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ROLE OF EVOLVED HOST BREADTH IN THE INITIAL EMERGENCE OF AN RNA VIRUS

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Understanding how evolution promotes pathogen emergence would aid disease management, and prediction of future host shifts. Increased pathogen infectiousness of different hosts may occur through direct selection, or fortuitously via indirect selection. However, it is unclear which type of selection tends to produce host breadth promoting pathogen emergence. We predicted that direct selection for host breadth should foster emergence by causing higher population growth on new hosts, lower among-population variance in growth on new hosts, and lower population variance in growth across new hosts. We tested the predictions using experimentally evolved vesicular stomatitis virus populations, containing groups of host-use specialists, directly selected generalists, and indirectly selected generalists. In novel-host challenges, viruses directly selected for generalism showed relatively higher or equivalent host growth, lower among-population variance in host growth, and lower population variance in growth across hosts. Thus, two of three outcomes supported our prediction that directly selected host breadth should favor host colonization. Also, we observed that indirectly selected generalists were advantaged over specialist viruses, indicating that fortuitous changes in host breadth may also promote emergence. We discuss evolution of phenotypic plasticity versus environmental robustness in viruses, virus avoidance of extinction, and surveillance of pathogen niche breadth to predict future likelihood of emergence.

KEY WORDS: **Experimental evolution, generalist, host shift, niche expansion, specialist, vesicular stomatitis virus.**

Temporal variability of resources can determine whether a biological population is likely to evolve resource specialization versus generalization (Levins 1962, 1968; Lynch and Gabriel 1987; see reviews by Wilson and Yoshimura 1994; Kassen 2002). Specialists possess a relatively narrow resource niche, whereas generalists have a relatively broad niche. Adaptive generalization is a favored evolutionary strategy if a population faces ecological change that is complete, deterministic, and spans multiple generations. Examples might include organisms with short generation times that encounter seasonal changeover of resources, and micro-parasites that alternate between host species. Because such temporally heterogeneous environments lack spatial refuges, individuals in the population are forced either to reproduce in the environment or to perish. Thus, the classic prediction is that temporal variability causes a population to chase a "moving target," which favors

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direct selection for generalists that best tolerate all of the environments encountered (Roughgarden 1972).

The antithesis would be a population residing in a constant environment in which selection only requires the ability to survive and reproduce under a single condition. It is often assumed that these populations will tend to be dominated by niche specialists that evolve strong performance in the current environment at the expense of reduced performance under other circumstances (Levins 1968; Futuyma and Moreno 1988; Stearns 1992; Palaima 2007). Mechanistic explanations for such performance trade-offs include antagonistic pleiotropy (a negative genetic correlation between performance across environments; Levins 1968; Rausher 1984; Lynch and Gabriel 1987; Elena and Lenski 2003), and the accumulation of neutral mutations that are deleterious in alternate environments (Kawecki 1994). Regardless of the underlying mechanism, if phenotypic trade-offs are common then the genetic architecture of a generalist selected in environments A and B, should differ from that of a specialist selected in environments A or B alone. The reason is that a population selected in both A and B habitats is unlikely to fix mutations involved in antagonistic pleiotropy or mutation accumulation, because these alleles would impede the process of natural selection in the dual-environment context.

However, a population evolving in a constant environment is not necessarily destined to become dominated by specialist genotypes. Rather, increased niche breadth may also evolve in a homogeneous environment, via indirect effects of selection. Here a population may fortuitously evolve improved performance in an alternate environment due to a correlated response to selection, where traits important for fitness in the current environment also happen to improve performance under other conditions, not experienced during selection (e.g., Hoffman and Parsons 1989). Although it is well established that virtually any environmental circumstance may cause traits to evolve through correlated selection (Darwin 1859; see also Price and Langen 1992), our main point is that populations in temporally variable environments should be directly selected for niche breadth, whereas those in less-variable environments may fortuitously achieve niche breadth through indirect selection.

Whether niche breadth evolves directly or indirectly, generalists are predicted to be advantaged over specialists when environments change unexpectedly. The general idea is that evolution of generalism tends to somehow shape the genetic architectures underlying generalist traits to foster improved performance in novel environments. This prediction mostly derives from theory on direct selection for niche breadth (Lynch and Gabriel 1987), suggesting that indirect selection for generalism is less often beneficial in novel contexts. This assumed hierarchy creates the a priori prediction that selected generalists should (on average) outperform indirectly selected generalists when encountering novel environments, and specialists should be relatively disadvantaged.

IMPORTANCE OF HOST BREADTH IN PARASITE EVOLUTION

A parasite population that is challenged to infect a new host species is fundamentally an invading population, and is one specific example of the general case of a biological population attempting to exploit a novel resource (Woolhouse and Gowtage-Sequeria 2005). As is theoretically true for the general case of niche invasion, in some cases, the specificity of a parasite for its current host constrains the ability for the parasite to shift (i.e., emerge) onto another host species. These constraints may be ecological, such as the lack of environmental opportunities to transmit to alternative hosts. Or there may be evolutionary constraints, such as the inability for a parasite to use its adapted host-specific traits when attempting to infect a new host. The consequence of extreme host specificity is exemplified by variola virus that causes the human disease smallpox. Because variola virus is unknown to infect species other than humans, a global vaccine campaign was highly effective in eliminating the virus from human populations, apparently forcing the virus into extinction (Fenner 1982). By contrast, the host range of influenza virus includes several species of birds and mammals, creating the possibility that rare variants of the virus could evolve that directly transmit between birds and humans (e.g., Kawaoka et al. 1989; Suarez et al. 1992; Baigent and McCauley 2003).

An emerging pathogen is defined as a disease parasite that newly appears in a host population, or that rapidly expands its host range (Morse and Schluederberg 1990). Infectious RNA viruses have emerged to cause some of the most deadly pandemics in human history, and are regarded as major threats for future disease emergence. One well studied contributing mechanism may be the usually high error rates in RNA replication, which allow RNA virus populations to access rare mutations such as altered capsid proteins governing attachment and entry of novel cells (Taylor et al. 2001).

Ecological contributions to viral emergence are less explored, especially historical adaptive niche breadth and its potential role in promoting future host shifts. Viruses—like all biological entities—experience environmental change. The change may be anthropogenic, such as tropical deforestation and urbanization that may select for RNA viruses that evolve increased human transmission (Weaver 2006). Also, virus movement via vectors can expose viruses to novel hosts, perhaps explaining adaptation by Venezuelan equine, and chikungunya viruses to new mosquito vectors and domesticated animals that increase viral amplification (Brault et al. 2004; Weaver and Barrett 2004; Schuffenecker et al. 2006; Tsetsarkin et al. 2007). Environmental changes can also occur within the primary host, especially through immune

function (Herbeck et al. 2006; Worobey et al. 2007). The goal of the current study is to empirically determine whether evolution under a greater *extent* of environmental change causes some viruses to emerge more easily than others. Indirect support from data on emerging diseases in humans and domesticated animals suggests that prior host breadth may be a strong predictor of future emergence (Cleaveland et al. 2001; Taylor et al. 2001; Fenton and Pedersen 2005; Woolhouse and Gowtage-Sequeria 2005; Jones et al. 2008).

