Cloning, characterization and expression analysis of coagulation factor *II* gene in grass carp (*Ctenopharyngodon idella*)

B.H. Xu[1]*, K.J. Chen[1]*, Y.B. Yao[1], Q.L. Liu[1], T.Y. Xiao[1], J.M. Su[2] and H.Z. Peng[1]

[1]College of Animal Science and Technology, Hunan Agricultural University, Changsha, China
[2]College of Animal Veterinary and Medicine, Hunan Agricultural University, Changsha, China

*These authors contributed equally to the study.
Corresponding author: T.Y. Xiao
E-mail: xiaotiaoyi605@163.com

Genet. Mol. Res. 14 (4): 13764-13778 (2015)
Received April 15, 2015
Accepted August 18, 2015
Published October 28, 2015
DOI http://dx.doi.org/10.4238/2015.October.28.39

ABSTRACT. Here, we characterized the structure and function of the coagulation factor *II* (*FII*) gene in grass carp and determined its role in coagulation mechanisms. The *FII* gene EST was obtained using a constructed splenic transcriptome database; the full-length *FII* gene sequence was obtained by 3' and 5' RACE. The open reading frame (ORF) of *FII* was cloned and the full-length gene was found to be 1718 bp, with an ORF of 1572 bp; the gene contained a 25 bp 5'-untranslated region (UTR) and 108 bp 3'-UTR. The ORF encoded 524 amino acids, including 74 alkaline amino acids (arginine and lysine) and 69 acidic amino acids (aspartic acid and glutamic acid). The theoretical pI was 6.22. The calculated instability index (II) was 39.81, indicating that FII was a stable protein; the half-life period was predicted to be approximately 30 h. Amino acid sequence comparisons indicated that grass carp FII showed most similarity (71%) to FII of *Takifugu rubripes*, followed by *Oplegnathus fasciatus* (48% similarity) and *Larimichthys crocea* (47% similarity). A real-time reverse transcription PCR analysis showed that under normal

 ©FUNPEC-RP www.funpecrp.com.br

circumstances, *FII* was most highly expressed in the liver, followed by the gill, spleen, thymus, and head-kidney ($P < 0.001$). After injection of the grass carp reovirus 873 (GCRV873), the pattern of *FII* expression was significantly altered ($P < 0.001$); gene expression was high after injection, suggesting a response involving the initiation of the coagulation system and defense of the body in combination with the platelet and complement system.

Key words: Grass carp; Coagulation factor; Grass carp reovirus 873; Prothrombin; Grass carp hemorrhagic disease; Coagulation

INTRODUCTION

Grass carp (*Ctenopharyngodon idella*), family Cyprinidae, is an herbivorous freshwater fish widely distributed in the major river systems of China (Chen et al., 2012). The species is also used for fish farming in China where it has an annual production of over 1 million tons; the fish is characterized as delicious and nutritious, and it grows rapidly and has a low cost of breeding (Ye et al., 2012). Recently, increased disease outbreaks, such as bacterial, viral, and protozoan infections have become a major problem for grass carp farming (Zhang et al., 2013). Grass carp hemorrhagic disease, which is caused by the grass carp reovirus (GCRV), is the one of the most severe of the infectious diseases that affect the species and is characterized by a long season, fast transmission, and high risk. Infected fish can suffer severe hemorrhages and have an increased risk of mortality; these effects cause significant economic losses (Su et al., 2012).

The immune system of grass carp has been extensively studied; this system includes processes for specific immunity (e.g., lysozyme, superoxide dismutase, complements C3, C4, and interferon) and non-specific immunity (e.g., leukocytes, phagocytes, and leukomonocytes in the blood circulation system) (Jin et al., 2013). Vertebrates are known to have thirteen types of coagulation factor (factors I - XIII) in their blood, and these factors play important roles in the process of coagulation, anticoagulation and fibrinolysis in their tissues (Liu et al., 2013). Coagulation factors are produced in the liver (Abel et al., 2011). The factors can act on most types of physical damage resulting from bacterial or viral infection and have important functions in innate immunity and adaptive responses in the body (Tassanakajon et al., 2013). Coagulation factor II, also known as prothrombin, is a vitamin-K-dependent blood coagulating protein that can be converted into thrombin by coagulation factors X, V and others (Eerenberg et al., 2011). The gene for coagulation factor II (*FII*) is located on grass carp chromosome 11 (11p-q12) and has 14 exons and 13 introns and encodes a protein with a relative molecular mass of approximately 72,000 Da (Kalamchi, 1987). Prothrombin can not only activate thrombin with the help of factor *VII* and calcium (Ca^{2+}) factor, but also functions in a negative feedback mechanism to avoid thrombosis due to excessive thrombin formation (Peyvandi et al., 2012). Coagulation is one of the principal body defense systems in grass carp. Coagulation factors are mainly found in the blood lymph and blood cells and are divided two types, main coagulation factors and auxiliary coagulation factors (Liu et al., 2013). Although the *FII* gene sequence and the structure and function of FII protein in dogs, mice, cattle, rabbits, and other mammals is known, there is considerably less understanding of coagulation processes and coagulation factors in fish (Wong et al., 2010; Solomon et al., 2011). In the present study, we characterize the structure and function of the grass carp *FII* gene in order to elucidate its role in coagulation mechanisms in this species.

MATERIAL AND METHODS

Ethics statement

Approval for the study was obtained from the Institutional Animal Ethics Committee of Hunan Agricultural University. All experiments were conducted in strict accordance with the established Guide for the Care and Use of Laboratory Animals of the National Institutes of Health

Subjects

A one-year-old healthy grass carp was collected from the aquaculture base of the Hunan Agricultural University. This fish was temporarily kept in an aquarium in the laboratory for 2 days without feeding. The aquarium was filled with tap water (pH 7.0) that had been aerated for 4 days and contained 7.5-8.2 mg/L dissolved oxygen, at a temperature of 20 ± 1.5°C. The tools (scissors, tweezers, and blade) for vivisection were washed and wrapped up with tin box, baked at 180 °C for 8h and then cooled to room temperature. The fish was killed humanely, and spleen, liver, head-kidney, thymus, and gill tissues were collected. These tissues were used for extraction of total RNA and to obtain the full sequence of the grass carp *FII* gene.

