Emergence of Multi-Dideoxynucleoside–Resistant Human Immunodeficiency Virus Type 1 Variants, Viral Sequence Variation, and Disease Progression in Patients Receiving Antiretroviral Chemotherapy

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A set of five reverse transcriptase mutations, which include Q151M, is known to confer multidideoxynucleoside resistance (MDR) in human immunodeficiency virus type 1 (HIV-1). MDR mutations were found in 6 (17%) HIV-1 isolates from 36 patients, most of whom were receiving long-term combination therapy. Q151M was among the first of the substitutions to appear. Additional substitutions were observed, although none were common among all 6 patients. Certain zidovudine-related mutations were not observed together with the MDR mutations, indicating possible enzymatic constraint. During chemotherapy, the HIV-1 RNA levels in the 6 patients initially decreased and then rose. Initially, CD4 cell counts also responded favorably but were near or below baseline beyond 40 months of therapy. Such loss of clinical benefits appeared to coincide with the appearance of the MDR mutations. A common background genotype was not observed among HIV-1 isolates with or without MDR.

Human immunodeficiency virus type 1 (HIV-1) develops resistance against virtually any currently available therapeutic reverse transcriptase (RT) and protease inhibitors [1–6]. In an attempt to delay or block the development of drug-resistant HIV-1 variants and alleviate drug toxicities, multidrug antiviral therapy has been widely used [7, 8]. However, a set of five mutations in the pol gene (including Ala 62→Val substitution [A62V], V75I, F77L, F116Y, and Q151M) has been identified in HIV-1 isolates from patients receiving long-term combination chemotherapy with zidovudine plus zalcitabine or zidovudine plus didanosine. This set of mutations confers onto HIV-1 reduced sensitivity to multiple dideoxynucleosides [5, 9–13]. Detailed studies on sequentially obtained clinical HIV-1 isolates have shown that these mutations develop in a relatively ordered manner and that a stepwise increase in resistance is associated with the accumulated mutations.

The alteration of RT’s substrate recognition, caused by the five mutations, appears to account for the viral multidideoxynucleoside resistance (MDR) [14, 15]. Indeed, a marked difference has been noted in inhibition constants (K_i; e.g., the K_i of a mutant RT carrying the five mutations is 62-fold higher for AZTTP than is the K_i of wild-type RT [RTwt]); however, steady-state kinetic studies have shown that RTwt and RTs carrying combined mutations have comparable catalytic efficiency (k_cat/K_m) as examined using homopolymeric DNA and heteropolymeric RNA templates [14, 15]. Furthermore, it has been demonstrated that, as assessed in vitro in the presence of zidovudine or didanosine, HIV-1 with the five amino acid substitutions has a replication advantage over that of HIV-1, with fewer MDR-associated amino acid substitutions [16].

The frequency of the emergence of these MDR-associated amino acid substitutions appears to be relatively low, although many of the patients examined to date have received combination chemotherapy for ≤2 years [11, 17]. It is conceivable that the frequency may increase in the future, considering that longer periods of combination chemotherapy consisting of multiple RT inhibitors with or without protease inhibitor(s) will be used more frequently. Therefore, in this study, we examined the RT-encoding gene of HIV-1 isolates from 36 patients, most of whom were receiving combination therapy with zidovudine plus didanosine for >36 months. We also examined whether the emergence of the MDR-associated amino acid substitutions was related to immunologic, virologic, and clinical manifestations.

Materials and Methods

Patients. All patients were enrolled in research protocols of the National Cancer Institute and were seen in the outpatient department of the Warren Grant Magnuson Clinical Center, National Institutes of Health. At each clinical visit, patients underwent a variety of clinical and laboratory evaluations, including assessment of lymphocyte subsets and measurement of serum HIV p24 antigen. Clinical aspects of most patients have been reported elsewhere [18]. Of the 36 study subjects (31 adults and 5 children), 30 received zidovudine plus didanosine combination therapy, while 4 received zidovudine plus zalcitabine. One patient received dida-
nosine monotherapy followed by zidovudine monotherapy, and the remaining patient received consecutive monotherapies of didanosine, zidovudine, lamivudine, and zalcitabine.

