Evolution of Increased Survival in RNA Viruses Specialized on Cancer-Derived Cells

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ABSTRACT: Viruses and other pathogens can diverge in their evolved host-use strategies because of exposure to different host types and conflicts between within-host reproduction and between-host survival. Most host-pathogen studies have emphasized the role of intrahost reproduction in the evolution of pathogen virulence, whereas the role of extra-host survival has received less attention. Here, we examine the evolution of free-living virion survival in RNA virus populations differing in their histories of host use. To do so, we used lineages of vesicular stomatitis virus (VSV) that were experimentally evolved in laboratory tissue culture for 100 generations on cancer-derived cells, noncancerous cells, or alternating passages of the two host types. We observed that free-living survival improved when VSV populations specialized on human epithelial carcinoma (HeLa) cells, whereas this trait was not associated with selection on noncancer cells or combinations of the cell types. We attributed this finding to shorter-lived HeLa monolayers and/or rapid cell-to-cell spread of viruses on HeLa cells in tissue culture, both of which could select for enhanced virus stability between host-cell replenishment. We also showed evidence that increases in virion survival were associated with decreases in virulence, which suggests a trade-off between survival and virulence for the VSV populations on one cell type. Our results shed new light on the causes and consequences of "sit and wait" infection strategies in RNA viruses.

Keywords: experimental evolution, host-parasite interaction, specialization, free-living survival, virus.

Introduction

Differential reproduction and survival are the cornerstones of evolution via natural selection. Intrahost reproduction as a determinant of parasite virulence is often the focus of studies in the evolution of infectious disease (Bull 1994; Ebert and Bull 2003; Ewald 2004), whereas the role of free-living (extracellular) survival has received relatively less attention (e.g., Walther and Ewald 2004; de Paepe and Taddei 2006). Some theory has attempted to combine free-living survival as well as reproduction into mathematical models for the evolution of parasite virulence (Bonhoeffer et al. 1996; Gandon 1998; Kamo and Boots 2004). Although these studies have proposed a range of intriguing explanations for the relationship between a parasite's free-living survival and its virulence, theoretical models have been limited in their ability to accurately predict the ecological conditions through which improved free-living survival should evolve and whether such selection may constrain parasites from maximizing other traits. De Paepe and Taddei (2006) used a comparative approach in studying lytic bacteriophages to show that free-living survival tended to trade off with phage reproduction on *Escherichia coli* bacteria, perhaps because stability achieved through more densely packed viral DNA slows the rate of phage genome replication. However, it is unclear how ecological circumstances may cause closely related populations of a single virus to diverge in free-living survival.

Although the underlying ecological drivers are poorly studied, it is evident that viruses of eukaryotes can differ dramatically in free-living virion stability (Halfon 1996; Ausar et al. 2006; Leschonsky et al. 2007; Mateo et al. 2008; Caciagli et al. 2009; Kennedy and Parks 2009; Alto and Turner 2010). One possibility is that free-living particle stability reflects selection under differing ecological histories. It is commonly acknowledged that greater extracellular exposure should increase the likelihood of virion degradation due to stressors such as desiccation, acidity, and elevated temperature. Thus, a logical prediction is that (all else being equal) viruses that spend longer periods outside of cells should be selected for greater free-living stability relative to viruses that are less often subjected to

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extracellular exposure (even in the absence of abiotic stressors, such as extreme temperature and pH).

Immortalized cells grown in tissue culture are essential models for understanding basics of cell biology, virus genetics, and evolution (Harrison 1912; Skloot 2010). It is obvious to researchers using these systems that different tissue culture cell lines vary in monolayer permanence between serial propagation; some cell types become detached (die or senesce) faster than others in the absence of infecting viruses owing to myriad possible differences in cellular physiology (Freshney 2000). Furthermore, the growth rate for a virus population may vary among cell types due to differential rates of cell-to-cell spread. Thus, depending on the host cell type, a virus population may exhaust its cell resources quickly and experience a long wait time before host cells are available for additional infection. Here, we examine whether these differing properties of cell types in tissue culture may translate to differential selection for evolved free-living virion survival.

High levels of population growth on a wide variety of hosts define a virus as generalized in host use, relative to a virus specialist that grows well on fewer hosts. In vitro studies have concerned the evolution of host-use generalism versus specialism by experimentally evolving viruses in the laboratory (Turner and Elena 2000; Greene et al. 2005; Duffy et al. 2006, 2007; Remold et al. 2008). Turner and Elena (2000; see also Remold et al. 2008) examined the problem using vesicular stomatitis virus (VSV), a negative-sense, single-stranded RNA *Vesiculovirus* in the family *Rhabdoviridae* that naturally infects mammals and insect vectors (Lyles and Rupprecht 2007). The VSV is usually cultured in the laboratory on baby hamster kidney (BHK) cells. Turner and Elena (2000) used an ancestral population of VSV Indiana serotype to found 4 lineages per treatment in a 100-generation (25 passages; 48 h per passage) evolution experiment. One treatment cultured viruses strictly on human epithelial carcinoma (HeLa) cells, a second treatment only on Madin Darby canine kidney (MDCK) cells, and a third treatment on alternating (MDCK and HeLa) cell types (see "Material and Methods" for additional details). As summarized by Turner et al. (2010), because the HeLa-evolved viruses (those exposed only to HeLa hosts for 25 passages) improved in HeLa performance at the expense of performance on MDCK and ancestral BHK hosts, these populations were deemed to be host-use specialists. In contrast, viruses evolved via alternating host passages improved in HeLa and MDCK growth but lost performance on the original BHK host, which is consistent with directly selected generalization. Last, the MDCK-evolved viruses did not gain fitness on HeLa cells but improved on MDCK and were the only populations to show correlated improvement on the original BHK host, classifying these viruses as indirectly selected generalists.

