In vivo virus growth competition assays demonstrate equal fitness of fish rhabdovirus strains that co-circulate in aquaculture

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A novel virus growth competition assay for determining relative fitness of RNA virus variants in vivo has been developed using the fish rhabdovirus, Infectious hematopoietic necrosis virus (IHNV), in juvenile rainbow trout (Oncorhynchus mykiss). We have conducted assays with IHNV isolates designated B, C, and D, representing the three most common genetic subtypes that co-circulate in Idaho trout farm aquaculture. In each assay, groups of 30 fish were immersed in a 1:1 mixture of two genotypes of IHNV, and then held in individual beakers for a 72 h period of in vivo competitive virus replication. Progeny virus populations in each fish were analyzed for the presence and proportion of each viral genotype. In two independent assays of the B:C isolate pair, and two assays of the B:D pair, all fish were co-infected and there was a high level of fish-to-fish variation in the ratio of the two competing genotypes. However, in each assay the average ratio in the 30-fish group was not significantly different from the input ratio of 1:1, indicating equal or nearly equal viral fitness on a host population basis, under the conditions tested.

1. Introduction

Fitness is one of the most important parameters thought to influence viral diversity, spatial distribution, and evolution. Viral fitness is often measured as the relative ability of two competing viruses to produce infectious progeny during co-infection in a given environment (Domingo et al., 1999; Domingo and Holland, 1997; Holland et al., 1991). To date, the vast majority of literature on fitness of vertebrate RNA viruses has been generated in cell culture systems with model viruses such as vesicular stomatitis virus and foot and mouth disease virus. These in vitro systems have provided an extensive foundation of information and theory on RNA virus fitness and population biology (Chao, 1990; Clarke et al., 1994; Domingo et al., 2001; Duarte et al., 1992, 1994a,b; Escamis et al., 1996, 1999; Holland et al., 1991; Miralles et al., 1999; Novella et al., 1995a,b, 1999; Turner and Chao, 1998, 1999; Weaver et al., 1999). However, their relevance to fitness in intact vertebrate hosts remains to be demonstrated, partly due to a lack of in vivo systems for virus growth competition assays. Recently several research groups have reported competition studies with human immunodeficiency virus (HIV) strains in cell culture systems (Dykes et al., 2006; Harrigan et al., 1998; Lu and Kuritzkes, 2001; Martinez-Picado et al., 2006) and in ex vivo peripheral blood mononuclear cells (Abrahm et al., 2005; Arien et al., 2005; Neumann et al., 2005; Quinones-Mateu et al., 2000; Troyer et al., 2005). For in vivo competition between viral strains in intact living vertebrate hosts there are relatively few publications, mostly in mice and bird hosts, and they typically use small group sizes of less than ten animals (Burke et al., 2006; Carrillo et al., 1998; Doczek et al., 1996; Lee and Suarez, 2004; Lenhoff et al., 1998). Therefore, to study the relative fitness of RNA virus variants in vivo, we sought to develop a system to measure competitive fitness of selected isolates of infectious hematopoietic necrosis virus (IHNV) in its natural host, rainbow trout (Oncorhynchus mykiss).

IHNV is an economically important rhabdoviral pathogen that causes epidemics in both cultured and wild salmonid fishes throughout western North America (Bootland and Leong, 1999; Wolf, 1988). Characterization of hundreds of field isolates has defined three major genetic groups of IHNV in North America, designated the U, M, and L genogroups (Kurath et al., 2003). The M genogroup is endemic, and also frequently epidemic, in a localized intensive rainbow trout farming region in Idaho where phylogenetically distinct sub-groups, designated MA-MF have been designated the U, M, and L genogroups (Kurath et al., 2003). We have conducted assays with IHNV isolates designated B, C, and D, representing the three most common genetic subtypes that co-circulate in Idaho trout farm aquaculture. In each assay, groups of 30 fish were immersed in a 1:1 mixture of two genotypes of IHNV, and then held in individual beakers for a 72 h period of in vivo competitive virus replication. Progeny virus populations in each fish were analyzed for the presence and proportion of each viral genotype. In two independent assays of the B:C isolate pair, and two assays of the B:D pair, all fish were co-infected and there was a high level of fish-to-fish variation in the ratio of the two competing genotypes. However, in each assay the average ratio in the 30-fish group was not significantly different from the input ratio of 1:1, indicating equal or nearly equal viral fitness on a host population basis, under the conditions tested.
documented (Troyer and Kurath, 2003; Troyer et al., 2000). The MB, MC, and MD sub-groups are the three most prevalent and widely distributed types of IHNV in the virus-endemic region, and they have been shown to co-circulate in the field for over 20 years (Troyer et al., 2000). Therefore, we hypothesized that representative isolates from these subgroups would have equal or nearly equal fitness.

