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RESEARCH ARTICLE

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Investigating the effect of ribavirin treatment on genetic mutations in Crimean–Congo haemorrhagic fever virus (CCHFV) through next-generation sequencing

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Abstract

Crimean–Congo haemorrhagic fever (CCHF) is the most widespread tick-borne viral haemorrhagic fever affecting humans, and yet a licensed drug against the virus (CCHFV) is still not available. While several studies have suggested the efficacy of ribavirin against CCHFV, current literature remains inconclusive. In this study, we have utilised next-generation sequencing to investigate the mutagenic effect of ribavirin on the CCHFV genome during clinical disease. Samples collected from CCHF patients receiving ribavirin treatment or supportive care only at Sivas Cumhuriyet University Hospital, Turkey, were analysed. By comparing the frequency of mutations in each group, we found little evidence of an overall mutagenic effect. This suggests that ribavirin, administered at the acute stages of CCHFV infection (at the World Health Organization-recommended dose) is unable to induce lethal mutagenesis that would cause an extinction event in the CCHFV population and reduce viremia.

KEYWORDS
Crimean–Congo haemorrhagic fever virus, mutation, ribavirin, variant, whole genome next-generation sequencing

Jake D'Addiego, Nazif Elaldi, and Nadina Wand contributed equally to this study.

1 | INTRODUCTION

Crimean–Congo haemorrhagic fever (CCHF) is a widespread tick-borne zoonotic disease, causing haemorrhagic symptoms exclusively in humans, with reported case fatality between 5% and 40%.[1] The causative agent, Crimean–Congo haemorrhagic fever virus (CCHFV), an *Orthonairovirus* within the *Nairoviridae* family, has a negative-sense single-stranded tripartite RNA genome, consisting of a 1.7 kb small (S) segment, a 5.4 kb medium (M) segment, and a 12.2 kb large (L) segment.[2]

CCHFV is endemic to the Balkans, southern Europe, the Middle East, Africa, and parts of Asia.[1] It can infect a number of tick genera, but its principal vectors are ticks of the *Hyalomma* genus.[3–6] The expanding geographical range of the CCHFV tick vector due to climate change, the carriage of infected ticks on migratory birds and the movement of livestock and wild animals are a serious cause for concern due to the continued lack of licenced therapeutics and vaccines for the disease.[7,8] Consequently, the World Health Organization (WHO) has identified CCHFV as a priority pathogen and outlined a CCHF Research and Product Development Roadmap, which aims to accelerate the development of diagnostics, therapeutics and vaccines, as well as strategies for vector control in affected areas.[9]

Treatment of patients diagnosed with CCHF is generally supportive, with basic symptom management focusing on replacing lost blood, maintaining the correct balance of fluids and electrolytes, as well as sustaining heart and respiratory function while the patient is recovering.[10] Two severity scoring systems (SGSs), based on standard blood analysis and age, have been developed to help estimate the prognosis of CCHF patients, which can help predict an unfavorable outcome with 96%–100% sensitivity and 93%–100% specificity.[11,12] In Turkey these algorithms are employed by clinicians in endemic regions to identify the urgency of patient transfer to a tertiary care center, where a treatment plan and infection control procedures can be implemented.[11,12] While not part of the SGS, a viral load exceeding 10^8 copies/mL is associated with poor prognosis.[13,14] The only therapeutic agent currently recommended by the WHO for the treatment of CCHF is ribavirin, administered either orally or intravenously.[1] However, its effectiveness in vitro and in vivo remains controversial, and a case-controlled clinical trial has never been conducted.[12,15–20]

Ribavirin is a broad-spectrum antiviral guanosine analog, which has been routinely used in combination with interferon-α to treat hepatitis C. It has also been indicated for the treatment of Lassa fever and respiratory-syncytial virus infections[10,15,21–23] and has efficacy against herpes, vesicular stomatitis, and influenza viruses.[24]

Several mechanisms of action have been proposed for ribavirin, including inhibition of mRNA capping, impact on host cell gene expression, inflammation and immunomodulation, through its ability to decrease intracellular guanosine triphosphate (GTP).[25,26] In addition, ribavirin may inhibit viral RNA-dependent RNA polymerases, leading to an increase in viral mutagenesis.[21] The latter is suggested to work by erroneous substitutions of ribavirin triphosphates (RTPs) for guanosine triphosphates (GTPs), which have ambiguous base-pairing properties with uridine triphosphate or cytidine triphosphate, leading to an increase in G-to-A and C-to-T (equivalent to C-to-U in RNA sequence) mutations.[27–30] While this mechanism of action has been found to occur in vitro for hepatitis C virus,[22] other studies have undermined this hypothesis in vivo.[31] A recent clinical study inferred that ribavirin treatment increased the rate of CCHFV genome mutagenesis, however, this was based only on a single case report and lacked appropriate controls.[28]

In this study, we investigated the mutagenic effect of ribavirin on CCHFV genomes using next-generation sequencing (NGS) coupled with a sequence-independent, single-primer amplification (SISPA) protocol. This approach was used to eliminate sequence bias and encompass multiple strains of CCHFV circulating in Turkey, which guided us away from a targeted sequencing scheme. Complete CCHFV consensus genomes from patients, who received either ribavirin or supportive care only were determined on Day 1 post hospitalisation. CCHFV genomes were sequenced over the subsequent three sampling days, and sequencing reads were mapped against the Day 1 consensus genomes to determine whether ribavirin treatment increased mutation rates in the CCHFV populations. Viral loads of all patients were also monitored to establish if increased mutation rates were accompanied by a detectable reduction in viremia post ribavirin treatment.

2 | METHOD

2.1 | Sample collection and ribavirin treatment

Serum samples were taken from six adult patients (four males and two females), between the ages of 18 and 64 years, admitted to Sivas Cumhuriyet University Hospital between April and May 2020. Samples were collected on the day of admission and then daily until discharge (between 8 and 11 days). Samples were immediately stored at −80°C for further analysis. Initial CCHF diagnosis was based on a positive real-time reverse-transcriptase polymerase chain reaction (RT-PCR) test (Altona Diagnostics) result for patients presenting with symptoms of viral haemorrhagic fever (VHF). The study was part of a joint project (Newton-TUBITAK Katip Celebi) between the UK Health Security Agency (UKHSA) and Turkey, approved by the local Ethics Committee of Sivas Cumhuriyet University, Turkey (Protocol # 2019-09/04). All patients provided an informed consent form.

