Quantitative in vivo and in vitro characterization of co-infection by two genetically distant grass carp reoviruses

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Grass carp reovirus (GCRV) is one of the most serious pathogens threatening grass carp (Ctenopharyngodon idella) production in China. Through sequence analysis, the co-existence of two genetically distant grass carp reoviruses, named GCRV-JX01 and GCRV-JX02, was revealed in the same diseased grass carp sample collected in 2011. GCRV-JX01 and GCRV-JX02 shared high levels of homology with GCRV-873 and GCRV-GD108, respectively. In contrast to GCRV-JX01, GCRV-JX02 induced no cytopathic effect in infected cells. A quantitative real-time PCR assay was employed to monitor the replication efficiency of both virus strains in either Ctenopharyngodon idella kidney (CIK) cells or infected cell supernatant. The results demonstrated that, although GCRV-JX02 did reduce the cellular replication level of GCRV-JX01 up to 10-fold during co-infection, there was no significant impact on the productive virus progeny level in supernatant compared to that of cells infected by GCRV-JX01 alone. To validate the hypothesis that both viruses might co-infect grass carp without significant interference in the field, we collected clinical samples from two different fish farms in 2012 and monitored virus loads for each fish. The data showed that 55% of the collected fish samples were co-infected by GCRV-JX01 and GCRV-JX02, and the single virus infection rate was 10% for GCRV-JX01 and 20% for GCRV-JX02. For both viruses, the in vivo viral loads under co-infection and single viral infection were similar. No serological cross-reaction or cross-protection occurred between GCRV-JX01 and JX02 in our immunization and challenge tests. This new information on co-infection by two genetically distant virus strains should be helpful for designing vaccines targeting the causative agents of grass carp haemorrhagic disease.

INTRODUCTION

Grass carp (Ctenopharyngodon idella) is one of the four major fish species that are crucial to freshwater aquaculture in China (Ke et al., 1990; Ding et al., 1991; Wang et al., 1994). Grass carp reovirus (GCRV) is a major fish pathogen that causes yearly severe epidemic outbreaks of haemorrhagic disease affecting fingerling and yearling grass carp in Southern China; other aquatic animals including black carp (Mylopharyngodon piceus), topmouth gudgeon (Pseudorasbora parva) and rare minnow (Gobiocypris rarus) were also reported to be sensitive to GCRV (Wang et al., 1994; Rangel et al., 1999; Zhang et al., 2010a). Currently, there is no effective treatment against GCRV infection except vaccination, which has been regarded as the only promising method in reducing the loss of the grass carp farming industry (Lu et al., 2011). A chemically attenuated virus strain (GCHV-892), licensed for commercial production in 2011 (Veterinary Drug Permit no. 190986021, China), has been the only legal vaccine for grass carp haemorrhagic disease. Yet the effectiveness of a single-vaccine approach in preventing the disease could be undermined if two reoviruses were found to independently cause the disease. Co-infection could further render the single-vaccine approach ineffective.

Aquareovirus and Orthoreovirus are two genera in the family Reoviridae. Studies on GCRV and other aquareovirus genomes have revealed that there is a close evolutionary relationship between members of genera Aquareovirus and Orthoreovirus (Attoui et al., 2002; Fang et al., 2000). Aquareoviruses (AQRV), affecting a wide variety of aquatic animals including fish and crustaceans, are a significant threat to aquaculture industries throughout the world. GCRV and grass carp haemorrhagic disease also play a major role in the study of viral replication and pathogenesis of aquareoviruses (Lu et al., 2011). GCRV is a dsRNA virus that has been assigned to the genus...
**Aquareovirus** in the family *Reoviridae* (Mertens et al., 2005). The genome of GCRV consists of 11 segments that are packaged into a double-layered capsid without an envelope, and encodes 7 structural proteins (VP1–VP7) and 5 nonstructural proteins (Attoui et al., 2002; Fang et al., 2005, 2008; Cheng et al., 2008, 2010). The type strain, GCRV-873, can propagate in fish cell lines and produce observable cytopathic effects (CPFs) under the microscope during infection (Ke et al., 1990). Yet recently, it was found that two newly isolated GCRV strains, GCRV-HZ08 and GCRV-GD108, propagated in a grass carp cell line without detectable CPE (Zhang et al., 2010a; Ye et al., 2012).