Our previous research focused on the effects of ecological history (constant vs. alternating hosts) on reproduction of viruses on selected and unselected hosts (Turner and Elena 2000; Remold et al. 2008; Turner et al. 2010), whereas here we examine whether differences in viral ecological history are associated with changes in free-living virion stability. First, we examined characteristics of the host-cell environments that comprised the recent evolutionary histories of the virus populations and observed that HeLa and MDCK cells fundamentally differ in the average duration of cell monolayer viability. We then tested whether these differences translated to divergence in virion stability among virus specialists (HeLa evolved), directly selected generalists (alternating evolved), and indirectly selected generalists (MDCK evolved). Last, to examine whether a trade-off occurred between traits affecting free-living survival and those determining virulence, we measured relative plaque size (a proxy for cell-to-cell spread, viral fecundity, and virulence) among viruses that differed in survival.

Material and Methods

Strains and Culture Conditions

We obtained HeLa cells from American Type Culture Collection (CCL-2), MDCK cells from European Collection of Cell Cultures (85011435), and BHK cells from the laboratory of Esteban Domingo (University of Madrid). Cells grew in Dulbecco modified Eagle's minimum essential medium (DMEM) at 37°C, 95% relative humidity, and 5% CO_2. Viruses were derived from the Mudd Summer strain of VSV Indiana serotype. The ancestor used in experimental evolution (Turner and Elena 2000) was a population polymorphic for an Asp^{259}→Ala substitution that alters the VSV glycoprotein (Holland et al. 1991), causing resistance to a mouse I_1-monoclonal antibody.

In the experimental evolution study (Turner and Elena 2000), the ancestor founded 12 lineages subjected to 25 serial passages (4 generations per passage; 100 generations total) of in vitro evolution in 25-cm^2 plastic flasks containing approximately 10^5 cells/cm^2 cultured in DMEM under the above conditions. Four lineages (M1–M4) evolved on MDCK cells, four (H1–H4) evolved on HeLa cells, and four (A1–A4) evolved via alternating host passages. Virus passage was initiated by multiplicity-of-infection (MOI) of 0.01 viruses per cell, followed by 48-h incubation. Harvested supernatant contained roughly 4 generations (Miralles et al. 1999) of viral progeny. Serial transfer occurred via a 10^{-4} dilution (bottleneck) of the supernatant containing virus progeny placed in a new flask

containing freshly grown cells. We repeated this process 25 times per lineage. We stored virus population samples at −80°C for later use. We obtained the consensus sequence for the common ancestor and for each evolved population near the end point (passage 24); Remold et al. (2008) described molecular substitutions separating the lineages from the ancestor.

Before starting the current experiment, we amplified HeLa-evolved and MDCK-evolved viruses on HeLa and MDCK cells, respectively; we inoculated approximately 10^4 plaque-forming units (pfu) of frozen-stock virus onto a monolayer containing approximately 10^5 cells/cm^2 and allowed 48 h of viral infection under the above culture conditions. We amplified alternating-host-evolved viruses separately on each cell type; we showed that these populations did not differ in survival responses across amplification hosts ($F = 0.02$, df $= 1, 6$, $P = .88$), which allowed us to pool these separate amplifications. We saved supernatants from these infections to use as sources of virus for subsequent experiments. We amplified the ancestral virus on BHK cells. Despite evolved changes in BHK performance, all viruses were capable of infecting these cells, which provided an unbiased cell type to enumerate virus titers (pfu/mL) for virion stability measurements (Alto and Turner 2010; Turner et al. 2010).

Host Mortality

Within each of 5 assay blocks, we cultured replicate ($n = 5$) uninfected monolayers of HeLa and MDCK cells at 37°C as described above. We destructively sampled monolayers at 5 time points (0, 24, 28, 32, and 48 h) using standard methods of cell wash with phosphate-buffered saline, followed by trypsin incubation (3 min for HeLa and 5–10 min for MDCK) to detach cells from plastic surfaces to allow collection. We diluted cell samples, mixed them 1 : 1 (volumetric) with trypan blue stain, and counted cells via microscopy using a hemocytometer. Trypan blue is a vital stain that differentiates live and dead cells by absorbing to dead-cell membranes. Unstained counts estimated densities of live cells, whereas stained cells measured accumulation of dead cells through time.