For the prediction of mortality risk, CCHF patients were grouped using the severity grading score (SGS) system as follows: low (0–5), intermediate (6–10), and high risk (11–16). The system uses age and standard clinical and routine laboratory parameters made on hospital admission.[11,12] Assessed clinical symptoms underpinning SGS designation include, but are not limited to, fever, myalgia, nausea, vomiting, diarrhea, and bleeding (Table S1). Daily clinical chemistry results evaluating liver function, platelet, red blood cell, and white blood cells count, as well as clotting factors, were also obtained for each patient (Figure S3). All six patients received supportive care and three out of the six patients also received ribavirin treatment. The initiation of oral ribavirin treatment was a decision made by the ward clinicians. When used, oral ribavirin was administered on Day 2

of hospitalisation at the dose schedule recommended by the WHO (30 mg/kg initial loading dose, followed by 15 mg/kg every 6 h for 4 days, followed by 7.5 mg/kg every 8 h for 6 days).[32]

2.2 | Sample processing and RNA extraction

Serum samples were inactivated in a containment level (CL) 4 laboratory; 140 µL of each sample were added to 560 µL of buffer AVL and 560 µL of ethanol. RNA was extracted utilising the QIAamp Viral RNA Mini Kit (Cat. No. 52904; Qiagen) following the manufacturer's instructions. RNA was eluted in 60 µL of nuclease-free water and stored at −80°C.

2.3 | CCHFV RT-PCR

Before sequencing, viral RNA levels (copy/mL) were determined using an in-house quantitative real-time RT-PCR assay optimized to accommodate recently published Turkish sequences.[33] Reactions were prepared in a final volume of 20 µl and included 1X TaqMan Fast Virus 1-Step Master Mix (Cat. No. 4444436; Thermo Fisher Scientific), 0.5 µM of forward primer CCHF-F1 (5′-CCCTTTTTAAACTCCTCAAACC-3′), 0.5 µM of forward primer CCHF-F2 (5′-CCTTTTTTAAACTCCTCAAACC-3′), 1 µM of reverse primer CCHF-R (5′-TCTCAAAGAAACACGTGCC-3′), 0.5 µM of probe CCHF-P (5′FAM-ACTCAAGAGAACACTGT GGGCGTAAG-3′MGBEQ) and 5 µL of template RNA.

Quantitative real-time RT-PCR was performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) platform with the following cycling parameters: 50°C for 10 min, 95°C for 2 min and 45 cycles of 95°C for 10 s and 60°C for 30 s.

Copies of CCHFV genomic RNA in the extracted clinical samples were determined, based on a quantified synthetic RNA standard curve and normalized to copies per mL of patient serum, taking into account extraction and elution volumes. The rate of viral load decrease was determined as the difference between the quantified genomic CCHFV RNA copies calculated on two consecutive days, divided by the earlier time point viral load.

2.4 | CCHFV sequencing

Randomly amplified cDNA was prepared for three ribavirin-treated patients and three control patients on the first day of hospitalisation (Day 1), and subsequently on the day of treatment initiation (Day 2) and 1 and 2 days post treatment initiation using a SISPA approach, according to a previously described protocol.[34,35] Random PCR enrichment of each patient sample was performed in triplicates and sequenced from a single sequencing replicate.

Illumina sequencing libraries were prepared utilising a Nextera XT V2 kit (Cat. No. FC-131-1096; Illumina) and sequenced on a 2 × 150-bp paired-end Illumina MiSeq instrument operated by the UK Health Security Agency (UKHSA) Genomics Services Development Unit (Colindale).

2.5 | CCHFV variants analysis

Forward and reverse sequencing reads in FASTQ files were mapped to reference S (KY362517), M (KY362519), and L (KY362515) segment sequences utilising BWA-MEM with default settings. Consensus sequences and variants details were derived

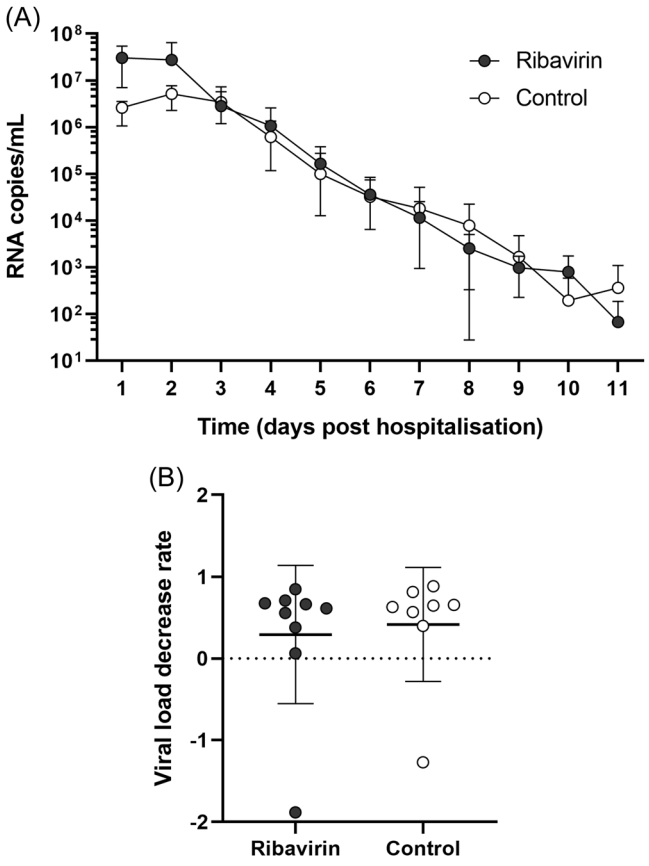

FIGURE 1 Daily monitoring of serum Crimean–Congo haemorrhagic fever virus (CCHFV) viral load. (A) CCHFV genome copies in patients' serum samples were determined using a specific in-house real-time reverse-transcriptase polymerase chain reaction analysis for 11 days post hospitalisation. The average CCHFV viral load in ribavirin-treated (dark grey circles) and control group patients (white circles) is expressed in RNA copies per mL of serum. The error bars represent the range of the viral load for each day in the two patient groups. (B) The rate of CCHFV viral load daily decrease in ribavirin-treated patients (dark grey circles) was compared to that in patients (white circles) who received supportive care only. The individual data points are the rate of decrease between every two consecutive days in each group. The horizontal lines represent the mean CCHF viral rate of decrease in both patient groups. The standard deviation is shown as error bars.

from BAM files utilising an in-house C++ program QuasiBAM.[36] IUPAC ambiguity codes were utilised for base frequencies above 20%. Variants were called in cDNA sequences, where Thymine (T) was reported to represent Uracil (U) in the original RNA sequence.[27–30] High-frequency variants (≥10%) were analysed in all regions with a sequencing depth above ×30. Low-frequency variants (2%–10%) were called for regions with a sequencing depth of at least ×100. Mapping metrics were derived with SAMtools flagstat, depth, and coverage functions. Variants frequencies between ribavirin-treated and control groups were compared using the Mann–Whitney *U* test. Each mutation frequency rate was assigned a rank, and critical values were determined using two-tailed testing. Reported *p* values are significant at the 5% level. Charts were plotted by GraphPad Prism v6.0 (GraphPad Prism Software).