Among all GCRV isolates, complete genome sequences of GCRV-873 and GCRV-GD108 strains have been reported (Attoui et al., 2002; Ye et al., 2012). Only partial genome segments of other GCRV isolates (i.e. GCRV-991, GCRV-875 and GCRV-876) have been published. Epidemiological surveys and RT-PCR assays of clinical samples collected from different regions suggested that GCRV-873 and GCRV-GD108 represented the two dominant grass carp reovirus genotypes in Southern China (Ye et al., 2012). However, no information was available on the co-existence or co-infection of the two genetically distant genotypes before this study. Lacking this information, the single attenuated virus-based vaccine strategy poses an unknown and uncontrollable risk for grass carp farmers.

Rapid cDNA synthesis and sequencing techniques have been developed for dsRNA viruses (Maan et al., 2007). They make the sequence analysis of reovirus genomic dsRNA fragments no longer technically demanding. Through the use of the established FLAC (full-length amplification of cDNAs) technique and sequence analysis, the co-existence of two genetically distant grass carp reovirus genotypes was revealed from diseased grass carp in the Jiangxi province of China in 2011. To probe the interference between these two viral strains both in vivo and in vitro, a quantitative real-time RT-PCR method was employed to monitor the replication level of both viruses during co-infection in grass carp and its cell lines. This paper reports new findings on the epidemiology of grass carp reoviruses and discusses the significance of these findings with respect to designing vaccination approaches for disease control.

**RESULTS AND DISCUSSION**

Detection and isolation of two genetically distant GCRV strains from the same batch of clinical samples in 2011

Clinical samples from fish with typical haemorrhagic symptoms from a grass carp farm in Jiangxi province were collected for molecular diagnosis of GCRV infection in May 2011. Through the use of FLAC technology (Maan et al., 2007), full-length cDNA copies of the viral dsRNA genomic segments were synthesized and amplified by PCR. Complete sequences of five genomic segments were initially identified through this method (GenBank accession numbers JQ042805, JQ042806, JQ042807, JQ042808 and JX26303). Surprisingly, three of them were over 99% identical with the corresponding genomic segments of GCRV-873, and the other two sequences were over 99% identical with the genome of GCRV-GD108. Since GCRV-873 and GCRV-GD108 shared little homology, the chance for recombination between these two viruses should be very low. Considering that GCRV-873 and GCRV-GD108 represented two major and distinct reovirus genotypes in grass carp (Ye et al., 2012), we hypothesized that the two genetically distant virus strains might co-infect grass carp during disease pandemic. To prove it, the infinite dilution method was used to purify the two virus strains, hereby named GCRV-JX01 and GCRV-JX02, from grass carp *Ctenopharyngodon idella* kidney (CIK) cells in 96-well plates. As expected, GCRV-JX01 caused an extensive CPE, similar to the CPE caused by GCRV-873 in infected CIK cells. Similar to GCRV-GD108 infection, GCRV-JX02 infection resulted in no observable CPE (Fig. 1a). At 24 h post-virus infection with an m.o.i. of 5 of each virus, dsRNA was extracted from the infected cells for both viruses and analysed by agarose gel electrophoresis. Typical reoviral genomic patterns (represented by large, medium and low molecular mass dsRNAs) demonstrated that both viruses seemed to replicate well in the infected CIK cells (Fig. 1b).