Free-Living Virion Survival

We amplified a virus strain for 48 h on its selected host and harvested the supernatant to obtain a cell-free lysate. We then diluted the lysate for each test virus to achieve approximately 10^6 pfu/mL of virus in 10 mL of cell-free DMEM, which was placed in a 25-cm^2 culture flask. We prepared a total of 12 flasks for each test virus, allowing threefold replication of an assay that monitored survival (remaining titer) of the virus by destructively sampling flasks at four time points (12, 24, 36, and 48 h) during 37°C incubation. We estimated percentage virus survival using the formula $(N_t/N_0) \times 100$, where N_0 is the initial density and N_t is the remaining density measured at each of the four postincubation time points (t).

We compared virion survival between the three virus groups using two different methods. First, we used a three-way mixed model analysis of variance (PROC MIXED, ANOVA; SAS Institute 2005), treating virus strain and time as fixed-effect variables and virus lineage (1–4) as a random-effect variable. We analyzed the main effects of each fixed-effect variable as well as interactions between VSV and time. The test for an interaction between evolved virus type and time enables us to determine whether there may be temporal effects of survival during the experiment. For example, because we anticipate substantial mortality to occur by the final sampling period, there may be an effect of evolved virus type on survival at early times but not at late times. Subsequently, we analyzed significant treatment effects by contrasts of means adjusting for multiple comparisons, within each postincubation time point, using the sequential Bonferroni method (Rice 1989).

Second, although the ANOVA segregated the effects of virus lineage on survival, we sought to test the survival distributions of the various virus populations through time. To this end, we used the Kaplan-Meier estimator (XLSTAT 2011) to interrogate differences in death curve trajectories and used multiple tests (log-rank, Wilcoxon rank-sum, and Tarone-Ware tests) to compare the equivalence of the survival distribution functions of the different virus populations.

Plaque Diameter

To measure plaque diameter, we allowed a sample of VSV containing 200 pfu to infect a confluent monolayer of HeLa or MDCK cells, which caused single virions to initiate individual plaques. After 1-h incubation at 37°C (95% relative humidity and 5% CO_2), we added a 7% agarose overlay. After 23 h, we removed the overlay and stained with crystal violet to visualize plaques.

We scanned plates with lids removed and a light source placed underneath the scanner. Using Adobe Photoshop, we magnified each plate to 200% and increased the contrast by adding a brightness/contrast filter of +20 points. We measured plaque diameter using the Ruler tool (set at data collection in millimeters with standard error of 0.1 mm), which calculates the distance between any two points in the work space. We measured 15 plaques for each test virus on each host (HeLa and MDCK) for a total for 30 measurements for each test virus. We used nested ANOVAs with virus lineage nested within host-evolved virus treatment to test whether average plaque diameter differed

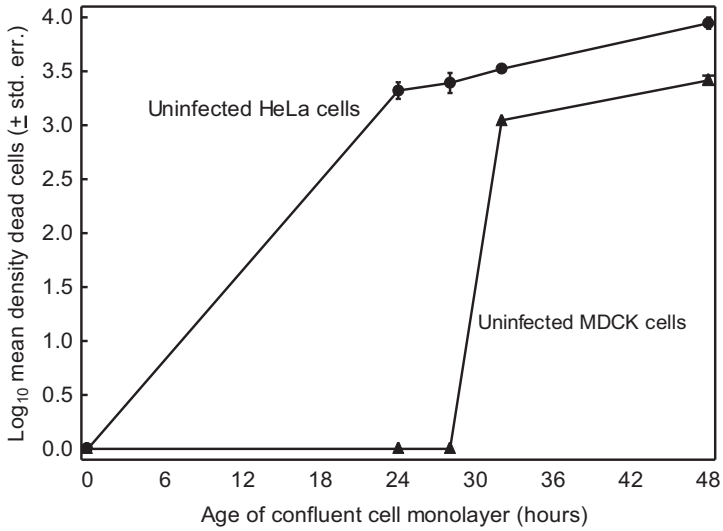

Figure 1: Human epithelial carcinoma (HeLa) and Madin Darby canine kidney (MDCK) tissue culture cells differed in mortality, when grown in the absence of viruses at 37°C. We conducted five blocks of cell death assays, which used a vital stain to estimate the percentage accumulation of dead cells in monolayers sampled at five time points (0, 12, 24, 36, and 48 h). Cell monolayers were initially confluent and composed entirely of live cells. Dead cells appeared earlier and accumulated faster in uninfected monolayers of HeLa cells (filled circles), compared with those of MDCK cells (filled triangles); see text for statistical analysis. Each symbol is the mean (±SE) of five assay blocks, and error bars too small to be visualized are omitted.

across hosts for all evolved populations and the ancestor. Separate analyses were performed for measurements on HeLa and MDCK host cells.

Results

Differences in Host Mortality

In the earlier experimental evolution (Turner and Elena 2000), virus populations infected 24-h-old confluent monolayers of HeLa or MDCK cells. To compare the average initial densities (cells/mL) of these uninfected monolayers, we grew each host type for 24 h with replication ($n = 5$). Results showed that the mean density (± SD) of cells harvested from HeLa monolayers ($9.06 \times 10^6 \pm 1.82 \times 10^6$ cells/mL) exceeded that of MDCK monolayers ($5.56 \times 10^6 \pm 8.08 \times 10^5$ cells/mL) and that the difference was statistically significant (t-test with $t = 3.93$, $\mathrm{df} = 8$, $P = .004$).