Although these correlative studies are highly suggestive, we lack empirical tests of the proposed relationship between evolved host breadth and emergence potential; support for this idea would inform surveillance practices, and provide greater power to predict which pathogens pose the greatest risk of future emergence in humans and other organisms (Holmes and Rambaut 2004; Moya et al. 2004; Fenton and Pedersen 2005).

STUDY SYSTEM

We sought quantitative evidence that pathogens directly selected for increased host breadth were relatively advantaged in emergence, owing to higher average growth, and lower variance in growth, when attacking novel hosts. To do so, we used a model system of RNA viruses experimentally evolved in laboratory tissue-culture environments. Vesicular stomatitis virus (VSV) is a negative-sense ssRNA *Vesiculovirus* in the family *Rhabdoviridae* that naturally infects mammals and insect hosts, and that provides a powerful model for addressing hypotheses in ecology, evolution, and biomedicine (e.g., Holland et al. 1991; Duarte et al. 1992; Clarke et al. 1994; Miralles et al. 1999; Turner and Elena 2000; Elena et al. 2001; Sanjuan et al. 2004; Sanjuan et al. 2007; Remold et al. 2008; Alto and Turner 2010). Because VSV is an arthropod-borne virus (arbovirus) naturally capable of infecting various host species (Lyles and Rupprecht 2007), it is possible to experimentally evolve VSV under different host-use regimes and to examine the consequences of this prior evolution for future performance in novel host environments (see also studies by Weaver et al. 1992; Novella et al. 1999; Greene et al. 2005; Ciota et al. 2007a,b).

Previously, we allowed experimental lineages of VSV to evolve for 100 generations (25 passages; 48 h per passage) on novel host cell environments in tissue culture (Turner and Elena 2000; see also Remold et al. 2008). An ancestral population of VSV Indiana serotype was used to found four lineages in each experimental treatment (Fig. 1). One treatment allowed viruses to evolve on human epithelial carcinoma (HeLa) cells, a second treatment allowed adaptation to Madin Darby canine kidney (MDCK) cells, and a third treatment involved alternating passages on the two hosts (alternating-host-evolved). At the end of the study, the 48-h growth of each evolved population was gauged

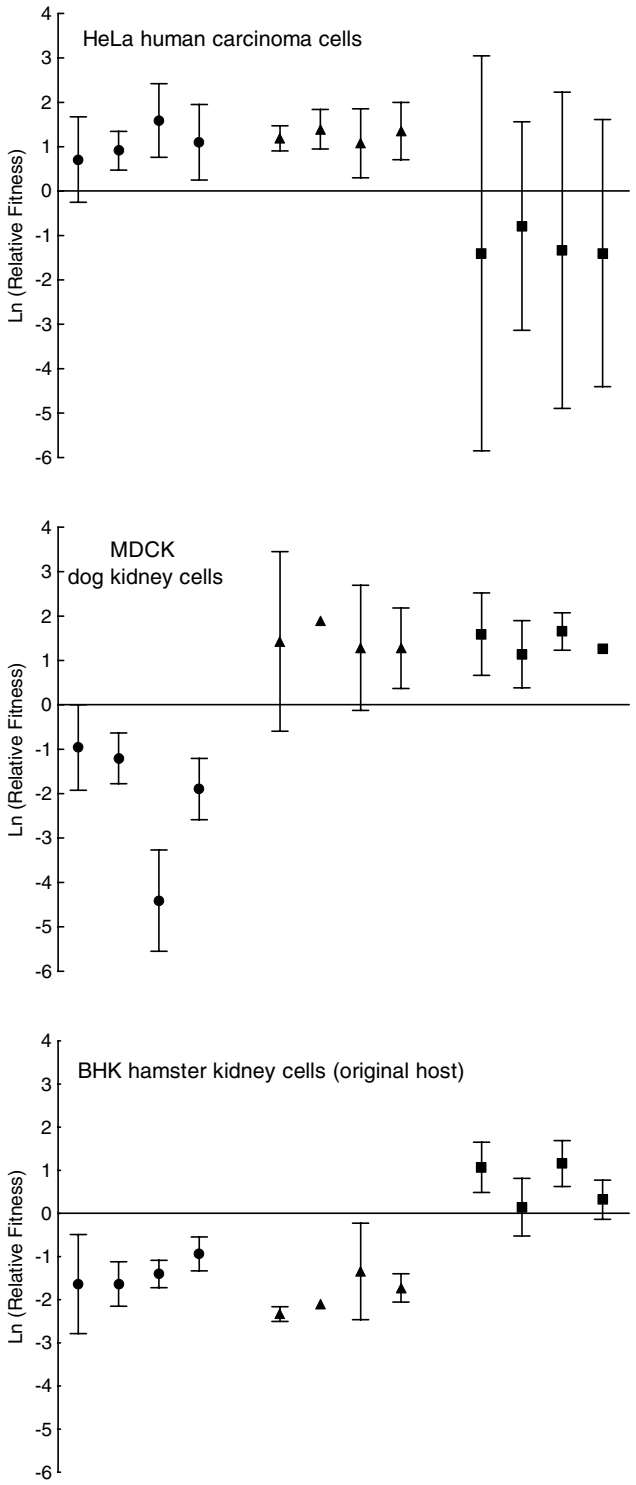

Figure 1. Fitness of VSV populations after 100 generations of experimental evolution on HeLa cells, MDCK cells, or alternating-host passages. Each point is mean log fitness (change in virus titer in pfu/mL after 48 h) measured relative to a common competitor with threefold replication on HeLa, MDCK and BHK (original host) cells. Error bars indicate 95% confidence limits. Filled circles: HeLa-evolved viruses; filled triangles: alternating-host evolved viruses; filled squares: MDCK-evolved viruses.

relative to the common ancestor on HeLa and MDCK cells, and on the original laboratory host: baby hamster kidney (BHK) cells. In virus studies, these measures are often called "relative fitness assays," but they are more accurately termed paired-growth assays that compare relative titer production of virus strains (Turner and Chao 1998).

These data revealed that the three treatments led to evolution of differing host-use strategies (Fig. 1; Turner and Elena 2000; Remold et al. 2008). First, viruses selected strictly on HeLa cells improved in growth on this cell type, at the expense of reduced performance on the other novel host MDCK, and on the ancestral BHK host. Consistent with theory, this evidence suggests these populations can be termed specialists. Second, viruses selected via alternating-host passages (a temporally heterogeneous environment) improved in growth on both selected hosts: HeLa and MDCK cells. This outcome is also theoretically predicted, because the populations became more tolerant of the environments experienced during selection. However, assays on the original host, BHK cells, revealed that alternating-host passage did not universally improve performance on all known hosts. Third, viruses selected strictly on MDCK cells improved in fitness on the selected host, but did not gain fitness on the unselected novel host, HeLa cells. Interestingly, however, the MDCK-selected lineages were the only populations to show fortuitous correlated improvement in performance on the original BHK host. We therefore defined the MDCK-adapted populations as "indirectly selected generalists." In summary, the experiment yielded three groups of virus populations that evolved different host-use strategies: specialists (HeLa-passage), directly selected generalists (alternating-host-passage), and indirectly selected generalists (MDCK-passage).