Sequence analysis indicated that the outermost VP7 capsid protein encoded by the Seg-10 segment of the GCRV genome was the most divergent of all the structural proteins (Attoui et al., 2002; Cheng et al., 2008). As a newly isolated strain, the Seg-10 of GCRV-GD108 encoded a protein that was not homologous to the VP7 of any known AQRVs (Ye et al., 2012). Phylogenetic trees were constructed by the neighbour-joining method based on the amino acid sequences of Seg-10 for both GCRV-JX01 and JX02. As shown in Fig. 1c, GCRV-JX01 was closest to GCRV-873, which was regarded as the GCRV standard strain. GCRV-JX02 was closest to GCRV-GD108, which was only very recently sequenced and reported (Ye et al., 2012). The trees apparently displayed a distant genetic relationship between GCRV-JX01 and GCRV-JX02.

Plaque purification of CPE-causing virus was also performed and followed by amplification of each picked plaque in CIK cells. By using primers targeting all of the 12 ORFs encoded by the 11 genomic segments of GCRV-873, we were able to show that all randomly picked viral colonies possessed the 12 ORFs (see Table S1 and Fig. S1, available in JGV Online). Since this was believed to be the first discovery of the co-existence of two genetically distant reoviral strains in the same batch of clinical samples, further sequencing of all the encoding ORFs of GCRV-JX01 and GCRV-JX02 was performed to confirm that these two viral strains respectively shared sequence identity of above 99% with GCRV-873 and GCRV-GD108 (data not shown). These data demonstrated the existence of two genotypes of viruses in the clinical sample.
The chance of producing reassortments from the co-infection seemed very low; however, we could not completely exclude the possibility at this time. Generally, our results supported the idea that GCRV-873 and GCRV-GD108 represent two major reovirus genotypes that cause haemorrhagic disease in grass carp.

Establishment of a real-time RT-PCR method for virus quantification

A classical TCID_{50} analysis was performed to titrate the infectious viral particles in infected supernatants. Real-time RT-PCR had been proved effective in the diagnosis of grass carp reovirus infection (Escaffre et al., 2010). A novel real-time RT-PCR system was applied to investigate the in vivo and in vitro co-infection of GCRV-JX01 and GCRV-JX02 in both the grass carp and its cell lines. Since the Seg-10 of GCRV-JX01 and the Seg-11 of GCRV-JX02 were similar in length (around 950 bp), they were chosen to be the target fragments for real-time RT-PCR amplification. Two sets of real-time PCR primers were designed with the DNAssist program to detect the GCRV-JX01 and JX02 genomes (see Table S1). For absolute quantification of the genomic dsRNA, RNAs representing the Seg-10 of GCRV-JX01 and the Seg-11 of GCRV-JX02 were synthesized as the templates for real-time RT-PCR. To quantify the genomic RNA level in infected cells, synthesized RNA standards were diluted in total cellular RNA that was normalized to the same concentration as sample RNAs. Thus, two specific standard curves were obtained by running five 10-fold serial dilutions of in vitro transcribed RNAs (Fig. 2a). The correlation coefficients (R^2) were above 0.980 and reaction efficiencies (E) covered a range from 98.1 to 100.1 %.

The specificity of the products amplified using SYBR Green I was monitored by the dissociation curve of each amplicon. A single peak in the dissociation curve at the expected temperature for a certain amplicon indicates specific amplification. In our analysis, GCRV-JX01 and GCRV-JX02 amplicons could be easily distinguished by their melt temperature (T_m) values. Melting curve analysis revealed that the GCRV-JX01-specific melting curve displayed a T_m of 80.5 °C, whereas the GCRV-JX02-specific curve showed a
$T_m$ of 78.5 °C (Fig. 2b). Both amplicons were sequenced to further confirm the specificity of the real-time RT-PCR assay (data not shown).