Monolayers do not measurably increase in cell density after becoming confluent, but the cells will eventually die unless harvested and passaged to seed a new monolayer. We compared uninfected 24-h-old monolayers of HeLa and MDCK cells to determine whether they differed in the accumulation of dead cells during subsequent 48-h incubation. We estimated the densities of dead cells at 5 time points (0, 24, 28, 32, and 48 h) in monolayers that previously grew for 24 h to become confluent; thus, the assay reflected the 48-h window that allowed virus infection of monolayers that previously grew for 24 h to become confluent (Turner and Elena 2000; see also "Material and Methods"). We conducted 5 blocks of assays, where each block contained the two cell types. Results (fig. 1) from all five blocks showed that, after 24 h, the uninfected HeLa monolayers contained approximately 50% dead cells (cf. initial density above) and that the density of dead cells increased steadily between 24 and 48 h. In all five assay blocks, the slope of the increase in dead cells through time in HeLa monolayers was significantly positive using linear regression ($\mathrm{df} = 3$, $.019 > P > .037$). In contrast, the data for dead-cell accumulation through time in MDCK monolayers differed markedly (fig. 1). Dead cells were not detected in any of the uninfected MDCK monolayers until 32 h, followed by an increase in dead cells between 32 and 48 h. These results matched anecdotal observations in the laboratory (N. Morales, personal communication), in which HeLa cells were seen to naturally degrade (i.e., become detached from surfaces) faster than MDCK cell monolayers. Also, the findings matched the observed greater durability of MDCK monolayers, which require relatively longer trypsin incubation to release cells from adhering to plastic surfaces (see "Material and Methods"). We concluded that HeLa cell monolayers physiologically degraded (died) faster than MDCK cells within the 48-h transfer cycle used in experimental evolution of the VSV populations.

Differences in Free-Living Virion Survival

Within the 48-h transfer cycle used in experimental evolution, effects of VSV infection and natural cell death caused cell monolayers to be completely destroyed (Turner and Elena 2000). In addition, Turner and Elena (2000) reported that, by passage 5 of the 25-passage experiment, the VSV populations achieved maximum titer within 24 h of the 48-h transfer cycle. These findings indicated that culture conditions required the viruses to generally survive in the supernatant for at least 24 h before serial passage. Although this caused virus survival to be generally important in the experimental evolution, we hypothesized that the HeLa-specialized viruses should be especially selected for improved survival owing to strict passage on HeLa cells, which died relatively faster within the initial 24 h of the transfer cycle (fig. 1). To test this idea, we conducted repeated ($n = 3$) survival assays for each of the 12 evolved populations and the ancestral strain. We placed viruses in cell-free culture medium at 37°C and estimated what percentage of virions remained infectious (able to form plaques) at four elapsed-time points (12, 24, 36, and 48 h). Unlike other studies, in which an ANOVA to measure time effects may increase, decrease, or remain the same, survival in the current study must decrease with time. Therefore, the observed significant effect of time was expected and not surprising (table 1). However, the inclusion of time in the context of an evolved virus type (i.e., interaction) is highly relevant and enabled us to determine temporal effects of survival that were specific to each evolved virus type. We performed a total of 144 measures (3 replicates × 4 time points × 12 populations).

We observed that mean survival of the wild-type ancestor was rather low across the times assayed, ranging between roughly 5% and 30% (fig. 2). Also, results (fig. 2) showed that, in most cases, the evolved viruses presented survival measures similar to those of the ancestor. However, the exception was the HeLa-evolved viruses, which showed equivalent or significantly higher mean survival values than the ancestor and equal or better survival than the other evolved groups of viruses (fig. 2). Importantly, this survival advantage occurred at 12 and 24 h, which corresponded to the typical durations when viruses had to sit in the supernatant following monolayer destruction and before serial passage onto a fresh monolayer (Turner and Elena 2000). However, for assays at 36 and 48 h, we observed that the vast majority of viruses did not survive to remain infectious, regardless of treatment group. These extreme time points exceeded durations spent outside of cells by any of the viruses during their experimental evolution and indicated that the survival advantage of HeLa-specialized viruses at early times (12–24 h) did not translate to measurable differences at late times (36–48 h).

Table 1: ANOVA for effects of virus strain, time, and interaction on vesicular stomatitis virus (VSV) free-living survival

Effect	df	*F*	*P*
VSV	2, 6	3.77	.0872
Time	3, 168	44.12	<.0001
VSV × time	6, 168	5.87	<.0001

Follow-up pairwise contrasts for a significant interaction between VSV group and time in our ANOVA model (table 1) showed that HeLa specialists survived significantly better than the other virus groups at the 12-h and 24-h time points (HeLa-specialist viruses, H; MDCK-specialist viruses, M; alternating host viruses, A; H vs. M at 12 h, $P < .0001$; H vs. A at 12 h, $P = .0002$; H vs. M at 24 h, $P = .002$, H vs. A at 24 h, $P = .0005$). The survival of alternating-host-evolved viruses was similar and not significantly different from that of MDCK-evolved lineages at time points after incubation (all contrasts showed $P > .43$; fig. 2).

