

Multiple infection dynamics has pronounced effects on the fitness of RNA viruses

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Abstract

Several factors play a role during the replication and transmission of RNA viruses. First, as a consequence of their enormous mutation rate, complex mixtures of genomes are generated immediately after infection of a new host. Secondly, differences in growth and competition rates drive the selection of certain genetic variants within an infected host. Thirdly, but not less important, a random sampling occurs at the moment of viral infectious passage from an infected to a healthy host. In addition, the availability of hosts also influences the fate of a given viral genotype. When new hosts are scarce, different viral genotypes might infect the same host, adding an extra complexity to the competition among genetic variants. We have employed a two-fold approach to analyse the role played by each of these factors in the evolution of RNA viruses. First, we have derived a model that takes into account all the preceding factors. This model employs the classic Lotka-Volterra competition equations but it also incorporates the effect of mutation during RNA replication, the effect of the stochastic sampling at the moment of infectious passage among hosts and, the effect of the type of infection (single, coinfection or superinfection). Secondly, the predictions of the model have been tested in an *in vitro* evolution experiment. Both theoretical and experimental results show that in infection passages with coinfection viral fitness increased more than in single infections. In contrast, infection passages with superinfection did not differ from the single infection. The coinfection frequency also affected the outcome: the larger the proportion of viruses coinfecting a host, the larger increase in fitness observed.

Introduction

A hallmark of RNA viruses is their error-prone replication, with mutation rates estimated to be 10^{-3} – 10^{-5} substitutions per nucleotide per round of replication (Drake & Holland, 1999). In addition, many RNA viruses display high frequencies of both homologous and heterologous recombination (Lai, 1995). Consequently, replicating populations of RNA viruses, even clonal

populations, rapidly evolve to become complex mixtures of variants. This results in the coexistence of multiple genotypes during disease outbreaks, even within infected individuals (reviewed in Domingo & Holland, 1997).

Recently, the role played by long-term competition among genetic variants during the adaptation of the RNA virus vesicular stomatitis virus (VSV) to cell cultures has been shown (Clarke *et al.*, 1994; Quer *et al.*, 1996; Miralles *et al.*, 1999a, 2000). Competition between two viral genotypes always resulted in a fitness improvement in both clones relative to their ancestors but in no gain relative to one another, an arms race known as the Red Queen (Van Valen, 1973). On the other side, it has been demonstrated that two variants cannot coexist

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indefinitely, as a consequence of competitive exclusion (Clarke *et al.*, 1994; Quer *et al.*, 1996). The Red Queen dynamics of coexistence between clones is thought to be disrupted because of the appearance of a beneficial mutation in one of the competitors, such that a high competitive advantage is conferred to its carrier (Elena *et al.*, 1997). Also, population complexity (i.e. the number of genetic variants) shows diminishing return on the rate of viral adaptation as a consequence of excessive competition among variants (Miralles *et al.*, 1999a, 2000).

During natural virus outbreaks, the infection of available hosts can occur in three ways. (i) When the density of available hosts is high and viral density is low, a host is usually infected by a single variant at a time. (ii) As the epidemic progresses, more and more hosts become infected and the density of virus in the population increases as well. Hence, the probability that a newly released virus migrates to an empty host decreases with time. Thus an already infected host can be superinfected by a different immigrating strain of the virus (Nowak & May, 1994). In spite of fitness differences between variants, at the moment of the invasion, the resident has a numerical advantage for exploiting the limiting resources. (iii) A different situation arises when two strains of the virus coinfect a susceptible host (May & Nowak, 1995). In this case, the ecological niche is available for both variants and neither has a numerical advantage, their fate will be mainly determined by their relative fitnesses.

In experiments that simulated the migration of VSV between hosts, Miralles *et al.* (1999b) detected a positive relationship between the rate of migration and the fitness of the viral meta-population. Obviously, the higher the migration rate, the more chances of sampling clones bearing beneficial mutations from the original viral population and the faster the spread to other lineages and sweep through the population. In these experiments, infectious passages always gave rise to coinfection.

A competition model for multiple infections

To make testable predictions about the role of each of these factors in the evolution of RNA virus populations, we have developed a model that takes into account: (i) mutation and competition within hosts, (ii) the rate and type (single, coinfection and superinfection) of infection passages (migration) between hosts, and (iii) the stochastic sampling during infection passages. The model will be developed in detail elsewhere, and here we only present its most relevant features.

For simplicity, we simulated the infectious process dividing it into two nonoverlapping steps. First, after a host becomes infected by a mixture of virus strains, the viral population starts to grow. During viral growth, competition between strains and mutation occurs. Once all the available resources within the host are exhausted,

growth stops. Secondly, the resulting viral population is sampled at random and the sample, small compared with the final population size, used to infect a new host. Hence, competition only happens within the host whereas drift is only important at the moment of transmission and can be ignored afterwards.