**Co-infection of GCRV-JX01 and JX02 in CIK cells displayed no significant interference in progeny production**

To produce virus stock for our infection and quantification test, CIK cells were infected with GCRV-JX01 or JX02 with a low m.o.i. At 48 h post-infection (p.i.), infected supernatants were harvested and parts of them were subjected for TCID$_{50}$ titration. For co-infection analysis, $10^7$ TCID$_{50}$ GCRV-JX01 and $10^7$ TCID$_{50}$ GCRV-JX02 were mixed and inoculated to CIK cells followed by 1 h of virus adsorption. As an infection control, the same amount of GCRV-JX01 or GCRV-JX02 was individually used to infect CIK cells. At 0, 4, 8, 12, 18, 24 and 36 h p.i., supernatant was sampled for virus titration by TCID$_{50}$ assay. For GCRV-JX01, cells in 96-well plates were observed under a light microscope for typical CPE. For GCRV-JX02, RT-PCR analysis targeting Seg-11 was performed on total RNA from infected CIK cells in 96-well plates for positive infection. At these time points, infected cells were harvested for total RNA extraction and purification. Viral genomic RNA copy numbers were further quantified using the real-time RT-PCR system. The mean and the SD of the copy numbers for each sample were determined and data points considered valid when falling within 1 SD of the mean. The viral RNA copies in the cells and supernatant samples were calculated through the extrapolation of the threshold cycle (C$_t$) values to the standard curves in the real-time analysis. Quantification data were analysed using the Cochran test ($P<0.05$) and transformed to log base 10. A $t$ test was used to compare means of the virus copy numbers in the assay. Statistical analyses were conducted using the SPSS program (version 16.0).

Titration of virus at the indicated time points showed that co-infection resulted in identical supernatant-progeny production patterns as infection of individual virus for both GCRV-JX01 and JX02 (Fig. 3a). At all the tested time points, infected cultures were subjected to TCID$_{50}$ titration. The mean linear correlation coefficient ($R^2$) and the mean percentage PCR efficiency ($E$) are indicated. (b) The specificity of two pairs of primers used in the quantitative real-time RT-PCR. Melt peaks of PCR products and negative no template controls (NTCs) are indicated.

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**Fig. 2.** Standard curve and melting curve analysis of the established real-time quantitative RT-PCR. (a) Two standard curves for cellular viral RNA quantification. The mean linear correlation coefficient ($R^2$) and the mean percentage PCR efficiency ($E$) are indicated. (b) The specificity of two pairs of primers used in the quantitative real-time RT-PCR. Melt peaks of PCR products and negative no template controls (NTCs) are indicated.
points, no significant variation was observed between the individual infection and co-infection, as verified by the \( t \) test \((P>0.05)\). A difference in virus-producing patterns was noticed for these two viruses during either co-infection or separate infection. GCRV-JX02 replicated faster than GCRV-JX01 during early infection. At 4 h p.i., the titre of GCRV-JX02 was 100 times higher than that of GCRV-JX01. The titre of GCRV-JX01 continued to grow to a peak level of \(10^7\) at 18 h p.i., while GCRV-JX02 remained stable from 4 to 18 h p.i.

The different viral progeny production patterns for these two genotypes might indicate a difference in their replication efficiencies inside the infected cells. Thus, we continued to investigate the amplification efficiency of cellular viral genomic RNAs. Since total cellular RNAs were used as templates for our assay, the quantitative data might represent the combination number of both genomic dsRNA and transcribed mRNA. In CIK cells co-infected with the two genotype viruses, GCRV-JX02 exhibited a sharp increase \((P<0.01)\) inside cells from 4 to 12 h p.i. until reaching a peak level of \(10^{10.45}\). Cells infected with only GCRV-JX02 maintained a stable RNA level of \(10^8.82\) from 4 to 12 h p.i., and during late infection, a sharp increase of viral RNA copy number to as high as \(10^{10}\) was noticed (Fig. 3b). In CIK cells co-infected with both GCRV-JX01 and JX02, RNA copies of GCRV-JX01 remained at a stable level of \(10^{10.8}\) in the first 12 h. GCRV-JX01 infection alone resulted in a continuous increase in viral-specific RNA numbers to a peak level of \(10^{8.75}\) (Fig. 3b). Due to the cell necrosis effect caused by GCRV-JX01, co-infection of both genotypes resulted in a sharp decrease in viral RNA copy numbers from 12 h p.i., which was similar to the result from infecting with GCRV-JX01 only.