In contrast to ANOVA that compared the differences in the least square means of the virus populations, we used Kaplan-Meier survival analysis to compare their relative death curve trajectories and tested differences in how these treatment populations decreased through time. Populations could conceivably have differed in their least square means across time and still not differed in overall death trajectory. The Kaplan-Meier estimator with log-rank, Wilcoxon, and Tarone-Ware tests all revealed significant differences (observed values = 60.93, 47.27, and 54.98; critical value = 7.81; df = 3; $P < .0001$) between the survival distribution functions of the treatment populations.

Differences in Plaque Diameter

We used estimates of plaque diameters on HeLa and MDCK cells to compare how quickly VSV strains spread from cell to cell to form plaques in the time allowed. Using nested ANOVAs, we determined that there were significant differences in plaque diameters among the host-evolved viruses on HeLa but not MDCK host cells (table 2). MDCK-evolved viruses yielded the largest mean plaque diameters on HeLa cells (fig. 3). The alternating-host-evolved viruses were intermediate in average plaque size, with the HeLa-evolved viruses presenting the smallest mean plaque sizes on HeLa cells. The relative plaque sizes for the three host-evolved virus treatments were significantly different from each other on HeLa cells, the shorter-lived host type. Virus lineages varied significantly within host-evolved viruses for plaque diameter on the HeLa and MDCK cells (table 2).

We used a *t*-test (two tailed) to compare the means of

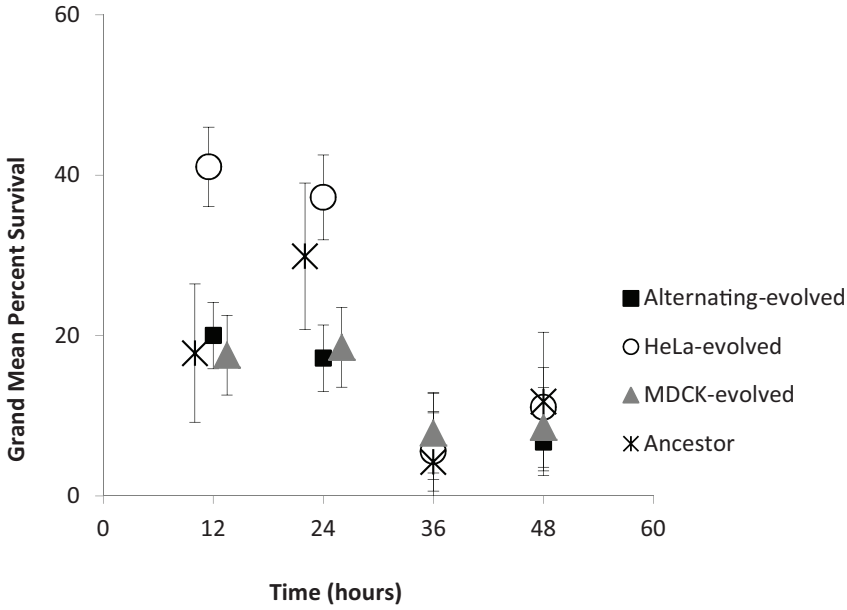

Figure 2: Viruses evolved solely on human epithelial carcinoma (HeLa) host cells showed improved extracellular survival at 37°C. In replicated ($n = 3$) assays, we estimated the mean percentage of infectious particles (survivors) in virus populations exposed to 37°C incubation and sampled at four time points (12, 24, 36, and 48 h). Each point is the least squares mean (±SE) for the four virus populations in each treatment group; ancestor values are means (±SE) for a representative clone of wild-type vesicular stomatitis virus (VSV). See text for statistics.

the plaque diameters of all viruses (evolved and ancestor) on HeLa cells versus MDCK cells. Results demonstrated that, on average, plaque diameter was significantly greater on HeLa cells than MDCK cells ($t_s = 13.43$, df = 388, $P < .001$). Because HeLa monolayers were denser than MDCK monolayers (see above), these combined results indicated that a greater number of HeLa cells must be destroyed by the VSV strains per unit time, compared with growth on MDCK cells.

Trade-Off between Survival and Virulence

The above results showed that HeLa-evolved viruses tended to improve in survival, whereas MDCK-evolved viruses tended to show the largest plaque sizes; taken together, these data suggested that the two traits related through an evolved trade-off. We therefore inquired whether there was a trade-off between virus survival (virion stability) and plaque diameter (virulence). We adjusted the significance level using the sequential Bonferroni method to apply a more stringent cutoff for significance, given that trade-offs between survival and virulence are gauged on different hosts. Figure 4 depicts the relationship between free-living survival at 12 and 24 h (the time points where significant differences were observed) and plaque diameter for the individual lineages of all virus groups on the two hosts: HeLa (fig. 4*A*) and MDCK (fig. 4*B*). A Pearson product-moment correlation test demonstrated a significant negative correlation between survival and fecundity for the VSV populations on MDCK cells ($r = -0.58$, $n = 12$, $P = .047$) but not on HeLa cells ($r = -0.14$, $n = 12$, $P = .68$).