Here we compared and contrasted mean and variance in 48-h growth of the VSV populations on four cell types, which to our knowledge consisted of completely novel hosts for their common ancestor in the laboratory. In particular, we tested three interrelated hypotheses for the advantage of selected generalists in new environments, with no expectation that one of these predictions should be deemed most important.

First, we predicted that selected-generalist viruses should show higher mean growth upon immediate invasion of a novel host environment, relative to the other two virus groups. This prediction follows the assumed greater tendency for selected generalism to promote increased survival and/or reproduction under new circumstances (Janzen 1967; Levins 1968; Roughgarden 1972; Taylor et al. 2001; Vazquez and Simberloff 2002; but see Bennett and Lenski 1999).

Second, we predicted that a population of selected-generalist viruses should tend to be more consistent in performance across multiple new environments, relative to populations of the other two groups. This prediction assumes that direct selection for generalism tends to promote robust performance across new environments, due to adaptive phenotypic plasticity (phenotypic change via environmental tracking) or environmental robustness (phenotypic constancy despite environmental change). This advantage would be evidenced by lower variance in growth across novel habitats for a given selected-generalist population.

Third, owing to their assumed adaptation for constant performance, we predicted that selected-generalist viruses as a group should show lower variance in performance in a new environment, relative to the other two virus groups. This prediction relates to the idea that a group of populations may commonly experience correlated responses to selection, but this indirect selection may differ among them. For example, the magnitude of indirect selection acting on a trait will change if the correlation between directly and indirectly selected traits varies as selection proceeds. Also, the effect of epistasis on correlated characters may change as new mutations become fixed in a population (Remold and Lenski 2001; 2004; Rice 2000). Although these examples suggest that populations may diverge due to particulars of indirect selection, parallel correlated responses have been observed in bacteria (e.g., Cooper et al. 2003), and our MDCK-selected VSV populations seem to have responded similarly with respect to indirect fitness gains on BHK (Fig. 1).

In evaluating the three separate but related predictions described above, we observed that virus populations directly selected for host breadth had either equivalent or higher mean fecundity on new hosts, as well as lower variance in progeny production among populations and across novel hosts, relative to fortuitous generalists and to specialists. These results supported two of the three predictions that VSV strains historically adapted to alternating hosts should be relatively advantaged in novel-host emergence. In a separate comparison, we also observed that indirectly selected generalists emerged better than specialists, suggesting that fortuitous changes in host breadth may benefit emergence. The results suggested that determination of current niche breadth should be further investigated as a potentially useful indicator in predicting pathogen emergence.

Materials and Methods

STRAINS AND CULTURE CONDITIONS

MarmU ancestor virus is derived from Mudd Summer strain of VSV Indiana serotype and is a mouse I_1-monoclonal antibody-resistant mutant, containing an $Asp^{259} \rightarrow Ala$ substitution that alters the VSV glycoprotein (Holland et al. 1991; Turner and Elena 2000). Previously (Turner and Elena 2000) a MarmU population founded 12 lineages subjected to 25 passages (100 generations)

of in vitro evolution in 25 cm^2 plastic flasks containing $\sim 10^5$ cells/cm^2 in Dulbecco modified Eagle's minimum essential medium (DMEM), 10% heat-inactivated fetal calf serum, 37°C incubation, 95% relative humidity, and 5% CO_2 atmosphere. Four lineages (M1–M4) were evolved on Madin-Darby canine kidney cells (MDCK; European Collection of Cell Cultures #85011435), four (H1–H4) on human carcinoma cells (HeLa; American Type Culture Collection #CCL-2), and four (A1–A4) 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 four generations (Miralles et al. 1999) of viral progeny; diluted supernatant was serially transferred to a new flask containing freshly grown cells. The process was repeated 25 times per lineage. Virus population samples were stored at −80°C for later use. The consensus sequence was obtained near each endpoint (passage 24) and for the common ancestor, and molecular substitutions separating the lineages from the ancestor are described elsewhere (Remold et al. 2008).

For the current study, we obtained four mammalian cell types described as permissive for VSV Indiana serotype infection: NCTC Clone 929 (American Type Culture Collection #CCL-1) derived from mouse connective tissue; BS-C-1 (ATCC #CCL-26) derived from African green monkey kidney tissue; C6 (ATCC #CCL-107) derived from rat glioma; PK-15 (ATCC #CCL-33) derived from pig kidney tissue. Monolayers were grown in DMEM with 5% heat inactivated newborn bovine calf serum, except C6 cells that were grown in Ham's F12K medium with 82.5% horse serum and 15% heat inactivated newborn bovine calf serum. Cells were grown to 10^5 cells/cm^2 in 48-well plastic plates, 37°C incubation, 95% humidity and 5% CO_2 atmosphere.

ASSAY CONDITIONS

Monolayers were infected at MOI = 0.01, and supernatants were obtained after 48 h incubation and stored at −80°C for later use. Three replicate assays on all four novel hosts were performed for each virus population in a single temporal block. Three replicate assays on the "home" host were also performed for each population in a single temporal block; H1–H4 were amplified on HeLa, M1–M4 were amplified on MDCK, and A1–A4 were amplified on both HeLa and MDCK. Virus amplification on "home" hosts was used to standardize growth of viruses on novel hosts.

Viral titers (plaque-forming units [pfu] per mL) in each 48 h supernatant were estimated using plaque assays, in which serially diluted samples were plated on BHK cells under DMEM with 10% heat-inactivated fetal calf serum and solidified with 2% agarose, 37°C incubation, 95% relative humidity, and 5% CO_2 atmosphere. After 24 h incubation, media and agarose were removed and plates were stained with crystal violet to visualize plaques. All populations were assayed in a single block, and a second block with a lower range of dilutions was performed for H2 and H3 whose titers were too low to generate accurate estimates under the original dilution conditions. Each plaque was assumed to have originated from a single infecting virus. BHK cells are typically used to propagate VSV in our laboratory and are highly permissive for virus infection; thus, BHK cells provided a consistent and sensitive host type for gauging viral titers. To confirm that BHK cells provided unbiased titer estimates, additional assays compared enumeration of a single virus stock of each virus population on BHK versus on its selected host(s) (HeLa and/or MDCK cells).