For initial development of an in vivo virus growth competition assay, virus isolates of equal or similar fitness were desirable because they would allow us to test whether we could reliably generate, detect, and quantify mixed infections in individual fish. The three IHNV isolates chosen for this work were obtained from clinically infected rainbow trout at the same aquaculture facility in the same year (1991). The competition system developed here (Fig. 1) and referred to as the “MISFIT” system (for multiple isolated fish infection test) involved challenging juvenile rainbow trout in batch with a mixture of two different IHNV isolates that could be distinguished genetically. Fish were then separated into individual beakers and held for a 3-day period of competitive in-host virus replication. Progeny virus populations in each infected fish were then analyzed to determine whether one or both of the input genotypes were present, and in what proportions. Each competition assay was conducted in approximately 30 individual fish, so that the calculated average of the resulting ratios in the 30 progeny virus populations provided a host population-scale estimate of the relative fitness of the two virus isolates being competed. We report here development and validation of this novel in vivo virus competition assay, and show that two pairs of IHNV isolates (MB20:MC30 and MB20:MD39) that co-circulated in the field have equal or nearly equal competitive fitness.

2. Materials and methods

2.1. Cells and viruses

*Infectious hematopoietic necrosis virus* (IHNV) is the type species of the genus *Novirhabdovirus*, within the family *Rhabdoviridae* (Tordo et al., 2005). The IHNV isolates referred to here as MB20, MC30, and MD39 have been described previously as isolates FF020-91, FF030-91, and FF039-91, respectively (Troyer et al., 2000). These isolates are members of genetic subgroups MB, MC, and MD, respectively, all within the M genogroup of IHNV. Competition assays conducted between isolates MB20 and MC30 will be referred to as B:C assays, and the progeny of these isolates are referred to as geno-

![Flow diagram of in vivo virus growth competition assay for determining relative fitness of infectious hematopoietic necrosis virus (IHNV) variants.](image-url)

**Fig. 1.** Flow diagram of in vivo virus growth competition assay for determining relative fitness of infectious hematopoietic necrosis virus (IHNV) variants. The assay uses multiple isolated fish infection test (MISFIT) conditions for challenging groups of fish with two viral isolates, and progeny virus populations are analyzed by two methods as shown.
type B (gtB) and genotype C (gtC), respectively. Similarly, assays conducted between isolates MB20 and MD39 will be referred to as B:D assays, and the progeny of these isolates are gtB and genotype D (gtD), respectively. Viruses were propagated in the epithelioma papulosum cyprini (EPC) cell line with minimal essential medium supplemented with 10% fetal calf serum as described (Anderson et al., 2000). The viral strains used in the competition assays were passaged three times in cell culture at a multiplicity of infection (MOI) of 0.001 to minimize defective interfering particles. High-titer viral stocks were stored as 1 ml aliquots at −80 °C. Virus stocks were precisely titered by plaque assay (Batts and Winton, 1989), with a minimum of seven replicate dilution series, each counted in triplicate wells. Stock virus titers were: MB20, 2.79 ± 0.20 × 10⁸ pfu/ml; MC30, 2.14 ± 0.18 × 10⁸ pfu/ml; and MD39, 1.44 ± 0.22 × 10⁸ pfu/ml (mean ± 1 S.E.M.).

2.2. Fish maintenance and batch challenge to determine virulence of individual virus strains

All fish were research grade rainbow trout from Clear Springs Foods, Inc., generously provided by Dr. S.E. LaPatra. Juvenile fish were received and maintained at 15 °C in sand-filtered, UV-irradiated lake water, and fed a moist pelleted diet from Bio-Oregon. Fish were restricted from feeding for 24 h prior to challenge to reduce stress (Wedemeyer, 1996). For virulence assays of individual virus strains, triplicate groups of 25 rainbow trout (average weight 1.3 g) were batch challenged by immersion in water containing each virus isolate (MB20, MC30, and MD39). Control groups were mock challenged with cell culture media containing no virus. Challenge exposure was for 1 h at 1 × 10⁶ pfu virus/ml in 11 of static water with aeration. Following challenge, water flow was resumed and fish were held in batch in 41 tanks with flow-through water for 30 days, with mortalities recorded daily. Cumulative percent mortality among triplicate tanks within each treatment was tested by chi-square analysis and no significant difference was found. Average mortality curves were estimated with a Kaplan–Meier method and compared with a log-rank test using the Statistical Package for the Social Sciences (SPSS), Version 11.5.