**Preparation of peripheral blood mononuclear cells (PBMC).** PBMC were isolated from heparinized blood obtained during scheduled clinical visits through differential centrifugation with lymphocyte separation medium (Organon Teknika, Durham, NC) or Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden), washed twice with RPMI 1640, and counted. Cells were then washed with PBS and resuspended in lysis buffer (containing NP-40, Tween 20, polymerase chain reaction [PCR] buffer, and protease K) to 7.5 × 10^6–10^7 cells/mL, depending on the available number of cells. Cell lysates were heated at 56°C for 2 h or overnight, then protease K was inactivated at 95°C for 5 min, followed by a 1-min centrifugation to remove cellular debris. Frozen PBMC were rapidly thawed, washed twice with complete medium, counted, and further processed as described above.

**Determination of nucleotide sequences of HIV-1.** Cell lysates were subjected to nested PCR. The first round of PCR was 35 cycles with a 55°C annealing temperature, and utilized primers SA009 (5'-TTTTAATTTCATGGCCTAT-3') and SA015 (5'-ACTCCTAGTCTGGTTCCTTTAGA-3'), which generated a fragment including codons 1–272 of RT. First-round PCR products (1 µL) were used directly in the second round of PCR, which consisted of 25 cycles at a 55°C annealing temperature, with primers 881MF (5'-TGTTAAAACGAGCGGCTCCG-GGATGTAGGGCCAAAAAGTAAACCA-3') and 891MR (5'-CAGGGAAACGCTATGACCGCTAGCCCAATTACATTTT- TCCCCACTAA-3'), which included the M13 forward and M13 reverse standard sequences, respectively. This generated a fragment that spanned codons 16–266 of RT and contained an M13 tail at each end.

Second-round PCR products were first purified with PCR Select III columns (5 Prime—3 Prime, Boulder, CO) and directly sequenced using both M13-forward and M13-reverse dye-labeled primers on an automated DNA sequencer (model 373; Perkin-Elmer, Foster City, CA). Following electrophoresis, sample files were processed by use of Factura software (Perkin-Elmer). Heterozygous base sequences were identified when electropherograms showed a minor peak at ≥50% of the major peak. Sample files were aligned by use of AutoAssembler software (Perkin-Elmer) to generate a deduced nucleotide sequence, which was translated to yield the amino acid sequence.

**Molecular cloning.** Several HIV-1 isolates were subjected to molecular cloning followed by sequence determination as previously described [5, 10]. The second-round PCR products were digested with XmnI and NheI, purified, and inserted into the pTZ19R vector [5]. Competent cells (DH5α; Life Technologies Gibco BRL, Gaithersburg, MD) were transformed, and individual colonies were grown in LB broth containing ampicillin. Plasmid DNA was purified with Tip 20 columns (Qiagen, Chatsworth, CA). Alternatively, PCR products were purified with PCR Select III columns and cloned directly (TA Cloning Kit; Invitrogen, San Diego). Molecular clones were sequenced as described above.

**HIV-1 RNA quantification.** Serum levels of HIV RNA were determined for patients A–E by use of an assay (Amplicor; Roche Diagnostic Systems, Somerville, NJ) following the manufacturer’s protocol. The HIV-1 RNA level for patient F was determined by RT-PCR assay as previously described [5, 10].

**Results**

**Development of MDR mutations among patients receiving combination therapy.** We examined HIV-1 sequences from 36 patients to identify the prevalence of the Q151M mutation: ~700 bp of the pol gene, spanning codons 30–260 of RT, was examined. Of the 36 patients, 6 (17%; patients A–F) were infected with HIV-1 that contained one or more of the MDR mutations. Treatment regimens, dosages, and relevant clinical information for these patients are shown in table 1. Patients A, B, D, and E had received a simultaneous or alternating therapy with zidovudine and didanosine for 28, 16, 39, and 38 months, respectively, when the MDR mutations were first identified. Patient F had received an alternating therapy with zidovudine and zalcitabine for 16 months when Q151M was identified, as previously described [5, 10]. However, in patient C, Q151M was first identified following 57 months of didanosine monotherapy. The only prior antiviral chemotherapy given to this patient was 3 months of zidovudine therapy, which had concluded 12 months prior to commencement of didanosine.

