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Murine norovirus (MNV-1) exposure *in vitro* to the purine nucleoside analog Ribavirin increases quasispecies diversity.

For Publication as an Original Research Article in Virus Research

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Title Running Head: Ribavirin impacts *in vitro* MNV-1 quasispecies diversity

HIGHLIGHTS:

- Exposure to Ribavirin statistically significantly increases Mouse Norovirus (MNV-1) quasispecies diversity
- Ribavirin-induced nucleotide substitution rates are estimated using time series of site-dependent nucleoside-composition vectors obtained from deep sequencing data
- Observed mutations in MNV-1 due to Ribavirin treatment cannot be explained by Ribavirin's activity as purine nucleoside analog alone.

KEYWORDS: norovirus, MNV-1, quasispecies, Ribavirin, serial passaging

0. ABSTRACT

Ribavirin is a pharmaceutical antiviral used for the treatment of RNA virus infections including norovirus, hepatitis C virus, hepatitis E virus, Lassa virus, respiratory syncytial virus, and rhinovirus. Despite the drug's history and documented efficacy, the antiviral mechanism of Ribavirin remains unclear.

Mechanisms proposed include depletion of the intracellular GTP pool, immunomodulatory effects, induction of error catastrophe, inhibition of viral polymerase activity, and/or inhibition of viral capping. In the present study, we leveraged deep sequencing data to demonstrate that Ribavirin increases murine norovirus (MNV-1) viral diversity. By serial passaging MNV-1 in RAW 264.7 cells for twenty generations in the presence of Ribavirin, we demonstrated statistically significant increases in both the number of unique haplotypes and the average pairwise difference (APD). Based on statistically significant differences in the probability of nucleotide mutations based on Roche 454 sequencing, we also demonstrated that single nucleotide substitutions are increased in the presence of Ribavirin. Finally, we demonstrated Ribavirin's impact on statistically significantly reducing the relative proportion of the dominant sequence within the quasispecies.

1. INTRODUCTION

Ribavirin is a pharmaceutical antiviral used for the reduction of RNA virus titers in both cell culture (*in vitro*) and animals (*in vivo*) (e.g., (Chang and George, 2007; Hall et al., 1983; Hruska et al., 1980; Li et al., 2008)). Ribavirin has been used in humans to treat infections for norovirus, hepatitis C virus, hepatitis E virus, Lassa virus, respiratory syncytial virus, and rhinovirus (Gane et al., 2013; Gerolami et al., 2011; Hall et al., 1983; McCormick et al., 1986; Ruuskanen et al., 2014; Woodward et al., 2012). Despite the drug's history and documented efficacy, the antiviral mechanism of Ribavirin remains unclear (Graci and Cameron, 2002). Mechanisms that have been proposed include depletion of the intracellular GTP pool, immunomodulatory effects, induction of error catastrophe, inhibition of viral polymerase activity, and/or inhibition of viral capping (Graci and Cameron, 2006). Results from studies seeking to identify the specific mechanism are mixed. GTP pool depletion, for example, was suggested as the primary mechanism for reduction of infection with flavivirus, paramyxovirus, and calicivirus, but not for Lassa virus (Chang and George, 2007; Leyssen et al., 2005; Ölschläger et al., 2011). These findings suggest that either the mechanism is specific to the target virus or more than one mechanism is responsible for antiviral efficacy (Graci and Cameron, 2002; Graci and Cameron, 2006).

The mechanism of error catastrophe has been suggested based on Ribavirin's role as a purine nucleoside analog (Crotty et al., 2001; Crotty et al., 2000; Graci and Cameron, 2006). Purposeful induction of error catastrophe is a promising antiviral strategy for the treatment of viral infections where the etiological agent is characterized by a high intrinsic mutation rate (which provides a mechanism for evading immunological defenses) (Anderson et al., 2004; Domingo, 2005; Graci et al., 2008; Harris et al., 2005). Error catastrophe theory suggests that the viral population, referred to as a quasispecies, can be pushed into extinction by increasing the mutation rate such that viral progeny are no longer capable of carrying out necessary functions (e.g., host cell invasion, RNA transcription) (Domingo and Holland, 1997). The theory relies on the finding that the high intrinsic mutation rates of retroviruses and riboviruses are close to the error threshold and population extinction can be induced by increasing the relative abundance of defective virus within the quasispecies (Anderson et al., 2004; Grande-Pérez et al., 2005). For example, two or more mutations per genome (compared to the normal 1.5 mutations) were sufficient to significantly decrease poliovirus infectivity (Crotty et al., 2001; Graci and Cameron, 2002). Many other studies have similarly demonstrated reduced viral fitness after introduction of (Loeb et al., 1999; Ortega-Prieto et al., 2013; Ruiz-Jarabo et al., 2003; Sierra et al., 2000).

The perceived mechanism of Ribavirin-induced error catastrophe is based on studies of Ribavirin incorporation into RNA in place of either adenosine or guanosine (Anderson et al., 2004; Crotty et al., 2000). Experimental studies suggest less than three molecules of Ribavirin are incorporated per viral genome per replication cycle (though this is dependent on intracellular RTP concentrations)(Graci and Cameron, 2002). Subsequent RNA-dependent replication may introduce random single nucleotide mutations, with Ribavirin pairing with either cytosine or uridine. Studies have proposed that Ribavirin's activity of inducing increased G-to-A and C-to-U transition mutations induces error catastrophe sufficiently to reduce viral titers based on studies comparing mutation frequency with viral titer (e.g., (Anderson et al., 2004; Contreras et al., 2002; Crotty et al., 2001; Crotty et al., 2000; Hofmann et al., 2007)

Interestingly, the conceptual model of Ribavirin misincorporation does not explain empirically observed mutations. The conceptual model suggests that Ribavirin triphosphate pairs with cytosine or uridine, causing misincorporation in place of guanosine or adenosine (Crotty et al., 2000; Vignuzzi et al., 2005). Subsequent pairing of Ribavirin with cytosine or uridine would result in C-to-U or U-to-C mutations. Reverse complement mutations of G-to-A and A-to-G should also be observed. However, studies of sequence data evaluating the impact of virus exposure to Ribavirin consistently demonstrate statistically significant increases in C-to-U and G-to-A transitions but not U-to-C or A-to-G mutations (Beaucourt and Vignuzzi, 2014; Crotty et al., 2000; Jin et al., 2013).