Discussion

Laboratory studies show that VSV populations can readily diverge in their ability to reproduce on host cell types, which allows VSV to serve as a useful model for exploring evolution of host-use specialization and generalization in RNA viruses (Novella et al. 1995, 1999; Turner and Elena 2000; Ogbunugafor et al. 2010; Turner et al. 2010). Here we showed that previous evolution of cell tropism (Turner and Elena 2000) led to observed divergence in virion stability among the groups of VSV populations. We observed that the VSV lineages independently evolved on HeLa cells showed improved survival at durations associated with time spent outside of cells (12 and 24 h) during the experimental evolution. In contrast, the evolved viruses and wild-type ancestor showed equivalent low survivability at extreme durations (36 and 48 h), beyond the previous selection conditions. We concluded that the evolution of

Table 2: Nested ANOVA on plaque diameter of host-evolved viruses grown on Madin Darby canine kidney (MDCK) and human epithelial carcinoma (HeLa) cell types

	MDCK			HeLa		
Source	*F*	df	*P*	*F*	df	*P*
Vesicular stomatitis virus	1.78	2, 9	.2235	32.86	2, 9	<.0001
Lineage (vesicular stomatitis virus)	3.67	9, 168	.0003	1.95	9, 168	.0485

increased free-living survival coincided specifically with VSV specialization on HeLa cells.

Virulence is typically defined as the negative effect of a parasite on host fitness. The plaque diameter of a virus is a convenient metric to gauge its ability to infectiously spread and destroy hosts, allowing plaque size comparisons among virus strains to be used as estimators of relative differences in viral virulence, fecundity, and fitness (e.g., Burch and Chao 2000; Abedon and Culler 2007; Sanjuán et al. 2007). Mathematical models have examined the relationship between traits for free-living survival and virulence in parasites (Bonhoeffer et al. 1996; Gandon 1998; Kamo and Boots 2004). Interestingly, our study suggested that free-living survival can trade-off with virulence in the VSV populations. Although the HeLa-specialized lineages were the best free-living survivors, they produced the measurably smallest mean plaque diameters when grown on both cell types, although the difference was only significant on HeLa cells. In contrast, MDCK-selected viruses evolved the largest plaque sizes and alternating-host-selected viruses were intermediate in plaque size when measured on HeLa cells. These data indicated that the time that viruses spent on HeLa versus MDCK cells during experimental evolution dictated how traits relating to survival and virulence evolved. We determined that these two traits were inversely correlated on MDCK cells; although the correlation coefficient was negative on HeLa cells, this result was not significant. These analyses hinted that a genotype × environment interaction governed the trade-off, but this idea is speculative and is the subject of our ongoing research.

Two lines of evidence demonstrated how propagation of VSV populations on HeLa cells could produce selection for greater free-living survival. First, we observed that HeLa monolayers degraded faster than MDCK monolayers in the absence of infecting viruses. Virus replication is only possible in living (metabolically active) cells. Our data indicated that measurable differences in mortality existed for the two cell types in the absence of virus infection. Second, across all of the evolved populations, the sizes of VSV plaques on HeLa cells were much larger than those produced on MDCK cells. Although methods exist for accurately measuring cell-to-cell spread of viruses in tissue culture using inserted markers, such as green fluorescent protein, we chose to employ plaque size as a convenient method for gauging the rate of virus growth, leaving the virus genome unaltered. Still, this gross method showed a substantial host-related difference in VSV spread between cells, which indicated that the "wave-front" of epidemiological spread occurred faster for the viruses on relatively denser HeLa cell monolayers (i.e., more HeLa cells than MDCK cells are destroyed per unit time). These findings match reports that cancer-derived cells are generally more susceptible to VSV infection (e.g., Russell and Peng 2007). Thus, we showed that viruses propagated on HeLa cells experienced relatively longer periods of time outside of host cells due to (i) effects of the cell type's mortality and (ii) the rate of viral infectious spread on this host during the period preceding monolayer destruction or detachment. Either (or both) mechanism could have favored HeLa-evolved variants that were advantaged in extracellular stability.

Differences in the timing of transmission offer one plausible mechanism for the observed divergence in virus stability. Other possibilities exist, however, especially different intracellular interactions occurring between VSV and HeLa cells versus MDCK cells. HeLa cells are derived from cancerous tumors, whereas MDCK cells are not. Cancer and ordinary cells often fundamentally differ; for example, differing innate defense mechanisms, such as lesser interferon production and/or sensitivity in cancer cells, would create different selection pressures for the virus as it interacts with host cells during infection. Also, tumor-derived cells are neoplastic, which should cause HeLa cell monolayers to be relatively more heterogeneous than MDCK monolayers, on average. These differences could cause HeLa-specialized viruses to evolve traits particularly suited for replication within HeLa cells, leading to divergence in virion stability as a purely pleiotropic consequence. Using whole-genome sequencing, Remold et al. (2008) identified many synonymous and nonsynonymous nucleotide substitutions separating the three virus groups (HeLa evolved, MDCK evolved, and alternating evolved) from each other and the common ancestor. Interestingly, of the five genes constituting the VSV genome, the HeLa-evolved lineages showed no changes in the N (nucleoprotein) or P (phosphoprotein) genes, which ruled out two of the five VSV genes as candidate loci for the evolution