To model the growth within a host, we used a one-dimensional discretized fitness-space model that has been previously used to describe VSV evolution *in vitro* (Tsimring *et al.*, 1996). Let $P_n^f(r_i, t)$ represent the composition of the viral population in the f -host ($f = 1, \dots, F$) and at infection transfer n ($n = 1, \dots, N$). Within a host, the population is composed, at a given moment t (measured in viral generations), of a mixture of viruses and each one is characterized by its growth rate, r_i . The chosen fitness space assumes that growth rate increases linearly between C arbitrary fitness classes ($i = 1, \dots, C$), i.e. $r_i = \alpha_i$ (α being a positive integer). Mutations occur, with rate μ , in a stepwise-manner (Kimura & Ohta, 1973), which means that a virus in the i th fitness class can mutate only to one of the two adjacent classes $i + 1$ or $i - 1$. (It is straightforward to generalize the model to the case of infinite fitness classes which, in fact, eliminates the awkwardness of boundaries.)

The growth dynamics is thus described by the following set of $C \times F$ differential equations which are a generalized, discrete version of Fisher's diffusion model (Murray, 1989):

$$\frac{\partial P_n^f(r_i, t)}{\partial t} = r_i P_n^f(r_i, t) \left[1 - P_n^f(r_i, t) - \sum_{j \neq i}^{C-1} \beta_{ij} P_n^f(r_j, t) \right] + \mu \left[P_n^f(r_{i+1}, t) + P_n^f(r_{i-1}, t) - 2P_n^f(r_i, t) \right] \quad (1)$$

The first term is a generalization of Lotka-Volterra's competition equations (Bulmer, 1994) for C -competitors, whereas the second is the diffusion in a one-dimensional fitness space caused by mutations (Tsimring *et al.*, 1996).

It has been shown that for competing viral populations the following relationship between competition rates, β_{ij} , holds (Solé *et al.*, 1999):

$$\beta_{ij} = 1/\beta_{ji} = r_j/r_i \quad (2)$$

Therefore, mutation between discrete fitness classes not only affects the growth rate of the viral genotypes, r_i , but also their competitive abilities, β_{ij} .

All the hosts start each infection cycle with an initial viral population of size $P_0 = P_n^f(r_i, 0)$. After T generations of replication the viral population reaches the within-host carrying capacity size, K , and growth ceases:

$$K = \sum_{i=1}^C P_n^f(r_i, T) = 1. \quad (3)$$

At this moment, the viral population is allowed to migrate into a new host, completing the infection cycle. To do so, $P_0 \ll K$ viruses are randomly sampled from the population and transferred to the new host. The initial

conditions applied to the $(n + 1)$ th infection cycle are given by:

- (a) Single-infection: $P_{n+1}^f(r_i, 0) = P_0 P_n^f(r_i, T)$.
 (b) Coinfection: $P_{n+1}^f(r_i, 0) = (1 - \phi) P_0 P_n^f(r_i, T) + \phi P_0 P_n^g(r_i, T)$; where ϕ represents the proportion of virus immigrated from host $g \neq f$.
 (c) Superinfection: $P_{n+1}^f(r_i, 0) = (1 - \phi) P_0 P_n^f(r_i, T)$ represents the resident viral population in the host f and $P_{n+1}^f(r_i, \tau) = P_{n+1}^f(r_i, \tau) + \phi P_0 P_n^g(r_i, T)$ the population that has immigrated after τ arbitrary units of time from the host g .

To test whether the prediction from this model hold in real populations, we have made an experimental evolution assay using VSV as a model virus.

Materials and methods

Biological materials

The two VSV clones used in this study were derived from the Mudd-Summer strain of the Indiana serotype. The clone employed in the evolution experiments, MARM C, has an Asp₂₅₉ → Ala substitution in the G surface protein. [The same clone has been named MARM U elsewhere (Novella *et al.*, 1999)]. This substitution allowed the replication under I₁ monoclonal antibody concentrations that neutralize wild-type viruses (VandePol *et al.*, 1986). The second clone was an I₁-mAb sensitive, surrogate wild-type and was used as a common competitor in the competition experiments. To avoid any additional genetic changes in this clone, a large volume with a high titre ($\sim 10^{10}$ PFU mL⁻¹) was produced and kept at -80 °C. The fitness of MARM C relative to this wild-type strain has been determined previously (Holland *et al.*, 1991; Novella *et al.*, 1995, 1999; Miralles *et al.*, 1999a, b, 2000) as well as 24 times during the course of the present experiment (1.00 ± 0.03 , Student's $t_{23} = 0.001$, $P = 0.999$), and always has a relative value of 1. Thus, the mutation conferring the MARM phenotype can be considered selectively neutral.