STATISTICAL ANALYSIS

Titers and standardized titers were $\log_{10}$-transformed to improve normality. Mixed linear models assessing effects of the fixed effect evolutionary history and the random effect population nested within history on virus titer were performed (PROC MIXED, SAS Institute 2004). Because alternating-host-evolved populations differed in titer on their two home hosts, two analyses comparing alternating- and MDCK-evolved populations and alternating- and HeLa-evolved populations were performed, keeping the historical evolutionary host constant within each analysis. These analyses showed variability between evolutionary histories; populations from different histories reach unequal titers when grown on their home host. Therefore, each measure of virus titer on a novel host was standardized by dividing by the tested population's average titer on its home host. Analyses comparing HeLa- and MDCK-evolved viruses necessarily used unstandardized titers because these viruses lacked a shared evolutionary host.

Standardized or unstandardized titers of paired groups of virus populations were compared as described above, using mixed linear models in which evolutionary history, novel host identity, and their interaction were included as fixed factors and population nested within evolutionary host was included as a random factor (means effect, testing first prediction). In these models, the nested random factor was fit with and without the assumption of equal variances among levels of the grouping variable to test the second and third predictions involving variability in performance among populations and among novel hosts. These analyses required likelihood ratio tests generated using submodels, from which parameters of interest were removed from the models. One potential consequence of complex model structures in PROC MIXED is the inability for submodels to converge, preventing tests of one or more possible effects. This occurs when the iterative computer algorithms required for the restricted maximum-likelihood approach we employed, fail to converge on parameter estimates. We reported all relevant instances where submodels did not converge.

Results

Whereas our previous experiments with the evolved virus populations addressed their relative changes in growth compared to a common competitor (Turner and Elena 2000; Remold et al. 2008), the current study concerns comparisons among the virus populations in their abilities to achieve high titer (i.e., absolute fitness measured through particle production). The logic is that competition against conspecifics is perhaps less likely to occur during a host shift event compared to infection of an established host. Therefore, we used estimates of viral titers to address the main hypotheses that direct selection for host breadth promotes (1) higher average titer on novel hosts, (2) lower variance of population titer across novel hosts, and (3) less variable titer among populations on novel hosts.

GROWTH ON SELECTED (HOME) HOSTS

In theory, differences in titers detected in novel hosts could be due to two nonmutually exclusive causes. First, they could reflect differences in the potential to establish a population under novel circumstances. This is the type of difference in titer we aim to quantify. But universal differences in the growth capability of populations arising over the experimental evolution would also affect titer in the novel hosts. The latter is not specific to novel environments, and would also be detected in the populations' selected environment(s). For these reasons, we began the current study by quantifying titer of each evolved VSV population after a single passage on the host(s) experienced during experimental evolution: HeLa and/or MDCK cells (Turner and Elena 2000). Titer on a host was measured as mean titer ($\log_{10}$ plaque-forming units per mL), estimated by plaque counts visualized on the permissive host, BHK cells. Replicated ($n = 3$) assays for all 12 populations in the relevant host(s) were performed in a single experimental block, and titer was estimated at 48 h post infection. The objective was to identify any baseline differences in titer among the VSV populations when they were allowed to infect host(s) experienced during prior selection.

We performed three separate analyses contrasting titers for pairs of grouped VSV populations, using mixed linear models allowing unequal variances (PROC MIXED, SAS Institute 2004). Titers were compared at 48 h post inoculation, which was the passage duration in the experimental evolutions that generated these populations. Two of these preliminary analyses compared titers of alternating-host versus single-host adapted virus populations on the host that they had shared during their evolutionary history (either HeLa or MDCK). Both analyses showed that populations experiencing alternating-host selection had a slight but statistically significant difference in virus titer, relative to populations evolved in either of the single-host regimes (Table 1). To adjust for this difference in titer that is not novel-environment specific we therefore used titers in the selected (home) hosts to standardize titers in the novel hosts when comparing these virus groups (see below). The third analysis comparing titers of MDCK- versus HeLa-adapted virus populations necessarily prevented separating the effects of evolutionary host and assay host, because these were completely confounded (i.e., there was no shared evolutionary host). Here we found a significantly higher titer for HeLa-adapted viruses (Table 1). This result indicated that either an intrinsic difference existed between HeLa and MDCK cells as assay hosts, or that HeLa versus MDCK cells led to repeatable differences in their effects on virus selection, on average causing HeLa and MDCK evolved populations to differ in yield on their "native" hosts. Comparisons between these two groups (see below) necessarily used unstandardized titers, but any mean titer differences detected between MDCK- and HeLa-adapted virus populations must be interpreted in the context of these results.

Table 1. Results from mixed general linear models testing the effect of evolutionary history on VSV $\log_{10}$ titer (pfu/mL), when viruses are grown for 48 h on the host type used in selection. Separate comparisons are made between HeLa-evolved viruses versus alternating-host-evolved viruses, MDCK-evolved viruses versus alternating host-evolved viruses, and HeLa-evolved viruses versus MDCK-evolved viruses.

Source	df[1]	Test statistic[2]
HeLa-adapted versus alternating-host adapted[3]		
History	1, 6	72.0^{***}
Population (history) [*means*]	1	0.8^{NS}
Population (history) [*variances*]	1	0.0^{NS}
MDCK adapted versus alternating-host adapted		
History	1, 6	27.6^{**}
Population (history) [*means*]	1	1.1^{NS}
Population (history) [*variances*]	1	0.2^{NS}
HeLa adapted versus MDCK adapted		
History	1, 4.8	52.0^{***}
Population (history) [*means*]	1	1.0^{NS}
Population (history) [*variances*]	1	0.2^{NS}

[1]df indicates degrees of freedom, denominator df for *F* test is estimated using the Satterthwaite approximation. df for likelihood ratio (LR) tests are equal to the difference in the number of parameters in the full and reduced models.

[2]The fixed effect is tested with an approximate *F* test. Random effects are tested using likelihood ratio LR tests; the LR test statistic is −2×(maximum likelihood of the test's full model − maximum likelihood of the restricted model, from which the variance component being tested has been removed), and is distributed approximately chi-squared. In the tests of variance effects, variances are constrained to be equal in the reduced model.

[3]All models accommodate differences in within-population variances between histories.

$^{NS}P > 0.1$; $^{*}0.01 < P < 0.05$; $^{**}0.001 < P < 0.01$; $^{***}P < 0.001$.