Here, we employed murine norovirus (MNV-1) infection of RAW 267.4 cells as a model for assessing the effects of Ribavirin on norovirus (NoV) infection. MNV-1 is a positive-sense RNA virus with an icosahedral capsid that is 28-35 nm in diameter and is commonly used as a surrogate for human norovirus due to *in vitro* culturability (Bae and Schwab, 2008). Human noroviruses are the leading cause of epidemic gastroenteritis globally (Patel et al., 2008), and there is currently no effective vaccine or treatment. Furthermore, NoV mutation rate is high, estimated at 10^{-5} – 10^{-3} substitutions per nucleotide site (Bull et al., 2010). The mutation rate of NoV is likely near the error threshold; increasing the mutation rate can lead to lethal mutagenesis (Arias et al., 2014; Crotty and Andino, 2002). Therefore, Ribavirin and similar nucleoside analogs capable of inducing lethal mutagenesis are increasingly investigated as treatment options for norovirus infections (Arias et al., 2014; Woodward et al., 2015).

In the present study, we investigated the impacts of Ribavirin on MNV-1 quasispecies diversity. Using data obtained from deep sequencing, we determined whether or not Ribavirin influences quasispecies diversity and identified the single nucleotide substitutions most responsible for the increase in diversity. In order to estimate the effect of Ribavirin on single nucleotide substitutions, a novel algorithm was developed, based on a time series of site-dependent nucleoside composition vectors obtained from deep sequencing. These data demonstrate that Ribavirin increased quasispecies diversity primarily through C to U and G to A single nucleotide substitutions.

2. MATERIALS AND METHODS

2.1. Cells, Viruses, and Reagents. Murine macrophage-like RAW 267.4 cells were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum (FBS) and antibiotics (penicillin

[250 U/ml] and streptomycin [250 µg/ml]). Ribavirin was purchased from Sigma Aldrich (St. Louis, MO) and dissolved in distilled water.

2.2. MNV-1 Plaque Assay. Murine norovirus (MNV-1) virus stocks were prepared as described (Wobus et al., 2004). The generation numbers used heretofore refer to number of passages since the start of the present experiment; the 0th generation refers to the 6th passage of MNV-1.CW3 (referred to as MNV-1 throughout) (Thackray et al., 2007).

MNV-1 samples were titrated by 10-fold dilution plaque assays in duplicate as previously described (Gonzalez-Hernandez et al., 2012; Wobus et al., 2004) in Lab 1. Lab 2 employed a protocol with the following modifications. Two hundred microliters of MNV-1 samples, in tenfold dilutions using complete D-MEM with 2% FBS, were inoculated onto wells for titrating. Plates were incubated for an hour at 37°C (Lab 2) and plates were overlaid with 2 ml of D-MEM with 0.7% NuSieve GTG agarose (Lonza Inc., Allendale, NJ). Plates were incubated for 24 hours before a second overlay of 2 ml of D-MEM with 0.7% NuSieve GTG agarose and 0.07% neutral red (Fisher Scientific, Pittsburgh, PA) was added. Plaques were counted following an additional 48 hours of incubation.

2.4. MNV-1 Serial Passaging With and Without Ribavirin. To assess the impact of Ribavirin on MNV-1 replication, we followed the procedure described in Chang and George (Chang and George, 2007). Both Chang and George (2007) and Arias et al. (2014) demonstrated minimal cell toxicity (over 80-90% cell viability) for RAW 264.7 cells exposed to Ribavirin at concentrations at or below 200 µM (Arias et al., 2014; Chang and George, 2007). Ribavirin treatment was continued for twenty serial passages alongside a vehicle control of D-MEM with 10% FCS (Lab 1) or 2% FBS (Lab 2). The experiment was performed in triplicate in two separate laboratories. In brief, confluent RAW 264.7 cells in six-well plates were incubated for 6 h at 37°C with 5% CO₂ with 2 ml of either complete D-MEM with 2% FBS or complete D-MEM with 2% FBS and Ribavirin (100 µM for Lab 1, 50 µM for Lab 2). Cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1.0, first based on the titer of the initial stock and then based on the titer of the previous passage. Following infection, plates were incubated overnight for an additional 12-18 hours. Cells were then frozen and thawed twice at -80°C and 37°C, respectively, and MNV-1 in the supernatant was titrated for use in the next passage.

2.5. MNV-1 Sequencing. Viral RNA was isolated from samples collected at the 0th, 5th, 10th, 15th, and 20th passage for Ribavirin treatment and the vehicle control. RNA was extracted, transcribed, and amplified using QIAamp Viral RNA Mini Kit (Qiagen) with DNase (Qiagen), SuperScript III Reverse Transcriptase (Invitrogen), and Fast Start High Fidelity PCR System (Roche) according to the manufacturer's instructions. Amplification was performed on three amplicons in the P-domain of MNV-1 (See Table 1). Sequencing was performed using the 454 platform (Roche) per manufacturer's instructions. Samples were stored and shipped at -80 C from the University of Michigan (Ann Arbor, MI) and Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) to the Johns Hopkins Applied Physics Laboratory (Laurel, MD) where they were sent to a third party lab for sequencing preparation. They were prepared in batches of between 1 and 18. The samples were then sequenced at the Applied Physics Laboratory from 7 August 2012 to 20 June 2013 in batches of 1 to 4 on 23 FLX plates (see Table 2).