Baby hamster kidney cells (BHK-K) were grown as monolayers in Dulbecco modified Eagle's minimum essential medium (DMEM) containing 5% newborn calf serum and 0.06% proteose peptone 3. Cells were grown in 25 cm² plastic flasks (containing 5 mL of medium) for infections or in 100 cm² petri plates (containing 15 mL of medium) for routine maintenance. Cells grew at a density of $(3.1 \pm 0.2) \times 10^5$ cells cm⁻².

To produce the I₁-mAb (Lefrancois & Lyles, 1982) needed for the competition experiments, we propagated hybridoma cells in DMEM containing 20% fetal calf serum, 2 µg mL⁻¹ of thymidine, 0.1 µg mL⁻¹ of glycine, 14 µg mL⁻¹ of hypoxanthine, and 1.3 µL mL⁻¹ of Opti-Mab (GibcoBRL, Rockville, USA) in large volumes to obtain litres of high-titre neutralizing MAb, which was stored at -80 °C.

All cell lines were maintained in incubators at 37 °C and with 7% CO₂ atmosphere.

Long-term evolution conditions

In our experiments, multicellular hosts were simulated by cells cultured in flasks. Each multicellular host was simulated by a flask containing 7.7×10^6 BHK-K cells. For each treatment, the host population size was 20 flasks. Each flask was labelled with an identification (f), and a transfer (n) numbers.

The experiment was initiated by infecting the first set of flasks with $\sim 1.5 \times 10^5$ MARM C virus/flask (MOI ≈ 0.02). In a day, the cytopathic effect was complete and yielded $\sim 7.5 \times 10^9$ virus mL⁻¹. Flask (f , n) was sampled, diluted 10⁴-fold and used to infect flask (f , $n + 1$), and so on. For the case of no migration (single infection), the flask (f , $n + 1$) was infected with 200 µL from the flask (f , n). In the case of infection passage with coinfection, the flask (f , $n + 1$) was infected with virus from two different sources: from flasks (f , n) (resident virus) and ($g \neq f$, n) (immigrant virus), g being randomly chosen without replacement. In the case of infection passage with superinfection, the (f , $n + 1$)-flask was infected with virus from the (f , n)-flask (resident virus). Then, they sat in the incubator for 6 h and, at this moment, virus from ($g \neq f$, n)-flask immigrated.

Preliminary experiments have shown that the end of lag phase and the beginning of exponential growth is reached about 6 h after infection with MARM C (Fig. 1). This ensures that any immigrant virus will have a chance to find empty live cells, although the resident virus is almost ready to generate the first burst.

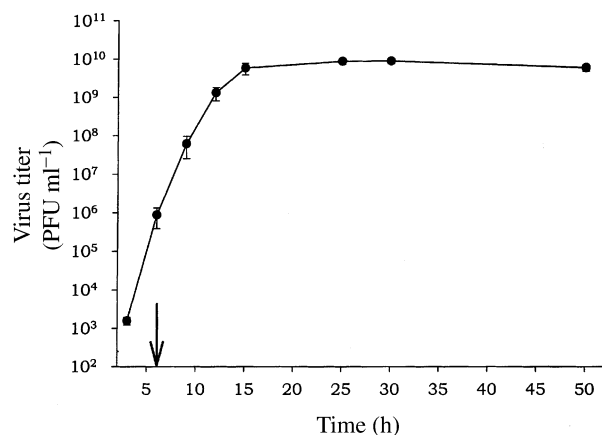


Fig. 1 One-step growth curves carried out for VSV MARM C. The arrow indicates the moment when superinfections were carried out: 6 h after the first infection. See Materials and methods for a more detailed description. Error bars represent 95% confidence intervals ($n = 12$).

Two levels of proportional representation of immigrant virus, ϕ , were employed (5 and 50%). These five transfer procedures (two types of migrations \times two frequencies of migration + one single infection) were repeated during 25 consecutive days, which is equivalent to a minimum of 100 generations of viral replication (Miralles *et al.*, 2000).

Relative fitness assays

At the end of each experiment, MARM C evolved populations were assayed for relative fitness (Holland *et al.*, 1991). Relative fitness measures the degree of adaptation of viral populations to the experimental environment. Three replicates of each evolving MARM C population were each mixed with a known amount of wild-type, and the initial ratio for each replicate mixture, R_0 , was determined by plaque assays with and without I₁-mAb in the agarose overlay medium. Incorporation of antibody into the plaque overlay medium (after virus penetration), instead of standard virus neutralization, eliminates the problem of phenotypic mixing and hiding (i.e. the encapsidation of MARM RNAs within phenotypically wild-type envelopes) (Holland *et al.*, 1989). Each competition mixture was then serially transferred to obtain reliable estimates of relative fitness as follows: at each transfer, the resulting virus mixture was diluted by a 10^4 factor and used to initiate the next competition passage by infection of a fresh monolayer. The ratio of MARM C to wild-type was determined by plating with and without I₁-mAb in the overlay agarose medium at different transfers. These determinations gave the proportion of MARM C to wild-type at transfer t , $R(t)$. The antilogarithm of the regression slope, $\ln R(t) = \ln R(0) + t \ln W$, was taken as an estimate of the fitness of the MARM C competitor relative to wild-type (Elena *et al.*, 1998).