Analysis of *FII* expression was analyzed using one-year-old healthy grass carp (N = 180, mean body weight = 120 ± 10 g) that had been temporarily housed in an aquarium in the laboratory for 2 days. The fish were randomly divided into 2 equally-sized groups (control and infected groups) and were placed into 2 aquaria for each group. Control grass carp were injected with 0.65% physiological fish saline at the base of pectoral fin; the infected group was injected with 0.5 mL standard strain GCRV873, also at the base of the pectoral fin. Both groups were maintained in well-aerated water at 28°C. Fifteen fish from each group were selected at random at six different time points after injection: 0, 24, 48, 72, 96, and 138 h. Spleen, liver, head-kidney, thymus, and gill tissues were collected. Total RNAs were extracted from the tissues. Samples from 3 fish per treatment group and time interval were used for analysis of gene expression.

Primer design

Primers were designed using published conserved *FII* gene sequences: FII-Mid (F) and FII-Mid (R) were used cloning the conserved region of *FII*; FII-3'' and 3'-Adaptor were used for 3' RACE; FII-5' and 5'-Adaptor were used for 5' RACE. FII was the gene specific primer used for quantitative expression analysis, and β-actin was used as the internal control. The above primers were designed by Primer 5.0 and synthesized by the Shanghai Sangon Biotechnology Co. Ltd. (Table 1).

Extraction of total RNA from grass carp tissues

The extraction of total RNAs from grass carp tissues was carried out as described in the experimental procedures of the column animal RNA-out Kit (Beijing Tianenze Gene Technology Co., Ltd, Beijing, China). The collected spleen, liver, head-kidney, thymus, and gill tissues were placed separately in 10-mL centrifuge tubes and 100 mg/mL Trizol lysate was added. The tissues were homogenized on ice and 200 µL of each lysate was transferred to a centrifuge tube (pre-treated with 1.5 mL diethyl pyrocarbonate, DEPC). Then, 800 µL RNA extraction solution A for animal tissues, and 200 µL chloroform was added. After mixing, the suspension was centrifuged at

2000 rpm at 4°C for 5 min. A 200-300 μL aliquot of the supernatant was placed into a DEPC-treated centrifuge tube and an equal volume of RNA extraction solution B for animal tissues was added. Half of the solution was added to an adsorption column in a collection tube; this was centrifuged for 30 s at 12000 rpm and the flow-through liquid was discarded. The other half of the solution was then added to the adsorption column and the procedure was repeated. A column washing liquid (700 μL) was added and centrifuged for 30 s at 12000 rpm; this step was repeated with 300 μL of the column washing liquid. The adsorption column was transferred to another DEPC-treated centrifuge tube and 50 μL RNA eluent was added; the tube was centrifuged for 30 s at 12000 rpm, and the eluate, containing the RNA, was collected. After extraction of the RNA, the quality of the isolated material was checked using 1% gel electrophoresis (Wang et al., 2015) .

Table 1. Primer sequences used in the present study.

Primer name	Sequences (5'-3')
FII-Mid (F)	AAGTTTGTGGAGGAAGGTGGTG
FII-Mid (R)	TGACCGAGGTGGAATTGCGACA
FII-3' (F)	TGAAATCATTGTCCACCCTA
FII-3' (R)	CCTGTCTGTCTGCCCACCAA
3'-Adaptor	CTGATCTAGAGGTACCGGATCC
3'-Adaptor (dT)	CTGATCTAGAGGTACCGGATCC$(T)_{14}$
FII-5' (F)	TGGGCAGACAGACAGGATG
FII-5' (R)	AGAGCAATGTCTCGGTTCAGG
5'-Adaptor	GACTCGAGTCGACATCGA
5'-Adaptor (dT)	GACTCGAGTCGACATCGA$(T)_{17}$
M13/pUC (F)	GTTGTAAAACGACGGCCAG
M13/pUC (R)	CAGGAAACAGCTATGACC
FII (F)	TGGGCAGACAGACAGGA
FII (R)	CCCTAAATACAACTGGAAGGAA
β-actin (F)	GCCGTGACCTGACTGACTA
β-actin (R)	TCAAGAGCCACATAGCAGAG

F = Forward; R = Reverse.

Amplification of FII gene cDNAs

High-quality cDNAs were synthesized as described in the instructions for the Revert Aid First Strand cDNA Synthesis Kit reverse transcription Kit (MBI, America). Briefly, the RNA samples were placed in 0.2 mL centrifuge tubes on ice; a reaction mix of 20 μL was prepared by adding 6 μL nuclease-free water, 5 μL RNA, 1 μL o1igo(dT) 18, 4 μL 5X reaction buffer, 2 μL 10 mM dNTP mix, 1 μL RNase inhibitor (20 μ/μL), and 1 μL reverse transcriptase (200 μ/μL).

The synthesized cDNAs were used as templates for PCR amplification using the FII-Mid (F) and FII-Mid (R) primers, supplemented with Taq enzyme and premix (Promega Company). The reaction mix of 25 μL contained 12.5 μL premix, 1 μL cDNA, 0.5 μL FII-Mid (F), 0.5 μL FII-Mid (R), and 10.5 μL nuclease-free water. The reaction conditions were pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min. The quality of the amplification products were checked using 1.0% agarose gel electrophoresis; unused products were stored at -80°C.

3'RACE of grass carp FII gene 3'-end cDNA

High-quality cDNA was synthesized following the manufacturer instructions using a Revert Aid First Strand cDNA Synthesis Kit reverse transcription Kit (MBI, America). Ten μL nuclease-free

water was added to a sterile 0.2-mL centrifuge tube, along with 1 μL 3' RACE O1igo(T)-Adaptor, and 1 μL total RNA; the tube was centrifuged for 30 s at 12000 rpm, then incubated in a water-bath for 5 min at 65°C. After 1 min on ice, 4 μL 5X reaction buffer, 2 μL 10 mM dNTP Mix, 1 μL Ribolock RNase inhibitor, and 1 μL Revert Aid M-Mulv Reverse was added to the tube and centrifuged for 30 s at 12000 rpm. The tube was then incubated in a water-bath for 90 min at 42°C, extended at 70°C for 5 min and finally terminate reactions. The cDNA was used as the template for PCR with the specific primers and universal primers for 3' RACE O1igo (T)-Adaptor. The reaction mix of 25 μL contained 12.5 μL premix, 1 μL cDNA, 0.5 μL FII-3' (N), 0.5 μL Adaptor (dT), and 10.5 μL nuclease-free water. The reaction conditions were: pre-denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. FII-3'(W) and 3' RACE Adaptor nested primers were used with the same reaction conditions using the PCR product (diluted 50 times) as the template. The quality of the amplified products was confirmed using 1.0% agarose gel electrophoresis; unused product was stored at -80°C.