2.6. Sequence preprocessing. Programs were written in the C programming language to filter and parse raw 454 sequencing reads after format conversion using sffinfo (454 Life Sciences). Quality filtering and trimming were performed with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were aligned against an MNV-1 reference genome (Accession# NC008311). Deletions were replaced with the corresponding base(s) in the reference genome. Insertions were deleted. To reject sequences that were likely to have been created by errors in reverse transcription, amplification, and/or sequencing, we calculated the likelihood of each haplotype sequence being generated from random mutations in sequences with higher observed copy numbers. A haplotype is defined here as an amplicon with a unique sequence (i.e., one or more single nucleotide substitutions relative to other sequences in the population). Only sequences that were less than 0.1% likely to be generated at the observed frequency by random error ($\alpha = 0.001$) were considered statistically significant and accepted. An α of 0.001 implies that less than 1 false sequence was admitted per sample. As part of the Average Pairwise Difference (APD) and nucleotide substitution analyses, the following outlier alignment positions were ignored due to unusually high amplicon variability: amplicon 3 positions 11 and 292; amplicon 4 positions 326, 361, and 393.

2.7. Diversity estimation. Three metrics were used to characterize the quasispecies diversity across serial passages: 1) Dominant Strain (DS) diversity, 2) Haplotype Number (HN) diversity and 3) APD diversity. Diversities were calculated from datasets collected every 5 passages. The dominant strain was defined as the most abundant haplotype in a sample. The DS diversity was estimated as the proportion of sequences with the dominant sequence. Lower proportions correspond to higher diversity. We defined HN diversity as $(1/L_A)\log_{10}(N_H)$, where L_A is amplicon length and N_H is the number of distinct haplotypes. The normalization factor (L_A) accounts for the dependency of the number of observed haplotypes on the sequencing length when modeling single nucleotide mutations was independent and identically distributed.

Finally, APD was estimated empirically as the expected proportion of differences per nucleotide over the distribution of sequence pairs. The empirical estimate is evaluated using the Bio::fastAPD Perl module v1.1.0 (Baughner, J.D., Pineda, F.J., 2014. Bio::fastAPD. At <https://metacpan.org/pod/Bio::fastAPD>) and is similar to, but distinct from, commonly used nucleotide diversity estimation methods (for example, Nei, M (1987))(Nei, 1987). The software workflow for estimating nucleotide diversity using average pairwise difference has been made publicly available (Baughner, J.D., Pineda, F.J., 2014. fastAPD_workflow - Nucleotide diversity (average pairwise difference) workflow. At https://github.com/jdbaughner/fastAPD_workflow).

2.8. Characterization of Ribavirin-induced nucleotide substitutions. We leveraged measured time series of site-dependent nucleoside-composition vectors to estimate Ribavirin-induced nucleotide substitution rates. Specifically, sequencing runs from each set of passages yielded K sets of T nucleoside proportion vectors, where T is the number of sequenced passages and K is the length of the amplicon. At each nucleoside site, k , within the $T \times K$ matrix, $p_k(t)$ is the vector of observed nucleoside proportions for each nucleoside adenosine (a), cytidine (c), guanosine (g), and uracil (u) at each passage, t , such that:

$$p_k(t) = [p_{k,a}(t), p_{k,c}(t), p_{k,g}(t), p_{k,u}(t)].$$

The nucleoside proportions after Δ passages can be modeled as a linear transformation of the initial proportions, i.e.:

$$q_k(t + \Delta) = M(\Delta)p_k(t)$$

where $q_k(t + \Delta)$ is a vector of predicted nucleoside proportions and where $M(\Delta)$ is a 4 x 4 Markov matrix of nucleotide substitution probabilities X :

$$M = \begin{bmatrix} X_{a \rightarrow a} & X_{a \rightarrow c} & X_{a \rightarrow g} & X_{a \rightarrow u} \\ X_{c \rightarrow a} & X_{c \rightarrow c} & X_{c \rightarrow g} & X_{c \rightarrow u} \\ X_{g \rightarrow a} & X_{g \rightarrow c} & X_{g \rightarrow g} & X_{g \rightarrow u} \\ X_{u \rightarrow a} & X_{u \rightarrow c} & X_{u \rightarrow g} & X_{u \rightarrow u} \end{bmatrix}.$$

Under the assumption that the nucleotide substitution rates between serial passages are small and independent of local composition, $M(\Delta)$ can be estimated for each time series by minimizing the expected Kullback-Liebler (KL) divergence between the predicted and the observed proportions for each site across the each amplicon length:

$$E\{D(q|p)\} = \sum_k \sum_{\alpha} p_{k,\alpha}(t) \log \frac{p_{k,\alpha}(t)}{q_{k,\alpha}(t)}$$

where the sum is over the sites in the amplicon of interest and where $\alpha=[A,C,G,U]$.

All calculations were performed in R. The R package *optimx* was used to perform the requisite minimization with respect to the substitution probabilities. The default Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm with an analytical gradient is used by *optimx* (Nash and Varadhan, 2011). The matrix elements of $M(\Delta)$ were normalized prior to each iteration and after the final iteration, to maintain the Markov property. In total, 105 transition matrices were estimated, corresponding to 51 Ribavirin-treated time series and 54 control time series. The transition matrices elements of the treatment and control groups were compared using the Wilcoxon signed-rank test, with family-wise error rate conservatively controlled using the Bonferroni correction. For each group and each type of substitution (e.g. A->C), outlier substitution rates were identified and removed using median(log₁₀(rate)) $\pm$ 3 MADs (median absolute deviation). The software workflow used for this analysis has been made publicly available (Baugher, J.D., Pineda, F.J., 2014. estimate_sub_rate - Nucleotide substitution rate estimation workflow. At https://github.com/jdbaugh/estimate_sub_rate).