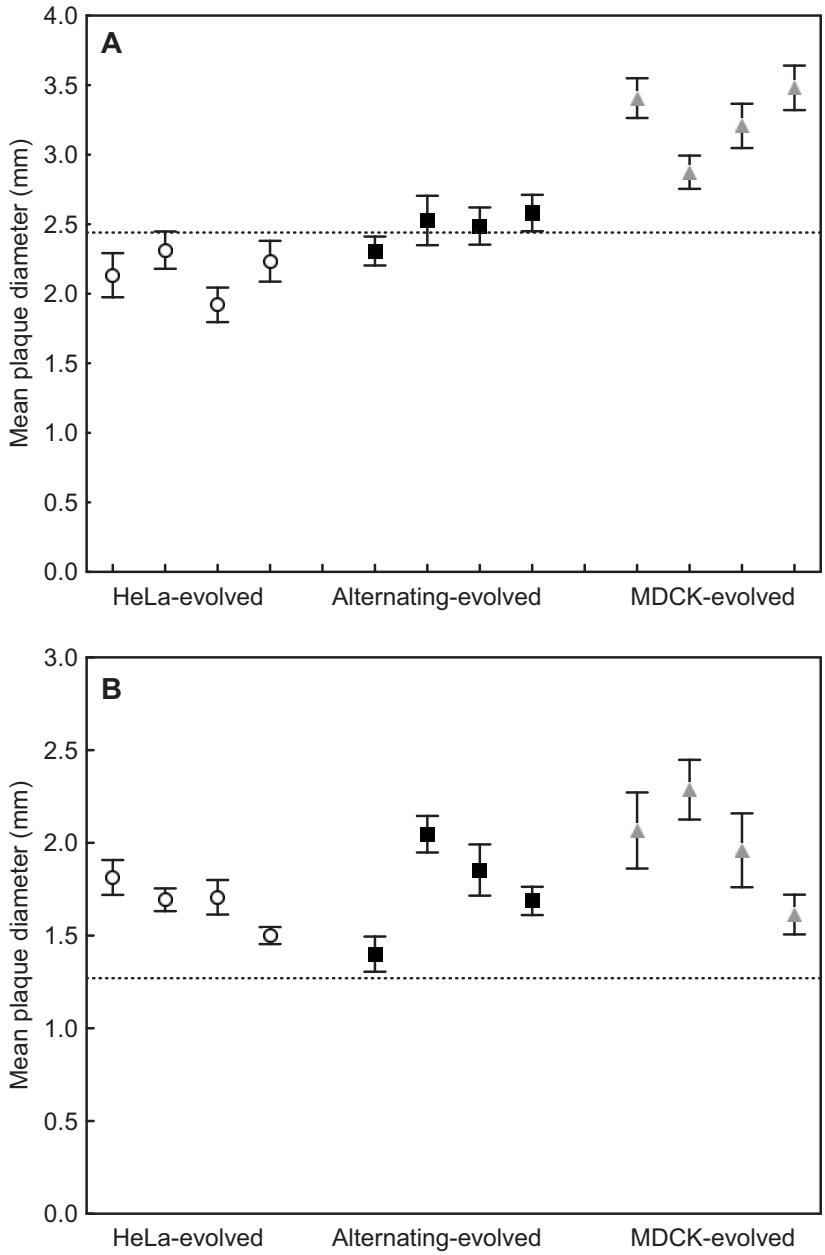

Figure 3: Viruses evolved solely on Madin Darby canine kidney (MDCK) cells formed larger plaques on both hosts, relative to viruses evolved on human epithelial carcinoma (HeLa) cells and on alternating hosts. We measured the mean diameter of 15 plaques for each virus population when grown on HeLa cells (*A*) and MDCK cells (*B*). Each symbol represents the mean (±95% confidence interval), and the dashed line is the mean plaque diameter of the ancestral vesicular stomatitis virus (VSV) population.

of enhanced stability. All four HeLa specialists showed identical nonsynonymous changes in the M (matrix protein) and L (polymerase) genes, hinting that individual or epistatic effects of these mutations may be responsible. These loci are involved in formation and condensation of the ribonucleocapsid (RNP) core in VSV (Kaptur et al. 1995; Lenard 1996; Lyles and McKenzie 1997) and the possibility exists that a change in one or both genes could lead to a more densely packed RNA genome, analogous to the stability mechanism that De Paepe and Taddei (2006) suggested for DNA phages. However, this proposed mechanism in VSV is highly speculative and is the subject