5' RACE

The 3' RACE O1igo (T)-Adaptor for 3'-end cDNA synthesis was replaced with FII-5' (N), and the 5'-end cDNA synthesis used the experience of the first chain of the 3' RACE end cDNA synthesis. cDNA was purified using the manufacturer instructions for the DNA Fragment Purification Kit Purification Kit (TaKaRa Company), and 3' poly A was added and amplified according to the instructions for the Terminal deoxynucleotidyl transferase Kit (TaKaRa Company). The reaction mix of 50 μL contained 20 μL diluted cDNA, 5 μL PCR Buffer, 10 μL 10 mM dNTPs, 1.6 μL 10 mM (dT) 17-Adaptor, 3.2 μL 100 mM Adaptor, 3.2 μL FII-5' (W), 1 μL Taq, and 6 μL nuclease-free water. The amplification conditions were: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 5 min, and extension at 74°C for 40 min; then 30 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 1 min, extension at 72°C for 3 min; finally, pre-denaturation at 94°C for 40 s, annealing at 58°C for 1 min, and extension at 72°C for 5 min. The quality of the amplification products was checked using 1.0% agarose gel electrophoresis; unused product was stored at -80°C.

Recovery, cloning and sequencing of target bands

PCR products were recovered using an agarose gel DNA extraction kit. The recovered PCR products were connected to pUCm-T carrier and then transformed into competent cells of Escherichia coli DH5a; successful transformation was confirmed using the standard screen on a plate with Amp/IPrG/x-Gal. Positive clones were amplified by culturing and then sent to the Shanghai Sangon Biotechnology Co. Ltd. for sequencing.

Analysis of grass carp FII gene cDNA full-length sequences and the deduced amino acid sequences

A BLAST analysis was performed using the NCBI database to determine the sequences of the positive clones; a final sequence was constructed using Primer5.0 and DNAMAN software. The full-length sequence of *FII* gene cDNA was placed in the NCBI database, and combined DNA STAR, DNAMAN, Primer5.0 and other biological software were used to identify the open reading frame

(ORF) sequence, and the sequence of the predicted protein. The protein sequence was placed into the NCBI database to identify homologous protein sequences. Predict Protein, HMM2.0 and other online software was used to predict the two- dimensional and three- dimensional structures of the protein. MEGA4.0 software and the neighbor joining method were combined to construct a phylogenetic tree. A phylogenetic tree of the *FII* gene of grass carp and 15 other species was edited by the Tree view program with a bootstrap value of 500.

Reverse transcription PCR (RT-PCR) expression analysis of grass carp *FII* gene

RNA samples were obtained from tissues of 3 grass crap that had been injected with the toxin and from three control fish at every sampling interval. After reverse transcription, the cDNAs were checked for concentration and purity. The amplification conditions were as described by the operating manual for SYBRJ Green Real time PCR Master Mix and the fluorescence quantitative PCR instrument (ABI 7300hT). The primer sequences are shown in Table 1. Each reaction mix of 25 µL contained 1 µL cDNA template, 12.5 µL SYBR Green Real time PCR Master Mix, 0.5 µL *FII* (F), 0.5 µL *FII* (R), and 10.5 µL nuclease-free water. The amplification conditions were pre-denaturation at 94°C for 5 min, and then 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s; finally, extension at 72°C for 10 min. The sequence detection system (SDS version 1.4) of the fluorescence quantitative PCR instrument was applied for data collection and analysis and the establishment of a standard curve for each sample. The Ct value, measured by the $2^{-\Delta Ct}$ method, was used to detect the amplification efficiency of the grass crap *FII* gene and β-actin. The relative expression level was calculated by analyzing the expression in different tissues after resolving the amplification specificity of the curve primer.

Statistical analysis

Statistical analysis was performed using SPSS version 19.0. Data are presented as mean ± standard deviation (mean ± SD) and were tested for normality. Comparison between two groups was performed using *t*-tests, and comparisons among groups using one-way analysis of variance (ANOVA); homogeneity of variance test was carried out before the analysis. LSD-*t*-test was applied for the paired-comparisons among multiple groups. $P < 0.05$ was selected as statistically significant.

RESULTS

RNA quality detection

The quality of the extracted total RNA was checked using 1% agarose gel electrophoresis. The analysis identified two obvious bands, one at 28S and the other at 18S (Figure 1), suggesting the recovery of high quality RNA without degradation that could be used for synthesis of first strand cDNA. The OD260/OD280 and OD260/OD230 values were approximately 2.0, and were therefore consistent with the purity requirements.

3' RACE and 5' RACE results

The 3' RACE and 5' RACE yielded PCR products of 400-500 bp and 700-800 bp, respectively (Figure 2).

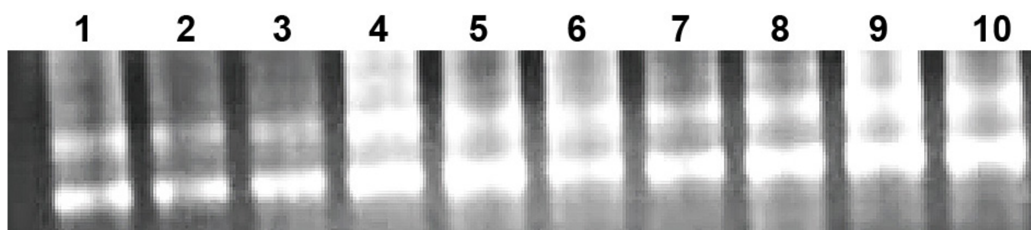

Figure 1. Total RNA from various tissues of grass carp (1 and 2, gill; 3 and 4, head-kidney; 5 and 6, thymus; 7 and 8, spleen; 9 and 10, liver).

