

Identification of Recombinant Human Rhinovirus A and C in Circulating Strains from Upper and Lower Respiratory Infections

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Abstract

Human rhinoviruses (HRVs), in the *Enterovirus* genus within the family *Picornaviridae*, are a highly prevalent cause of acute respiratory infection (ARI). Enteroviruses are genetically highly variable, and recombination between serotypes is known to be a major contribution to their diversity. Recently it was reported that recombination events in HRVs cause the diversity of HRV-C. This study analyzed parts of the viral genes spanning the 5' non-coding region (NCR) through to the viral protein (VP) encoding sequences of 105 HRV field isolates from 51 outpatient cases of Acute Respiratory Infection Network (ARINET) and 54 inpatient cases of severe lower respiratory infection (SLRI) surveillance, in order to identify recombination in field samples. When analyzing parts of the 5'NCR and VP4/VP2 encoding sequences, we found intra- and interspecies recombinants in field strains of HRV-A and -C. Nineteen cases of recombination events (18.1%) were found among 105 field strains. For HRV-A, there were five cases (4.8%) of intraspecies recombination events and three cases (2.8%) of interspecies recombination events. For HRV-C, there were four cases (3.8%) of intraspecies recombination events and seven cases (6.7%) of interspecies recombination events. Recombination events were significantly more frequently observed in the ARINET samples (18 cases) than in the SLRI samples (1 case; $P < 0.0001$). The recombination breakpoints were located in nucleotides (nt) 472–554, which comprise stem-loop 5 in the internal ribosomal entry site (IRES), based on the HRV-B 35 sequence (accession no. FJ445187). Our findings regarding genomic recombination in circulating HRV-A and -C strains suggest that recombination might play a role in HRV fitness and could be a possible determinant of disease severity caused by various HRV infections in patients with ARI.

Citation: Kim H, Kim K, Kim D, Jung H, Min Cheong H, et al. (2013) Identification of Recombinant Human Rhinovirus A and C in Circulating Strains from Upper and Lower Respiratory Infections. PLoS ONE 8(6): e68081. doi:10.1371/journal.pone.0068081

Editor: Thomas F. Schulz, Hannover Medical School, Germany

Received: January 24, 2013; **Accepted:** May 24, 2013; **Published:** June 27, 2013

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Funding: This study was supported with funds from the intramural research fund of Korea NIH (2011-N47001-00). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Human rhinoviruses (HRVs), first discovered in 1953, are nonenveloped, positive single-strand RNA viruses of the genus *Enterovirus* in the family *Picornaviridae* [1,2]. HRVs are the major cause of upper and lower respiratory tract infections in humans [3]. In particular, HRVs are the second main cause of bronchiolitis and wheezing illnesses in infancy, which are strongly associated with a high risk of developing asthma in childhood [4]. It is recognized that 50–85% of most asthma exacerbations are caused by HRV infections [5,6,7,8]. HRVs are transmitted commonly by the respiratory–salivary route, both by contact and airborne transmission [9].

HRVs have a genome of approximately 7,200 base pairs (bp) containing a single reading frame that encodes four viral capsid proteins (VP1, VP2, VP3 and VP4) and seven nonstructural proteins (2A^{pro}, 2B, 2C, 3A, 3B, 3C^{pro} and 3D^{pol}) [8,10]. HRVs have a 5' non-coding region (NCR) of about 650 bp that consists of a cloverleaf-like (CL) motif and an internal ribosome entry site (IRES) at the 5' end of the genome, with roles in viral replication and translation initiation, respectively. The IRES of HRVs contains five secondary RNA structures called stem-loops (SLs) 2–6 and a polypyrimidine tract (PPT) located between SL5 and SL6 [11].

Currently, 153 proposed types of HRVs have been identified and classified into three species (A, B and C) based on the

Table 1. Designed Primer sets of 5' NCR and VP4/VP2 regions.