2.3. MISFIT challenge conditions for fish infection

For the MISFIT protocol fish (approximately 1–2 g in size) were challenged in batch by immersion in static water containing a total virus titer of 1 × 10⁴ pfu/ml. The challenge exposure was for 12 h with aeration, and the total amount of static water in each challenge was standardized to 50 ml/fish with a minimum volume per tank of 1 l. After challenge fish were moved into a rinse tank and held in flowing water for 1 h to flush out excess virus. Each fish was then placed into an individual beaker with 150 ml static water and maintained at 15 °C by circulating temperature-controlled water outside the beakers for a 3-day period of virus replication. Fish were then euthanized by adding tricaine methansulfonate (MS-222, final concentration 200 mg/l) to the beakers, and whole fish were stored frozen at −80 °C.

2.4. In vivo virus growth curves under MISFIT conditions

Using the MISFIT challenge protocol fish were exposed to individual virus isolates MB20, MC30, and MD39. At specified sampling times, three fish from each group were euthanized and frozen at −80 °C. The virus titer in each whole fish was subsequently determined by plaque assay (Batts and Winton, 1989). Fish were assigned to each sampling time based on a random numbering scheme. These growth curve experiments were performed twice for each virus isolate. In the first experiment (mean fish weight 0.8 g), the earliest sampling point was 16 h after the initiation of challenge and subsequent time points were at 24 h intervals out to 7 days post-challenge. In this experiment there were significant titers (3.5–4 log pfu/g) in some fish at the first (16 h) time point. Therefore, the second experiment (mean fish weight 1.4 g) included earlier time points at 1, 6, and 12 h after initiation of challenge, followed by sampling at 24 h intervals out to 7 days.

2.5. In vivo competition assays with IHNV isolate pairs MB:MC and MB:MD

For MB:MC competition assays 30 juvenile trout were challenged using the MISFIT protocol with a 1:1 mixture of virus isolates MB20 and MC30. The challenge virus mixture was based on the pfu/ml of each virus stock, and the total challenge virus concentration was 10⁴ pfu virus/ml (i.e. 0.5 × 10⁴ pfu/ml of each virus genotype). Assays of the MB:MD virus pair were performed in the same way using isolates MB20 and MD39. Assays were not performed between MC30 and MD39 due to the lack of differential restriction sites appropriate for the gt-spec RE digest method (described below) in the cDNA sequences of these two isolates. For each competition assay groups of 10 fish were also challenged with each individual isolate alone at a total concentration of 10⁴ pfu virus/ml, and 10 fish were mock challenged with a similar volume of cell culture media containing no virus. After a 72 h co-replication period fish were euthanized as described above and stored frozen at −80 °C.

2.6. RNA extraction, reverse transcription, and first round PCR amplification for progeny virus population analysis

For analysis of progeny virus populations total RNA was extracted from whole fish using a guanidinium thiocyanate protocol modified from Anderson et al. (2000), as follows. Fish in sampling bags were thawed and homogenized in 5 ml denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% N-laurylsarcosine, and 0.1 M β-mercaptoethanol) using a Stomacher Lab-Blender 80 (Tekmar). One ml of homogenate was transferred to a fresh tube and vortexed after each addition, in sequence, of 125 µl 2 M sodium acetate pH 4.0, 1 ml water-saturated phenol (molecular biology grade, Sigma), and 250 µl water-saturated chloroform:isoamyl alcohol (49:1). Samples were then incubated on ice for 15 min and centrifuged 30 min at 2200 × g. One milliliter of supernatant was transferred to a fresh microfuge tube, mixed with 1 ml isopropanol, and precipitated overnight at −20 °C. Samples were then centrifuged for 15 min in a microfuge at 12,000 × g and RNA pellets were resuspended in 500 µl of autoclaved, 0.45 micron-filtered water. After addition of 50 µl 8 M LiCl samples were incubated at 4 °C for 30 min and centrifuged for 15 min in a microfuge. Pellets were washed with cold 70% ethanol, re-centrifuged, and dried. Final pellets were suspended in 25 µl water and stored at −70 °C.

Reverse transcription of minus-sense genomic viral RNA was carried out using 3 µl of total RNA, diluted with 2 µl water and heated at 95 °C for 5 min, in a 20 µl reaction containing 200 units MMLV-RT (USB), 1X MMLV buffer, 1 mM dNTPs, and 20 units RNAsin (Promega). Glycoprotein (G) gene sense primer 558+ (5′-TGG-AGG-AAA-ATG-CAC-CAC-ATC-3′) was used in the reaction at a final concentration of 1 µM. The reaction was incubated at 42 °C for 45 min, followed by 95 °C for 5 min. The nucleotide sequence of primer 558+ was conserved among isolates MB20, MC30, and MD39. The cDNA produced in this reaction was then used as a
2.7. Progeny virus population analysis by genotype-specific restriction enzyme digest (gt-spec RE digest)