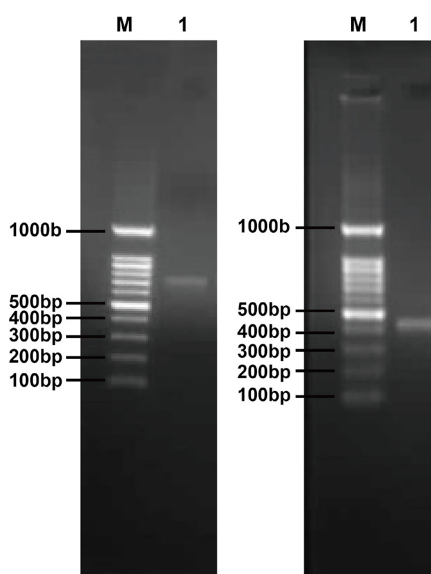

Figure 2. Grass carp coagulation factor (F) *II* gene: A1: 5' RACE products. B1: 3' RACE products. M: 100 bp DNA ladder.

Splicing and analysis of grass carp FII gene full-length sequences

The data from PCR and RACE analyses on the grass carp *FII* gene were submitted to GenBank (accession No. KF937388). The constructed full-length sequence of the gene was 1718 bp, with an ORF of 1572 bp a 25 bp 5'-UTR and a 108 bp 3'-UTR. There were 2 AATAAA motifs in the mRNA and 2 signal ATTTA sequences related to the stability of mRNA. The contents and ratios of the various bases were as follows: A, 516 (30.05%); G, 438 (25.51%); T, 414 (24.11%); C, 349 (20.33%); C+G, 787 (45.84%); Tm = 83.74°C. The cDNA sequence and the deduced amino acid sequence are shown in Figure 3.

Analysis of grass carp FII gene amino acid sequence

The grass carp FII protein sequence was interrogated using ExPasy online software (http://web.expasy.org/protparam). This showed that the 524 transcribed and translated protein sequences of grass carp *FII* gene contained $C_{2658\ h4147}N_{739}O_{774}S_{28,}$ among which there were 69

negatively charged amino acid residues (aspartic acid and glutamic acid) and 74 positively charged amino acid residues (arginine and lysine). The theoretical pI was 6.22. The calculated instability index (*II*) was 39.81, indicating a stable protein; the half-life period was estimated at approximately 30 h. The predicted protein sequence of grass carp *FII* contained 20 different amino acids, with a relatively high proportion of lysine (Lys) (8.2%) and glutamate (7.8%) and a low proportion of methionine (1.7%) (Table 2, Figure 4).

```
1     tta cag gct cag tca ctc tca agg atg gga gcg aaa cta gca cct ctt cta ctc ttc tta ctt
1                                      M   G   A   K   L   A   P   L   L   L   F   L   L
64    ttt gga caa gtt ttc cat ctc aca ctg tgt cat aat gtg ttt atc aat aat aag gaa gcc tct
14     F   G   Q   V   F   H   L   T   L   C   H   N   V   F   I   N   N   K   E   A   S
127   cag att att cgt gcg aag agg gcc aac act gtt ttt gag gag tta aag cct ggt aat ctg gag
35     Q   I   I   R   A   K   R   A   N   T   V   F   E   E   L   K   P   G   N   L   E
190   aga gag tgt gtg gaa gag atc tgt gac cat gag gaa gct cga gaa gtg ttt gag aga gtt gat
56     R   E   C   V   E   E   I   C   D   H   E   E   A   R   E   V   F   E   R   V   D
253   aaa acg gaa ata ttt tgg gcg aaa tat tta ggc tgt gaa gga aca acc ctg tcc aga aca ccc
77     K   T   E   I   F   W   A   K   Y   L   G   C   E   G   T   T   L   S   R   T   P
316   caa aat att aac agt ttg aga ata tgt gcg act aca gag ggg gac tgt ttc ata aat att gga
98     Q   N   I   N   S   L   R   I   C   A   T   T   E   G   D   C   F   I   N   I   G
379   gca aag tat gct ggt aaa gta tct gtc acc aag tct gga aag gca tgc cag tac tgg aaa agc
119    A   K   Y   A   G   K   V   S   V   T   K   S   G   K   A   C   Q   Y   W   K   S
442   aac ttt ccc cac aag att gac gaa ttt aat gtg aca cag ctg aag cta cag gag aac ttc tgc
140    N   F   P   H   K   I   D   E   F   N   V   T   Q   L   K   L   Q   E   N   F   C
505   agg aac cca gat aag cac aaa gat ggc cct tgg tgt ttt acc aga gac ccg act gtc agg agg
161    R   N   P   D   K   H   K   D   G   P   W   C   F   T   R   D   P   T   V   R   R
568   gag acc tgc aat gtg cca aaa tgc ggt gag gct gtt gtt cct cct cca aaa gct cct ttg gac
182    E   T   C   N   V   P   K   C   G   E   A   V   V   P   P   P   K   A   P   L   D
631   aag ttt gtg gag gaa ggt ggt gga cga gaa aga aca act cta gac caa agg aaa gct ttc ttt
203    K   F   V   E   E   G   G   G   R   E   R   T   T   L   D   Q   R   K   A   F   F
694   aac ccc cgc agc ttt ggc aac gga gag cta gac tgt gga gag cgc ccc ttg ttt gag aag atc
224    N   P   R   S   F   G   N   G   E   L   D   C   G   E   R   P   L   F   E   K   I
757   aac aaa gcg gac aag aat gag aag gag ctt ctg atg tcc tat act gga agc aga att gtg gga
245    N   K   A   D   K   N   E   K   E   L   L   M   S   Y   T   G   S   R   I   V   G
820   gga gac gag gct gaa gtg gct agc gct cca tgg cag gtg atg ctg tat aag cgt agc cct cag
266    G   D   E   A   E   V   A   S   A   P   W   Q   V   M   L   Y   K   R   S   P   Q
883   gag ctg ctg tgt gga gcc agt ctg atc agt gat gaa tgg atc ctt act gcg gcc cac tgc att
287    E   L   L   C   G   A   S   L   I   S   D   E   W   I   L   T   A   A   H   C   I
946   ttc tac cca ccg tgg aac aag aac ttc acc atc aat gat atc atc gtc cgc ctg gga aaa cac
308    F   Y   P   P   W   N   K   N   F   T   I   N   D   I   I   V   R   L   G   K   H
1009  tct cgt atc aag tat gag agg ggc act gag aag att gtg gcc att gat gaa atc att gtc cac
329    S   R   I   K   Y   E   R   G   T   E   K   I   V   A   I   D   E   I   I   V   H
1072  cct aaa tac aac tgg aag gaa aac ctg aac cga gac att gct ctt ctg cac atg aag aag cct
350    P   K   Y   N   W   K   E   N   L   N   R   D   I   A   L   L   H   M   K   K   P
1135  gta gtc ttt acg aat gag atc cat cct gtc tgt ctg ccc acc aag agc atc gca aag aat ttg
371    V   V   F   T   N   E   I   H   P   V   C   L   P   T   K   S   I   A   K   N   L
1198  atg ttt gca ggt tac aag ggc cgt gta act ggc tgg ggg aac ctc aga gaa tcg tgg aca tca
392    M   F   A   G   Y   K   G   R   V   T   G   W   G   N   L   R   E   S   W   T   S
1261  aac cca aaa gat ctt cca tca gtg ctg caa cag att cac ttg ccc att gtg gac cag agc atc
413    N   P   K   D   L   P   S   V   L   Q   Q   I   H   L   P   I   V   D   Q   S   I
1324  tgt cgc aat tcc acc tcg gtc ata atc act gac aac atg ttc tgt gct ggt tac cag cca gat
434    C   R   N   S   T   S   V   I   I   T   D   N   M   F   C   A   G   Y   Q   P   D
1387  gat tca aaa aga ggt gat gct tgt gaa gga gac agt gga ggg cct ttt gtg atg aag agt cct
455    D   S   K   R   G   D   A   C   E   G   D   S   G   G   P   F   V   M   K   S   P
1450  aca gat aaa cgg tgg tat cag att ggc att gtg tcg tgg ggt gag ggc tgt gat cgc gat ggc
476    T   D   K   R   W   Y   Q   I   G   I   V   S   W   G   E   G   C   D   R   D   G
1513  aaa tat gga ttc tac acg cac tta tat cgt atg cgt cgc tgg atg aaa aaa gtc att gag aaa
497    K   Y   G   F   Y   T   H   L   Y   R   M   R   R   W   M   K   K   V   I   E   K
1576  aca ggc tcg gaa gat gat gag tga tct tcc aag ctg gaa aga gac ctt tgc cac aca aga aat
517    T   G   S   E   D   D   E   *
1639  aaa cac tta cgt caa atg tga tca aac ctg caa atg gat gaa taa atc tga att ctt tgt atg
1702  ttg aaa aaa aaa aaa
```