Primer I.D.	Sequence(5'→3')	Polarity	Position ^a
RV-OL 26	GCACTTCTGTTTCCCC	Sense	177-192
RV-OL 27	AGGACACCCAAAGTA	Antisense	544-559
RV-KH_F	CCTCCGGCCCTGAATGYGGCTAAYC	Sense	447-472
RV-KH_R	GCATCIGGYARYTTCCACCACCANCCYTT	Antisense	1055-1083

a Position referred by Genbank accession no. FJ445187

b. Bold letters mark: mix-base.

nucleotide sequences that encode the VP1 protein, as HRV-C species are difficult to isolate by *in vitro* culture and to serotype [9,12,13,14,15,16,17,18,19]. HRVs share many features of their genome organization and structure with other picornaviruses. The genus *Enterovirus* in the family *Picornaviridae* has undergone much evolutionary genetic diversification through recombination events [20,21,22,23]. HRVs have also developed genetic diversity by recombination near the 5' NCR/P1, P1/P2 and P2/P3 boundaries [8,10,24,25]. Recombination events between the 5' NCR and the VP4 encoding sequences have mainly been observed in HRV-C, and the recombination breakpoints have been identified at SL5 and the PPT region [10]. In addition, some HRV-C that are closely related to HRV-A based on sequence analysis of the 5' NCR have been designated "HRV Ca", and these HRV Ca subspecies have been suggested to arise from interspecies recombination [10,25,26]. However, the inter- or intraspecies recombination events of field HRV-A and intraspecies recombination events of field HRV-C have not been studied until recently.

After finding of HRV-C, numerous studies on epidemiological and clinical manifestations have been conducted to elucidate the pathogenicity of HRV-C infection associated with disease severity. However the correlation between disease severity and HRV-C is still controversial [27,28,29,30,31]. In addition, HRVs are genetically heterogeneous and recombination events between or within species could cause complicate the identification and typing of HRVs, as well as a differentiation of clinical consequences.

This study aimed to understand and characterize the various recombination events between 5' NCR and VP4/VP2 region of field strains of HRV including species A, B and C, using 105 HRVs identified from two distinctive laboratory surveillance systems, the Acute Respiratory Infectious Network (ARINET) and Severe Lower Respiratory Tract Infections (SLRI) surveillances undertaken from October 2008 to March 2009. We investigated the occurrence and location of recombination events in these field strains of HRVs by phylogenetic analysis and by applying the Recombination Detection Program and described comparative analysis of unbiased recombination events in two surveillance systems with different disease severity.

Materials and Methods

Ethics statement

For specimens from ARINET, this study was approved by the Institutional Review Board of Korea Centers for Disease Control and Prevention (KCDC; 2012-09CON-03-4C) as it involved de-identified remaining respiratory tract samples which were not related to human gene study and collected for the respiratory virus diagnosis with written informed consent from patients, their parents or legal guardian. De-identification was performed except for each subject's age, gender, reported diagnosis, time of collection and virus detection results.

In the case of specimens from SLRI, ethical clearance was obtained from Yonsei University Health System Institutional Review Board, Seoul, Korea (4-2008-0649). Target population was the total population of children less than 5 years needed to be admitted for their lower respiratory infections. Patients who had their parents or legal guardians' written consent to participate in surveillance were enrolled. We obtained their nasopharyngeal aspirate specimens and their clinical information without personal ones.

Specimen collection and virus detection

Nasal aspirate specimens from patients with ARI ($n = 3082$) and nasopharyngeal aspirate specimens from patients with SLRI ($n = 381$) were collected in the ARINET and SLRI surveillances and marked as KA and KL in sample name respectively, in South Korea from October 2008 to March 2009 [32]. Among the HRV-positive samples-827 from ARINET and 85 from SLRI—51 and 54 samples, respectively, were selected by random sampling method. The viral RNAs of collected specimens were extracted using the QIAamp Viral RNA Mini Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and stored at -70°C until used for experiments.

The extracted RNA was applied to one-step reverse transcription-polymerase chain reaction (RT-PCR) reagents and the Labopass™ RV Detection kit (Cosmo Genetech, Seoul, South Korea) for detection of HRV. The kit was developed by the division of influenza and respiratory virus in KCDC with Cosmo Genetech [32].

RT-PCR system for sequencing of the 5' NCR and VP4/VP2 sequences

cDNA was synthesized using Superscript™ [0428] Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo-dT primers according to the manufacturer[2019]s instructions. A previously reported 5[2032] NCR primer set was used to amplify part of the IRES containing SL3, SL4 and SL5 from nucleotides (nt) 176[2013]559 : based on accession number FJ445187, as shown in Table 1 [33]. From the analysis of 131 genomes of reference HRVs from Genbank and modification of previously reported primer sequences, a new primer set was designed covering the VP4/VP2 sequences (nt 447–1083).