To quantify the relative proportion of each genotype in mixed RNA populations of isolate pairs B:C and B:D, we first performed a second round of conserved PCR to amplify sufficient template representing all genotypes in the populations, and then used genotype-specific restriction enzyme digestion (gt-spec RE digests) with enzymes EcoR1 and Xba1. For nested PCR the primary conserved PCR product (483 bp) was purified using the StrataPrep PCR Purification Kit (Stratagene) and used as a template in a second round of PCR with conserved primers 619+ (5′-GAT-GCA-GGA-ATA-CCA-GCC-TGT-3′) and 975- (5′-ACT-GTG-TGA-TAT-TAT-CTC-3′). Two microliters of the purified first round product was used in a 50 μl PCR containing 2.5 units Taq DNA polymerase (Promega) with 2.5 mM magnesium chloride, and 0.2 mM dNTPs. Primers 619+ and 975− were used in the reaction at final concentrations of 1 μM. The reaction conditions included denaturation at 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. This reaction produced amplified DNA which represented the whole virus population present in each fish.

2.8. Progeny virus population analysis by genotype-specific PCR

For maximum sensitivity in detecting presence or absence of each genotype we developed genotype-specific PCR primers that were complementary to one genotype (gtB, gtC, or gtD) but had at least one substitution relative to the other genotypes. These primers bind to the same sites within the mid-G region of iHNV, but were designed to be slightly different in length in order to equalize primer melting temperature (Tm) at 60°C. Primer sequences were: gtB+ (5′-ATG-ATT-GAA-TTC-TGT-GGG-GGA-3′), gtB− (5′-TCC-CAG-TCT-TGT-GGG-GGA-3′), gtC+ (5′-G-ATG-ACA-TTC-TGT-GGG-GGG-3′), gtC− (5′-TCC-CAG-TCT-TGT-GGG-GGG-3′), gtD+ (5′-T-ATT-GAA-TTC-TGT-GGG-GGA-3′), and gtD− (5′-TCC-CAG-TCT-TGT-GGG-GGA-3′). To confirm that these primers amplified only their complementary genotype, RT-PCR and nested PCR were performed using high-titer stocks of virus isolates MB20, MC30, and MD39 as templates. To perform genotype-specific PCR (gt-spec PCR) on virus progeny populations from misfit fish challenges, the first round conserved PCR product (483 bp) described above was used as a template for a second round of PCR using the genotype-specific primers. Two microliters of the first round PCR product was used in a 50 μl reaction containing 2.5 units Taq DNA polymerase (Promega) with 2.5 mM magnesium chloride, and 0.2 mM dNTPs. Gt-spec primers were used in the reaction at final concentrations of 1 μM. Reactions were denatured at 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. This produced a genotype-specific ~120 bp product that was visualized by agarose gel electrophoresis and ethidium bromide staining.

2.9. Statistical analyses

General linear models (GLM) were implemented for comparing the percent of B in the mixed infection populations between treatment groups. For GLM analysis, the dependent variable was the proportion of B, which was arc-sin square root transformed to meet the assumptions of homogeneity of variance and normal distribution. Fixed factors in the model included genotype pair (BC or BD) and experiment (1 or 2). Fully factored models were tested and non-significant terms dropped starting with highest order interactions, to obtain the minimal significant model. An underlying hypothesis of the study was that genotype B had equal fitness to genotypes C and D. To determine this, a two tailed t-test was utilized to calculate whether the ratio of B versus C (in B:C experiments) or B versus D (in B:D experiments) was significantly different from 50%. All statistics were carried out in SPSS Version 11.5.

a template for PCR with conserved primers 558+ and 1014− (5′-ATG-TGG-AGA-TCG-GAA-CTT-GGA-3′). These primers are complementary to all three virus isolates used in this study and they amplify a 483 bp DNA product from all genotypes present in the virus populations. This DNA product is near the middle of the G gene of iHNV and contains the previously described 303 bp “mid-G” region that has been used extensively for phylogeographic studies (Kurath et al., 2003; Troyer and Kurath, 2003; Troyer et al., 2000). This region was chosen because isolates MB20, MC30, and MD39 had a number of nucleotide differences that could be used as markers to distinguish them in mixed infections. PCR was carried out with 10 μl of the RT reaction in a 100 μl reaction using 5 units Taq polymerase (Promega). 1 × PCR buffer (Promega), 7.5 μg RNase A, and 0.11 mM dNTPs. An additional 11 pmol of sense primer 558+ was added along with 22 pmol of antisense primer 1014−. The reaction conditions included denaturation at 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. This reaction produced amplified DNA which represented the whole virus population present in each fish.