![Fig. 3](http://vir.sgmjournals.org) Time-course curves of viral loads in both infected supernatants and infected cells. CIK cells were infected with GCRV-JX01, GCRV-JX02 or both (as shown). (a) TCID\(_{50}\) assay of virus in supernatants of \(10^6\) infected cells. (b) RT-PCR analysis of viral RNA copy numbers from \(10^6\) infected cells. The supernatant and infected cells were sampled at 0 to 36 h p.i. in duplicate experiments. *, significant difference between infection alone and co-infection with the \( t \) test \((P<0.05)\); **, highly significant difference with the \( t \) test \((P<0.01)\).
These data indicated that both genotypes could replicate efficiently in grass carp cells during co-infection. There was no observable interference in the progeny production of both viruses in the infected supernatants. Compared with individual virus infection, certain differences had been noticed in the replication or transcription levels of viral-specific RNA inside infected cells during co-infection in vitro; but the viral replication of both genotypes proceeded successfully during either co-infection or separate infection. The quantification data for both supernatants and cells supported the general idea that the two viruses could co-exist in the same host cells and finish their replication cycle efficiently.

**Detection of co-infection by two genotype GCRVs from diseased carps during the pandemic season in 2012**

In May 2012, a grass carp haemorrhagic pandemic was reported in Xiantao of Hubei province. Twenty diseased carps of around 0.5 kg in size were collected from two isolated local fish farms. In Table 1, samples 1–10 were from farm A, and samples 11–20 were from farm B. The kidney tissue of collected fish was used for RNA purification and real-time RT-PCR analysis. The amplified products from clinical samples showed 'characteristic' amplification curves for either GCRV-JX01 or JX02 using our primer sets. The amplified products from tissue of collected fish was used for RNA purification and farm A, and samples 11 were isolated local fish farms. In Table 1, samples 1–10 were from farm A and 60% of carps from farm B showed positive for GCRV-JX01, 15 were positive for GCRV-JX02 and 11 were positive for both viruses. The fact that 50% of carps from farm A and 60% of carps from farm B showed co-infection confirmed our hypothesis that the two dominant reovirus genotypes might jointly contribute to the disease pandemic in grass carps.

Table 1 summarizes the data from real-time RT-PCR analysis for all the 20 grass carps. It was worth noting that in this analysis, primers specific for GCRV-JX01 and JX02 also represented the known GCRV-873 and GCRV-GD108 genotypes, respectively. Out of the 20 carps, 13 were positive for GCRV-JX01, 15 were positive for GCRV-JX02 and 11 were positive for both viruses. The fact that 50% of carps from farm A and 60% of carps from farm B showed co-infection confirmed our hypothesis that the two dominant reovirus genotypes might jointly contribute to the disease pandemic in grass carps.

Detailed quantification data as shown in Table 1 also revealed that in all the tested samples, the tissue-specific viral genomic RNA level of GCRV-JX01, ranging from $10^{2.44}$ to $10^{4.40}$, was significantly lower than that of GCRV-JX02, ranging from $10^{4.7}$ to $10^{6.01}$. This might be partially explained by different CPEs caused by these two viruses. Since either one of the two virus strains could cause disease in grass carps with typical haemorrhagic symptoms, it was hard to conclude that GCRV-JX01 was more virulent than GCRV-JX02.

**Grass carp immunized with either one of the two viral strains produced no cross-reactive antibody in dot blot assays and was susceptible to infection of another virus**

The low level of homology between GCRV-JX01 and JX02 might suggest a lack of serological cross-reactivity between these two viruses. To prove this hypothesis, an immunization and challenge test was performed. Twenty-one days post-immunization of grass carps with either inactive GCRV-JX01 or JX02, sera was sampled from each immunized carp to analyse its reactivity to both viral strains. Dot blot assays demonstrated that no serological cross-reactivity between these two viruses was detected in all the sera samples (Fig. 4a).