3. RESULTS

3.1. MNV-1 did not develop resistance after exposure to 50 or 100 μ M Ribavirin for 20 serial passages.

To quantify the impact of Ribavirin as an antiviral agent, we compared MNV-1 titers in the presence of Ribavirin (treatment) to the vehicle control (control). Over twenty serial passages of MNV-1, the average titers (average $\pm$ standard error) for the control and the treatment were 8.25 ± 0.06 and $7.36 \pm$

0.06 log₁₀ PFU/ml for lab 1; control and treatment titers for lab 2 were 8.39 ± 0.04 and 7.72 ± 0.05 log₁₀ PFU/ml (Figure 1). This corresponds to an average treatment effect size for Ribavirin on MNV-1 titers of 0.89 ± 0.09 and 0.66 ± 0.06 log₁₀ PFU/ml for Labs 1 and 2, respectively. Treatment effect size varied over the course of both experiments, ranging from a minimum of -0.11 ± 0.12 log₁₀ PFU/ml for the 18th passage of Lab 1 to a maximum of 1.60 ± 0.15 log₁₀ PFU/ml for the 19th passage in Lab 2.

By comparing the treatment effect size of Ribavirin on MNV-1 serially passaged over 20 generations with Ribavirin (treatment) and without Ribavirin (control), we determined whether or not MNV-1 developed resistance to Ribavirin in our study. Our data demonstrate no statistically significant difference in MNV-1 titers between the treatment and control samples for either Lab 1 (100 µM Ribavirin, two sample t-test, $p = 0.77$) or Lab 2 (50 µM Ribavirin, two sample t-test, $p = 0.29$). For Lab 1, MNV-1 control and treatment titers were reduced by 0.59 ± 0.10 and 0.55 ± 0.10 log₁₀ (PFU/ml). For Lab 2, titers were reduced by 0.69 ± 0.20 and 0.42 ± 0.09 log₁₀ (PFU/ml). These data show that under the tested conditions, MNV-1 does not develop resistance to Ribavirin after 20 serial passages.

3.2. Although the dominant MNV-1 strain sequence remained stable following Ribavirin treatment, its relative proportion decreased. To identify the impact of Ribavirin on MNV-1 viral quasispecies diversity, we first investigated changes in the relative proportion of the dominant virus strain sequence. Under Ribavirin treatment, the dominant strain diversity increases over 20 serial passages relative to the control group. The dominant sequence remained identical to the sequence of MNV-1 (Accession Number NC_008311) for all three amplicons over 20 serial passages for both treatment and control. However, the proportion of dominant strain sequences decreased under Ribavirin treatment (Table 3). For Lab 1, dominant strain diversity decreased by 3, 11, and 8% for amplicons 2, 3, and 4; for Lab 2, dominant strain diversity decreased by 11, 15, and 18%. The decrease was statistically significant for Lab 1, amplicon 2 and for Lab 2, amplicons 2, 3, and 4. ($p < 0.05$). The dominant proportions were not statistically significantly different between the stock virus at passage 0 and the control at passage 20 for most of the amplicons (Table 3, $p > 0.05$), with the notable exception of Lab 1, amplicon 2 ($p = 0.02$). Our data show that the dominant MNV-1 strain sequence is conserved following Ribavirin treatment, but its relative proportion within the MNV-1 quasispecies decreases. Notably, the proportion of the dominant strain is influenced by amplicon length, so the fraction of dominant strains is not directly comparable between amplicons.

The decreasing trend in the proportion of dominant sequences was not influenced by read depth, as determined by lack of statistically significant correlation. Specifically: Spearman's correlation coefficients for read depth and the proportion of dominant sequences were -0.21 ($p = 0.34$), -0.39 ($p = 0.06$), and -0.16 ($p = 0.48$) for Lab 1 amplicons 2, 3, and 4, respectively and -0.18 ($p = 0.40$), -0.23 ($p = 0.30$), and -0.17 ($p = 0.43$) for Lab 2 amplicons 2, 3, and 4, respectively.

3.3. Ribavirin increases haplotype diversity. To identify whether or not the decrease in the proportion of the dominant MNV-1 strain sequence coincided with an increase in MNV-1 quasispecies diversity, we investigated MNV-1 haplotype diversity. Under Ribavirin treatment, the number of haplotypes increased over serial passages relative to the control group. The average increase in the number of statistically significant haplotypes across all three amplicons for the vehicle control (per *in vitro* passage)

was 23 ± 16.3 for Lab 1 and 3.6 ± 6.2 for Lab 2. The increase was not statistically significantly greater than zero, (Wilcoxon Signed Rank Test, $p = 0.26$ for Lab 1 and $p = 0.48$ for Lab 2). However, Ribavirin treatment significantly increased the number of haplotypes: The per *in vitro* passage rate of haplotypes was 75 ± 27 for Lab 1 and 120 ± 21 for Lab 2. These increases are statistically significantly greater than both zero (Wilcoxon Signed Rank Test, $p = 0.01$ for Lab 1 and $p < 0.001$ for Lab 2) and the vehicle control (Wilcoxon Signed Rank Test, $p = 0.03$ for Lab 1 and $p < 0.001$ for Lab 2) (Figure 2).

Read depth was statistically significantly correlated with the number of haplotypes only for amplicon 2 of Lab 1 (Spearman's $\rho = 0.49$, $p = 0.02$). There was no statistical significance for amplicons 3 ($\rho = 0.41$, $p = 0.05$) and 4 ($\rho = 0.33$, $p = 0.12$) of Lab 1 or amplicons 2 ($\rho = 0.26$, $p = 0.23$), 3 ($\rho = 0.04$, $p = 0.83$), or 4 ($\rho = 0.22$, $p = 0.30$) of Lab 2.