3 | RESULTS

3.1 | Effect of ribavirin treatment on the CCHF viral load

CCHF viral load was determined daily in samples from six CCHF patients (three ribavirin-treated and three who received supportive care only) post hospitalisation using in-house quantitative real-time RT-PCR and expressed as average normalised CCHFV genomic RNA

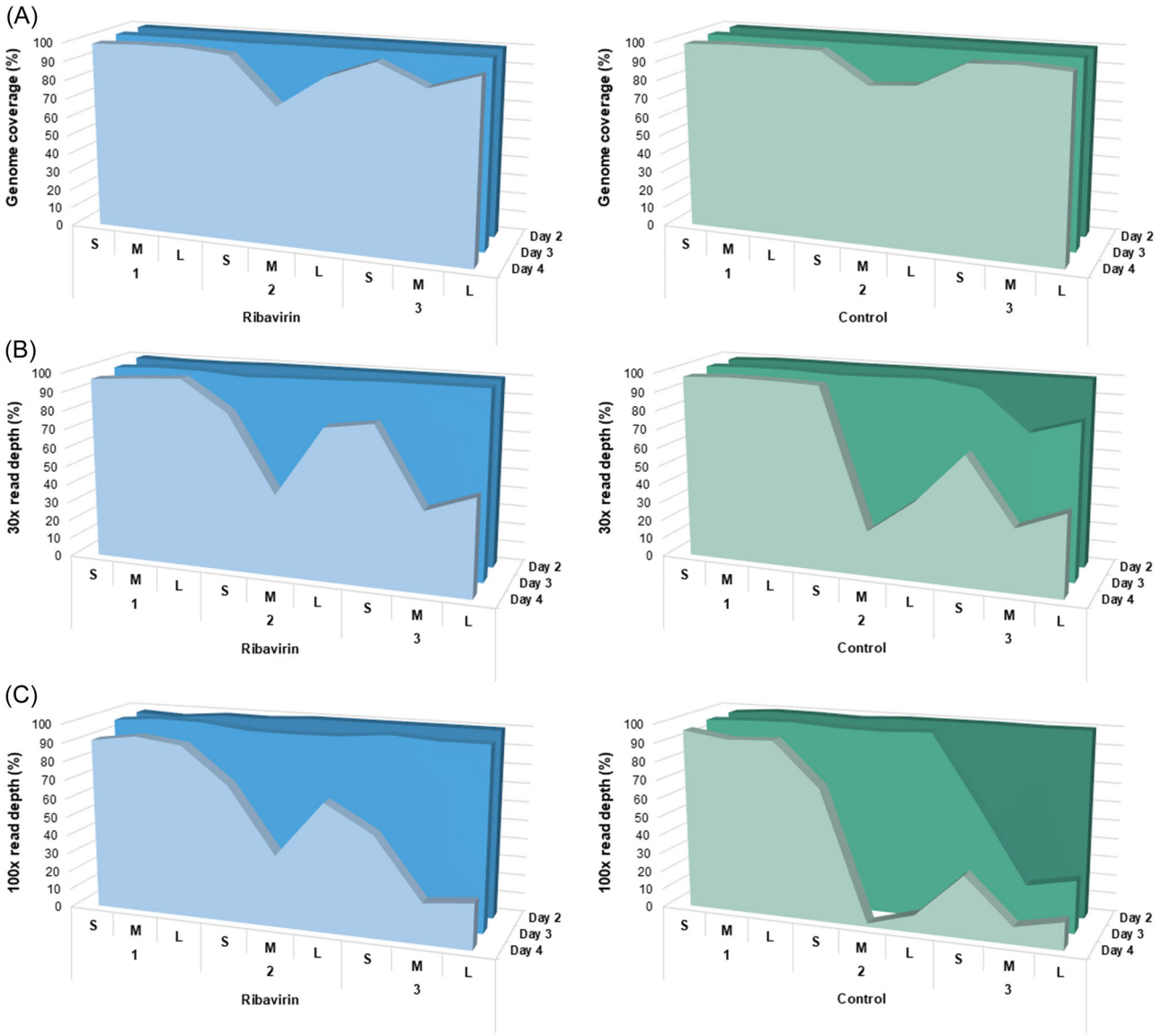

FIGURE 2 Reference coverage and sequencing depth of Crimean–Congo haemorrhagic fever virus (CCHFV) genomes extracted from patients' samples. Full CCHFV genome coverage recovery (A), ×100 (B), and ×30 (C) sequencing read depths detailed for the S, M, and L segments (represented as a percentage on the *x*-axis) for each ribavirin-treated patient (blue) and each control group patient (green), decreased from Day 2 through to Day 4 post hospitalization (from dark to light blue or green, respectively) with the decreasing viral load.

copies per mL of serum (Figure 1A). The SGS scores for the ribavirin-treated patients were 2 (low risk), 2 (low risk), and 8 (moderate risk), and for the untreated patients were 0 (low risk), 5 (low risk), and 7 (moderate risk), with no fatal outcomes for any of the patients (Figure S1).

Despite the similarity of the clinical severity scores between the two groups of patients, the viral load on Day 1 post hospitalisation in the patients treated with ribavirin was higher, ranging between 3.4×10^6 and 4.5×10^7 copies/mL, compared to the patients who received supportive care only with an average viral load between 1.1×10^6 and 3.6×10^6 copies/mL (Figure 1A). However, the viral load decreased at similar rates in both groups of patients, with an average daily rate of decrease in ribavirin-treated patients at a factor of 0.29 ± 0.85 (1 standard deviation [SD]), compared to 0.42 ± 0.69 (1 SD) in the control patients (Figure 1B) ($p = 0.7439$). On the final day of sampling, Day 11, only one patient within each group had detectable CCHFV RNA. The other two patients in the ribavirin-treated group had no CCHFV RNA detected on Days 10 and 11, whereas in the control group, no CCHFV RNA was detected on Days 9 and 11.