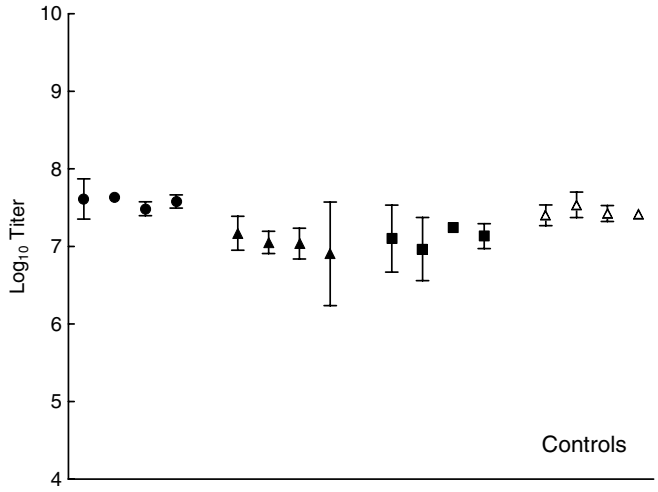

Figure 2. Growth of VSV populations on the host(s) experienced during their prior experimental evolution. Each point is the raw mean of $\log_{10}$ virus titer (in pfu/mL) after 48 h estimated with threefold replication; error bars indicate 95% confidence limits. Filled circles: HeLa-evolved viruses on HeLa; filled triangles: alternating-host evolved viruses on HeLa; filled squares: MDCK-evolved viruses on MDCK; open triangles: alternating-host evolved viruses on MDCK.

In contrast, we detected no differences in among-population variance in titer resulting from prior host adaptation (Fig. 2, Table 1). This result indicates that in comparisons between selective treatments any differences in variance detected in a population's titers across multiple novel-hosts (prediction ii) or among populations experiencing a shared or nonshared new host (prediction iii) are specific to the context of a new environment and do not reflect general differences in titer.

Last, we previously suggested that enumeration of the virus populations on BHK cells did not differ from titers assessed on their selected hosts (HeLa, and/or MDCK) (Alto and Turner 2010). That is, despite performance (relative 48-h growth) differences on BHK (Fig. 1), titer estimates of plaque-forming units per milliliter were equivalent on BHK versus selected hosts. To confirm this outcome, we grew each population on its home host(s) for 48 h with replication ($n = 3$), and then enumerated the resulting progeny on the home host(s) and on BHK. We found that the grand mean titer of HeLa-evolved populations was very similar whether enumerated on HeLa cells (7.08 $\log_{10}$ pfu, 0.07 SD) versus on BHK cells (7.07 $\log_{10}$ pfu, 0.08 SD). An analogous outcome was found for the MDCK-evolved populations (MDCK: 6.96 $\log_{10}$ pfu, 0.30 SD; BHK: 7.09 $\log_{10}$ pfu, 0.09 SD), and for the Alternating-host-evolved populations (HeLa: 7.05 $\log_{10}$ pfu, 0.06 SD; MDCK: 7.05 $\log_{10}$ pfu, 0.07 SD; BHK: 7.10 $\log_{10}$ pfu, 0.07 SD). Moreover, we conducted a series of *t*-tests in which the replicate titers for each population were compared across assay hosts. In all 16 tests, we found no significant effect of assay host on titer estimate ($P > 0.1$). For these reasons, BHK cells were used throughout the current study to measure viral titers produced on the novel hosts.

PERFORMANCE ON NOVEL HOSTS

We allowed the 12 evolved virus populations to infect four novel cell types derived from distinct mammalian species (monkey, mouse, pig, rat) and including tissue types different from those used in the prior experimental evolution. These assays were performed with threefold replication and involved estimates of virus titer at 48 h. All 144 virus titer samples (12 test viruses × four host types × threefold replication) were generated in a single experimental block, and samples were stored as cell-free virus supernatants at −80°C for later analysis. Where possible, to avoid biasing our analysis in favor of virus populations inherently advantaged in particle production (Fig. 2), we standardized each estimate of $\log_{10}$ titer on a novel host by dividing it by the mean $\log_{10}$ titer of the relevant population measured on its selected host (see above). Thus, we could standardize titers on HeLa when comparing alternating-host adapted viruses with HeLa-evolved populations (specialists), and similarly standardized on MDCK in the comparison with MDCK-evolved populations (indirectly selected generalists). However, the comparison between HeLa- and MDCK-adapted populations necessarily used unstandardized titers because these two groups lacked a shared selected host.

Results are shown in Figure 3. As above, we separately compared the pairs of grouped VSV populations, using mixed linear models allowing unequal variances (PROC MIXED, SAS Institute 2004) to address our three predictions. In comparison with the HeLa-evolved specialists, analyses showed that mean titers on novel hosts were significantly higher for directly selected generalist populations that evolved on alternating hosts (History effect, Table 2; prediction i). In addition, the virus populations evolved on alternating hosts had significantly lower among-population variance in titer in the novel hosts, compared to the HeLa-selected populations (Population (history) [variances] effect, Table 2; prediction iii), indicating that populations sharing the alternating-host history are more similar in their responses to novel environments. Finally, we tested whether populations from alternating-host populations were less variable in their titers on different novel hosts (Novel host × population (history) [variances], Table 2; prediction ii). This significant interaction indicates that populations selected on alternating hosts have a narrower range of titers across novel hosts than do the HeLa-adapted populations: in other words, they are more consistently robust with respect to titer. The model also accommodates other variability involving differences among the four novel hosts (Novel host × history effects, Table 2), but because these hosts were chosen merely to provide a range of challenge environments, these effects will not be further discussed.

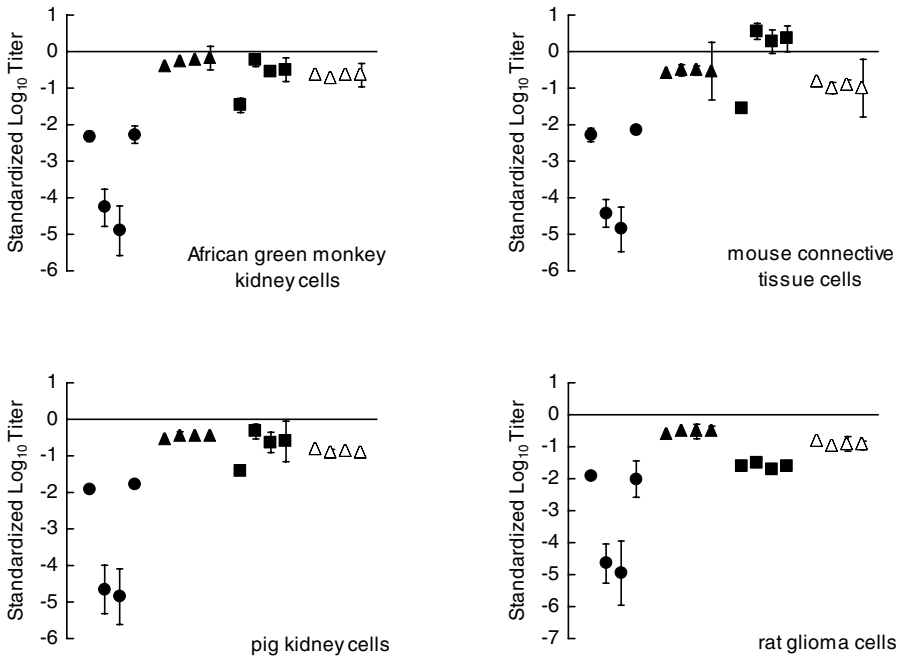

Figure 3. Growth of each evolved VSV population on cells derived from four different novel hosts. Each point is the mean of $\log_{10}$ virus titer (titer in pfu/mL) after 48 h estimated with threefold replication; error bars indicate 95% confidence limits. Growth estimates are standardized by each population's mean titer on its evolved host at 48 h post infection. Standardized virus growth equal to zero indicates the virus grows equally well on the novel host as on its evolved host. Filled circles: HeLa-evolved viruses; filled triangles: alternating-host evolved viruses amplified on HeLa; filled squares: MDCK-evolved viruses; open triangles: alternating-host evolved viruses amplified on MDCK.