Figure 3. Grass carp *FII* full-length cDNA sequence and protein sequence. The 3' UTR includes two poly-A tail signaling sequences (AATAAA; shown in red) and two rapid degradation of mRNA sequences (ATTTA; shown in blue).

Table 2. Amino acid composition of the FII protein of grass carp.

Name of amino acid	Triplet abbreviation	One-letter abbreviation	Number	Mol/mol (%)	Property
Aspartic acid	Asp	D	28	5.34	Acidic
Glutamic acid	Glu	E	41	7.82	Acidic
Arginine	Arg	R	31	5.92	Basic
Histidine	His	H	12	2.29	Basic
Lysine	Lys	K	43	8.21	Basic
Serine	Ser	S	26	4.96	Hydrophilic
Threonine	Thr	T	28	5.34	Hydrophilic
Glycine	Gly	G	38	7.25	Hydrophilic
Cysteine	Cys	C	19	3.63	Hydrophilic
Glutamine	Gln	Q	14	2.67	Hydrophilic
Asparagine	Asn	N	29	5.53	Hydrophilic
Tryptophan	Trp	W	12	2.29	Hydrophobic
Tyrosine	Tyr	Y	14	2.67	Hydrophobic
Alanine	Ala	A	27	5.15	Hydrophobic
Isoleucine	Ile	I	34	6.49	Hydrophobic
Leucine	Leu	L	37	7.06	Hydrophobic
Methionine	Met	M	9	1.72	Hydrophobic
Phenylalanine	Phe	G	24	4.58	Hydrophobic
Proline	Pro	P	28	5.34	Hydrophobic
Valine	Val	V	30	5.73	Hydrophobic

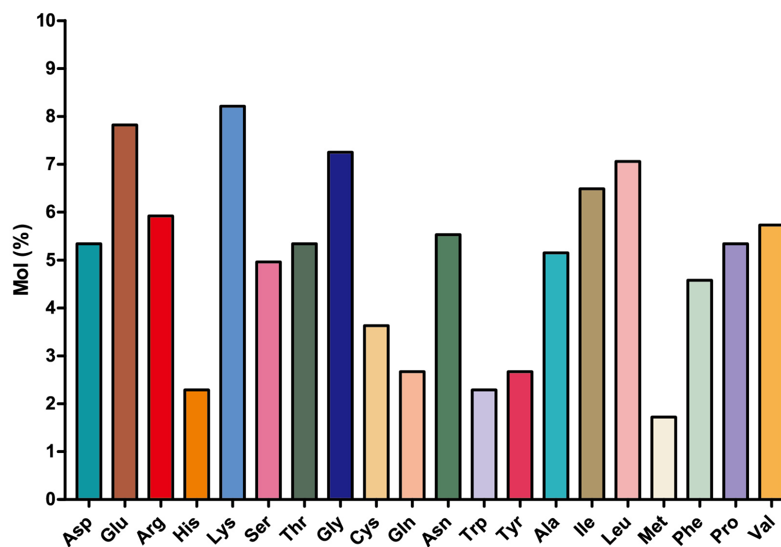

Figure 4. Amino acid frequencies in the protein encoded by the grass carp *FII* gene.

Prediction of the two-dimensional structure of grass carp FII protein sequence

The two-dimensional structure of grass carp FII protein sequence was predicted using http://www.predictprotein.org/. The analysis indicated that a β-fold accounted for 17.7%, an α-helix accounted for 14.5%, and the largest proportion of irregular ring structures accounted for 67.7% (Figure 5). In addition, there were some two-stage structures such as a β-angle.