3.4. Ribavirin increases average pairwise difference diversity. As an additional indicator of Ribavirin's impact on MNV-1 diversity, we compared APD diversity estimates of treatment and control groups. In the initial MNV-1 population, APD was estimated as $2.5 \times 10^{-4} \pm 6.4 \times 10^{-5}$. Over 20 serial passages in the vehicle control, the rate of increase of APD – as calculated using linear regression – as $2.5 \times 10^{-5} \pm 0.45 \times 10^{-5}$ ($p < 0.001$) and $1.4 \times 10^{-5} \pm 0.25 \times 10^{-5}$ ($p < 0.001$) per *in vitro* passage for Labs 1 and 2, respectively. (Figure 3). Treatment of MNV-1 with Ribavirin increased APD by an additional $2.5 \times 10^{-4} \pm 0.49 \times 10^{-4}$ and $2.5 \times 10^{-4} \pm 0.28 \times 10^{-4}$ per *in vitro* passage for Labs 1 and 2, which is statistically significantly greater than the APD increase in the vehicle control ($p < 0.001$ in both Labs 1 and 2). Therefore, Ribavirin treatment increases MNV-1 diversity as measured using APD.

Estimates of APD are not statistically significantly correlated with read depth. Specifically: Spearman's correlation coefficients between APD and read depth for amplicons 2, 3, and 4 were 0.17 ($p = 0.44$), 0.23 ($p = 0.29$), and 0.34 ($p = 0.12$) for Lab 1 and 0.21 ($p = 0.33$), -0.17 ($p = 0.42$), and 0.10 ($p = 0.63$) for Lab 2.

3.5. Ribavirin increases C-to-U, G-to-A, G-to-U, and C-to-A nucleotide substitutions. Based on our findings that Ribavirin increased MNV-1 quasispecies diversity, we identified the specific nucleotide substitutions responsible for the increase. Data from the transition matrices for both the control (M_c) and the treatment (M_t), empirically estimated by minimizing the expected Kullback-Liebler (KL) divergence, revealed a Ribavirin-dependent increase in nucleotide substitution rates (Figure 4).

A combined analysis of Labs 1 and 2 (Figure 4A) showed highly significant increases in, specifically, C-to-U, G-to-A, G-to-U, and C-to-A mutations, along with significant increases in most other mutations. However, upon separate analyses, we observed highly significant increases for only C-to-U and G-to-A mutations from Lab 1 (Figure 4B) and significant increases globally from Lab 2 (Figure 4C). We also observed that the estimated rate of transition mutations was greater than that commonly expected for transversion mutations.

3.6. Ribavirin decreases proportion of nonsynonymous amino acid substitutions. The relative proportion of nonsynonymous amino acid sequences was investigated to identify potential impacts of Ribavirin-induced mutation on subsequent translation. We observed a non-significant decrease in the relative proportion of nonsynonymous amino acid substitutions (and, conversely, an increase in

synonymous substitutions) after 20 passages in the control (Table 4, two sample t-test, $p = 0.33$). Following 20 passages in the presence of Ribavirin, we observed a statistically significant decrease in nonsynonymous mutations relative to both the stock virus (Table 4, two sample t-test, $p < 0.05$) and the vehicle control after 20 passages (Table 4, two sample t-test, $p < 0.05$). Notably, none of the nonsynonymous substitutions resulted in the generation of a stop codon (i.e., UAG, UAA, or UGA) (data not shown). Because the region sequenced was the essential P-domain of the capsid, the generation of a stop codon is necessarily associated with lethal mutagenesis.

4. DISCUSSION

Ribavirin increases quasispecies diversity in murine norovirus passaged in RAW 264.7 cells. In RNA viruses, quasispecies diversity is perceived as beneficial, as it increases the probability for the virus to persist within dynamic environments (Vignuzzi et al. 2006). The cost associated with quasispecies diversity, however, is an increase in nonviable progeny. One perceived mechanism of Ribavirin is the induction of error catastrophe by increasing the relative proportion of nonviable progeny such that the viral population is no longer sustainable (Vignuzzi et al., 2005). Increasing nonviable progeny, or otherwise defective virus, can lead to extinction at both low and high concentrations of mutagens (Grande-Pérez et al., 2005). Here, we conclusively demonstrate that Ribavirin increases MNV-1 quasispecies diversity via increased frequency of C-to-U, A-to-G, and possibly (to a lesser extent) global increases in nucleotide substitution rates. Simultaneously, MNV-1 titers in RAW 264.7 cells exposed to Ribavirin are reduced by $0.8 \log_{10}$. These findings align with previously published *in vitro* and *in vivo* which also report dose-dependent effect sizes (Arias et al., 2014; Chang and George, 2007). Our data provide additional support for Ribavirin's perceived ability to precipitate error catastrophe.

Previous findings that Ribavirin has little influence on virus mutation rates *in vitro* are explained by the conservation of the dominant amplicon sequence in the population. Our findings contrast with others who have noted no increased mutagenesis due to treatment with Ribavirin (Chang and George, 2007; Chevaliez et al., 2007; Lutchman et al., 2007). This discrepancy is resolved by noting that the studies that did not observe mutagenesis examined only the consensus sequence. Our findings show that despite an increase in mutagenesis for the viral population exposed to Ribavirin, the dominant MNV-1 strain sequence was conserved following twenty serial passages. Reliance on the consensus sequence alone would fail to detect nucleotide substitutions in virus treated with Ribavirin. Sanger sequencing, which is used to identify consensus sequences, is insufficient to observe impacts of Ribavirin on viral genomes. An exception is when there is strain drift within the population, such as occurs when mutagenesis provides an advantageous phenotype. One example is the emergence of Ribavirin-resistant poliovirus (Pfeiffer and Kirkegaard, 2003). It is important to note that both Sanger sequencing and the technique used here (based on deep sequencing) may also miss frame shift mutations, which have been shown to occur in foot-and-mouth disease virus exposed to Ribavirin (Perales et al., 2011).