An analysis using unstandardized titers yielded similar conclusions for tests of the three predictions. The test of prediction (2) remained significant ($P < 0.001$ became $P < 0.05$), and that of prediction (1) became marginally significant ($P < 0.05$ became $P = 0.052$). The test of prediction (3) could not be performed due to failure of model convergence (see Methods). The results from allowed tests suggested that titer standardization had no substantive effect.

The second comparison was between the alternating-host adapted viruses and the MDCK-selected populations that achieved generalism through indirect selection. Here, we found no differences in overall mean titer on the novel hosts (prediction i), but consistent with the comparison involving HeLa-selected populations, we found a significantly lower among-population variance for the alternating-host selected populations overall, across novel hosts, and a significantly smaller range of titers for these populations relative to the MDCK-adapted ones on individual novel hosts (Table 2, Fig. 2; predictions iii and ii, respectively). An analysis using unstandardized titers yielded similar conclusions for tests of prediction (1) (not significant) and prediction (3) ($P < 0.05$ became $P < 0.001$); but a test of prediction (2) was not performed because the required submodel did not converge (see Methods). These results showed that titer standardization did not affect the analysis, in allowable tests of relevant effects.

The third comparison was between the HeLa- and MDCK-adapted viruses, and used only unstandardized titers. We found that the MDCK-adapted viruses had a marginally significant higher mean titer on the novel hosts (prediction i), a significantly lower among-population variance across novel hosts (prediction iii), and a significantly smaller range of titers on individual novel hosts (prediction ii) (Table 2, Fig. 2). We stated above that the observed titer differences for these viruses on their selected hosts could not be attributed to effects of evolutionary host versus assay host, because the viruses were evolved on different hosts. Consequently, we noted that when comparing unstandardized mean titers of the two virus groups on a shared emergence host, we could not definitively conclude whether the overall difference in mean titer was attributable to evolutionary history and independent of a response to the current test environment. However, the variance comparisons were conclusive, because the associated control comparison showed no differences in variance between the two virus groups (Table 1); that is, variance differences detected on the emergence hosts were due to this new factor in the analysis, rather than due to intrinsic differences between the viruses that would be observable regardless of the test environment.

Last, we examined whether growth of viruses on their evolved host(s) was a strong predictor of performance on an emergence host. To do so, we conducted a simple linear regression with

Table 2. Results of mixed general linear models testing how 48-h VSV $\log_{10}$ yield (titer in pfu/mL) on four novel hosts is affected by evolutionary history, population within history, novel host type, and their interactions. Separate comparisons are made between HeLa-evolved viruses versus alternating-host-evolved viruses, MDCK-evolved viruses versus alternating host-evolved viruses, and HeLa-evolved viruses versus MDCK-evolved viruses. In the first two comparisons, population titers on novel hosts are standardized relative to a population's titer on its evolved host.

Source	df[1]	Test Statistic[2]
HeLa adapted versus alternating-host adapted[3]		
History	1, 3.05	14.8*
Novel host	1	23.4***
Population (history) [*means*]	1	129.6***
Population (history) [*variances*]	1	15.0***
Novel host×history [*means*]	1	8.8**
Novel host×history [*variances*]	1	2.1^{NS}
Novel host×population (history) [*means*]	1	4.1*
Novel host×population (history) [*variances*]	1	5.0*
MDCK adapted versus alternating-host adapted		
History	1, 5.12	0.0^{NS}
Novel host	1	31.6***
Population (history) [*means*]	1	68.9***
Population (history) [*variances*]	1	8.6**
Novel host×history [*means*]	1	62.5***
Novel host×history [*variances*]	1	05.6**
Novel host×population (history) [*means*]	1	92.7***
Novel host×population (history) [*variances*]	1	35.5***
HeLa adapted versus MDCK adapted		
History	1, 4.52	6.2^{+}
Novel host	1	22.8***
Population (history) [*means*]	1	200.2***
Population (history) [*variances*]	1	3.4*
Novel host×history [*means*]	1	49.6***
Novel host×history [*variances*]	1	9.6***
Novel host×population (history) [*means*]	1	53.0***
Novel host×population (history) [*variances*]	1	4.2*

[1]df indicates degrees of freedom, denominator df for *F* test is estimated using the Satterthwaite approximation. df for likelihood ratio (LR) tests are equal to the difference in the number of parameters in the full and reduced models.

[2]The fixed effect is tested with an approximate *F* test. Random effects are tested using likelihood ratio LR tests; the LR test statistic is −2×(maximum likelihood of the test's full model − maximum likelihood of the restricted model, from which the variance component being tested has been removed), and is distributed approximately chi-squared. In the tests of variance effects, variances are constrained to be equal in the reduced model.

[3]All models accommodate differences in within-population variances between histories.

$^{NS}P>0.1$; $^{+}0.1<P<0.05$; $^{*}0.01<P<0.05$; $^{**}0.001<P<0.01$; $^{***}P<0.001$.

repeated measures on populations, looking at the relationship between $\log_{10}$ mean titer in the controls ("home" host) and $\log_{10}$ mean titer on each novel host. We found highly significant relationships between $\log_{10}$ mean titers on home versus novel hosts in all cases: rat host, $P = 0.0067$; pig host, $P = 0.0003$; mouse host, $P < 0.0001$; monkey host, $P < 0.0001$. These results indicated that growth performance on the evolutionary host was strongly correlated with growth performance on the novel host. Also, we conducted a similar set of analyses, which included the covariate "evolutionary history" (virus group). The results showed that on all four novel hosts, after controlling for variability in titer on the host used in selection, there was no significant relationship between virus titer in the selected environment and that in the novel host. This outcome supported our above argument that titer standardization had no substantive effect in our analyses.

Discussion

We examined whether evolved host breadth improves the future ability of an RNA virus to emerge on unencountered hosts, and whether the type of selection for host breadth—direct versus indirect—altered this outcome. In particular, we tested three hypotheses that predicted RNA viruses directly selected for host use were relatively favored to emerge due to (1) higher mean growth on unencountered hosts, (2) lower variance (greater consistency) in mean growth of a population across novel hosts, and (3) lower variance (more consistent growth response) in new environments.