Computer simulations

The stochastic effects associated with the passage to a new host are taken into consideration by randomly sampling the final population $P_n^f(r_i, T)$. To illustrate the sampling method, let us consider the single-infection case. We obtained $P_{n+1}^f(r_i, 0)$ from $P_n^f(r_i, T)$ by repeating the following set of operations:

- (i) Choose a fitness class i at random.
- (ii) Generate a random number $\xi \in (0, 1)$.
- (iii) If $\xi < P_n^f(r_i, T)$, then add a small amount of virus $0 < \varepsilon \ll 1$ to $P_{n+1}^f(r_i, 0)$.
- (iv) Repeat (i)–(iii) until the initial population equals $P_{n+1}^f(r_i, 0) = P_0$.

Next, the population grows as described by eqn 1. Growth was stopped after T viral generations, when the population reached the carrying capacity size (eqn 3).

The same procedure was performed for the two types of migration sampling from different flasks at the

convenient proportions (ϕ). In accordance to the experimental design, the number of simulated hosts was set to $F = 20$, and the number of infectious passages, to $N = 25$. Other parameters employed in the simulations reported were: $C = 50$ fitness classes, $\mu = 0.05$ mutations per replication round, $P_0 = 0.2$, $T = 4$ rounds of viral replication within a host to reach the carrying capacity, $\tau = 1$ rounds of replication at the moment of superinfection and, $\varepsilon = 0.01$. (Computer programs are available upon request to R.V.S.)

Goodness of fit of the experimental data to the simulation results was tested by a correlation analysis (Haefner, 1996). A significant positive correlation indicates a match between both data sets; whereas, no correlation implies that the model does not account for the observations.

Results

Numerical analysis of the model and predictions

For illustrative purposes we only considered competition between two viral populations $x = P_{n+1}^f(r_x, t)$ and $y = P_{n+1}^f(r_y, t)$, where x and y represent the frequency of the immigrant and resident populations, respectively, in host f at infectious passage n . If $r_x > r_y$ (i.e. the immigrant virus is fitter than the resident) the ultimate winner should be the x strain, unless the nonlinearities in eqn 1 played a role against it. In particular, as the dynamics in each infection stopped at a cut-off point of $x + y = 1$, depending on the initial conditions, very different outcomes could be obtained.

Under the conditions of eqn 2, no stable internal equilibrium exists; one viral population drives the other(s) to extinction (Solé *et al.*, 1999). Figure 2a shows the behaviour for several test runs of the model. Despite an asymmetry imposed by the condition $r_x > r_y$, trajectories starting with initial pairs (x_0, y_0) from different sides of the diagonal $y = x$ always converged to the exclusion points.

Figure 2b shows the case where a host is coinfecting with two migrant viral populations and the one with higher fitness represents only $\phi = 5\%$ of the inoculum received by the host. From Fig. 2b, it is clear that when the frequency of the high fitness variant (x) was low at the time of coinfection, it had a high probability of winning the competition because of its faster replication, but in some cases (12%), the low fitness variant (y) won as a result of chance. Hence, in the long run, the fitness of a viral population under continuous infectious passage with a low frequency of coinfection (5% in this case) involves two components: a majority of cases where the most fit virus wins, plus a small fraction of cases where low fitness variants win the competition.

To explore how the relative frequencies of high fitness clones affected the outcome of the competition, we