The cause for the differential rate of C-to-U and G-to-A relative to U-to-C and A-to-G mutations is not clear. Our analysis, in agreement with previous studies, reveals an absence of U-to-C or A-to-G mutations despite a large increase in C-to-U and G-to-A mutations (Beaucourt and Vignuzzi, 2014; Crotty et al., 2000; Dietz et al., 2013; Jin et al., 2013). One proposed mechanism is that Ribavirin incorporation

opposite cytosine is more favorable than incorporation opposite uracil (Day et al., 2005; Graci et al., 2007). A similar potential mechanism is equal incorporation rates Ribavirin opposite both cytosine and uracil followed by preferential Ribavirin pairing with uracil. A third proposed mechanism is the combined influence of both depletion of intracellular GTP levels and misincorporation of Ribavirin opposite cytosine and uracil (Day et al., 2005; Graci and Cameron, 2006). Suppressed GTP concentrations would shift kinetics to favor Ribavirin incorporation with cytosine. Our data highlight the differential mutation rates, but are insufficient to differentiate between the two proposed mechanisms.

While the mutation rate analyses from both labs agreed on a significant increase in C-to-U and G-to-A mutations, they differed in regard to increased abundance of additional mutations. The conceptual model that Ribavirin incorporates opposite cytosine and uracil does not explain the observed increased abundance of additional mutations (e.g., mutations that are not C-to-U or G-to-A). However, by accounting for disruptions caused by depleted guanosine concentrations, then additional mutations would be expected to arise due to increased misincorporation of other nucleosides. As Ortega-Prieto et al. (2013) note, GTP pool depletion alone is unlikely to cause mutagenesis sufficient to decrease specific infectivity; Ribavirin's role as a purine nucleoside analogue is also needed (Ortega-Prieto et al., 2013). We note here that the frequency of additional mutations differed across the two laboratories, which may be explained by lab-dependent effects, including differences in protocols as discussed in Methods, differences in individual researcher practices, and/or Ribavirin concentrations (i.e., dose-dependent effects). It is of great interest to understand if the predominant mechanism of Ribavirin activity is largely influenced by dosage. Along these lines, our methods for estimating mutation transition matrices provide new opportunities for evaluating additional experimentally-validated models of possible mechanisms underlying Ribavirin-induced mutation, including GTP pool depletion and combinations of misincorporation and GTP depletion.

Observed increase in mutations due to Ribavirin treatment should be considered prior to use of Ribavirin as a treatment option for viral infections. Poliovirus was previously shown to develop resistance to Ribavirin following a G-to-A mutation in the polymerase (Pfeiffer and Kirkegaard, 2003). In our study, G-to-A mutations were statistically significantly more likely to occur in MNV-1 treated with Ribavirin. Therefore, it is not surprising that a resistant poliovirus emerged given that: there exists a G-to-A mutation for poliovirus that gives rise to resistance, and that Ribavirin increases G-to-A mutation rates. Linking potential mutations to Ribavirin resistance mechanisms, and understanding Ribavirin's role in inducing mutations, provides insight into whether or not treatment of an infection should include Ribavirin. In the absence of knowledge concerning the potential for a virus to develop resistance, awareness of Ribavirin's mutagenic properties (and ability to induce resistance) is necessary.

We observed increases in the relative proportion of nonsynonymous amino acid substitutions in MNV-1 exposed to Ribavirin coincident to increases in viral diversity. Synonymous amino acid substitutions likely impact viral fitness. The simultaneous increase in viral diversity and decrease in relative proportion of nonsynonymous mutations suggests that viral diversity is driven primarily by the fixing of nucleotide mutations with minimal impact on viral fitness. Supporting this assertion is the lack of observed stop codons which necessarily reduce fitness.

There are three limitations that should be noted for this study. The first is that we relied on frequency of haplotype reads for a number of our analyses. The preparation of the library for 454 sequencing differentially amplifies segments, distorting the relative abundance of haplotypes. Therefore, the reported relative abundance of each haplotype does not necessarily reflect the true relative abundance. Nevertheless, our analyses rely on direct comparisons between the treatment and the vehicle control, which suffer similarly from differential amplification. Conclusions drawn from comparisons, if not estimates of magnitude, are valid. In future research, barcoding cDNA fragments during reverse transcription would provide an opportunity to account for differential amplification (Jabara et al., 2011). A second limitation is that library preparation introduces mutations during sequencing that may not have occurred due to experimental conditions. Using a conservative filter to identify statistically significant haplotypes, as described in the methods, reduced the likelihood that a reported haplotype occurred due to library preparation to less than 0.1%. The impact of this bias is likely limited because all findings are drawn from a direct comparison between treatment and control samples, which should be impacted equally by this limitation. Finally, estimates of nucleotide diversity may be impacted by relative abundances and therefore may not be meaningful in the context of mutagenesis. The impact of this is likely minor, however, because we observed similar trends in our analyses of number of haplotypes which is not impacted by abundance (though is impacted by sequencing coverage).

Our work demonstrates that Ribavirin treatment increases viral quasispecies diversity. Ribavirin increases viral quasispecies diversity by inducing C-to-U and A-to-G, and possibly (to a lesser extent) global increases in nucleotide substitution. The mechanism for preferential mutagenesis is unclear. Future studies on these mechanisms can leverage targeted haplotype data obtained via deep sequencing to quantify mutation rates, viral quasispecies diversity measurements, and viral population dynamics through application of a time series of site-dependent nucleoside composition vectors as demonstrated here.

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TABLES

Table 1: Amplicons in the P-domain sequenced using the 454 platform.

Table 2: Date for preparation, date for sequencing, raw read depth, and read depth used for analysis for each sample.

Table 3: After 20 serial passages the proportion (standard error) of dominant strain sequences was unchanged in the control group (Control p20) compared to the initial stock (Stock p0) but decreased significantly in the Ribavirin treatment group (Treatment p20).

Table 4: After 20 serial passages the proportion (standard error) of nonsynonymous amino acid substitutions decreased in both the control (Control p20) and Ribavirin treatment group (Treatment p20) relative to the initial stock (Stock p0), but the effect size was greater in the treatment group.