We observed that these predictions were mostly upheld in comparative analyses using a collection of VSV populations that had experienced differing host-selection regimes in vitro (Turner and Elena 2000; see also Remold et al. 2008). Direct selection for host breadth facilitated initial emergence in response to environmental change, in that the viruses selected on alternating hosts had significantly lower among-population variance in growth (titer) on new hosts (prediction iii), as well as lower population variance in titer across novel host environments (prediction ii). In addition, titers from these directly selected viruses were higher than the specialist HeLa-selected populations, consistent with prediction (1). However, viruses selected on alternating hosts did not have higher titers than viruses selected on MDCK hosts, which were considered indirectly selected (fortuitous) generalists. Rather, growth of these two types of viruses was statistically indistinguishable. Thus, five of the six major comparisons supported the predicted early emergence advantage of viruses directly selected for host breadth, and the sixth comparison showed that viruses adapted to alternating host environments were not disadvantaged in relative emergence potential. An additional analysis compared emergence potential of indirectly selected generalists versus specialist viruses. Here we observed that fortuitous changes in evolved host breadth may also produce substantial advantages for viruses when

they attempt to emerge on previously unencountered hosts. We are unaware of any theory that explicitly relates to this role of correlated responses to selection in promoting emergence, suggesting avenues for future exploration. Below we further discuss the implications of direct versus indirect selection for host breadth, on emergence potential.

Overall, these results suggest that knowledge of historical pathogen adaptation may be informative when predicting the future likelihood for a pathogen to emerge on novel hosts. To our knowledge, the current study represents the most rigorous empirical test of the predicted relationship between evolved host breadth and future host-shift potential of pathogens such as RNA viruses (Taylor et al. 2001; Woolhouse and Gowtage-Sequeria 2005). A recent study by Vasilakis et al. (2009) found that dengue viruses evolved in vitro on alternating hosts (Huh-7 human cells, and C6/36 mosquito cells) were sometimes advantaged relative to single-host-evolved viruses when growing on a novel host: Vero monkey cells. However, it is difficult to compare the two studies; unlike our experiment, the goal of the Vasilakis et al. (2009) study was not to harness a variety of hosts to test for a general advantage of evolved host breadth on RNA virus emergence.

With a single exception, all known arboviruses are RNA viruses. The error-prone nature of RNA replication (due to lack of proofreading) affords RNA viruses a large degree of genetic flexibility to accommodate a life style that involves obligate alteration between disparate hosts (invertebrates, vertebrates). In turn, this host alternation may often provide the temporal environmental variability we predicted should promote emergence potential, further suggesting that arboviruses may be particularly prone to emerge relative to other parasites. This idea is speculative and warrants further investigation, because support for the idea in a single study system (VSV) does not necessarily mean that supportive evidence would be found in other arboviruses. Furthermore, we acknowledge that our data came from in vitro experiments using only one collection of evolved viruses. We hope that other researchers will be motivated by our study to examine analogous hypotheses in a variety of virus systems, ideally harnessing both in vivo and in vitro approaches.

CONSEQUENCES OF DIRECT VERSUS INDIRECT SELECTION FOR HOST BREADTH

In general, it is expected that differences in environmental specificity (i.e., degree of resource specialization) observed among natural populations reflect their differing historical patterns of selection according to resource availability (Kassen 2002). One mechanism proposed to drive this phenomenon is that temporal heterogeneity of resources should select for greater flexibility in the types of resources used by populations (Roughgarden 1972). Lynch and Gabriel (1987) showed mathematically that a population evolving in a temporally heterogeneous environment should experience strong selection to tolerate a broad niche, especially if the time spent in any one environment exceeds generation time. Our VSV populations selected on alternating hosts seem to provide an example of this phenomenon (Fig. 1; Turner and Elena 2000; see also Remold et al. 2008).

In contrast, it is less straightforward to predict evolved changes in niche tolerance for a population evolving in a constant environment. Although the population will not experience direct selection for increased niche breadth, broader niche tolerance relative to the ancestor is an evolutionary possibility. The likelihood of this outcome depends on the degree to which performance in different environments is genetically correlated with adaptation in the selected environment, as well as the rate of accumulation of conditionally deleterious mutations that are neutral in the selected environment. Thus, a pathogen population evolving in a homogenous host environment may improve in performance in one or more unselected host habitats, if it experiences a correlated response to selection that is not hindered by fixation of conditionally deleterious alleles.

Our prior work demonstrated that all four VSV populations selected solely on MDCK cells experienced a correlated fitness improvement on the original host BHK cells, whereas all populations evolved strictly on HeLa cells or on both hosts did not (Fig. 1; Turner and Elena 2000). Thus, it is evident that pure selection on MDCK cells results in a higher probability that a VSV population coincidentally improves in growth on an unselected host, than does pure selection on HeLa cells. Of course, pure selection on MDCK cells is not universally capable of leading to fortuitous correlated selection, otherwise we should have observed that these populations improved on HeLa cells in the original study, and this was not the case (Fig. 1; Turner and Elena 2000). Last, we note that partial selection on MDCK cells also did not lead to universal improvement on other cell types, because the alternating-host generalists suffered fitness losses on the original BHK host (Fig. 1; Turner and Elena 2000).

Other researchers have similarly observed that RNA viruses consistently evolve expanded niche breadth under temporally fluctuating host-selection conditions, but that variable changes in niche breadth can evolve when populations are selected in homogeneous single-host environments (e.g., Novella et al. 1999; Cooper and Scott 2001). We suggest that the type of prior selection (direct vs. indirect) for host breadth experienced by our evolved VSV populations accounts for the observed patterns in emergence potential seen in the current study.

The evolved niche breadth we observed in our VSV populations may have arisen through increased environmental robustness, or via phenotypic plasticity. Robustness is favored by selection when the optimum phenotype remains the same under environmental variation, whereas plasticity is favored when the phenotype must change according to the environment (de Visser

et al. 2003). Thus, phenotypic plasticity may be considered a subset of robustness, and should be invoked only if phenotypic constancy is achieved through environmental tracking. Either or both mechanisms may account for the evolved niche breadth seen in our virus collection, and determination of the responsible mechanism(s) is an intriguing possibility for future research. One method would be to examine components of growth across the VSV life cycle (e.g., attachment rate, particles produced per infected cell, extracellular survival of virus particles), and gauge whether some viruses achieve niche breadth by holding these characters constant (environmental robustness) versus altering them in response to the particular host type (phenotypic plasticity).

We note that we could have chosen for our study any four (or more) hosts described by the literature as permissive for infection by VSV Indiana serotype. Using four such hosts chosen at random, all three of our predictions were strongly supported in the case of viruses directly selected for host breadth when compared to HeLa-selected populations, consistent with the idea that historical selection for niche breadth predicts future emergence potential. Although the viruses historically evolved on alternating hosts did not differ in mean titer on new hosts compared to MDCK-selected lineages, as predicted they differed less among one another in their ability to grow in novel contexts, and they were more consistent in titers across the novel hosts. Furthermore, fortuitously evolved host breadth led to an advantage over specialist viruses in all three tests of emergence potential, an outcome not predicted by current theory. Overall, these results suggest that a detailed understanding of the current niche breadth of a pathogen can help to accurately predict its potential for host emergence. In particular, identifying the most recent pattern of niche use selection (i.e., temporally variable vs. temporally constant) can help distinguish among niche-tolerant pathogens in terms of their capacity to further expand the host niche.