3.2 | CCHFV genome reference coverage and sequencing depth

Only samples from Days 1 to 4 of hospitalisation were included in the study (Figures 2 and S2), because samples from subsequent days did not yield sufficient sequencing depth and genome coverage to perform accurate mutation rate analysis. The average genome coverage for the samples included in the analysis was 100% for both Day 2 and Day 3 of the sampling period and dropped to an average of 95% by Day 4 (Figure 2A).

Variant frequency rates were determined only in regions of the CCHFV genome that qualified, based on minimum sequencing depth criteria. High-frequency mutations, occurring at a rate equal or above 10% of all reads, were called only when at least ×30 sequencing

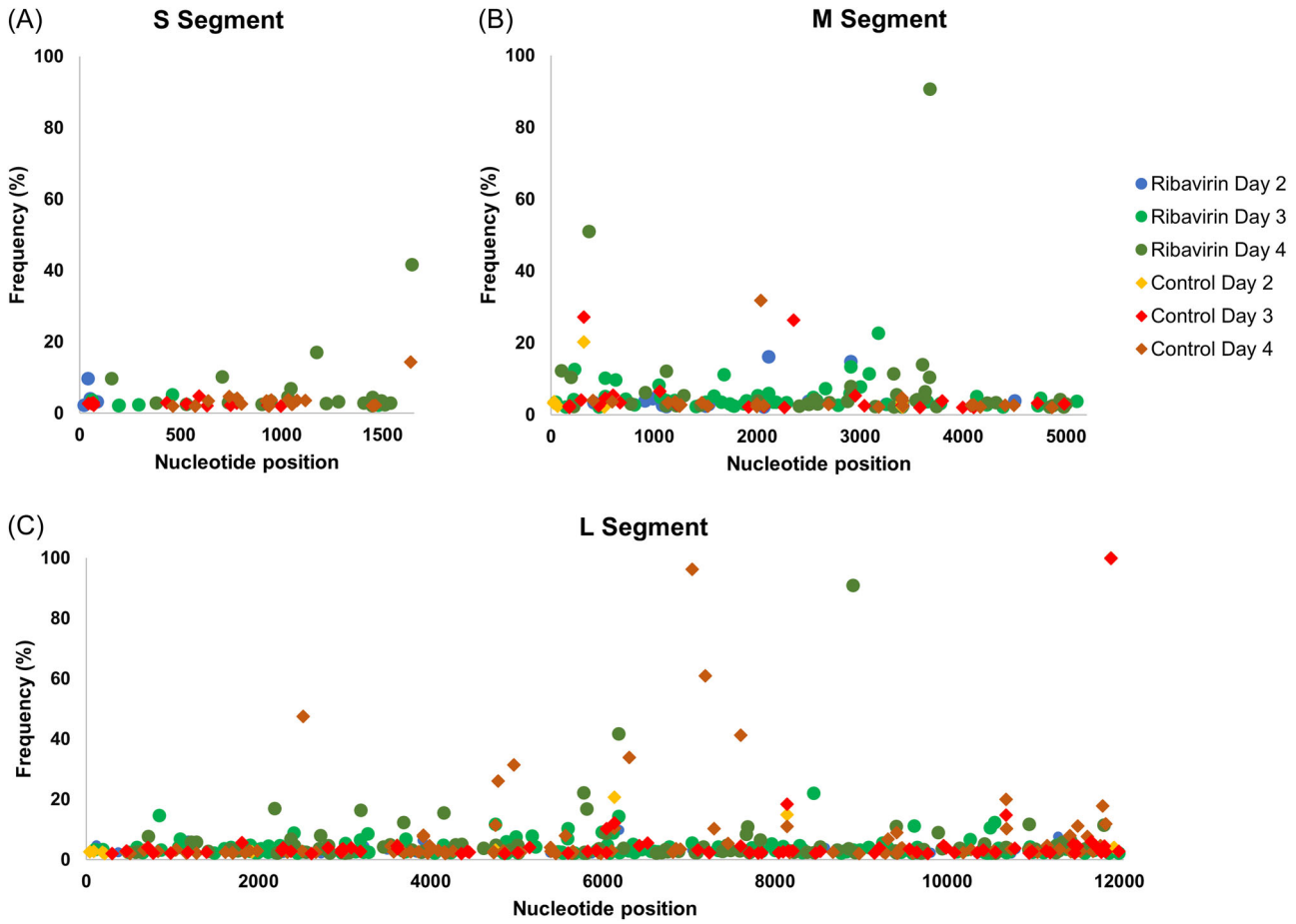

FIGURE 3 Distribution of all identified variants along the three segments of the Crimean–Congo haemorrhagic fever virus (CCHFV) genome in ribavirin-treated and control groups. Variant frequencies are shown at their absolute positions along the CCHFV S segment (A), M segment (B), and L segment (C). Variants with a frequency equal to or above 2% at each position were determined for all ribavirin-treated patients on Days 2 (blue), 3 (bright green), and 4 (dark green) and for control group patients on Days 2 (yellow), 3 (red), and 4 (orange) showing an even distribution of mutations across each segment of the complete CCHFV genome in both groups.

depth was obtained (Figure 2B). On average, on Day 4 88.03% of the S segment, 61.45% of the M segment, and 77.25% of the L segment presented with ≥×30 read depth in the ribavirin-treated group (Figure 2B, blue), whereas in the control group, the equivalent read depth was found in 88.01% of S segment, 52.23% of M segment, and 61.61% of L segment (Figure 2B, green).

Low-frequency variants, designated between 2% and 10% rate, were only called if ×100 sequencing read depth was obtained (Figure 2C). With decreasing CCHF viral loads, ≥×100 sequencing depth in the ribavirin-treated patient group had dropped to 72.50% of the S segment, 51.36% of the M segment, and 61.21% of the L segment (Figure 2C, blue). In the control patient group, the same sequencing depth was found in 66.73% of the S segment, 34.65% of the M segment, and 39.36% of the L segment (Figure 2C, green).

3.3 | Distribution of variants across the CCHFV genome

Subclonal diversity of CCHFV genomes was measured for each sample on the day of ribavirin treatment initiation (Day 2) and following a period of 2 days (Days 3 and 4). Diversity was measured as the rate of nucleotide changes (substitutions, insertions, and deletions) per site, per segment, which occurred between the samples' sequences and the first time point reference sequence (Day 1). A higher number of sequence variants (n = 457) were detected in the ribavirin treatment group across all segments than in the control group (n = 273) (Figure 3). The additional sequence variants in the ribavirin-treated group were present at a low frequency, between 2% and 10% of sequencing reads, equating to an average variant frequency of 4.96% across the CCHFV genome. In contrast, the control group contained a higher proportion of high-frequency mutation(s) (≥10%), resulting in an average variant frequency of 6.29%. The highest average frequency was observed in the M segment for the ribavirin-treated group (5.88%), compared to 5.18% for the control group (Figure 3B), and in the L segment for the control group (7.41%), compared to 4.40% for the ribavirin-treated group (Figure 3C). On average, the mutation frequency increased for the ribavirin-treated group by 0.71% between Day 2 and Day 3, and by 2.14% between Day 3 and Day 4. In the control group for the same time points, there was on average a decrease of 4.45% followed by an increase of 2.55% in variant frequencies observed, respectively.

