," *Fish and Shellfish Immunology*, Vol. 29, pp. 217-232 (2010).
18. Lee, N. S., Do, J. W., Park, J. W., and Kim, Y. C., "Characterization of virus distribution in rock bream (*Oplegnathus fasciatus*; Temminck and Schlegel) infected with megalocytivirus," *Journal of Comparative Pathology*, Vol. 141, pp. 63-69 (2009).
19. Li, Y. X., Sun, J. S., and Sun, L., "An inflammatory CC chemokine of *Cynoglossus semilaevis* is involved in immune defense against bacterial infection," *Fish & Shellfish Immunology*, Vol. 31, pp. 446-452 (2011).
20. Lin, C. H., Christopher John, J. A., Lin, C. H., and Chang, C. Y., "Inhibition of nervous necrosis virus propagation by fish Mx proteins," *Biochemical and Biophysical Research Communications*, Vol. 351, pp. 534-539 (2006).
21. Livak, K. J. and Schmittgen, T. D., "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method," *Methods*, Vol. 25, pp. 402-408 (2001).
22. Murali, S., Wu, M.-F., Guo, I.-C., Chen, S.-C., Yang, H.-W., and Chang, C.-Y., "Molecular characterization and pathogenicity of a grouper iridovirus (GIV) isolated from yellow grouper, *Epinephelus awoara* (Temminck & Schlegel)," *Journal of Fish Diseases*, Vol. 25, pp. 91-100 (2002).
23. Nagasawa, K. and Cruz-Lacierda, E. R., *Diseases of Cultured Groupers*, Southeast Asian Fisheries Development Center, Aquaculture Department, Iloilo, Philippines (2004).
24. Netherton, C. L., Simpson, J., Haller, O., Wileman, T. E., Takamatsu, H. H., Monaghan, P., and Taylor, G., "Inhibition of a large double-stranded DNA virus by MxA protein," *Journal of Virology*, Vol. 83, pp. 2310-2320 (2009).
25. Paperna, I., Vilenkin, M., and de Matos, A. P., "Iridovirus infections in farm-reared tropical ornamental fish," *Diseases of Aquatic Organisms*, Vol. 48, pp. 17-25 (2001).
26. Pavlovic, J., Haller, O., and Staeheli, P., "Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle," *Journal of Virology*, Vol. 66, pp. 2564-2569 (1992).
27. Peatman, P. and Liu, Z., "Evolution of CC chemokines in teleost fish: a case study in gene duplication and implications for immune diversity," *Immunogenetics*, Vol. 59, pp. 613-623 (2007).
28. Qin, Q. W., Chang, S. F., Ngoh, G. H., Gibson-Kueh, S., Shi, C., and Lam, T. J., "Characterization of a novel ranavirus isolated from grouper, *Epinephelus tauvina*," *Diseases of Aquatic Organisms*, Vol. 53, pp. 1-9 (2003).
29. Reddacliff, L. A. and Whittington, R. J., "Pathology of epizootic haematopoietic necrosis virus (EHNV) infection in rainbow trout (*Oncorhynchus mykiss* Walbaum) and redfin perch (*Perca fluviatilis* L)," *Journal of Comparative Pathology*, Vol. 115, pp. 103-115 (1996).
30. Reed, L. J. and Muench, H., "A simple method of estimating fifty percent endpoints," *The American Journal of Hygiene*, Vol. 27, pp. 493-497 (1938).
31. Samuel, C. E., "Antiviral actions of interferons," *Clinical Microbiology Reviews*, Vol. 14, pp. 778-809 (2001).
32. Sudthongkong, C., Miyata, M., and Miyazaki, T., "Iridovirus disease in two ornamental tropical freshwater fishes: African lampeye and dwarf gourami," *Diseases of Aquatic Organisms*, Vol. 48, pp. 163-173 (2002).
33. Trobridge, G. D. and Leong, J. A., "Characterization of a rainbow trout Mx gene," *Journal of Interferon & Cytokine Research*, Vol. 15, pp. 691-702 (1995).
34. Tsai, C. T., Ting, J. W., Wu, M. H., Wu, M. F., Guo, I. C., and Chang, C. Y., "Complete genome sequence of the grouper iridovirus and comparison of genomic organization with those of other iridoviruses," *Journal of Virology*, Vol. 79, pp. 2010-2023 (2005).
35. Walker, P. J. and Winton, J. R., "Emerging viral diseases of fish and shrimp," *Veterinary Research*, Vol. 41:51 (2010).
36. Wang, C. S., Chao, S. Y., Ku, C. C., Wen, C. M., and Shih, H. H., "PCR amplification and sequence analysis of the major capsid protein gene of megalocytiviruses isolated in Taiwan," *Journal of Fish Diseases*, Vol. 32, pp. 543-550 (2009).
37. Wang, C. S., Shih, H. H., Ku, C. C., and Chen, S. N., "Studies on epizootic iridovirus infection among red sea bream, *Pagrus major* (Temminck & Schlegel), cultured in Taiwan," *Journal of Fish Diseases*, Vol. 26, pp. 127-133 (2003).
38. Whittington, R. J., Becker, J. A., and Dennis, M. M., "Iridovirus infections in finfish – critical review with emphasis on ranaviruses," *Journal of Fish Diseases*, Vol. 33, pp. 95-122 (2010).
39. Whittington, R. J., Philbey, A., Reddacliff, G. L., and Macgown, A. R., "Epidemiology of epizootic haematopoietic necrosis virus (EHNV) infection in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum): findings based on virus isolation, antigen capture ELISA and serology," *Journal of Fish Diseases*, Vol. 17, pp. 205-218 (1994).
40. Williams, T., Barbosa-Solomieu, V., and Chinchar, V. G., "A decade of advances in iridovirus research," *Advances in Virus Research*, Vol. 65, pp. 173-248 (2005).
41. Wu, Y. C. and Chi, S. C., "Cloning and analysis of antiviral activity of a barramundi (Lates calcarifer) Mx gene," *Fish & Shellfish Immunology*, Vol. 23, pp. 97-108 (2007).
42. Zlotnik, A. and Yoshie, O., "Chemokines: a new classification system and their role in immunity," *Immunity*, Vol. 12, pp. 121-127 (2000).
43. Zlotnik, A., Yoshie, O., and Nomiyama, H., "The chemokine and chemokine receptor superfamilies and their molecular evolution," *Genome Biology*, Vol. 7, p. 243 (2006).