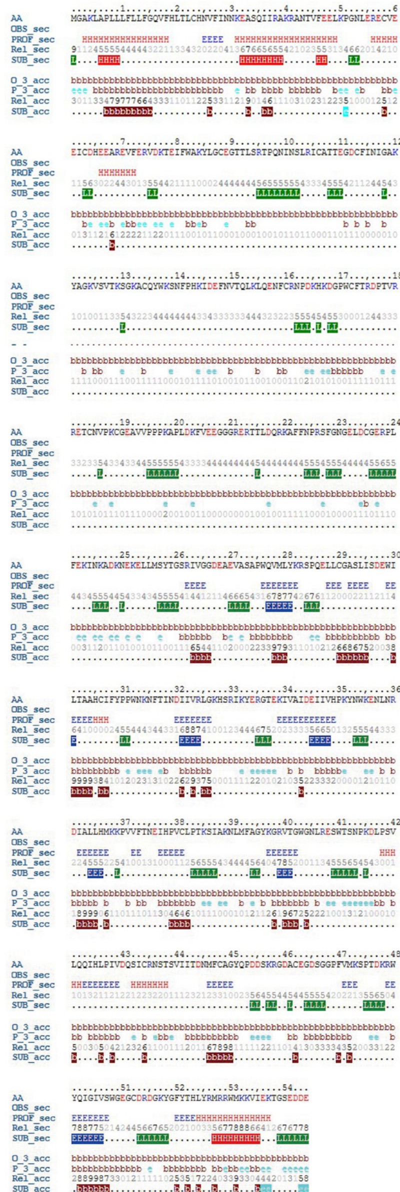

Figure 5. Prediction of the two-grade structure of the FII protein sequence.

Prediction of the three-dimensional structure of grass carp FII protein sequence

The three-dimensional structure of the grass carp FII protein sequence was predicted using http://pdb.rcsb.org/pdb/explore.do?structureId=2QML and a model was constructed. The analysis implied that the protein contained 7 right-handed α-helixes and 7 β-folds connected by a β-angle (Figure 6).

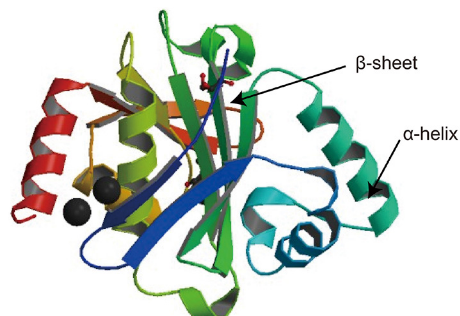

Figure 6. Model showing the predicted three-grade structure of the FII protein sequence.

Analysis of homology and molecular evolution of grass carp FII amino acid sequence

The phylogenetic analysis of the grass carp FII protein showed the highest similarity (71%) with the FII protein of *Takifugu rubripes* (NP_001027864.1), followed by *Oplegnathus fasciatus* (BAM36362.1) (48%) and *Larimichthys crocea* (ACA30405.1) (47%). Clustal W software was applied to analyze the grass carp FII amino acid sequence with the amino acid sequences of other species. A phylogenetic tree was constructed using the Mega 5.0 program and the neighbor-joining method (NJ). The number of bootstrap replications was set as 500. The phylogenetic tree is shown in Figure 7 and indicates that *T. rubripes*, *O. fasciatus,* and *L. crocea* formed a first branch, and then formed a second branch was formed with grass carp, and finally a branch with *Xenopus tropicalis*.

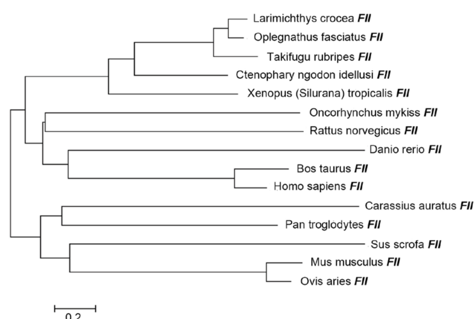

Figure 7. Phylogenetic tree of *FII* genes of selected vertebrates (*Larimichthys crocea* ACA30405.1, *Oplegnathus fasciatus* BAM36362.1, *Takifugu rubripes* NP_001027864.1, *Ctenopharyngodon idellusi* KF937388, *Xenopus* (*Silurana*) *tropicalis* NP_001015797.1, *Oncorhynchus mykiss* NP_001117856.1, *Rattus norvegicus* EDM10105.1, *Danio rerio* NP_001092248.1, *Bos taurus* AAI34503.1, *Homo sapiens* EAW95778.1, *Carassius auratus* AGO58838.1, *Pan troglodytes* JAA43518.1, *Sus scrofa* AAR99595.1, *Mus musculus* AAH13662.1, *Ovis aries* ACV04830.1).

Expression of grass carp *FII* gene in different tissues

A comparison of expression of the grass carp *FII* gene in different tissues showed that at time point 0 h, there were statistically different levels in the spleen, liver, head-kidney, thymus, and gill tissues ($P < 0.001$). Thus, the *FII* gene displayed a tissue-specific expression pattern. The highest level of expression was detected in the liver, followed by the gill, spleen, and thymus; the head-kidney had the lowest relative expression level (Table 3, Figure 8).

Table 3. Altered patterns of expression of the FII gene after injection of GCRV873.

Time (h)	Thymus		Gill		Liver		Spleen		Head-kidney	
	Control group	Infected group	Control group	Infected group	Control group	Infected group	Control group	Infected group	Control group	Infected group
0	1.19 ± 0.94	1.24 ± 0.98	1.93 ± 1.00	1.95 ± 0.99	2.68 ± 0.94	2.74 ± 1.00	1.80 ± 1.00	1.78 ± 1.00	1.34 ± 1.00	1.37 ± 1.00
24	1.27 ± 0.97	1.79 ± 1.04^{a}	1.98 ± 1.02	2.04 ± 1.02	2.69 ± 1.02	1.31 ± 1.00^{ab}	1.83 ± 0.97	1.21 ± 1.02^{a}	1.40 ± 0.97	1.13 ± 0.99
48	1.25 ± 1.00	1.30 ± 0.99	1.96 ± 1.02	2.90 ± 0.99^{abc}	2.71 ± 1.00	1.83 ± 1.03^{ab}	1.77 ± 0.94	1.08 ± 1.00^{ab}	1.34 ± 0.89	1.02 ± 0.97
72	1.28 ± 1.05	1.49 ± 0.99	1.93 ± 1.00	3.15 ± 0.97^{abc}	2.74 ± 1.00	5.81 ± 0.97^{abcd}	1.77 ± 1.00	1.88 ± 0.97^{cd}	1.34 ± 0.94	1.37 ± 0.98
96	1.23 ± 0.97	1.02 ± 0.99^{c}	1.96 ± 0.97	1.13 ± 1.00^{abcde}	2.74 ± 1.05	1.63 ± 1.17^{abe}	1.80 ± 0.94	1.27 ± 0.95^{ae}	1.37 ± 1.00	1.00 ± 0.94
138	1.29 ± 1.02	1.21 ± 0.97	1.91 ± 0.94	1.16 ± 0.97^{abcde}	2.75 ± 1.02	1.14 ± 0.98^{abe}	1.78 ± 1.00	2.99 ± 0.97^{abcdef}	1.43 ± 1.05	1.08 ± 0.97