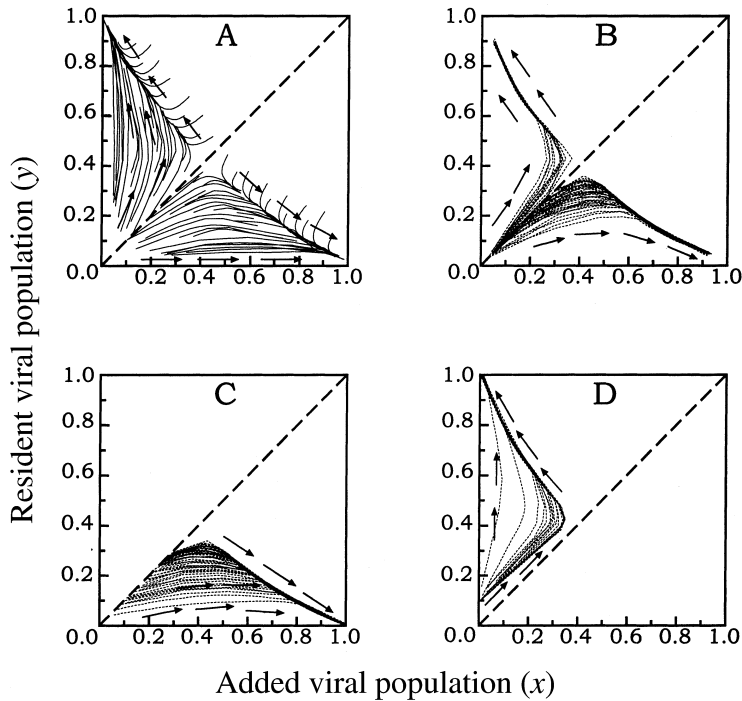


Fig. 2 Trajectories obtained by numerically solving the set of differential eqn (1). (a) The behaviour for several simulation trials for the system of differential equations. (b) The trajectories where $\phi = 5\%$ of the inoculum for an infection passage with coinfection has higher fitness. Although in the majority of cases the winner was the virus with the higher $r(x)$, because of the nonlinearities of the process, in 12% of cases the virus with the lower $r(y)$ won the competition. (c) The trajectories where $\phi = 50\%$ of the inoculum for an infection passage with coinfection has higher fitness: the virus with larger $r(x)$ always won the competition. (d) The case for infection passage with superinfection: regardless of the frequency of x nor its r , the y resident population always won. (In all cases, $r_x = 1.1$, $r_y = 1.0$).

simulated the case where $\phi = 50\%$ of the coinfecting viral particles have high fitness (Fig. 2c). In this case the faster replicating strain (x) always displaced its competitor and, as a result, the fitness of the viral population increased. These results lead to the prediction that migration with coinfection enables fixation of the fittest strains with a probability proportional to their initial frequency in the population (Hypothesis I). Furthermore, these simulations predict that the expected final fitness of a viral clone evolved under periodic infection passages with coinfection will be larger than the value observed without migration (single infections) (Hypothesis II), as observed by Miralles *et al.* (1999b).

Figure 2d presents the trajectories of the infectious passage under conditions of superinfection. In this case, initial demographic conditions are dominant over fitness differences. Regardless of the frequency of added x virus, the y resident viral population had all the tickets for the competition lottery. This was independent of whether fitness of the resident (y) strain was lower than the fitness of the immigrant (x) strain. This leads to a third prediction: migration with superinfection will not necessarily produce fitter virus than single-infections because the added virus may not have a chance to compete (Hypothesis III).

Results of the *in vitro* evolution experiments

Table 1 shows the fitness estimates for each flask and treatment. Figure 3 summarizes the results of the *in vitro* evolution experiment. Nonparametric methods were used

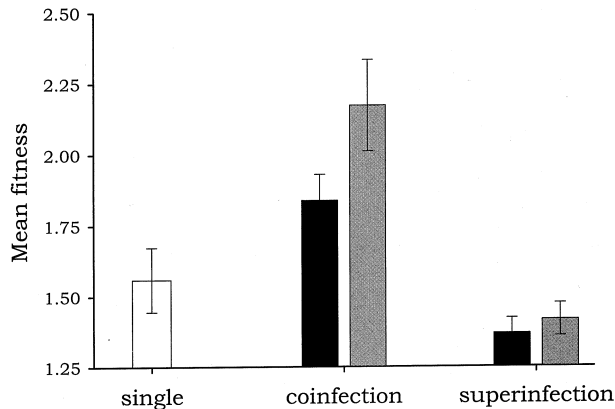
for statistical analysis (Sokal & Rohlf, 1995) because the fitness data violated the assumptions of normality (Kolmogorov–Smirnov's $D = 0.162$, $Z = 1.621$, $P = 0.010$) and homoscedasticity (Levene's $F_{4,95} = 10.992$, $P < 0.0001$). Under the migration model tested, we treated replicate cultures as independent statistical units because the null model is that migration makes no difference in the evolution of VSV, therefore the degrees of freedom (d.f.) for these tests are based on the 20 flasks per experimental block.

As expected from previous observations (Holland *et al.*, 1991; Novella *et al.*, 1995, 1999; Miralles *et al.*, 1999a, b, 2000), there was a significant increase in average relative fitness under all infection regimes. For example, under single-infection, the average fitness across the 20 infected hosts, after 100 generations of evolution, was 1.6 ± 0.1 , which is significantly higher than the fitness estimated for the ancestral clone (Mann–Whitney's $U = 30$, 24 and 20 d.f., one-tail $P < 0.001$).

Next, we were interested in testing whether the different types of infection resulted in significantly different increases in fitness. A Kruskal–Wallis test showed an overall effect of the type of infection for both values of ϕ employed ($H = 13.441$, $P = 0.001$ for 5% and $H = 12.980$, $P = 0.002$ for 50%; 2 d.f.). However, as it is evident from Fig. 3, this difference was mainly because of the large increases in average fitness of the viral populations evolved under coinfection regimes (Table 2). This result agrees with Hypothesis II, which predicted that the expected final fitness of a viral clone evolved under periodic infection passage with coinfection should be

Table 1 Fitness estimates (\pm SEM, $n = 3$) obtained for each flask and experimental treatment.