**Changes in AIDS-associated clinical parameters.** CD4 cell counts and viral RNA levels in patient serum samples were assessed over the course of therapy in the 6 patients (figure 1). CD4 cell counts initially showed a favorable response to therapy, with a declining trend as treatment ensued. None of the 6 patients showed a marked or sustained improvement in CD4 cell counts. At the end of study, CD4 cell counts were only slightly higher than at baseline for patients A (16%) and E (31%) and at baseline for patient D. However, CD4 cell counts had declined by 67%, 89%, and 60% for patients B, C, and F, respectively.

All patients (except patient F, whose pretherapy samples were not available) had a mean log decrease of 1.17 (range, 0.10–2.33) relative to baseline in HIV-1 RNA levels within 1 month of therapy. However, the viremia levels also rose as therapy ensued, and at the completion of study, the levels were close to baseline RNA levels in all patients, ranging from a 0.62 log decrease to a 1.52 log rise above baseline (mean, 0.08 log increase; figure 1). As mentioned above, baseline HIV-1 RNA was not determined for patient F; however, it rose from 50 copies/mL at 1 month of therapy to 11,500 copies/mL at the end of study after 42 months of therapy.

**Development of MDR mutations.** HIV-1 isolated from patient C showed the appearance of Q151M first among the set of five MDR mutations (figure 1), as previously reported for patient F [10]. In the other 4 patients (A, B, D, and E), Q151M was first identified together with two to four of the remaining MDR mutations; however, this simultaneous appearance may reflect the limitations of sample availability. HIV-1 isolated from patients B and C developed a set of four MDR mutations, which excluded the previously reported A62V [5, 9]. However, these HIV-1 populations did acquire the zalcitabine or didanosine resistance–associated mutation, K65R [19], and zidovudine resistance mutations, K70R and K219E or K219Q [20–22]
Table 1. Clinical profiles of 6 patients whose HIV-1 developed the MDR mutations.

<table>
<thead>
<tr>
<th>Patient, age (years)</th>
<th>Treatment dose (mg/day), and mode</th>
<th>Prior antiviral treatment</th>
<th>At study entry</th>
<th>At study end</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of CD4 cells (/mm$^3$)</td>
<td>Level of p24 (pg/mL)</td>
<td>Diagnosis</td>
<td>Length of therapy (months)</td>
</tr>
<tr>
<td>A, 30</td>
<td>Zidovudine (300) + didanosine (200–250), S</td>
<td>None</td>
<td>288</td>
<td>ND</td>
</tr>
<tr>
<td>B, 28</td>
<td>Zidovudine (300) + didanosine (250–500), S</td>
<td>None</td>
<td>309</td>
<td>70</td>
</tr>
<tr>
<td>C, 33</td>
<td>Didanosine (44–540) + zidovudine (1000–1200), C</td>
<td>Zidovudine + acyclovir</td>
<td>153</td>
<td>ND</td>
</tr>
<tr>
<td>D, 32</td>
<td>Zidovudine (600) + didanosine (500), A</td>
<td>Zidovudine (300 + didanosine (200), S</td>
<td>None</td>
<td>286</td>
</tr>
<tr>
<td>E, 39</td>
<td>Zidovudine (300) + didanosine (200–250), S</td>
<td>None</td>
<td>343</td>
<td>ND</td>
</tr>
<tr>
<td>F, 28</td>
<td>Zidovudine (1200) + zalcitabine (12), A</td>
<td>None</td>
<td>269</td>
<td>283</td>
</tr>
</tbody>
</table>

NOTE. All subjects were men. Treatment modes are expressed as S, simultaneous; C, consecutive; or A, alternating every 2 weeks (zidovudine / didanosine) or every week (zidovudine + zalcitabine). Patient C received 3 months of zidovudine (1200/day) + acyclovir concluding 12 months prior to didanosine monotherapy. Patient F was first reported by Shirasaka et al. (ERS103) [5, 10]. Acid-dissociated p24 levels in serum were determined at end of each therapy. ND = not detected, ARC = AIDS-related complex, OI = opportunistic infection. Determined at $^*$ 40, $^t$ 90, and $^t$ 38 months of therapy.