3.4 | Analysis of high-frequency mutation rates

An overall increase in high-frequency mutations was observed in both patient groups over the study period (Figure 4D). A total of 69 variants with a frequency ≥10% were called across the study.

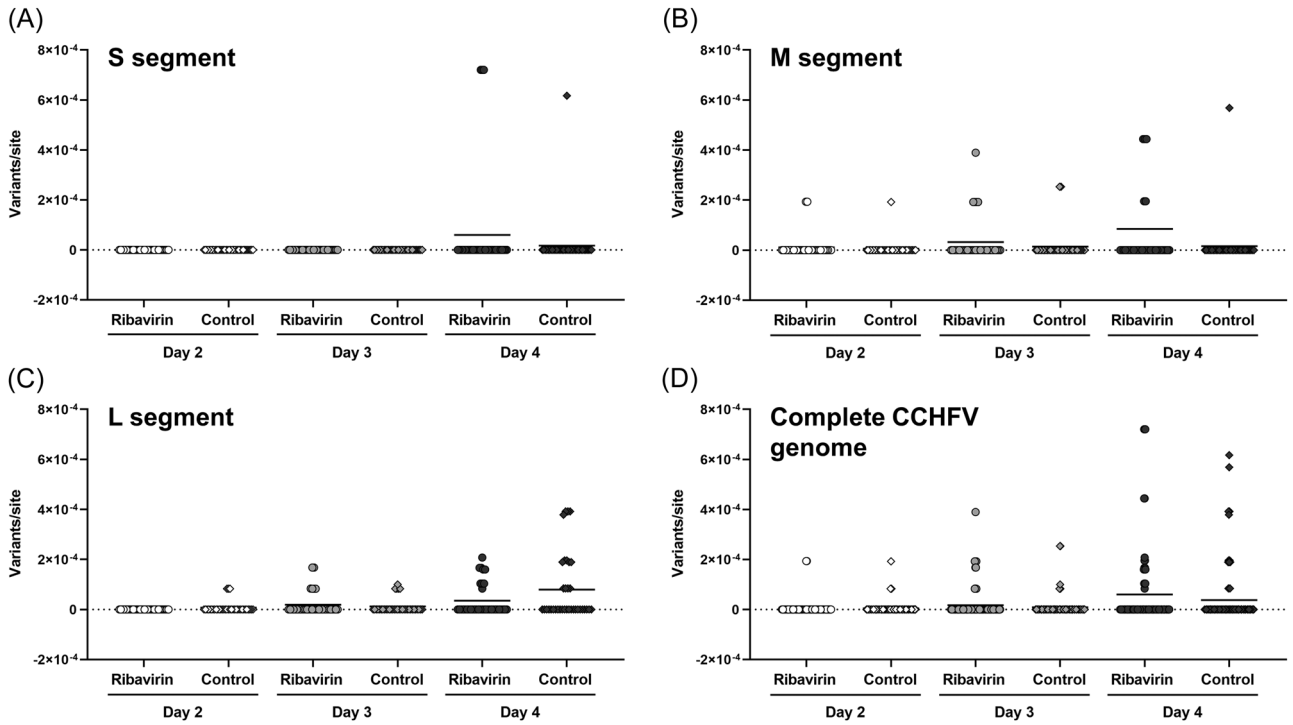

FIGURE 4 Comparison of high-frequency variants (≥10%) occurrence in the Crimean–Congo haemorrhagic fever virus (CCHFV) genome between the ribavirin-treated and control groups. Each high-frequency variant was reported for all ribavirin-treated (circles) and control group (diamonds) patients across the study period in the (A) S segment, (B) M segment, (C) L segment, and (D) cumulative for all segments. The rate of high-frequency variants was determined in all CCHFV segments from Day 2 (white circles and diamonds) through Day 3 (grey circles and diamonds) to Day 4 (black circles and diamonds) in both ribavirin-treated and control group patients. The horizontal bars indicate mean high-frequency variant rate values.

The highest proportion of high-frequency variants occurred in the L segment (65.2%) (Figure 4C), followed by the M segment (29%) (Figure 4B), and lastly the S segment (5.8%) (Figure 4A). On average, in the ribavirin-treated patients, high-frequency CCHFV mutants comprised 19.85% of the population, whereas in the control group, the average proportion of these mutants was 32%.

The ribavirin-treated group presented with an average mutations/site per segment of 6×10^{-5} two days post treatment initiation (Day 4), while at the same time point the control group had a mutations/site per segment of 3.7×10^{-5}. However, this difference was not statistically significant by Mann–Whitney *U* test ($p = 0.70$).

One day post treatment initiation (Day 3), the M segment had, on average, the highest occurrence of mutations at 2.3×10^{-5}, with the ribavirin-treated group having the highest frequency of mutations (Figure 4B). Two days post treatment initiation (Day 4) the L segment had on average a higher mutation rate/site than the S and the M segment, with a 5.7×10^{-5} variants/site (Figure 4C), compared to the S and M segment which presented respectively with 3.9×10^{-5} and 5×10^{-5} mutations/site (Figure 4A,B). High-frequency mutations were observed in the S segment only on Day 4 and were, on average, higher in the ribavirin-treated group, reaching a frequency of 6×10^{-5} (Figure 4A).

For the S segment, an average increase in A-to-G, T-to-C, and A-to-T mutations was observed in the ribavirin-treated group, whilst an increase in C-to-G mutations was observed in the control group (Figure 5A).

The M segment reported mostly an increase in C-to-T transitions, with the highest mutations/site per segment observed on Day 4 at 1.8×10^{-4} in the control group, and 1.2×10^{-4} in the ribavirin-treated group (Figure 5B).

The L segment reported a predominant increase in G-to-A and C-to-T transitions, with the highest mutations/site per segment recorded in the control group, reaching 2.2×10^{-4} for both types of mutations, and 8.9×10^{-5} and 5.5×10^{-5} for the ribavirin-treated group, respectively, for C-to-T and G-to-A mutations (Figure 5C). The observed average values were not significantly different, as determined by Mann–Whitney statistical analysis, between the ribavirin-treated and control patients for any of the segments ($p = 0.13$ for G-to-A mutations and $p = 0.28$ for C-to-T mutations in the L segment on Day 4; $p = 0.76$ for G-to-A and $p = 0.63$ for C-to-T mutations in all segments on Day 4).