Flask label	Single infection	Coinfection		Superinfection	
		$\phi = 5\%$	$\phi = 50\%$	$\phi = 5\%$	$\phi = 50\%$
1	1.3 \pm 0.2	2.7 \pm 0.9	1.6 \pm 0.2	1.41 \pm 0.09	1.29 \pm 0.05
2	1.03 \pm 0.09	1.7 \pm 0.1	1.5 \pm 0.2	1.5 \pm 0.3	1.60 \pm 0.05
3	1.7 \pm 0.3	1.9 \pm 0.5	1.6 \pm 0.1	1.10 \pm 0.04	1.5 \pm 0.2
4	1.5 \pm 0.2	1.3 \pm 0.2	1.5 \pm 0.1	1.37 \pm 0.09	1.6 \pm 0.3
5	1.1 \pm 0.1	1.30 \pm 0.09	1.42 \pm 0.07	1.3 \pm 0.1	1.6 \pm 0.1
6	1.1 \pm 0.2	1.8 \pm 0.1	2.51 \pm 0.08	1.14 \pm 0.06	1.3 \pm 0.2
7	1.5 \pm 0.1	1.9 \pm 0.2	2.51 \pm 0.06	1.18 \pm 0.05	1.3 \pm 0.2
8	1.5 \pm 0.3	1.6 \pm 0.2	2.5 \pm 0.1	1.8 \pm 0.1	1.4 \pm 0.2
9	1.6 \pm 0.2	1.75 \pm 0.06	2.27 \pm 0.09	1.13 \pm 0.09	1.3 \pm 0.2
10	1.2 \pm 0.1	1.8 \pm 0.1	2.6 \pm 0.2	1.20 \pm 0.09	1.0 \pm 0.2
11	1.2 \pm 0.2	1.6 \pm 0.2	1.6 \pm 0.3	1.6 \pm 0.3	1.6 \pm 0.1
12	1.1 \pm 0.1	1.5 \pm 0.1	1.4 \pm 0.1	1.5 \pm 0.3	1.5 \pm 0.3
13	1.8 \pm 0.3	1.7 \pm 0.3	1.2 \pm 0.2	1.0 \pm 0.1	2.1 \pm 0.9
14	1.1 \pm 0.2	1.6 \pm 0.2	2.0 \pm 0.3	1.2 \pm 0.2	1.17 \pm 0.08
15	1.1 \pm 0.2	1.26 \pm 0.09	1.4 \pm 0.1	1.8 \pm 0.2	1.2 \pm 0.2
16	1.8 \pm 0.1	2.2 \pm 0.3	3.22 \pm 0.07	1.9 \pm 0.4	1.7 \pm 0.2
17	2.1 \pm 0.2	2.5 \pm 0.5	3.1 \pm 0.2	1.3 \pm 0.1	1.19 \pm 0.03
18	2.7 \pm 0.4	2.4 \pm 0.3	3.3 \pm 0.5	1.2 \pm 0.3	1.0 \pm 0.2
19	1.7 \pm 0.2	2.1 \pm 0.2	3.0 \pm 0.3	1.19 \pm 0.07	1.6 \pm 0.5
20	2.7 \pm 0.9	2.3 \pm 0.4	3.2 \pm 0.2	1.5 \pm 0.1	1.3 \pm 0.2

**Fig. 3** Effect of different types of infectious passages. Each bar represents the average fitness value for 20 different viral isolates. Error bars represent standard errors. The black bars correspond with $\phi = 5\%$ immigrant virus per host, the grey bars, proportional representation with $\phi = 50\%$ immigrant virus per host.

larger than the value observed without migration. In accordance with Hypothesis I (i.e. infectious passage with coinfection results in the fixation of the fittest strain with a probability proportional to its initial frequency), the positive effect depended upon the magnitude of ϕ . The average fitness was 18% larger with coinfection than for single-infection when $\phi = 5\%$ ($P = 0.025$), and up to 39% larger when $\phi = 50\%$ ($P = 0.006$).

In contrast, infection passage with superinfection for both ϕ s tested did not differ from the single infection case (Table 2). Once again, these results support our competition model: infectious passage with superinfection does

Table 2 Comparisons among single infections (no-migration) and the two types of infectious passage (coinfection and superinfection) with two levels of proportional representation of immigrant virus (ϕ).

Test	Fitness (\pm SEM)	U^a	P	Combined P^b
Single vs. coinfection				
$\phi = 5\%$	1.84 \pm 0.09	117	0.025	0.001
$\phi = 50\%$	2.2 \pm 0.2	100	0.006	
Single vs. superinfection				
$\phi = 5\%$	1.37 \pm 0.05	171	0.445	0.684
$\phi = 50\%$	1.42 \pm 0.06	186	0.718	

^aMann-Whitney's U -test done with $n_1 = n_2 = 20$ (Sokal & Rohlf, 1995). In all comparisons, the fitness for the single infection was 1.6 ± 0.1 .