For the amplification of target genes, a 20 μl master mixture containing 2 μl of cDNA, 1 μl of each of the 10 pM target primers, 12 μl of DEPC-treated ddH₂O, 1 μl of 2.5 mM dNTP Mix (Cosmo Genetech), 2 μl of 10X SP-Taq buffer (Cosmo

Table 2. The age distribution of ARINET and SLRI patients.

Age	ARINET Number (%)	SLRI
		Number (%)
1-12 Month	16 (31.4%)	24 (44.4%)
1 year	14 (27.5%)	19 (35.2%)
2 year	5 (9.8%)	4 (7.4%)
3-10 year	9 (17.6%)	5 (9.3%)
11-20 year	0 (0%)	–
21-50 year	5 (9.8%)	–
51-70 year	2 (3.9%)	–
Total	51	52 ^a

^a Age of two patients in SLRI was not available

Table 3. The presumptive diagnosis of ARINET and SLRI patients.

	Diagnosis	Number (%)
ARINET ^a	pharyngitis	7 (12.5%)
	bronchitis	14 (25.0%)
	common cold	15 (26.8%)
	otitis media	6 (10.7%)
	pneumonia	1 (1.8%)
	sinusitis	13 (23.2%)
SLRI ^b	bronchiolitis	17 (34.0%)
	pneumonia	23 (46.0%)
	croup	6 (12.0%)
	asthma	4 (8.0%)

^a Multiple diagnosis included

^b Diagnosis of four patients in SLRI was not available

Genetech) and 1 µl of SP-Taq polymerase (Cosmo Genetech) was amplified for 40 cycles of 20 s at 95 °C, 40 s at 58 °C and 1 min at 72 °C. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit and Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis and recombination events prediction

The HRV sequences were aligned with those of 53 reference HRVs and previously published HRV strains: NY-074 from New York (Genbank accession number DQ875932); CL170085 from Geneva (Genbank accession number EU840952); QPM from Australia (Genbank accession number EF186077); C subtype 35 from Sweden (Genbank accession number JF436925); N10, N36 and N46 from Shanghai (Genbank accession numbers GQ223228, GQ213131 and GQ213134, respectively); LZ268, LZ508 and LZ101 from Beijing (Genbank accession numbers JF317013, JF317014, JF317015 and JF317017, respectively); and A21_p1177_sR3307, C36_p1075_s3911 and C43_p1154_sR1124 from Wisconsin (Genbank accession numbers JN837693, JN541267 and JX074056, respectively). The Clustal W method of MegAlign (ver. 8.0.2(13.4) 2) in the Lasergene 8 program suite (DNASTAR, Madison, WI, USA) was used [34].

The 5'NCR and VP4/VP2 sequence analyses were based on nt 193–470 and nt 623–1053, respectively (Genbank accession number FJ445187) [13,25]. The phylogenetic trees of the 5' NCR and VP4/VP2 were predicted using MEGA 4 (ver. 4.0.2) by the Neighbor-Joining method [35]. Bootstrap analysis was performed using 1000 replicates. In addition, each part of the 5' NCR and VP4/VP2 sequences was identified using Megablast. The sequences, covering nt 193–1053, were applied to SplitsTree 4 (<http://www.splitstree.org>) [36] and RDP 3 (<http://darwin.uvigo.es/rdp/rdp.html>) for predicting recombination events [37,38].

Nucleotide sequence accession numbers

The 19 genome sequences of recombinant viruses described in this study have been deposited in Genbank under accession numbers JX177615–JX177617, JX177619–JX177633 and JX177643.

Results

Clinical data and respiratory virus detection from patients

We obtained samples from the ARINET and SLRI surveillances as described in the Materials and Methods. The ARINET surveillance for outpatients with acute respiratory illness covers about 100 hospitals located all over Korea and includes all ages. The SLRI surveillance for inpatients covers four general hospitals in metropolitan areas and includes infants and children of less than 5 years of age [32]. These ARINET and SLRI surveillances represented mild and severe disease respectively, depending on clinical symptoms.