TABLE 1

Name	Forward Primer	Reverse Primer	Amplicon Size (bp)		Location (Relative to MNV-1 Accession# NC008311)
			With Primers	Primers Trimmed	
Amp2	5'-CAGATCACATGCTTCCACA-3'	5'-AAAGCAGGACACCCATGAAC-3'	450	420	5473-5922
Amp3	5'-GTTTCATGGGTGCTCTGCTT-3'	5'-GGGGAGAAAGGGACCAATT-3'	428	376	5903-6330
Amp4	5'-TTTTCAGGACACCATCCCTG-3'	5'-TTATTGTTTGAGCATTGCGC-3'	427	397	6255-6681

TABLE 2

ID	Passage	Ribavirin (μ M)	Lab	Rep.	Date Prepped	Date Sequenced	FLX Plate ID	Raw Reads	Analyzed Reads		
									Amp 2	Amp 3	Amp 4
1	0	-	1, 2	1	2012-08-01	2012-08-07	775484	217'421	68'546	49'001	57'275
2	0	-	1, 2	2	2013-03-01	2013-04-24	787397	36'334	5'091	4'703	14'649
3	0	-	1, 2	3	2013-03-01	2013-04-24	787397	49'156	18'615	3'419	11'071
4	0	-	1, 2	4	2013-03-01	2013-04-24	787397	64'491	13'461	7'710	23'774
5	5	0	1	1	2012-09-01	2012-09-27	775807	179'962	20'365	18'828	104'543
6	5	0	1	2	2013-03-01	2013-04-07	784364	208'038	39'863	49'319	63'766
7	5	0	1	3	2013-03-01	2013-04-13	785453	154'583	38'435	43'463	44'054
8	5	100	1	1	2011-01-11	2012-11-30	784219	291'092	82'889	67'179	61'034
9	5	100	1	2	2013-03-01	2013-03-22	784999	32'958	3'670	5'020	13'777
10	5	100	1	3	2013-03-01	2013-03-22	784999	12'440	910	2'038	4'738
11	10	0	1	1	2012-09-01	2012-09-28	777465	156'783	39'657	39'000	42'198
12	10	0	1	2	2013-03-01	2013-04-05	785607	149'677	46'136	40'716	36'871
13	10	0	1	3	2013-03-01	2013-04-13	785453	145'318	36'625	39'857	42'029
14	10	100	1	1	2012-09-01	2012-09-27	775807	166'324	30'778	44'210	48'933
15	10	100	1	2	2013-03-01	2013-03-22	784999	42'089	4'798	9'633	14'426
16	10	100	1	3	2013-03-01	2013-03-22	784999	80'109	10'213	21'106	25'889
17	15	0	1	1	2011-01-11	2012-11-30	784219	251'421	55'997	70'239	70'848
18	15	0	1	2	2013-03-01	2013-04-12	785012	144'142	33'804	36'394	45'637
19	15	0	1	3	2013-03-01	2013-04-12	785012	150'353	30'640	47'456	42'622
20	15	100	1	1	2012-09-01	2012-09-26	777511	177'962	37'384	43'099	48'409
21	15	100	1	2	2013-03-01	2013-04-07	784364	117'008	19'559	22'363	38'718
22	15	100	1	3	2013-03-01	2013-04-07	784364	60'682	5'612	8'126	22'110
23	20	0	1	1	2012-09-01	2012-09-28	777465	184'875	51'098	53'927	42'048
24	20	0	1	2	2013-03-01	2013-04-12	785012	161'082	45'051	86'083	23'104

25	20	0	1	3	2013-03-01	2013-04-12	785012	181'154	66'399	50'808	58'335
26	20	100	1	1	2012-09-01	2012-09-26	777511	190'064	43'162	43'032	57'044
27	20	100	1	2	2013-02-01	2013-04-05	785607	91'328	30'780	73'317	36'203
29	5	0	2	1	2013-03-01	2013-04-24	787397	74'487	15'277	10'914	29'685
30	5	0	2	2	2013-04-01	2013-04-27	787534	140'021	34'161	35'855	39'976
31	5	0	2	3	2013-04-01	2013-04-27	787534	195'174	73'541	86'781	977
32	5	50	2	1	2013-04-01	2013-04-27	787534	146'054	27'534	37'896	40'512
33	5	50	2	2	2013-04-01	2013-04-27	787534	140'526	29'454	41'121	32'983
34	5	50	2	3	2013-04-01	2013-05-29	788673	172'745	34'747	40'336	56'366
35	10	0	2	1	2013-04-01	2013-05-29	788673	154'689	21'601	30'791	69'196
36	10	0	2	2	2013-04-01	2013-05-29	788673	126'688	20'748	25'246	42'776
37	10	0	2	3	2013-04-01	2013-05-29	788673	150'295	28'442	29'665	53'281
38	10	50	2	1	2013-05-01	2013-06-13	788035	168'981	35'885	53'440	37'561
39	10	50	2	2	2013-05-01	2013-06-13	788035	153'814	28'667	37'874	47'883
40	10	50	2	3	2013-05-01	2013-06-13	788035	155'686	32'171	45'083	38'236
41	15	0	2	1	2013-04-01	2013-06-06	787730	209'243	47'057	62'206	61'385
42	15	0	2	2	2013-04-01	2013-06-06	787730	206'325	42'908	66'843	58'267
43	15	0	2	3	2013-05-01	2013-06-13	788035	154'176	35'406	42'082	46'433
44	15	50	2	1	2013-05-01	2013-06-15	787698	144'776	35'524	26'644	40'319
45	15	50	2	2	2013-05-01	2013-06-15	787698	186'538	46'209	34'164	54'413
46	15	50	2	3	2013-05-01	2013-06-15	787698	161'746	33'964	32'997	44'787
47	20	0	2	1	2013-05-01	2013-06-15	787698	156'741	51'098	53'927	42'048
48	20	0	2	2	2013-05-01	2013-06-20	788021	186'569	62'985	31'364	36'321
49	20	0	2	3	2013-05-01	2013-06-20	788021	212'086	39'888	58'500	47'858
50	20	50	2	1	2013-05-01	2013-06-20	788021	185'653	48'537	35'194	55'259
51	20	50	2	2	2013-05-01	2013-06-20	788021	183'855	18'694	27'643	19'106
52	20	50	2	3	2013-05-01	unknown	unknown	unknown	12'487	29'960	29'415