Additional mutations developed during therapy. We also asked whether other amino acid substitutions developed in addition to the five MDR mutations. Although no mutations were observed in common among the 6 patients, S68G was present in HIV-1 isolated from patients A, B, and C (table 2). The zalcitabine resistance–associated T69I substitution [23] was also identified in HIV-1 isolated from patients B and C, although these patients were zalcitabine naive. This also noted was a substitution K83R in patients B and E and differing amino acid substitutions at codon 162 in patients A, C, and D, to tyrosine, tryptophan, and histidine, respectively. We further asked whether other mutations might precede Q151M. This occurred only with HIV-1 isolated from patient C, in which A98S and T200A were observed before Q151M appeared.

MDR versus zidovudine resistance pathways. Finally, we asked whether a certain genetic background might predispose HIV-1 to develop MDR mutations as opposed to T215Y/F or other drug resistance–related mutations. When searching for residues in common between $\geq 2$ patients, only Lys at codon 122 and Phe at codon 214 were found among HIV-1 isolated from patients A, B, D, and E (data not shown). However these amino acids are common among published HIV-1 RT sequences [24] and are indeed present among 13 and 27, respectively, of the other 30 patients in this study who did not develop the MDR mutations. Conversely, among the 30 patients whose
Figure 1. Clinical correlates of disease progression relative to appearance of MDR in 6 HIV-1-infected patients. Serum HIV-1 RNA levels and CD4 cell counts are shown, as are MDR and zidovudine and didanosine resistance–related amino acid (aa) substitutions identified in polymerase-encoding region of HIV-1 isolates. Also noted is substitution S68G in patients A–C. Underlined aa substitutions indicate mixtures of wild-type (wt) and mutant aa. Treatment regimens and durations are indicated in bars. lam = lamivudine, did = didanosine. Patient F was first reported by Shirasaka et al. [5, 10].
Table 2. Reverse transcriptase amino acid (aa) substitutions in HIV-1 isolated from 6 patients following therapy.

<table>
<thead>
<tr>
<th>MDR substitutions</th>
<th>Zidovudine-related substitutions</th>
<th>Didanosine-related substitution</th>
<th>Additional substitutions identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>A62</td>
<td>V75</td>
<td>F77</td>
</tr>
<tr>
<td>A</td>
<td>V</td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td>B</td>
<td>—</td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td>C</td>
<td>—</td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td>D</td>
<td>V</td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td>E</td>
<td>V</td>
<td>I</td>
<td>L</td>
</tr>
<tr>
<td>F</td>
<td>V</td>
<td>I</td>
<td>L</td>
</tr>
</tbody>
</table>

NOTE: aa and positions in HIV-1 LAI strain are referenced at top. — indicates that aa is same as reference sequence. In patient C, A98S and T200A were identified prior to appearance of MDR mutations.

* Mixture.

Substitution from pretherapy aa different from reference sequence.

HIV-1 did not develop MDR, no apparent common genetic background was identified that might predispose those HIV-1 isolates to develop other drug resistance profiles, such as T215Y-mediated zidovudine resistance, as opposed to developing resistance via the MDR pathway.

Development of nucleoside resistance–related mutations in HIV-1 that did not show the MDR mutations. HIV-1 isolated from most of the remaining patients in this study showed the development of one or more zidovudine-resistance–related mutations (table 3). Of the 36 patients, 24 (67%) in this study developed at least one zidovudine-resistance mutation either alone or in combination with a zalcitabine or didanosine resistance–related mutation(s). The most prevalent zidovudine resistance–related mutations were M41L and T215Y, which were both present in isolates from 16 (44%) patients. Isolates from 13 (36%) patients had both M41L and T215Y. One patient’s HIV-1 had only a didanosine resistance-related genotypic change (L74V), and another’s had only a zalcitabine resistance-related genotypic change (T69D). Isolates from 4 (11%) of the 36 patients did not have substitutions associated with nucleoside resistance.

Discussion

In the present study, we identified a set or subset of amino acid substitutions (A62V, V75I, F77L, F116Y, and Q151M), which confer MDR on HIV-1, in 6 (17%) of 36 patients, most receiving long-term combination therapy of zidovudine plus didanosine. In isolates from 2 patients, the Q151M mutation...
Table 3. Mutations identified in HIV-1 isolates that did not exhibit MDR.