Among HRV-positive samples, 51 ARINET samples and 54 SLRI samples collected during the 2008–2009 winter season were selected for further analysis. Even though the age distributions of the ARINET and SLRI patients were not comparable directly (because the object of ARINET were patients from all ages but the SLRI from patients less than 5 years old), the majority of samples from both sets were from patients who were 1 year old or younger: 30/51 from ARINET (59%), and 43/54 from SLRI (80%), as shown in Table 2. There were no differences in the gender ratio between the ARINET (28 female and 23 male) and SLRI (28 female and 26 male) samples. Patients from the ARINET surveillance were diagnosed with pharyngitis, bronchitis, common cold, otitis media, pneumonia and sinusitis by the hospitals involved. Patients in the SLRI surveillance were diagnosed with bronchiolitis, pneumonia, croup or asthma, as shown in Table 3.

Phylogenetic analysis of the VP4/VP2 sequences and 5'NCR from ARINET and SLRI

The 5' NCR and VP4/VP2 sequences were applied to phylogenetic analysis using the MEGA 4 program with 53 reference HRVs and 14 previously isolated HRV strains, as described in the Materials and Methods. In this study, we only analyzed the sequences from 5' NCR to VP2 region, therefore the VP4/VP2 sequences were used to define the HRV types.

The phylogenetic trees predicted by the VP4/VP2 sequences were divided into HRV-A, -B and -C clusters, as shown in Figure S1A. The frequencies of presumed HRV-A, -B and -C were, respectively, 31 (60.8%), 1 (2.0%) and 19 (37.6%) in the 51 ARINET samples, and 21 (38.9%), 4 (7.4%) and 29 (53.7%) in the 54 SLRI samples. The ratios of these species differed slightly between the SLRI and ARINET samples, but the difference was not significant.

In analysis using the 5'NCR regions, the phylogenetic trees (Figure S1B) showed branches and cluster compositions differing from the VP4/VP2 sequence-based tree for both HRV-C and A. Fourteen HRV-C reference strains (QPM, QCE, NAT001, NY-074, C 24, C 25, C 26, N36, N46, C-43 p1154, CL170085, LZ269, LZY79 and LZY101), which were previously reported as Ca subspecies having a HRV-A 5'NCR, also clustered together with HRV-A reference strains. In addition, seven field strains classified as HRV-C by the VP4/VP2 tree were also clustered with HRV-A clusters in the 5' NCR-based tree. In contrast, three field strains classed as HRV-A by the VP4/VP2 tree were classified as HRV-C in the 5'NCR-based tree. Furthermore, we found that nine field strains of HRV-A and -C that were categorized as the same species but related to a different type at the 5'NCR and VP4/VP2 sequences had possible intraspecies recombination between the same species.

To further study these inconsistencies, all 5'NCR and VP4/VP2 sequences from these 19/105 (18.1%) strains were identified with the Megablast program, and showed high identities with different types in the same or different species, depending on the region analyzed. However, some reference sequences with the highest identities did not cover the entire region from the 5'NCR to the VP4/VP2 sequences. In these cases, the reference or previously published sequences having full coverage but showing slightly less identity were searched and selected as parent genomes for detecting recombination (Table 4). To summarize, only the 19 selected sequences in Table 4 were re-applied to the MEGA 4 program (Figure 1), and the phylogenetic trees showed the same results as in Figure S1. These results suggested the possibility of recombination events between the 5' NCR and the VP4/VP2 sequences.

Recombination events of HRV from field samples

Traditional bifurcating phylogenetic trees do not properly display the evolutionary history of different field strains, because one strain might be linked to more than one ancestral sequence. To confirm the possibility of inter- and intraspecies recombination, the 19 sequences were tested with the split decomposition network method and RDP3 using the regions from nt 193–1053 (from the 5'NCR to the VP2 sequence). Each sequence with a parent sequence selected from Megablast was applied to the SplitsTree 4 method [36]. All 19 selected field strains showed an interconnected relationship in the network, supporting recombination history between them, as shown in Figure S2.