TABLE 3

Amplicon*	Lab	p0 (Stock)	p5		p10		p15		p20	
			C	T	C	T	C	T	C	T
Amp2 (420 bp)	1	0.956 (0.009)	0.986 (0.002)	0.963 (0.036)	0.988 (0.003)	0.954 (0.023)	0.971 (0.013)	0.97 (0.017)	0.981 (0.003)	0.923 (0.001)
	2		0.983 (0.004)	0.984 (0.004)	0.980 (0.004)	0.949 (0.003)	0.984 (0.001)	0.915 (0.007)	0.969 (0.012)	0.859 (0.017)
Amp3 (376 bp)	1	0.909 (0.006)	0.944 (0.003)	0.922 (0.05)	0.913 (0.022)	0.881 (0.024)	0.849 (0.028)	0.860 (0.044)	0.844 (0.017)	0.797 (0.005)
	2		0.903 (0.004)	0.910 (0.002)	0.899 (0.005)	0.888 (0.004)	0.900 (0.013)	0.828 (0.006)	0.839 (0.049)	0.773 (0.017)
Amp4	1	0.940	0.975	0.931	0.971	0.887	0.944	0.849	0.968	0.865

(397 bp)		(0.015)	(0.000)	(0.047)	(0.003)	(0.026)	(0.010)	(0.035)	(0.00)	(0.007)
	2		0.979 (0.005)	0.961 (0.006)	0.962 (0.000)	0.915 (0.010)	0.970 (0.001)	0.869 (0.012)	0.870 (0.030)	0.771 (0.016)

*p0, p5, p10, and p20 refer to the 0th, 5th, 10th, 15th, and 20th passages, respectively. “C” and “T” refer to “control” and “treatment”. Fractions are proportion of sequences that are dominant strain. Paranthetical fractions are standard errors.

TABLE 4

Amplicon*	Lab	p0 (Stock)	p5		p10		p15		p20	
			C	T	C	T	C	T	C	T
Amp2	1	0.808 (0.031)	0.769 (0.076)	0.412 (0.217)	0.695 (0.026)	0.605 (0.053)	0.742 (0.075)	0.409 (0.215)	0.703 (0.054)	0.645 (0.016)
	2		0.762 (0.008)	0.8 (0.067)	0.775 (0.004)	0.747 (0.038)	0.772 (0.071)	0.655 (0.008)	0.735 (0.016)	0.533 (0.009)
Amp3	1	0.650 (0.098)	0.452 (0.103)	0.363 (0.075)	0.339 (0.053)	0.263 (0.037)	0.473 (0.077)	0.151 (0.077)	0.600 (0.080)	0.451 (0.000)
	2		0.643 (0.043)	0.566 (0.112)	0.647 (0.013)	0.474 (0.025)	0.575 (0.047)	0.436 (0.023)	0.501 (0.056)	0.265 (0.013)
Amp4	1	0.574 (0.086)	0.395 (0.084)	0.365 (0.057)	0.496 (0.038)	0.226 (0.02)	0.453 (0.011)	0.246 (0.031)	0.456 (0.041)	0.351 (0.03)
	2		0.289 (0.15)	0.402 (0.059)	0.471 (0.032)	0.419 (0.031)	0.438 (0.024)	0.39 (0.007)	0.449 (0.053)	0.219 (0.017)

*p0, p5, p10, and p20 refer to the 0th, 5th, 10th, 15th, and 20th passages, respectively. “C” and “T” refer to “control” and “treatment”. Fractions are proportion of sequences with nonsynonous substitutions. Paranthetical fractions are standard errors.

FIGURES:

Figure 1: Titer of MNV-1 following serial passages for both the vehicle control (0 μ M Ribavirin) and the treatment (100 μ M and 50 μ M Ribavirin for Labs 1 and 2, respectively) for two separate experiments (Lab 1 and Lab 2). Each data point represents the average of triplicate samples with error bars representing standard deviations. Effect size is represented as grey columns and is calculated as the log₁₀ difference between the vehicle control titer and the treatment titer. Data for the 12th serial passage in Lab 1 are missing.

Figure 2: The number of statistically significant haplotypes at the 0th, 5th, 10th, 15th, and 20th serial passages for control (open markers) and treatment (closed markers) groups from Lab 1 (triangle markers) and Lab 2 (circle markers). Each data point (error bar) represents the mean (standard

deviation) from three replicates ($n = 3$) except for the 20th passage for Lab 2, which represents data from two replicates ($n = 2$). The data from each replicate is visible individually in light gray in the background.

Figure 3: The average pairwise diversity at the 0th, 5th, 10th, 15th, and 20th serial passages for control (open markers) and treatment (closed markers) groups from Lab 1 (triangle markers) and Lab 2 (circle markers). Each data point (error bar) represents the mean (standard deviation) from three replicates ($n = 3$) except for the 20th passage for Lab 2, which represents data from two replicates ($n = 2$). The data from each replicate is visible individually in light gray in the background.

Figure 4: The off-diagonal results of the median Ribavirin-treated transition matrix, $\mathbf{M}_t$, (gray) and the median control, $\mathbf{M}_c$, (white). **A.** The results of Lab 1 (100 μM Ribavirin). **B.** The results of Lab 2 (50 μM Ribavirin). *** = $p\text{value} < 0.001$. ** = $p\text{value} < 0.01$. * = $p\text{value} < 0.05$.