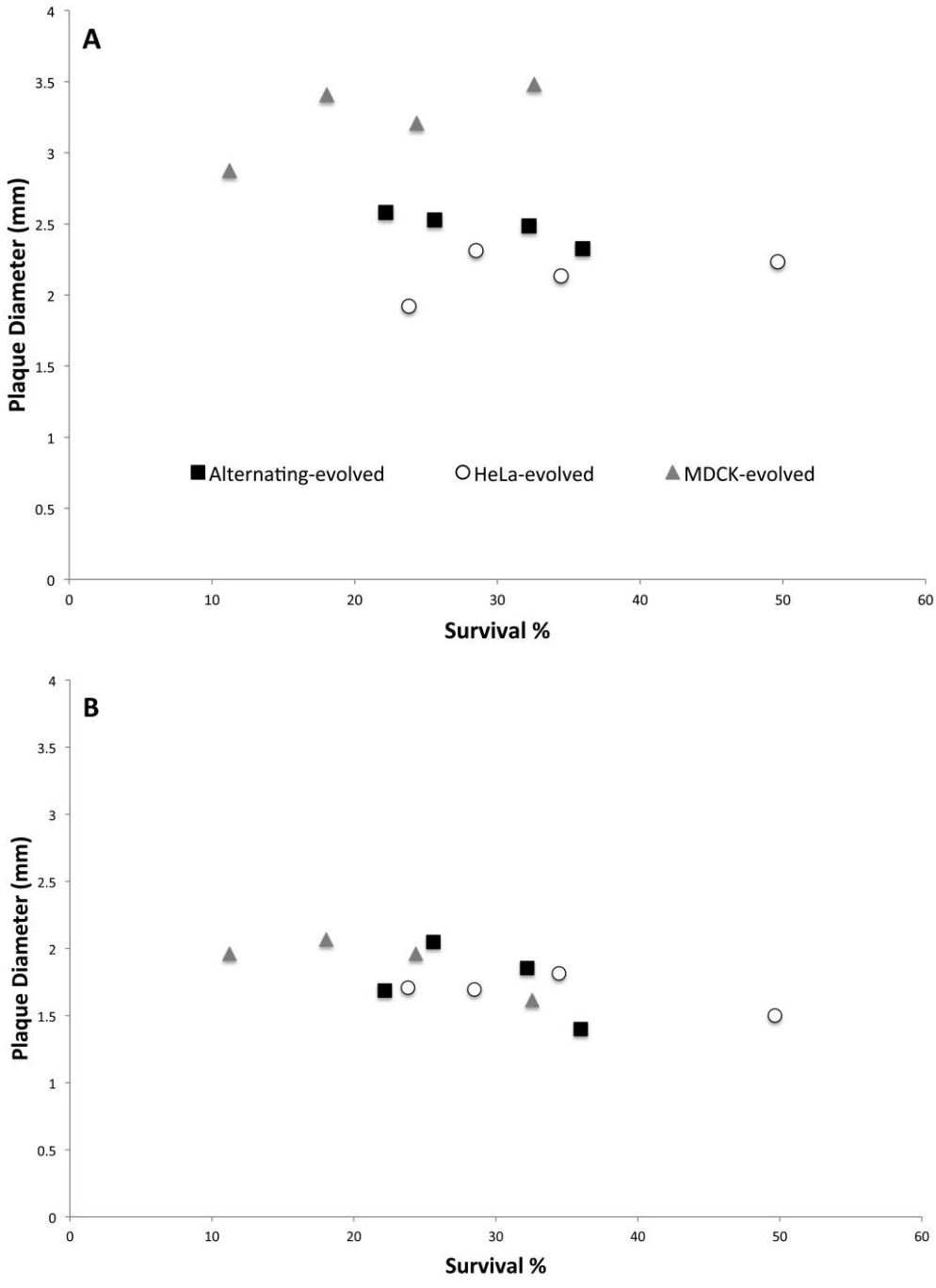

Figure 4: Virus survival tended to trade off with plaque diameter (virulence) in evolved vesicular stomatitis virus (VSV) populations on Madin Darby canine kidney (MDCK) hosts. Across all evolved populations, survival did not improve at the expense of decreased virus fecundity on human epithelial carcinoma (HeLa) cells (*A*) but did on MDCK cells (*B*). Survival estimates are means for each virus population across 12 and 24 h as depicted in figure 2. See text for details.

of ongoing investigation using reverse genetics in our laboratory. Regardless of the underlying mechanism, our data relate to the burgeoning interest in oncolytic virus therapy (Russell and Peng 2007), where VSV is suggested as a promising anticancer "drug" to target tumor destruction. Changes in the VSV M protein are implicated as important in developing this therapy, whereas L substitutions generally are not (Lichty et al. 2004; Stojdl et al. 2004; Lun et al. 2006); this result suggests that changes in L and/or epistasis between L and M mutations might also function in oncolytic properties of VSV.

The interplay of theoretical models and empirical studies can provide a more thorough understanding of the evolution of parasite biodiversity. Although theory is highly useful for identifying how different environmental factors should select for adaptive traits in parasites, the natural world is necessarily more complex than the most ambitious mathematical theory. Here, we show that divergence in virus free-living survival and virulence occurred in VSV populations subjected to differing host-use ecologies, even though free-living survival is not typically the focus of predictive models. These observations show that selection for virus survival is a useful component to add to existing models in the evolution of host-parasite interactions, especially RNA viruses. A more thorough understanding of selection pressures acting on free-living survival could inform how environmental stability contributes to evolutionary success of pathogenic viruses, such as avian influenza viruses transmitted between waterfowl in aquatic systems via the fecal-oral route and hepatitis C viruses transmitted via needles shared by injection drug users. Such knowledge could help in developing public health measures and pharmacological interventions to exploit the fragility of disease viruses when they exist in extracellular environments.

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