In addition, from the analysis using six methods in RDP, the 19 samples were predicted as being recombinant, and the recombination breakpoints were also suggested as expected

from the results of phylogenetic analysis (Figure 2 and Table 5). The breakpoint indicated by at least three methods was identified as the breakpoint of each recombination strain, as shown in Figure 3. Strains KA08-3505, KA08-4418, KA09-446, KA09-560 and KA09-822 had an intraspecies recombination of HRV-A at nt positions 547, 552, 506, 534 and 490, respectively. In interspecies recombination of HRV-A strains, the recombination breakpoints of the KA08-3539, KA09-4374 and KA09-864 strains were identified at nt positions 516, 522 and 516, respectively. The nt positions 591, 519, 538, 550, 554, 472 and 517 were identified as the recombination breakpoints of KA08-4010, KA08-4631, KA09-218, KA09-495, KA09-806, KA09-917 and KL0809-374, respectively, in interspecies recombination strains of the HRV-C virus. KA08-4189, KA09-101, KA09-756 and KA09-893 have breakpoints at nt positions 515, 519, 528 and 452, respectively, in intraspecies recombination strains of the HRV-C virus. Most of the breakpoints were scattered at the conserved region of SL5 from nt 515–554. SL5, along with the PPT, is known as the binding region for the cellular translation initiation protein: polypyrimidine tract binding protein (PTB).

To summarize, 105 field strains were characterized. Among these, five cases (4.8%) of intraspecies recombination strains and three cases (2.8%) of interspecies recombination strains were found among HRV-A viruses, and four cases (3.8%) of intraspecies recombination strains and seven cases (6.7%) of interspecies recombination strains were identified among HRV-C viruses. Recombination events were significantly more frequent in the ARINET samples (18/54; 33%) than in the SLRI samples (1/51; 2%; $P < 0.0001$).

Discussion

In our study, 52 strains of HRV-A (49.5%) and 48 strains of HRV-C (45.7%) were identified in 105 field samples, whereas only five HRV-B viruses (4.8%) were found. There was no clustering difference in the distribution of ARINET and SLRI strains representing acute, mild and severe illness. Coinfection with another respiratory virus—mainly RSV—was found in 10 cases of HRV-A, 15 cases of HRV-C and four cases of HRV-B.

HRVs have remarkable genetic and antigenic variability, 102 known serotypes of HRV-A and B, and new types of HRV-C are being discovered continually. It is known that recombination events in the HRV genome have increased the diversity of each virus in the family *Picornaviridae* [20,21,22,23]. In earlier studies, Lee et al. (2007) analyzed the 5'NCR of 103 HRVs from Wisconsin and confirmed nine novel field strains [13,25]. In 2009, Huang et al. referred to these results and studied recombinant HRV-C among 66 HRVs from cases of ARI. In that study, 14 of 34 strains of HRV-C were found to be related closely to HRV-A by analysis of the 5' NCR sequences, and these strains of HRV-C were designated as "HRV Ca". The authors suggested that this strain had arisen from interspecies recombination, and that the Cc subspecies containing the HRV-C 5'NCR and VP4/VP2 sequences had not experienced a recombination event [10,25]. Palmenberg et al. identified intraspecies recombination of the HRV-A strain in three HRV-A reference viruses and interspecies recombination events in

Table 4. Blast results of VP4/VP2 and 5' NCR regions.