^bThe combined P -level represents the Fisher's combined probability for the same null hypothesis (Sokal & Rohlf, 1995).

Table 3 Nonparametric two-way analysis of variance (Sokal & Rohlf, 1995) testing for differences between the two different regimes of infectious passage (coinfection and superinfection), the two levels of viral immigration per host (ϕ) and their interaction.

Source of variation	d.f.	H	P
Infectious passage	1	27.200	<0.001
ϕ	1	0.767	0.381
Infectious passage \times ϕ	1	0.007	0.931
Total	3	27.975	

not produce fitter viruses than single-infections (Hypothesis III). As a consequence of hypotheses II and III, it is expected that viral fitness under continuous infection

passages with coinfection is larger than under continuous superinfection. The nonparametric two-way analysis of variance shown in Table 3 confirmed this. We observed a net 'type of infectious passage' effect ($P < 0.001$) with lower fitness attained under continuous passage with superinfections than continuous passage with coinfections (Fig. 3).

Match between *in vitro* and *in silico* results

The association between the results of our numerical simulations and the empirical observations is shown in Fig. 4. There is a high correlation between the observed and simulated values (Spearman's $r_s = 1.000$, 3 d.f., $P < 0.001$) (Haefner, 1996). This match between bench and simulation results confirms the reliability of the model. Simulation results were robust to changes in parameter values, producing low variation in outcome and high correlation with observations. The simulation results reported were obtained with the parameters specified above.

Discussion

Our theoretical and experimental results show the effect of different infectious processes on the fitness and adaptation of RNA viruses. Infection passages with coinfection increased fitness compared with the single infection case. The magnitude of the increase was proportional to the frequency of the immigrant virus. In contrast, passages with superinfection did not generate fitness values different from those achieved with single infection, regardless of the frequency of the

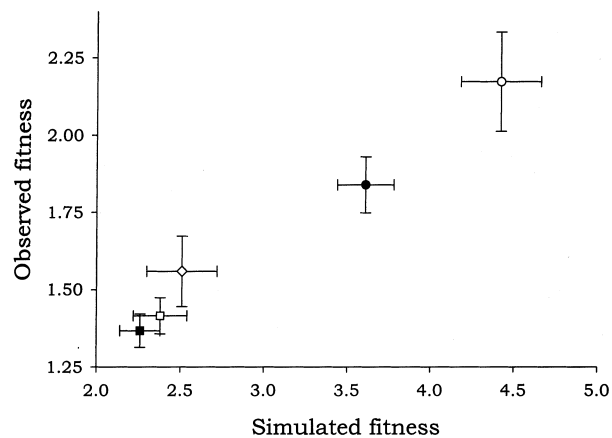


Fig. 4 Correlation between the results of numerical simulations carried out according to the fitness-space model described in the text and the experimental observations. Error bars represent standard errors. Single infection (\diamond), $\phi = 5\%$ coinfection (\bullet), $\phi = 50\%$ coinfection (\circ), $\phi = 5\%$ superinfection (\blacksquare), and $\phi = 50\%$ superinfection (\square).

immigrant virus. These results were also supported by simulations of viral evolution on a fitness landscape and explained by a modified Lotka–Volterra's competition model that incorporates mutation and migration. Competition among viral strains within a host and a noisy founder-effect induced by initial numbers of each strain are the major forces driving the evolution of fitness in these experiments. The more chances for competition, the higher the fitness achieved by the viral populations. Regular passages with coinfection provided more opportunities for such competition, and generated a higher degree of viral fitness than passages with single-infection.

Infection passages with superinfection did not generate increases in fitness larger than those obtained under single-infection regimes. This observation is best explained by the asymmetry induced by initial demographic conditions: once the system is out of equilibrium (Fig. 2d), its fate does not depend on the added viral population but is driven to exclusion to the point where the resident population was the only survivor.

The results here presented stress on the key role played by competition during viral evolution. Coexistence between different viral variants competing for a single resource is not possible in the long run. Even in the most favourable case, when both coinfecting competitors had equal fitness and abundances, coexistence is jeopardized by the fact that beneficial mutations will certainly arise in one competitor, improving its relative fitness, but not in the other. However, if the improvement can be compensated by another beneficial mutation in the second strain, a Red Queen process takes place, improving the fitness of both competitors relative to their ancestors but keeping them at the same point relative to each other (Clarke *et al.*, 1994; Quer *et al.*, 1996). When a fitness increase cannot be readily compensated, coexistence breaks down and the fitter strain dominates (Miralles *et al.*, 1999a, 2000). Beneficial mutations have been demonstrated to arise rarely in evolving viral populations ($\sim 6.4 \times 10^{-8}$ per genome and generation) but, more importantly, the average selective advantage associated with beneficial mutations is high ($W \approx 1.3$) (Miralles *et al.*, 1999a). By contrast, deleterious mutations are produced at a much higher rate (~ 1.2 per genome and generation), but with a small average selective disadvantage ($W \approx 0.998$) (Elena & Moya, 1999). The drift imposed by transmission events plays a role against low frequency variants. Almost every newly produced variant can be lost if its frequency in the population is still low at the moment of transmission. However, chances that a beneficial mutation is not lost by drift during its first few generations of life strongly depend on the selective advantage it confers [$\sim 2 s$ for viral populations (Gerrish & Lenski, 1998)]. Therefore, highly beneficial mutations are more likely to survive drift and, hence, transmission events should favour their spread.