To determine the reproducibility of the gt-spec RE digest quantification we performed repeated analyses of RNA from three different fish from a B:C competition assay. The fish were chosen because they had varying B:C ratios in their progeny virus populations (mostly B, mostly C, or approximately equal proportions of B and C). Each of these RNA samples was analyzed four independent times, beginning with the reverse-transcription and proceeding through gt-spec RE digest and densitometry to determine the percent gtB and gtC as described above.
significant difference (in a high proportion of the exposed fish. A virus concentration of doses was tested to determine a dose capable of initiating infections. The challenge was extended to 12 h. Similarly, a range of challenge virus (data not shown). Therefore, the duration of the immersion challenge was not sufficient to establish infection in a high proportion of fish. MC30, or MD39, followed by isolation of fish in separate beakers, standard 1 h batch exposure to individual virus isolates MB20, to prevent cross-infection. Preliminary studies indicated that the standard 1 h batch exposure to individual virus isolates MB20, MC30, or MD39, followed by isolation of fish in separate beakers, was not sufficient to establish infection in a high proportion of fish. Fish that were mock challenged had 4.0% total mortality.

3. Results

3.1. Virulence of IHNV isolates MB20, MC30, and MD39 in batch challenge

As an initial biological characterization of the three IHNV isolates selected for growth competition assays their virulence was compared in a batch challenge study. The resulting cumulative mortality curves for the three isolates were very similar (Fig. 2), with no significant difference (p < 0.05) among the average final cumulative percent mortalities: MB20, 74.7%; MC30, 82.4%; MD39, 80.3%. Fish that were mock challenged had 4.0% total mortality.

3.2. Development of MISFIT protocol for IHNV infections in individual fish

The batch challenge method used above is standard for initiating laboratory infections with IHNV in rainbow trout, but a disadvantage of this method for in vivo competition assays is that the interpretation of results would be confounded by the multiple cycles of infection that occur when infected fish shed virus that initiates subsequent rounds of infection in other fish held in batch. Therefore, we developed a modified challenge protocol in which fish were initially exposed in batch to 1:1 mixtures of selected pairs of IHNV isolates and then separated into individual beakers to prevent cross-infection. Preliminary studies indicated that the standard 1 h batch exposure to individual virus isolates MB20, MC30, or MD39, followed by isolation of fish in separate beakers, was not sufficient to establish infection in a high proportion of fish (data not shown). Therefore, the duration of the immersion challenge was extended to 12 h. Similarly, a range of challenge virus doses was tested to determine a dose capable of initiating infections in a high proportion of the exposed fish. A virus concentration of \(1 \times 10^3\) pfu/ml during the 12 h immersion challenge was selected as our standard challenge titer because it reliably produced infection in over 90% of the fish (data not shown). This modified challenge protocol is referred to as “MISFIT” conditions.

Selection of a time point at which to sacrifice fish for assay of the in-host virus competitions involved two main factors. First, it was desirable to end the competition period before mortality began in order to avoid complicating the analysis with the factor of fish death. The IHNV isolates used in this study began to cause mortality in juvenile rainbow trout at day 6 after batch challenge exposure (Fig. 2). Second, it was important that the competition period be sufficiently long that each of the viruses in the assay reached their respective maximum titers. Otherwise, the competition result might be artificially biased toward a virus with initially faster replication kinetics. Because of this concern, we performed in vivo virus growth curves using MISFIT conditions to challenge fish with individual isolates MB20, MC30, or MD39, and total virus titers were determined for three fish per group at time points between 0 and 210 h post-infection. These growth curve experiments were performed twice for each virus isolate and the mean growth curves are shown in Fig. 3. The growth curves showed no detectable virus (detection limit = 2.3 log pfu/g) through 12 h after initiation of challenge, then a quickly rising titer level which peaked around 70 h, followed by a plateau or gradual decline. The three isolates had similar growth kinetics, with peak titers occurring by 72 h after initiation of challenge. Based on this data, 72 h was chosen as the time point at which fish would be sacrificed and assayed in the MISFIT competition experiments.

3.3. Validation of methods to analyze progeny viral populations in fish

Progeny virus populations were first analyzed by the gt-spec RE digest method that differentiates virus strains based on sequence differences that occur in restriction enzyme target sites in a conserved nested PCR product. Accuracy of the gt-spec RE digest methodology was determined by assaysing reconstructed control mixtures of isolates MB20:MC30 and MB20:MD39 at a predetermined range of known ratios (Fig. 4). For MB20:MC30 control mixtures the observed ratios determined in these assays varied from the expected values by 6.6 ± 1.7% (average ± 1 S.E.M.), with a range of 1–13% difference. For MB20:MD39 control mixtures the observed ratios varied from expected values by 9.1 ± 1.3%, with a range of 5–14%. In addition, these results indicated approximately 5% as the lower limit of detection for the assay, i.e. gt-spec RE was able to detect genotypes that comprised a minimum of 5% of the total in each lane. Thus the system could quantitate up to a 20-fold difference between the levels of two genotypes in a mixture.