3.5 | Analysis of low-frequency mutation rates

On average, low-frequency (2%–10%) mutations increased for all segments in both patient groups throughout the study period (Figure 6D). A total of 661 low-frequency variants were called, of

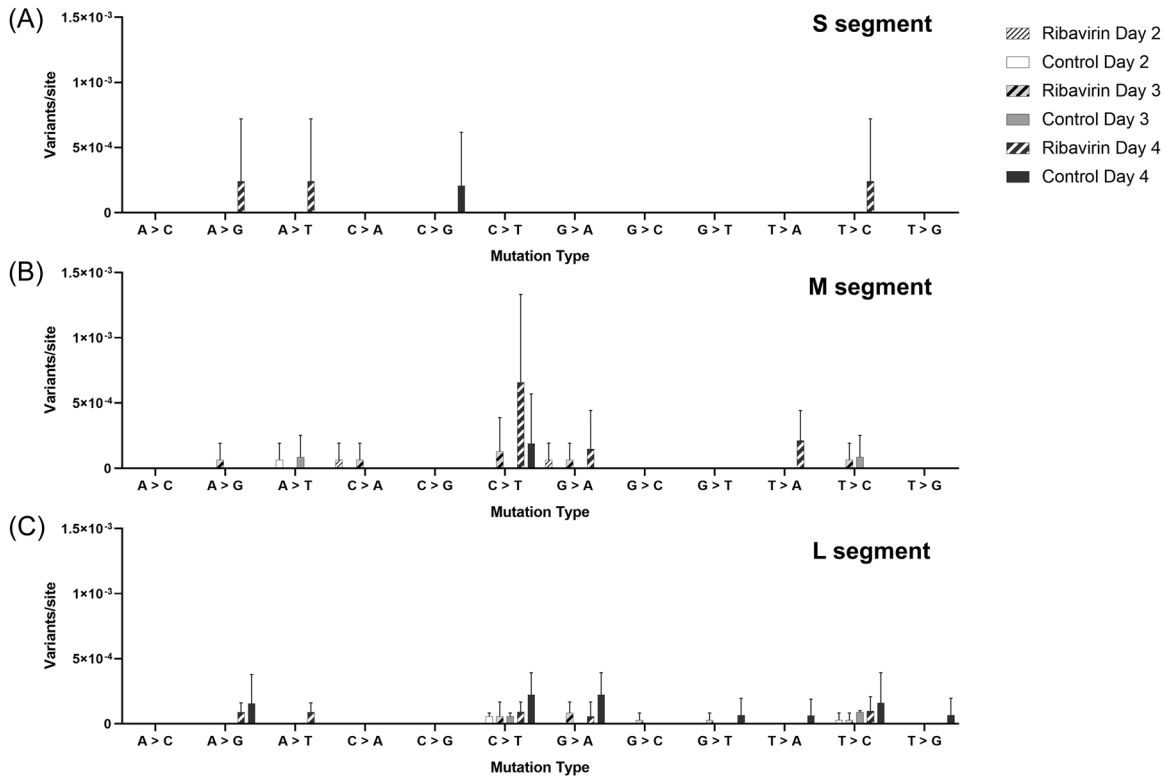

FIGURE 5 Comparison of the mutation rate of each type of high-frequency variant in the Crimean–Congo haemorrhagic fever virus (CCHFV) genome (≥10%) between the ribavirin-treated and control groups. Average rate of each high-frequency variant type was reported for the S (A), M (B), and L (C) segments. The frequency rate of each type of variant was determined for each day of the study in the ribavirin-treated patients (Day 2—black cross lines on white bars, Day 3—bold black cross lines on grey bars, Day 4—white cross lines on black bars) and control group patients (Day 2—white bars, Day 3—grey bars, Day 4—black bars). Error bars are included as a measure of the range of all determined rates of high-frequency mutation types.