Furthermore, we challenged the immunized grass carp with live virus. Fig. 4b demonstrates that GCRV-JX02 replicated efficiently in grass carps immunized with inactivated GCRV-JX01, and GCRV-JX02 completed its infectious life cycle in carps immunized with inactivated GCRV-JX-01. The lack of cross-protection between these two viruses could be explained by their low level of homology and was consistent with the discovery of their efficient co-infection in grass carp (Table 1) and its cell lines (Fig. 3).

Presently, vaccination against GCRV infection in grass carps is only based on one viral genotype. For an unidentified reason, the sequence information for the vaccine strain remains undisclosed. Nevertheless, our results on the lack of cross-protection suggest that a single-vaccine approach would be inadequate when grass carp are susceptible to

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**Table 1. RT-PCR results for samples of diseased grass carp from fish farms in Hubei province**

<table>
<thead>
<tr>
<th>Sample</th>
<th>log$_{10}$ (RNA copies per 200 µl)</th>
<th>Co-infection$^\dagger$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GCRV-JX01$^*$</td>
<td>GCRV-JX02$^*$</td>
</tr>
<tr>
<td>1</td>
<td>$10^{2.44}/35.28$</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>20</td>
<td>–</td>
<td>$10^{5.70}/33.15$</td>
</tr>
</tbody>
</table>

$^*$A minus sign indicates that grass carp reovirus was not detected in the sample.

$^\dagger$A minus sign indicates that co-infection was not detected in the sample.
infection by two reoviruses. The two dominant genotypes of GCRV should be simultaneously incorporated into a more effective disease prevention program.

**Conclusion**

We detected two genetically distant genotypes of grass carp reoviruses, named as GCRV-JX01 and GCRV-JX02, from the same clinical sample. TCID<sub>50</sub> assay and a real-time RT-PCR method were employed to quantify the virus replication levels in infected supernatants and cells, respectively. Co-infection of these two viruses in CIK cells resulted in the same time-course pattern of progeny production as individual virus infection. The fact that both virus genotypes successfully replicated in CIK cells during co-infection further supported that GCRV-JX01 and JX02 could co-exist in the same host cells and produce no significant interference on each other. Data on the virus RNA loads of both viral genotypes in clinical samples collected from a 2012 pandemic demonstrated that co-infection existed in over 50% of diseased grass carps. No cross-genotype protection occurred between GCRV-JX01 and JX02 in artificial vaccination and challenge analysis. These results collectively suggest that vaccination strategies based on one genotype of grass carp reovirus would be not adequate in disease prevention. Detection of both viral genotypes simultaneously had to be performed in pathogen diagnosis.

**METHODS**

**Sequencing and isolation of GCRV-JX01 and JX02.** Diseased grass carps with typical haemorrhagic symptoms were collected from...
a fish farm in Jiangxi province in 2011. Extracts of kidney were inoculated into the CIK cell line. The virus-infected cell culture supernatant was harvested and viral dsRNA was extracted through the use of TRIzol reagent (Invitrogen) following the manufacturer’s protocol. The FLAC technique (Maan et al., 2007) was employed to obtain the viral genomic nucleotide sequences. Alignments of all sequences were performed through the use of CLUSTAL W software (Thompson et al., 1994) and the National Center for Biotechnology Information (NCBI) BLAST program (http://blast.ncbi.nlm.nih.gov/BLAST.cgi). Phylogenetic analyses were performed with MEGA 4 software (Tamura et al., 2007).

Two GCRV genotypes were separately isolated from the same batch of clinical samples, further purified by infinite dilution to infect CIK cells, and confirmed by RT-PCR of viral RNA using primers designed according to the genome of GCRV-873 or GCRV-GD108 (data not shown). Purified virions were used to infect CIK cells for the observation of CPE under the microscope. Complete genomic sequences of GCRV-JX01 and JX02 were determined by RT-PCR and gene sequencing, and were compared to sequence information for sequences of GCRV-JX01 and JX02 were determined by RT-PCR of viral RNA using primers designed with the Primer 3 program (http://frodo.wi.mit.edu/Primer3).BLAST search. Primers for RT-PCR analysis of encoding regions within clinical samples, further purified by infinite dilution to infect CIK cells, and confirmed by RT-PCR of viral RNA using primers designed according to the genome of GCRV-873 or GCRV-GD108 (data not shown). Purified virions were used to infect CIK cells for the observation of CPE under the microscope. Complete genomic sequences of GCRV-JX01 and JX02 were determined by RT-PCR and gene sequencing, and were compared to sequence information for GCRV-873 and GCRV-GD108 from the NCBI nucleotide database.