Reproducibility of the gt-spec RE method was tested by performing four independent analyses of B:C ratios in each of three
mixed virus populations from fish that had varying B:C ratios (Fig. 5). In these assays quantification of relative proportions in virus progeny populations was highly reproducible, with less than 3.1% variation among the four replicates for each mixed virus population.

When gt-spec RE did not detect both genotypes in a potentially mixed population, nested gt-spec PCR was used for determining the presence or absence of genotypes with maximum sensitivity. Specificity of the gt-spec PCR assays was confirmed using high titer virus stocks as templates with primer pairs specific for amplification of gtB, gtC, and gtD. Each assay amplified only the intended template and not the other two genotypes (data not shown).

3.4. B:C competition assays with IHNV isolate pair MB20:MC30

Two independent B:C competition assays were conducted in which 30 juvenile trout were exposed to a 1:1 mixture of IHNV isolates MB20 and MC30 using the MISFIT protocol. In the first assay, analysis of progeny virus populations by gt-spec RE digest revealed that after 72 h of in-host competition 28 out of 30 fish had visible mixtures of gtB and gtC at varying ratios, one fish had only gtB visible, and one had only gtC visible. Analysis of the same progeny by nested gt-spec PCR produced equally bright gel bands for both genotypes B and C in all fish. Thus all fish contained co-infections of both gtB and gtC, including the ones in which only one genotype was visible by gt-spec RE digest. The proportions of gtB and gtC determined in all fish are given in Table 1. The average proportions for all fish in the first B:C assay was 46% gtB and 54% gtC ± 3.8 (S.E.M.), for an overall B:C ratio of 1:1.15 in this host population. There was wide variation in proportions within individual fish, but the majority of fish (23/30) had less than a twofold difference in the proportions of gtB and gtC. Overall, 15 fish had more gtB than gtC, and 15 had more gtC than gtB.

The second assay was performed using a different lot of juvenile rainbow trout. In this assay gt-spec RE digest indicated that progeny virus in 22 out of 30 fish had visible mixtures of gtB and gtC at varying ratios, four fish had only gtB visible, and four fish had only gtC visible (Table 1). Again all fish with only one genotype visible by gt-spec RE digest were shown to contain both genotypes by gt-spec PCR. The average proportions in the 30 fish were 48% gtB and 52% gtC ± 3.3 (S.E.M.), for a final B:C ratio of 1:1.09. There was wide variation among fish but 21 out of 30 had less than a twofold difference in the proportions of gtB and gtC. Overall, 15 fish had more gtB than gtC, and 15 had more gtC than gtB. An example of the electrophoresis results for 15 fish from the second assay is shown in Fig. 6.

The results of the two independent assays showed good reproducibility (Fig. 8). In both cases there was wide fish-to-fish variation, but the final average B:C ratios were not significantly different from the input ratio 1.0:1.0 (Table 1, p > 0.05), indicating equal or nearly equal fitness for IHNV isolates MB20 and MC30 under these conditions. In both assays control fish exposed to only MB20 or only MC30 showed the expected single genotype in their progeny by both gt-spec RE digest and gt-spec PCR, and control fish that were mock challenged had no virus. Representatives of these controls are shown in Fig. 6.

3.5. B:D Competition assays with IHNV isolate pair M20B:MD39

To assess whether results obtained in the B:C assays were specific to that particular pair of IHNV isolates, two assays were conducted between isolate MB20 and a different isolate, MD39. In the first B:D assay 25 out of 30 fish had visible mixtures of both genotypes by gt-spec RE digest, while three fish had only gtB visible and two fish had only gtD. As above, all fish that had only one visible genotype by gt-spec RE digest were found to contain both genotypes by gt-spec PCR. The proportions of gtB and gtD in all fish are shown in Table 1. In this first B:D assay the average for all 30 fish was 55.5% gtB and 45.5% gtD ± 4.4 (±1 S.E.M.), for a B:D ratio of 1.0:0.8. Again there was wide fish-to-fish variation in proportions, but 25 out of the 30 fish had less than a twofold difference in proportions of gtB and gtD. Eighteen out of thirty fish had more gtB than gtD, and 12 had more gtD than gtB.
Table 1
Percent viral genotype B* in mixed virus progeny populations in individual fish 3 days after immersion exposure to a 1:1 ratio of genotypes B and C (two experiments, 30 fish each), or B and D (two experiments, 28–30 fish each)