Are the results presented here general to other viral systems?

Turner & Chao (1998) observed that coinfection of a single cell by two strains of bacteriophage $\phi 6$ reduced viral replication rate because coinfection selected for within host competitive ability. Although these results appear to be in conflict with ours, two clarifications must be made: First, $\phi 6$ is a segmented virus that experiences segment reassortment (i.e. sex), whereas VSV is truly asexual (Pringle & Easton, 1997). Indeed, sex was demonstrated not to increase the rate of adaptation by Turner & Chao (1998). Secondly, our results are better explained in terms of the migration of mutant strains among multicellular hosts. Our multiplicity of infection (MOI ≈ 0.02) ensured that each cell was infected with a single virus 99.98% of the times, therefore, no within-cell competition could occur. In contrast, within-cell competition was a major factor in Turner & Chao's (1998) experiment.

There are other cases in the literature showing similar results. Multiple infections with the Rhabdoviridae pancreatic necrosis (IPNV) and haematopoietic necrosis viruses (IHNV) showed similar results (Alonso *et al.*, 1999). When BF-2 cells were coinfecting with IHNV and IPNV, the former had a lower growth rate and was systematically displaced by IPNV. In addition, the magnitude of the reduction in IHNV titre was dependent on the frequency of IPNV in the mixture: the lower the frequency of IPNV the less affected was IHNV replication. Furthermore, when cells were primarily infected with IHNV and later superinfected with IPNV, the titre reached by IHNV was equivalent to those concentrations found for IHNV in single infections. In other words, the resident IHNV enjoyed the numerical advantage of infecting first and grew without interference of IPNV.

Another example can be found in Baculoviridae nuclear polyhedrosis viruses (NPV) (Kamita & Maeda, 1993). When BmN cells were coinfecting with *Bombyx mori* NPV (BmNPV) and *Autographa californica* NPV (AcNPV), the replication of the former was completely inhibited by the later. In contrast, if the cells were already infected with BmNPV, superinfection with AcNPV did not produce inhibition, regardless of the exact moment in which superinfection occurred.

It is worth noting, however, that the inhibition of BmNPV replication was suggested to be caused at the translation level by a DNA helicase encoded in the AcNPV genome (Kamita & Maeda, 1993). This stresses the fact that interactions between two different viral species are not only governed by population parameters, but also by many other factors. Among others, we can list:

- (i) interactions between different viral-encoded macromolecules. HIV-1 replication is suppressed by the hepatitis C virus core protein, which might act as a

strong repressor of the long-terminal repeat of HIV-1 (Srinivas *et al.*, 1996).

- (ii) Kidnapping of cellular factors required for transcription and/or translation. Double infections with VSV and influenza results in a stop in the influenza-mRNA processing as a consequence of VSV-mediated inhibition of 3'-end processing and assembly of pre-U1 and -U2 snRNAs into the snRNPs required by influenza (Friele *et al.*, 1989). Also, VSV mRNAs are better competitors than influenza mRNAs for the initiation complexes (Friele *et al.*, 1989).
- (iii) Inhibition of superinfections by down-regulation of common receptors. HIV-1 and HIV-2 employ the same CD4 receptor; the latter protects against the former (Martin & Nayak, 1996).
- (iv) Nonspecific immune responses. Induction of β -chemokines by HIV-2 protect against HIV-1 (Kokkotou *et al.*, 2000).

The relative importance of these factors compared with the stochastic and competition effects observed in our experimental and simulation studies is not known. The proposed mathematical model applies to infections with two variants of the same virus, where competition should be for the same host factors although, ideally, the competition parameter, β_{ij} , could reflect any type of interaction.

Perspectives

These studies can be extended in several ways to provide a more valuable tool for the study of RNA virus evolution. For example, by considering more complex fitness landscapes with different degrees of epistasis (Solé *et al.*, 1999), by taking into consideration the presence of antibodies or antiviral drugs in the competition media, by adding extra levels of competition such as within-cell competition, by allowing migration before reaching the carrying capacity size (eqn 3), or by removing the restriction imposed by eqn 2, we may better describe the dynamics of viral populations and perhaps be able to predict their future behaviour accurately. Predictions should be compared with *in vitro* evolution experiments to test their validity. This combined strategy has been successfully employed in this report.

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