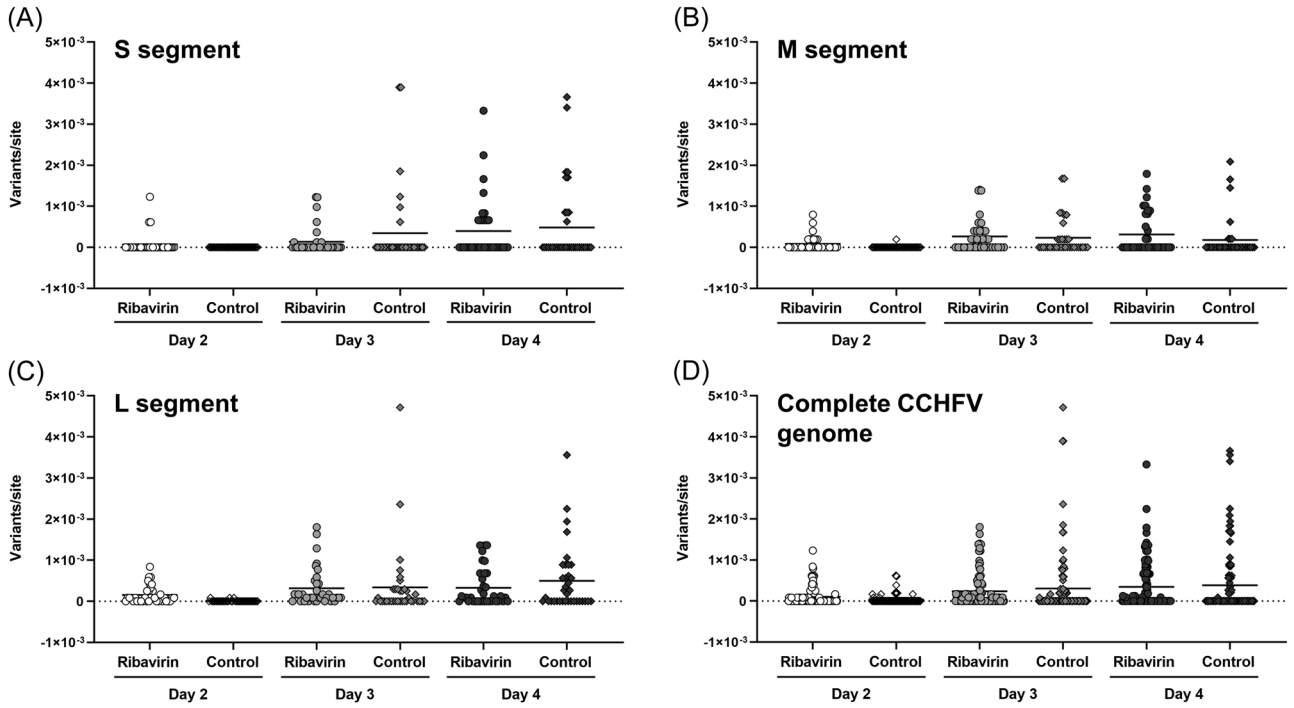

FIGURE 6 Comparison of low-frequency variants (2%–10%) occurrence in the Crimean–Congo haemorrhagic fever virus (CCHFV) genome between the ribavirin-treated and control groups. Each low-frequency variant was reported for all ribavirin-treated (circles) and control group (diamonds) patients across the study period in the (A) S segment, (B) M segment, (C) L segment, and (D) cumulative for all segments. The rate of high-frequency variants was determined in all CCHFV segments from Day 2 (white circles and diamonds) through Day 3 (grey circles and diamonds) to Day 4 (black circles and diamonds) in both ribavirin-treated and control group patients. The horizontal bars indicate mean high-frequency variant rate values.

which 67.5% were observed in the L segment, 23.1% in the M segment, and 9.4% in the S segment. 63.2% of all low-frequency variants were recorded in the ribavirin-treated group, while 36.8% were in the control group. Low-frequency mutants represented a greater proportion of the control group population on Day 3 and Day 4, compared to the ribavirin-treated group, although differences were not statistically significant ($p = 0.20$ and $p = 0.44$, respectively).

Mutation frequency decreased in the M segment for the control group between Day 3 and Day 4. The greatest frequency of mutations/site per segment was recorded in the M segment on the first day of treatment initiation (Day 2), with a frequency of 8.2×10^{-5} and 4.3×10^{-5} for the ribavirin-treated and control groups, respectively (Figure 6B). On Day 3 the rate of mutations was on average highest in the L segment, reaching 3.2×10^{-4} and 3.4×10^{-4} in the ribavirin-treated and control groups, respectively (Figure 6C). The S segment reported the highest average frequency of mutations on Day 4, reaching 3.9×10^{-4} and 4.8×10^{-4} mutations/site per segment for the ribavirin-treated and control group, respectively (Figure 6A).

An increase in A-to-G, T-to-C, C-to-T, and G-to-A transitions were observed in both ribavirin-treated and control groups and represented the most common mutation types observed in all three segments across the study period (Figure 7). The increase of C-to-T mutations in the ribavirin-treated patients' group in the M segment on Day 4 was statistically significant when compared to the untreated patients control group ($p = 0.05$) (Figure 7B).

4 | DISCUSSION

In this study, we examined the effect of ribavirin on the mutation rate of the CCHFV genome in patients treated with ribavirin during the acute stages of infection and compared it to patients who received supportive care only. This study was not a randomized control trial of the efficacy of ribavirin on CCHF disease outcome. Samples were collected from patients over the course of their hospital stay and subsequently grouped based on the patient's ribavirin treatment plan.

Conflicting arguments regarding the efficacy of ribavirin in treating CCHF have been published, suggesting that ribavirin may be effective in improving prognosis and as a prophylactic for healthcare workers,[16,19,37–39] while equally compelling evidence opposes this view, dismissing the usefulness of ribavirin in treating CCHF, reducing viral load and alleviating unfavorable outcomes.[17,18,20,40,41] In 2018, a review on the efficacy of ribavirin for treating CCHF was published in the Cochrane Database of Systematic Reviews and concluded that currently there is no strong evidence that ribavirin is effective at treating CCHF due to the large number of non-randomized studies which are often cited as evidence.[15] In the same year, a study was published that examined the effect of ribavirin on CCHF viral populations using NGS and alleged that ribavirin had a mutagenic effect on CCHFV due to a reported higher mutation rate when compared to the base error rate of RNA viruses polymerase.[28] However, this conclusion was based on