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Ave. %B 46  Ave. %B 48  Ave. %B 55  Ave. %B 51
S.E.M. 3.8  S.E.M. 5.3  S.E.M. 4.4  S.E.M. 4.9
t = -0.901  t = -0.393  t = 1.259  t = 0.309
p = 0.375  p = 0.697  p = 0.218  p = 0.760

*Asterisks denote virus populations that appeared to be single genotypes by the genotype-specific restriction enzyme digest but had both genotypes by genotype-specific nested PCR. These were designated as having 2% of the minor genotype because the actual proportion was above zero but below the detection limit of the genotype-specific restriction enzyme digest method, which was estimated at 5% of the population. t and p values are statistical results of t-tests showing the %B in each of the four experiments is not significantly different from 50% in C and B:D populations within boxed areas are shown in Figs. 6 and 7, respectively.

a Proportions were determined by genotype-specific restriction enzyme digest and expressed as percentages of the total population within each fish. Only %B is shown, but %C in B:C experiments is 100 – %B, and %D in B:D experiments is 100 – %B.

Fig. 6. Analysis of progeny virus populations in 15 fish from B:C experiment 2. Fish were exposed to a 1:1 mixture of IHNV genotypes B:C and held for 72 h of competitive virus replication in vivo. (A) Progeny analyzed by gt-spec RE digest. Arrows indicate genotype B-specific band (219 nt) and genotype C-specific band (281 nt) used for densitometry to determine proportions of each genotype in each fish, as shown in the bar graphs below each lane. (B) Same progeny as in panel A, analyzed by gt-spec PCR. Note uniform detection of both genotypes in all B:C infected fish, despite lack of visible genotype B in fish no. 5 and 6, or genotype C in fish no. 1, 3, or 15 in panel A. In both panels lanes labeled B and C indicate control fish exposed to a single virus isolate.
Fig. 7. Analysis of progeny virus populations in 14 fish from B:D experiment 2. Fish were exposed to a 1:1 mixture of IHNV genotypes B:D and held for 72 h of competitive virus replication in vivo. (A) Progeny analyzed by gt-spec RE digest. Arrows indicate genotype B-specific band (219 nt) and genotype D-specific band (281 nt) used for densitometry to determine proportions of each genotype in each fish, as shown in the bar graphs below each lane. (B) Same progeny as in panel A, analyzed by gt-spec PCR. Note uniform detection of both genotypes in all B:D infected fish, despite lack of visible genotype D in fish no. 12 and 14 in panel A. In both panels lanes labeled B and D indicate control fish exposed to a single virus isolate. There is no sample number 10 shown due to poor RNA recovery.

In a second B:D assay with 28 juvenile fish from a different lot, 24 fish had visible mixtures of gtB and gtD, 2 fish had only gtB visible, and 2 fish had only gtD visible. All fish contained both genotypes by gt-spec PCR. The average proportions (Table 1) were 51% gtB and 49% gtD ± 4.9, for a B:D ratio of 1.0:0.94. Fish to fish variation was high, but 22 out of the 28 fish had less than a twofold difference in proportions of the two genotypes. Progeny from 14 out of 28 fish had more gtB than gtD, and 14 had more gtD than gtB. Electrophoresis results for 15 fish from the second B:D assay are shown in Fig. 7.

In both of the B:D assays there was wide fish-to-fish variation, but the two assays showed good reproducibility (Fig. 8) and the final average B:D ratios were not significantly different from the input ratio 1.0:1.0 (Table 1, p > 0.05), indicating equal or nearly equal fitness for isolates MB20 and MD39 under these conditions. Controls in both assays performed as expected and representative controls are shown in Fig. 7.

4. Discussion

A novel system is described here that allows determination of relative fitness values for pairs of IHNV strains competing within live rainbow trout hosts. Advantages of this system as a model for vertebrate RNA virus fitness studies include the fact that IHNV infection of rainbow trout is a natural co-evolved host-pathogen relationship, fish can be challenged by immersion, which mimics the natural horizontal route of infection with IHNV, and numerous well characterized IHNV field isolates are available for study. Also, due to the small size of juvenile trout it is feasible to hold large numbers of individual fish in isolation to prevent cross-infection.

The MISFIT challenge protocol developed here performed well, generating mixed infections in all fish in each assay. The gt-spec RE digest for quantifying relative proportions of two genotypes in mixed virus populations was highly reproducible (within 3.1%), and reasonably accurate (within 14%). This level of performance is similar to that reported for analogous assays by other researchers (Dockter et al., 1996; Hall et al., 2001; Quinones-Mateu et al., 2000; Weaver et al., 1999; White and Wu, 2001). The gt-spec PCR also worked well and in several cases was able to detect low levels of a minority genotype that was not detectable by gt-spec RE digest.