<table>
<thead>
<tr>
<th>HIV-1 resistance mutations observed</th>
<th>No. of patients (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zidovudine-related mutation(s) alone</td>
<td>17</td>
</tr>
<tr>
<td>Zidovudine- and zalcitabine-related mutation(s)</td>
<td>3</td>
</tr>
<tr>
<td>Zidovudine- and didanosine-related mutation(s)</td>
<td>2</td>
</tr>
<tr>
<td>Zidovudine- and didanosine/zalcitabine related mutation(s)</td>
<td>2</td>
</tr>
<tr>
<td>Didanosine-related mutation (L74V) alone</td>
<td>1</td>
</tr>
<tr>
<td>Zalcitabine-related mutation (T69D) alone</td>
<td>1</td>
</tr>
<tr>
<td>No nucleoside-related mutations</td>
<td>4</td>
</tr>
</tbody>
</table>

NOTE. Substitution M41L was observed in HIV-1 isolates from 16 patients, that is, M41L (16), D67N (9), T69D (4), K70R (8), L74V (5), M184V (2), L210W (9), T215Y (16), T215F (5), K219Q (5), and K219E (3). Although not previously reported to be associated with drug resistance, the following were also observed: D67G (1), D67E (1), L74I (1), and V75M (1). Zalcitabine resistance-related mutation, T215C, was observed in 1 reportedly zalcitabine-naive patient. Two other zalcitabine-naive patients showed zalcitabine resistance-related mutation T68D. *L74V (HIV-1 from 2 patients) and M184V (HIV-1 from 1 patient) may have arisen in response to either zalcitabine or didanosine, since both antivirals were used by these patients.

appeared first, while in isolates from 4 other patients, Q151M plus two or more mutations appeared together (figure 1). In 2 of 3 patients whose HIV-1 developed the MDR mutations reported by Iversen et al. [25], the Q151M mutation also occurred first, while in the third patient, the Q151M mutation appeared with other mutations. However, Q151M is not always among the first of the MDR mutations to appear: Demeter et al. [26] reported that a patient receiving combination therapy with zidovudine, didanosine, and delavirdine developed A62V, V75I, and F116Y mutations but not the Q151M mutation [26].

Of note, HIV-1 in 1 of the 6 patients (patient C) developed the Q151M mutation after 57 months of didanosine monotherapy. This HIV-1 later developed additional MDR mutations while the patient was changing to a combination regimen of zidovudine plus lamivudine. Thus, assuming strict treatment compliance in patient C, it appears that the Q151M mutation can develop following prolonged didanosine monotherapy. While most previously reported cases of HIV-1 developing the Q151M mutation were from patients receiving combination chemotherapy [5, 6, 9–12, 17, 25], this particular case may not be so rare. Indeed, Schmit et al. [12] reported an HIV-1 isolate that first developed the Q151M mutation after the patient received zalcitabine monotherapy and then didanosine monotherapy. In addition, preliminary results of another study by Schmit et al. [17] showed the Q151M mutation alone developed in a patient who received stavudine, didanosine, and lamivudine consecutively. Furthermore, Van Rompay et al. [27] reported that a simian immunodeficiency virus (SIV) isolate developed the Q151M mutation when macaques received prolonged zidovudine treatment [27]. Thus, it appears that MDR may develop under combination nucleoside drug or mononucleoside drug pressure. However, it remains possible that this particular SIV isolate and the HIV-1 isolates mentioned above may share an as yet unidentified similarity that allows the development of the Q151M mutation following nucleoside monotherapy.

In the present study, HIV-1 isolated from patients B and C developed K65R, S68G, T69I, and K70R and a substitution at codon 219 to E or Q, in addition to the MDR substitutions during therapy. HIV-1 from patient A also developed the S68G mutation. In patients A, C, and D, three different amino acid substitutions at codon 162 were seen following therapy but not at baseline: A162Y, C162W, and S162H (table 2). It is noteworthy that the substitutions S68G, T69I, and those at codon 162 have not been reported to date. The significance of substitutions at these codons, however, awaits further investigation.