POTENTIAL ROLE OF MOLECULAR CHANGES SEPARATING VIRUS TYPES

Similar emergence potential as measured by mean virus titer between groups of VSV populations evolved on alternating hosts versus MDCK cells may reflect the observed similarities in genetic substitutions separating these viruses from their common ancestor, as described previously (Remold et al. 2008). The ~11 kb VSV genome contains five genes that code for proteins of known function (Wagner and Rose 1996): the nucleoprotein (N) that encapsidates viral genomic RNA; the phosphoprotein (P); the matrix protein (M), with both structural and nonstructural functions; the glycoprotein (G) that protrudes through the envelope of mature virions; and the large polymerase protein (L) that functions in conjunction with the P protein. We have shown that whereas the HeLa-evolved populations are phenotypically and genetically distinct from the other two sets of populations, the MDCK-evolved and alternating-host evolved viruses are quite similar genotypically despite their differing fitness on HeLa cells. This observation suggests that the partial overlap in emergence capability of these viruses may be explained by one or more of the shared nucleotide substitutions separating the MDCK-evolved and alternating-host evolved genomes from that of the common ancestor, and distinct from changes that occurred in the HeLa-selected populations (Remold et al. 2008). Notably, these shared substitutions occurred in all five genes constituting the VSV genome.

For sake of brevity, we discuss the potential importance of molecular substitutions that occurred in the G (glycoprotein) gene. The VSV G protein is important in cell attachment and entry, and contributes to the general ability of VSV to infect cells of differing host origin (Coll 1995). This existing capacity for the wild-type G protein to foster infection of various cell types perhaps suggests that evolved modifications to the G protein are unlikely to explain the emergence differences described in the current study. Nevertheless, we observed that there are four nucleotide substitutions in the G gene occurring in one or more alternating-host adapted and MDCK-selected populations, and these evolved alleles were entirely absent from all four HeLa-selected populations, suggesting a possible host-specific benefit (Remold et al. 2008). Therefore, one possibility is that similar changes in the G protein allowed the alternating-host and MDCK-selected viruses to improve in virus/cell interactions (through direct and indirect selection for niche tolerance, respectively). In turn, a pleiotropic benefit experienced through these changes to the G protein might explain the success of these viruses when attempting to emerge on novel hosts. The existence of shared new alleles separating the alternating-host and MDCK-selected viruses from their HeLa-adapted counterparts at the other four genes underscores the importance of further investigation to determine the molecular mechanisms for the current results.

LINKING EMERGENCE PROBABILITY TO PATHOGEN EVOLVABILITY

All three of the predictions we explored highlight how evolved niche breadth may proximately foster superior performance across new environments. It is suggested that ultimately such differences in performance may translate to differing risks of population extinction, assuming that greater current niche breadth positively correlates with evolvability in novel environments (Simpson 1944; Moran 1988). Thus, although in the present study we focus on the effects of ecological history on the immediate probability of successful virus replication within and among new hosts, past adaptation may influence success in novel hosts via effects on evolvability as well. This effect of strong initial emergence potential on evolvability could occur as follows (Antia et al. 2003; Andre and Day 2005). In a novel host, pathogens may produce sufficient progeny to establish an infection, but the size of the

pathogen population will generally be low. Genotypes with greater emergence potential will result in larger initial population sizes, and will therefore experience critical rare beneficial mutations earlier in the course of the new infection. This temporal advantage is crucial for adaptation to the novel host, where the pathogen population faces collapse due to challenges such as transmission bottlenecks, evolved host resistance, and the immune response. Early fixation of beneficial mutation(s) strengthens the population's replication advantage, and can result in a successful shift in host range. Thus, as a result of their high initial population sizes in novel hosts, viruses with an environmental robustness advantage, such as the alternating-host adapted populations described here, may have greater evolvability with respect to successful completion of a host shift. It is unknown whether the differences in mean and variance of virus titers observed in our study would truly lead to evolvability differences among our virus populations on new hosts. This idea merits further examination using mathematical modeling or experimental evolution *in vitro*.

The ability of a population to undergo adaptation in a new environment (evolvability) is clearly related to its ability to avoid extinction. Are viruses with relatively broad host ranges better able to avoid extinction? This is a provocative question, but it is difficult to test through historical methods applied in other biological systems. Jablonski (1986) used fossil evidence to conclude that increased geographic range (a proxy for niche breadth) allowed bivalve and gastropod species to experience reduced probability of extinction, thereby showing that greater habitat-colonizing ability seems to reduce the frequency of extinction in these marine invertebrates. Unfortunately it is difficult to determine whether pathogens such as RNA viruses share this relationship between geographic (or host) range and probability of extinction, because a direct test of this hypothesis using fossil evidence is not possible.

However, the following indirect method could be used to evaluate this intriguing idea. Methods of whole-genome sequencing are becoming increasingly affordable, and are improving in efficiency. As a result, it is now apparent that a variety of organisms carry genes that reflect past infection events by viruses. For example, data from the human-genome project revealed that a substantial fraction of our genetic material is virus-derived (e.g., endogenous retroviruses) (International Human Genome Sequencing Consortium 2001). Another recent study showed that associations between retroviruses and their sloth hosts were ancient owing to their congruent phylogenies spanning a geological era (Katzourakis et al. 2009). By using similar data from multiple potential host species, it may be possible to test whether extant viruses characterized as having broader current host range have been resident in the genomes of their hosts for longer times than viruses with narrow current host ranges. Such evidence may suggest that families of viruses with broad host ranges are more evolutionarily ancient, and may have benefited from a greater ability to avoid extinction.

CONCLUDING REMARKS

Our results are relevant for the pressing need to better foresee disease emergence. We are a long way from achieving accurate predictions, but evolutionary genetics studies of viruses provide some basic rules (Moya et al. 2004), and analyses of global trends in emerging infectious diseases highlight ecological factors that may promote emergence (Cleaveland et al. 2001; Woolhouse and Gowtage-Sequeria 2005; Jones et al. 2008). Our study successfully bridged these two approaches, and harnessed the power of laboratory experimental evolution to provide empirical evidence for a basic rule of emergence suggested in global disease trends. In particular, we showed that explicit knowledge of the ecological history of host use by a pathogen informed the prediction that direct selection for host breadth fosters emergence on completely novel hosts. Although precise mechanisms responsible for emerging disease are likely to be case specific, our empirical study is a starting point to identify generalities that are useful in building a predictive framework to aid infectious disease management and epidemiology (Pulliam 2008).

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