Letters indicate significant differences ($P < 0.05$): a, compared with control group at the same interval; b, compared with 0 h; c, compared with 24 h; d, compared with 48 h; e, compared with 72 h; f, compared with 96 h).

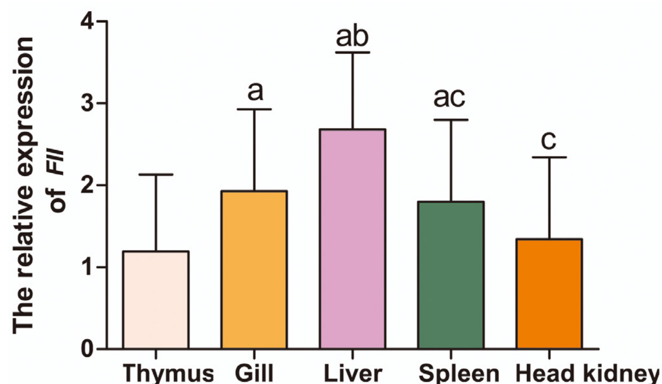

Figure 8. *FII* expression levels in various tissues by quantitative RT-PCR. Different letters indicate significant differences (P < 0.05): a, compared with thymus tissue; b, compared with gill tissue; c, compared with liver tissue; d, compared with head-kidney.

Changes in grass carp *FII* expression after GCRV873 injection

Immediately after injection, there was no significant difference in *FII* expression in the thymus after GCRV873 injection (P > 0.05); however, at 24 h, expression in the thymus of the infected group was significantly increased compared to the control group (P < 0.05). In the infected group, expression in gill tissues varied significantly among time points (P < 0.05). There was a sharp increase at 48 to 72 h, and then a sharp decline at 96 to 138 h; the difference was statistically significant compared with the control group (P < 0.05). After GCRV873 injection, *FII* expression increased significantly at 72 h in liver tissues compared to the control, but decreased at 24, 48, 96, and 138 h (P < 0.05). Expression of *FII* differed significantly in spleen tissues of the infected group between time points (P < 0.05); expression was significantly lower within 24 to 48 h compared to the control group (P < 0.05). There was a slight increase in *FII* expression at 72 h, and no significant difference was found with the control group (P > 0.05); the level of expression fell at 96 h, but reached its highest level at 138 h and showed a significant difference to the control group (P < 0.05). In the head-kidney, expression did not change significantly after GCRV873 injection (P > 0.05); the level of expression fell at 48 to 72 h, increased slightly at 72 h, and fell again at 96 to 138 h at which time there was no significant difference compared to the control group (P < 0.05) (Table 3, Figure 9).

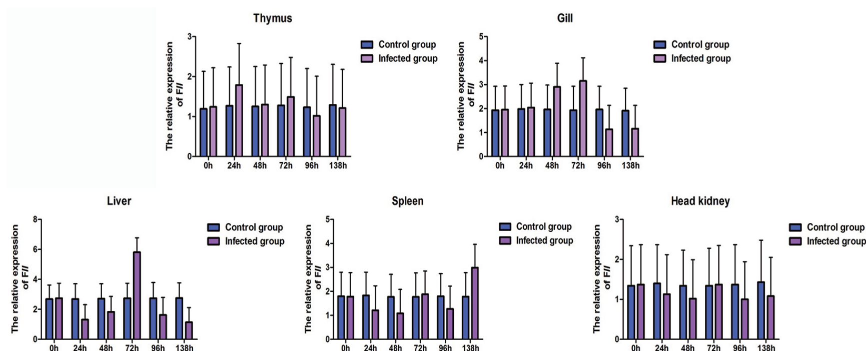

Figure 9. Altered patterns of *FII* expression in tissues after the injection of GCRV873.

 ©FUNPEC-RP www.funpecrp.com.br

DISCUSSION

Grass carp is a very important aquaculture species in China. However, its susceptibility to disease, such as hemorrhagic disease caused by GCRV, is a considerable problem (Su et al., 2012). The innate immune system in fish is a fundamental mechanism against disease agents. To date, traditional methods have proved incapable of providing adequate control of infectious diseases; however, development of molecular markers and identifying genes associated with the immune system has become a key to future development of the fishery (Zhang et al., 2013). In this study, we characterized the structure and function of the grass carp *FII* gene and determined its role in the coagulation process. Cloning of related genes from other aquatic species will undoubtedly enable a better understanding of disease-resistance, be of value for breeding resistant varieties, and provide important materials and a theoretical basis for future research.

FII/prothrombin, the precursor of thrombin, is the final effector in the clotting cascade resulting in the formation of fibrin. It is the key enzyme in the balance of procoagulation and anticoagulation; it acts by enhancing coagulation through positive feedback loops and also promotes anticoagulation via the protein C pathway (Gessoni et al., 2012). In the coagulation mechanism in vertebrates, prothrombin plays a vital role in coagulation, anticoagulation and soluble protein fibrosis. Prothrombin is encoded by a housekeeping gene that is closely involved in the coagulation mechanisms of most fish, poultry, and mammals (Kang et al., 2011).

Comparison of amino acid sequences showed that the grass carp FII protein showed similarity to that of *T. rubripes*, *O. fasciatus* and *L. crocea*. Thus, the grass carp FII protein is similar to the prothrombin of most fish, as the primary structure determines the advanced structure and further influences its function. The comparative similarities of the grass carp FII protein to those of *T. rubripes*, *O. fasciatus,* and *L. crocea* mirrors the direction of species evolution, which indicates that the grass carp *FII* gene has a highly conserved sequence (Tang et al., 2013). In our study, the phylogenetic tree analysis showed that *T. rubripes*, *O. fasciatus,* and *L. crocea* were located in one branch, and then formed a larger branch with grass carp, and finally formed a branch with *Xenopus tropicalis*. This analysis confirmed conservation of the grass carp *FII* gene, and was consistent with the direction of evolution of these species.