It has been noted that despite long-term chemotherapy with zidovudine, HIV-1 variants carrying all or subsets of the five mutations do not possess the T215Y/F mutation, which is associated with viral resistance to zidovudine [5, 9–12, 26]. In this study, HIV-1 isolates from none of the 6 patients stably possessed the Q151M mutation together with the T215Y/F mutation, in agreement with previous findings [5, 10, 14]. In this respect, it is possible that these two mutations effect an enzymatic constraint toward each other. However, an HIV-1 infectious clone carrying both Q151M and T215Y in an HXB2D background actively replicates in vitro [16], and a recombinant RT with these two mutations have an enzymatic activity comparable to that of RTwt and is less susceptible to ddNTPs tested, ddATP, ddCTP, ddGTP, and AZTTP [14, 15], suggesting that the two mutations do not impair the replicative ability of HIV-1. It is possible however, that Q151M and T215Y in the background of a clinical isolate may have a different replication profile and may be incompetent.

It is also possible that a certain nucleotide or amino acid sequence(s) predisposes HIV-1 to develop the Q151M substitution and ultimately the rest of the MDR mutations or to develop the T215Y or T215F mutation. However, a comparison of RT sequences of HIV-1 from the 6 patients prior to therapy did not reveal a common background genotype. Similarly, among non-MDR HIV-1 isolates, a common genotype was not identified. It appears that the lack of coexistence of Q151M and T215Y/F among HIV-1 populations within individuals alludes to a model of two distinct pathways of development of resistance to zidovudine, both conveying high-level zidovudine resistance but the MDR pathway also providing resistance to other dideoxynucleosides.

In the present study, all patients (except patient F) had a mean 1.17 log decrease (range, 0.10–2.33) in HIV-1 RNA levels within 1 month of therapy (figure 1). Initially, CD4 cell counts also responded favorably (figure 1), but levels were near or below baseline beyond 40 months of therapy. Such loss of clinical benefits generally appeared to coincide with the appearance of the MDR mutations.

In consideration of studies demonstrating the in vitro phenotypic drug resistance of HIV-1 carrying MDR mutations [5,
9–12], the growth kinetics of MDR HIV-1 clones in the presence of drugs [16], and the expansion of MDR clones when cocultured with a wild-type clone in the presence of drugs (Kosalaraksa P, Mitsuya H; unpublished observations), there is a strong suggestion of a direct correlation between the development of MDR mutations and the loss of clinical benefits. Enzyme kinetic studies also corroborate this notion [14, 15]. It was noted that in patients A and C, HIV-1 had not developed detectable MDR mutations at the moment when viremia levels rose after the initial drop, but the mutations developed soon thereafter. However, this finding may be due in part to the fact that proviral DNA rather than circulating viral RNA was sequenced in this study. Furthermore, it could be a reflection of the limitations of direct PCR sequencing methodology or to falsely high viral RNA values at these time points.

It was noted that in 4 of the remaining 30 patients whose HIV-1 did not develop the MDR mutations, HIV-1 possessed no significant mutations in the pol gene sequences examined (approximately codons 35–260 of RT). This occurred despite HIV-1 viremia levels that rose during the course of therapy. It is possible that an as yet unidentified mutation(s) had developed outside the pol region examined in the present study. Indeed, HIV-1 has been reported to develop mutations in the area of RT codons 315–359 in individuals receiving zidovudine and lamivudine [28].

In this study, the 17% prevalence of the MDR mutations among patients receiving 16–39 months of therapy is noteworthy. Larder et al. [29] recently reported no evidence of Q151M among 100 patients; however, that survey examined HIV-1 from patients who had received only 48 weeks of therapy. The possibility that some of those patients may yet develop the Q151M mutation should not be excluded. It should be noted that the development of MDR resistance substantially reduces the therapeutic options available, and this mutation pattern, if not detectable MDR mutations at the moment when viremia levels rose after the initial drop, but the mutations developed soon thereafter. However, this finding may be due in part to the fact that proviral DNA rather than circulating viral RNA was sequenced in this study. Furthermore, it could be a reflection of the limitations of direct PCR sequencing methodology or to falsely high viral RNA values at these time points.

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References