**Titration of virus in infected supernatant.** The titre of GCRV was determined as TCID₅₀ on CIK cells based on a typical CPE produced by viral infection (LaBarre & Lowy, 2001). For GCRV-JX01, 96-well plates were observed under a light microscope for typical CPE. For GCRV-JX02, RT-PCR analysis targeting Seg-11 was performed on total RNA from infected CIK cells in 96-well plates to determine positive infection. The TCID₅₀ value was calculated using the Reed–Muench method. A classical plaque assay was also performed on CIK monolayers to isolate virus with CPE from the supernatant of co-infected cells (Ridinger et al., 1982). RT-PCR analysis for the existence of all the 11 genomic segments of GCRV-JX01 was performed on total RNA from infected CIK cells in 96-well plates.

**Design of primers.** The nucleotide sequences of GCRV-JX01 Seg-10 and GCRV-JX02 Seg-11 were analysed separately with the DNAassist program for similarity comparison. Primer sets for both two viruses were designed with the Primer 3 program (http://frodo.wi.mit.edu/ primer3/). The specificity of the selected primers was evaluated with a BLAST search. Primers for RT-PCR analysis of encoding regions within viral genomic dsRNA segments were designed using the genome information for each virus. The sequences and details of the primers are shown in Table S1.

**Preparation of RNA standards.** Standard RNAs were generated as described previously (Guo et al., 2012). Briefly, to produce *in vitro* transcribed RNA with identical sequences of GCRV-JX01 Seg-10 (828 bp) or GCRV-JX02 Seg-11 (932 bp), RT-PCR was performed to amplify corresponding cDNAs from total RNA extracts of CIK cells infected with GCRV-JX01 or GCRV-JX02. The cDNA products of JX01-S10 and JX02-S10 contained the T7 promoter that was included in the primer sequence. They served as the templates for the *in vitro* transcription, conducted with the RibopMax large scale RNA production system-T7 (Promega). After incubation at 37 °C for 4 h, the DNA templates were removed by digestion with DNase and the transcribed RNAs were purified through the use of the RNeasy mini kit (Qiagen). After dilution, the concentrations of the RNAs were quantified through the measurement of absorbance at 260 nm with a NanoDrop system (Thermo) and the exact number of RNA molecules was calculated with the formula described by Escaffre et al. (2010). The calculated standard RNAs were serially diluted 10-fold in either water or cellular RNA solution to generate four standard curves for quantitative real-time PCR. For cellular viral RNA quantification, RNAs were diluted with extracted RNA solution of mock-infected cells (10⁶). For the quantification of virus in infected supernatants, RNAs were diluted with RNase-free water.

**Quantitative real-time RT-PCR assay.** cDNAs of purified RNAs, from 10⁶ infected cells or supernatants, were synthesized with the PrimeScript reverse transcription system (TaKaRa). Real-time PCR was carried out in 20 µl reaction mixture with SsoAdvanced SYBR Green Supermix (Bio-Rad) on a CFX96 Touch real-time PCR detection system (Bio-Rad). All samples in standard curves were carried out in duplicate to ensure reproducibility. Data acquisition, analyses and the quantification of the viral load of each sample were performed with CFX Manager software data analysis (Bio-Rad) and the SPS program (version 16.0).