The results of the RT-PCR analysis showed that under normal circumstances, the *FII* gene showed highest expression in the liver, followed by the gill, spleen, thymus, and head-kidney. After injection of GCRV873, *FII* expression levels in the liver, gill, and spleen were significantly altered. One possible explanation is that the immune system of grass carp is nonspecific. Thus, in the first 24 h after injection, the decrease of *FII* gene expression level was mainly due to the initiation of nonspecific immune system responses to GCRV873. However, as the period of infection continued, the limited ability of the non-specific immune system made it necessary to induce the blood coagulation system to resist the virus. In blood coagulation, thrombus formation requires the thrombin-mediated transformation of fibrinogen into fibrin; fibrous material in the inflammatory tissue clearance area plays a significant role in preventing the spread of bacteria or viruses (Li et al., 2011). The liver and spleen are known to be key organs for the blood system, so at 24 to 72 h after injection of GCRV873, when the nonspecific immune system was exhausted, the *FII* gene would be upregulated. Similar to other vertebrates, the coagulation system, complement system, and immune system of grass carp show mutual promotion and adjustment (Rhee et al., 2014). Therefore, we speculate that the grass carp *FII* gene may encode a protein for control of hemorrhagic diseases and may thus play an important role in resistance to GCRV873.

In conclusion, the grass carp *FII* gene has a high expression level after injection of

GCRV873; this increased expression may be associated with initiation of the coagulation system along with the platelet and complement systems. Our study provides a foundation for studying the mechanism and function of the *FII* gene and a theoretical support for analysis of coagulation mechanisms.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank our researchers for their hard work and reviewers for their valuable advice. This study was supported by a fund of the National Natural Science Foundation of China (#31272652), National High Technology Research and Development Program of China (#2011AA100404) and National technology System for Conventional Freshwater Fish industries of China Agriculture Research System (#CARS-46-42).

REFERENCES

Abel R, Salam NK, Shelley J, Farid R, et al. (2011). Contribution of explicit solvent effects to the binding affinity of small-molecule inhibitors in blood coagulation factor serine proteases. *Chem Med. Chem.* 6: 1049-1066.

Chen J, Li C, Huang R, Du F, et al. (2012). Transcriptome analysis of head kidney in grass carp and discovery of immune-related genes. *BMC Vet. Res.* 8: 108.

Eerenberg ES, Kamphuisen PW, Sijpkens MK, Meijers JC, et al. (2011). Reversal of rivaroxaban and dabigatran by prothrombin complex concentrate: a randomized, placebo-controlled, crossover study in healthy subjects. *Circulation* 124: 1573-1579.

Gessoni G, Valverde S and Manoni F (2012). Evaluation of the GeneXpert assay in the detection of Factor V Leiden and prothrombin 20210 in stored, previously classified samples. *Clin. Chim. Acta* 413: 814-816.

Jin Y, Tian LX, Zeng SL, Xie SW, et al. (2013). Dietary lipid requirement on non-specific immune responses in juvenile grass carp (Ctenopharyngodon idella). *Fish Shellfish Immunol*. 34: 1202-1208.

Kalamchi S (1987). Mental nerve anaesthesia secondary to breast cancer. *Dent. Update* 14: 127-128.

Kang TS, Georgieva D, Genov N, Murakami MT, et al. (2011). Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. *FEBS J*. 278: 4544-4576.

Li CH, Chen J, Shi YH and Lu XJ (2011). Use of suppressive subtractive hybridization to identify differentially expressed genes in ayu (Plecoglossus altivelis) associated with Listonella anguillarum infection. *Fish Shellfish Immunol*. 31: 500-506.

Liu Q, Xu B, Xiao T, Su J, et al. (2013). Molecular cloning, characterization and expression analysis of coagulation factor VII gene in grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol*. 35: 618-622.

Peyvandi F, Palla R, Menegatti M, Siboni SM, et al. (2012). Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European Network of Rare Bleeding Disorders. J. *Thromb. Haemost*. 10: 615-621.

Rhee JS, Jeong CB, Kim DH, Kim IC, et al. (2014). Immune gene discovery in the crucian carp Carassius auratus. *Fish Shellfish Immunol*. 36: 240-251.

Solomon D, Kim B, Scultetus A, Arnaud F, et al. (2011). The effect of rFVIIa on pro- and anti-inflammatory cytokines in serum and cerebrospinal fluid in a swine model of traumatic brain injury. *Cytokine* 54: 20-23.

Su J, Heng J, Huang T, Peng L, et al. (2012). Identification, mRNA expression and genomic structure of TLR22 and its association with GCRV susceptibility/resistance in grass carp (*Ctenopharyngodon idella*). *Dev. Comp. Immunol.* 36: 450-462.

Tang Y, Yu J, Li H, Xu P, et al. (2013). Molecular cloning, characterization and expression analysis of multiple leptin genes in Jian carp (*Cyprinus carpio* var. Jian). *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. 166: 133-140.

Tassanakajon A, Somboonwiwat K, Supungul P and Tang S (2013). Discovery of immune molecules and their crucial functions in shrimp immunity. *Fish Shellfish Immunol*. 34: 954-967.

Wang C, Dai J, Sun Z, Shi C, et al. (2015). Targeted inhibition of disheveled PDZ domain via NSC668036 depresses fibrotic process. Exp Cell Res. 331: 115-122.

Wong PC, Luettgen JM, Rendina AR, Kettner CA, et al. (2010). BMS-593214, an active site-directed factor VIIa inhibitor: enzyme kinetics, antithrombotic and antihaemostatic studies. *Thromb. Haemost*. 104: 261-269.

Ye X, Tian YY, Deng GC, Chi YY, et al. (2012). Complete genomic sequence of a reovirus isolated from grass carp in China. *Virus Res*. 163: 275-283.

Zhang QL, Yan Y, Shen JY, Hao GJ, et al. (2013). Development of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of grass carp reovirus. *J. Virol. Methods* 187: 384-389.