The first application of this novel system, as described here, was to perform competition assays between two pairs of virus strains that co-circulated in Idaho aquaculture. Extensive field studies have shown that multiple IHNV lineages co-circulate within and among the aquaculture facilities of the trout farming region of Idaho, and that virus traffic occurs between facilities. This suggests that viral variants likely must compete with each other in order to be maintained within this system, and that the virus types from the most prevalent and widely distributed subgroups MB, MC, and MD are likely to have similar fitness. In the assay results presented here mixed infection was the typical outcome of challenge with representative MB and MC, or MB and MD isolates under the MISFIT conditions. All four assays produced relative fitness ratio estimates

Fig. 8. Mean percent genotype B in mixed virus progeny populations from two independent B:C growth competition experiments and two B:D experiments. Means were calculated from data in Table 1, and standard error of the means is shown. In each assay the percent B was not significantly different from 50%, indicating equal or nearly equal fitness of the two viral genotypes tested.
indicating equal or nearly equal fitness. Although not statistically significant, final average ratios in both B:C assays showed gtC slightly favored, and both B:D assays showed gtB slightly favored. At present we cannot distinguish whether these slight deviations from equal ratios are really due to random variation around an equal fitness mean, or if they indicate small but real fitness differences. Differences of this magnitude could well have consequences in nature, but our current assay does not assess them with sufficient accuracy. We are currently developing genotype-specific quantitative real-time reverse-transcriptase PCR as an alternative method for quantifying proportions of genotypes in mixed virus progeny: this may provide higher accuracy in the future. Also, ongoing studies using different IHNV strain pairs in our competition assays have indicated unequal fitness ratios (Wargo, Garver, and Kurath, unpublished). This demonstrates that the assay does not always find equal fitness, supporting the results presented here for B:C and B:D assays as valid indications of equal or nearly equal fitness. The MB, MC, and MD IHNV isolates tested here had equal virulence in standard batch challenge. It is not known if IHNV virulence correlates with viral load or fitness, but this is also currently under investigation.

A notable feature of the data from all four assays described here was the high variation of genotype ratios in progeny from individual fish within a group. This was anticipated for several reasons. Previous virus competition studies conducted in animal and plant hosts have shown significant variability between individual host organisms (Dockter et al., 1996; Hall et al., 2001; Kurath and Dodds, 1994; Lenhoff et al., 1998). Viral infection of a host organism is a complex process that is likely to involve many stochastic events that may allow one or a few virus particles to find the progeny population observed within the host (Bergstrom et al., 1999; Domingo et al., 2001; Domingo and Holland, 1997; Zhang et al., 1993; Zhu et al., 1993). These bottleneck events may occur upon transmission or during spread and replication within the host (Duarte et al., 1994b). One contributing mechanism for bottlenecks within the host may be the phenomenon of viral interference, which has been demonstrated for the rhabdoviruses VSV and rabies virus in cell culture (Simon et al., 1990; Whitaker-Dowling et al., 1983). Thus we expected to observe significant fish-to-fish variation in the proportions of gtB:gtC or gtB:gtD after competition. This is exactly what we observed, with some fish having >95% one genotype, and some >95% the other genotype. This variability is strong evidence for the existence of stochastic events at some point in the transmission and replication cycle in this system, an observation that may not be evident in cell culture-based competition assays. Another alternative explanation for these results is that each fish has a different selective environment, with gtB being more fit in some fish and gtC more fit in others. This might be at least partially due to host genetic variation in individual fish since these assays used a typical outbred aquaculture fish stock. Regardless of the mechanism, the wide variation seen among progeny of competitions in individual host is an important observation that is likely to reflect the reality of virus competition processes in nature. This emphasizes the need to do these studies with sufficiently high numbers of animals to get a mean that indicates host population-scale fitness differences.

5. Conclusions

We have developed and tested a novel in vivo virus growth competition assay and shown that it does generate and characterize co-infections of two IHNV strains in living rainbow trout. The data presented here support our working hypothesis that IHNV strains that co-circulate stably in aquaculture have equal or nearly equal fitness. Although this in vivo assay does not recreate all the complex factors likely to determine viral fitness in the field, it represents an important step beyond viral fitness assays in cell culture, and toward understanding viral fitness within living hosts that have intact tissue and organ structure and mount a natural immune response to infection. In the future we will utilize this assay to explore many aspects of viral fitness including correlations with virulence, influence of individual stages of the viral infection cycle, and contribution of host genetics.

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References