**Co-infection of GCRV-JX01 and GCRV-JX02 in vitro.** The 90 % confluent CIK (10⁵) monolayers in 96-well plates were co-infected with GCRV-JX01 and GCRV-JX02 at an m.o.i. of 5 (5 × 10⁶ copies per flask). The same amount of GCRV-JX01 or GCRV-JX02 was used to infect CIK cells separately as positive controls. After 1 h of virus adsorption, the cells were washed twice with PBS (pH 7.5) and incubated with 5 ml fresh medium at 28 °C (Fang et al., 2008). Cells and supernatants were harvested separately at 0, 4, 8, 12, 18, 24 and 36 h post-adsorption and each time point was performed in duplicate. RNA extraction from cells or supernatants and cDNA synthesis were conducted as indicated above.

**Analysis of co-infection in clinical samples.** The quantitative real-time RT-PCR assay was employed to detect virus infection in kidney tissue of 20 diseased grass carps (0.5 kg each), which were collected during a pandemic in May 2012 from two fish farms in Hubei, China. Total RNA extracted from kidney tissue using the TRIzol method was used as the template for the real-time RT-PCR assay. To exclude an influence of potential inhibitors of PCR, synthesized cDNAs from clinical samples were diluted 10 and 100 times, amplified and compared to the results from undiluted cDNA samples. RNase-free water-template amplification was also conducted as a negative control.

**Immunization and challenge of grass carp with virus.** GCRV-JX01 or JX02 was concentrated to 1 × 10¹⁰ TCID₅₀ ml⁻¹. The virus was UV inactivated with a 30 W general electric germicidal lamp placed at a distance of 15 cm. The effectiveness of virus inactivation was determined by TCID₅₀ assay using CIK cells. A 200 µl preparation of the inactivated GCRV particles were emulsified in equal ratio with Freund’s complete adjuvant (Sigma) and intra-peritoneally vaccinated to 10 grass carp (200 g each) for each virus. The animals were monitored after immunization to ensure that there were no adverse effects. Sera were collected from the caudal vein with a 10-gauge needle at day 21 post-treatment. The pooled polyclonal sera of the 10 carps were subjected for further dot blot analysis for a test of serological cross-reactivity. The immunized carps were further challenged with GCRV-JX01 or JX02 at a dose of 10¹⁰ TCID₅₀ per 200 µl per fish through abdomen injection. The surviving carps (over 50 % at the end of the experiments) were anaesthetized and bled at day 1, 2, 3, 4, 7, 10, 14, 17 and 21 post-viral challenge. To serve as positive controls, non-immunized carps were challenged with the same dose of JX01 or JX02 and bled at the same time points as the immunized carps. The cross-protection against GCRV infection was indexed by a calculation of serum viral load through TCID₅₀ assay, which was performed as described above for titration of virus in infected supernatant.

**The immuno-dot blot assay.** Blots were performed using a Bio-Rad Bio-Dot apparatus. 10⁻⁶–10⁻¹⁰ TCID₅₀ virus (in a volume of 500 µl) per spot was spotted onto PVDF membrane and incubated at room temperature for 2 h. The membranes were removed from the apparatus and quenched with 5 % (w/v) dried non-fat milk in PBS containing 0.05 % Tween 20 (PBST) for 2 h at room temperature followed by a quick wash in PBST. Blood samples collected 4 weeks after immunization were used as primary antibody, and were added
to the membranes and incubated at 4 °C overnight. After washing three times with PBST for 5 min each, a monoclonal anti-grass carp IgM made in the laboratory was added and incubated for another 1 h. The blots were again washed three times with PBST for 5 min each, alkaline phosphate-conjugated rabbit anti-mouse antibody diluted at 1:3000 in PBST was added at 37 °C for 1 h. Washing was repeated and the blots were developed using Bio-Rad alkaline phosphatase conjugate substrate kit according to the directions supplied with the kit. Serum from normal grass carp was used as a negative control.

**ACKNOWLEDGEMENTS**

This work was supported by grants from the National Natural Science Foundation of China (no. 31072244) and the Earmarked Fund for China Agriculture Research System (no. CARS-46-12).

**REFERENCES**


Co-infection by two grass carp reoviruses