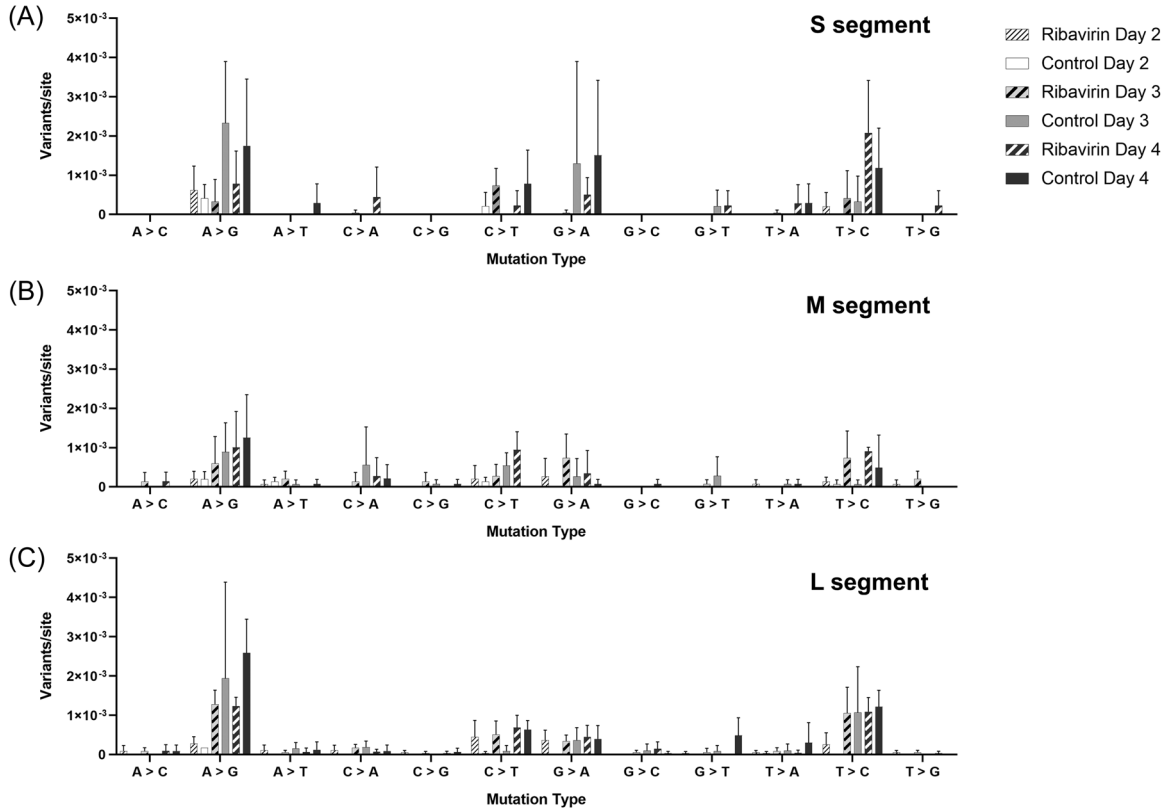

FIGURE 7 Comparison of mutation rate of each type of variant in the Crimean–Congo haemorrhagic fever virus (CCHFV) genome (2%–10%) between the ribavirin-treated and control groups. Average rate of each low-frequency variant type was reported for the S (A), M (B), and L (C) segments. The frequency rate of each type of variant was determined for each day of the study in the ribavirin-treated patients (Day 2—black cross lines on white bars, Day 3—bold black cross lines on grey bars, Day 4—white cross lines on black bars) and control group patients (Day 2—white bars, Day 3—grey bars, Day 4—black bars). Error bars are included as a measure of the range of all determined rates of high-frequency mutation types.

data from a single CCHF patient, furthermore it was not controlled by a related patient group that did not receive ribavirin, despite the longer study period (daily sampling up to Day 9).[28] In the current investigation, we had the opportunity to study more CCHF patients and to include a control group, who did not receive ribavirin, supported by exhaustive clinical data and real-time PCR analysis. In contrast to the approach used by Espy et al.[28] who employed a probe-enrichment protocol, we examined the mutation rates of the CCHFV genome from clinical samples using an unbiased SISPA sequence enrichment methodology, combined with paired-end Illumina sequencing. While it has been suggested that the SISPA sequencing library enrichment method has the potential to introduce sequencing errors,[42] recently it was shown that the methodology affects the recovery of sufficient sequencing depth in genome areas of high complexity and rich GC content, rather than bias.[43,44] Thus, to negate this issue, we only called high-frequency variants (appearing in ≥10% of obtained reads) in regions with at least ×30 sequencing depth, while low-frequency mutations (appearing in 2%–10% of obtained reads) were only recorded in areas with at least ×100 sequencing depth, bolstered by high-confidence paired-end Illumina sequencing data.[28] Furthermore, we had the unique opportunity to compare the dynamic CCHFV genome mutagenic rate in clinical samples of ribavirin-treated patients (on average 1.27×10^{-4} mutations/site) with that in a control group (on average 1.3×10^{-4} mutations/site) of CCHF patients who did not receive ribavirin treatment. The observed mutation rates are comparable to those reported for RNA viruses during infection and for purified viral RNA polymerases, which range from 10^{-3} to 10^{-6} mutations per nucleotide.[45]

RNA viruses have exceptionally high mutation rates, up to a million-fold higher than those of their hosts' genome, which can be advantageous for adaptation to changing environments, immune evasion, host switching, and transmissibility and may also affect virulence.[45–47] However, these mutation rates are almost ruinously high, with RNA viruses existing at the threshold of extinction level mutation frequency[48]—a weakness that could be exploited during antiviral treatment by tipping the viral population into lethal mutagenesis and causing a reduction in fitness level.[49–52] The broad-spectrum guanosine analog ribavirin has been shown to induce a mutation "error catastrophe" event in RNA viruses in vitro and in vivo by inducing an increase of C-to-T and G-to-A mutations.[53–57] In accordance with this proposed mechanism, we detected a statistically significant increase of low-frequency C-to-T transitions in the M segment of the ribavirin-treated group by Day 4. However, an

increase in G-to-A transitions (another mutation associated with ribavirin) was observed in both groups at levels that were not significantly different.

We observed a similar rate of decrease in virus titers for both ribavirin-treated and control groups, suggesting that ribavirin treatment did not contribute to a reduction of the viral load and therefore other factors must be involved in CCHFV clearance. Patients were monitored over a period of 11 days, although viral load post Day 4 following hospitalization was insufficient to achieve the required CCHFV genome coverage and depth to identify low- and high-frequency mutations. Nevertheless, we were able to observe the immediate effect of ribavirin and assess its mechanism of action as a mutagen of CCHFV nucleic acid.

It appears that ribavirin administered at the WHO-recommended dose and at the acute stage of viremia is unlikely to have a significant lethal mutagenic effect on the CCHFV genomes to affect viremia by tipping the mutagenic balance of the CCHFV population to extinction level. This is consistent with previous observations that ribavirin incorporation in the viral genome may be of insufficient frequency to induce fitness-reducing mutations in the viral nucleic acid[56] and cause a decrease of the viral load. The detected C-to-T low-frequency mutations in the M segment, encoding the viral glycoprotein, were likely tolerated at a low level in the viral population and were not lethal. The inclusion of the control group enabled us to infer that the rate of reduction of the viral load could not be attributed to ribavirin treatment alone.[56]

In conclusion, after comparing the rate of mutation of the CCHFV genome in patients treated with ribavirin in the acute stages of infection and in patients who did not receive ribavirin, we found little evidence of an overall mutagenic effect. While there was an increase in only low-frequency C-to-T transitions in the M segment of the ribavirin-treated patients' group on Day 4, this frequency was too low to affect error catastrophe. Further studies on a larger set of samples over a longer sampling period during ribavirin treatment in different endemic regions with different circulating CCHFV clades, employing targeted sequencing approaches, will help to ascertain whether ribavirin treatment can induce persistent fitness-damaging mutations in the CCHFV genome during later stages of human infection. Indeed, this approach offers a convenient advantage over the many difficulties encountered previously[15] in devising randomly controlled clinical trials of ribavirin.

AUTHOR CONTRIBUTIONS

Conceptualization and administration of the project: Roger Hewson, Nazif Elaldi, Gillian Slack, and Nadina Wand. *Designed the study*: Roger Hewson, Nazif Elaldi, Nadina Wand, and Jake D'Addiego. *Collected and synthesized the data*: Jake D'Addiego, Nadina Wand, Roger Hewson, Nazif Elaldi, Binnur Koksal Bagci, Karen Osman, Emma Kennedy, and Ayse Nur Pektas. *Interpreted the data*: Jake D'Addiego, Nadina Wand, Nazif Elaldi, Karen Osman, Ayse Nur Pektas, Binnur Koksal Bagci, and Roger Hewson. *Prepared the first draft of this manuscript*: Nadina Wand, Jake D'Addiego, Roger Hewson, and Nazif Elaldi. *Edited the final manuscript*: Nadina Wand, Jake D'Addiego, Roger Hewson, Nazif Elaldi, and Gillian Slack. All authors have read and approved the final draft of this manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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