Samples	VP4/VP2 Blast Results	5'NCR Blast Results	Classification of recombination
	Nearest strain (% ^a)	Nearest strain (%)	
KA08-3505	HRV-A 78 (92)	HRV-A 12 (98)	Intraspecies recombination of HRV-A
KA08-4418	HRV-A 56 (92)	HRV-C 26 (99) ^b	
KA09-446	HRV-A 85 (91)	HRV-A 78 (96)	
KA09-560	HRV-A 12 (94)	HRV-A 9 (97)	
KA09-822	HRV-A 45 (92)	HRV-A 21 (97)	
KA08-3539	HRV-A 43 (91)	HRV-C isolate LZ508 (95)	Interspecies recombination of HRV-A
KA08-4374	HRV-A isolate LZ172(98), HRV-A 41 (88) ^c	HRV-C isolate Resp_4817/07 (99), HRV-C subtype 35 (93) ^c	
KA09-864	HRV-A strain HRV-A21_p1177_sR3307_2010 (96)	HRV-C isolate Resp_5153/07 (99), HRV-C strain HRV-C36_p1093_sR548_2008 (83) ^c	
KA08-4189	HRV-C isolate LZ101 (99) ^b	HRV-C isolate LZ508 (95)	Intraspecies recombination of HRV-C
KA09-101	HRV-C 26 (98) ^b	HRV-C isolate N10 (99)	
KA09-756	HRV-C isolate LZ269 (97) ^a	HRV-C isolate Resp_4817/07 (99), HRV-C subtype 35 (93) ^c	
KA09-893	HRV-C strain CL-170085 (96) ^a	HRV-C isolate Resp_5789/07 (97), HRV-C strain HRV-C36_p1093_sR548_2008 (84) ^c	
KA08-4010	HRV-C PHL TTA425s (98), HRV-C isolate N46 (96) ^{b,c}	HRV-C strain HRV-C43_p1154_sR1124_2009 (98) ^a	Interspecies recombination of HRV-C
KA08-4631	HRV-C isolate Resp_4122(98)HRV-C isolate N10 (96) ^c	HRV-C 26 (99) ^a	
KA09-218	HRV-C isolate LZ179 (99) ^a	HRV-C 26 (99) ^a	
KA09-495	HRV-C isolate N36 (93) ^a	HRV-A 40 (98)	
KA09-806	HRV-C strain CU136(98), HRV-C sub-type 35 (82) ^c	HRV-A 53 (99)	
KA09-917	HRV-C strain NY-074 (98) ^a	HRV-A 53 (96)	
KL0809-374	HRV-C strain Ca09-0309-U (98), HRV-C strain CL-170085 (94) ^{a,c}	HRV-A 46 (98)	

The amplified sequences of VP4/VP2 and 5'NCR were identified by megablast program. 11 strains of HRV-C recombination events were identified compared to 8 strains of HRV-A recombination events. Interestingly, the recombinant viruses were mainly found in ARINET samples (94.7%: 18 of 19 strains).

a Identity

b. These reference strains clustered with HRV-A in the 5'NCR phylogenetic tree in this study and previous report [10,25].

c The parent genomes, that contained 5'NCR and VP4/VP2 region, of low identity for confirming recombination events

HRV-C viruses by phylogenetic and recombinant detection analysis [8,10,24,25]. In the present study, intraspecies recombination events of HRV-A and interspecies recombination events of HRV-C were also detected using similar methods. Surprisingly, we also detected three cases (2.8%) of interspecies recombination in HRV-A and four cases (3.8%) of intraspecies recombination in HRV-C viruses. The incidence of recombination events was found to be similarly distributed in both HRV-C (11/48 cases) and HRV-A (8/52 cases) strains ($P>0.05$) by testing for any difference between the two proportions [39]. We also confirmed that interspecies recombination events had occurred in 14 reference strains of HRV-C reference strains: QPM, QCE, NAT001, NY-074, C 24, C 25, C 26, N36, N46, C-43 p1154, CL170085, LZ269, LZ179 and LZ101. Interestingly, KA08-3539 and KA08-4189 have the same nucleotides in the 5'NCR regions which clustered with the C isolate LZ508, but have the different VP4/VP2 sequences. Thus, KA08-3539 (interspecies recombination in HRV-A) and KA08-4189 (intraspecies recombination in HRV-C) were related to HRV-A 42 and to the C isolate LZ101,

respectively. KA08-4374 (interspecies recombination in HRV-A) and KA09-756 (intraspecies recombination in HRV-C) also had the same 5' NCR and differences in the VP4/VP2 sequences. We assume that recombination events in the 5'NCR between VP4 and VP2 could have led to this diversity of HRVs.

The recombinant breakpoint was located at the IRES in SL5 at nt 472–554, as shown in Table 5 and Figure 3. McIntyre et al. have identified the recombination breakpoints, which were at SL5 (nt 484–548, based on accession number FJ445187) and PPT–SL6 (nt 560–581), of an interspecies recombinant rhinovirus of HRV-C [10]. In our study, although the breakpoint was not identified at PPT, the breakpoints in SL5 were similar to McIntyre's results. In addition, most of the breakpoints were scattered at nt 515–554 (the conserved nucleotide region in SL5), as shown in Figure 3. The 5'NCR including the IRES plays an important role during viral replication, transcription and translation through the construction of a secondary RNA structure. The SL5 region of the 5' NCR forms an RNA–protein complex with PTB, the cellular translation initiation protein, and

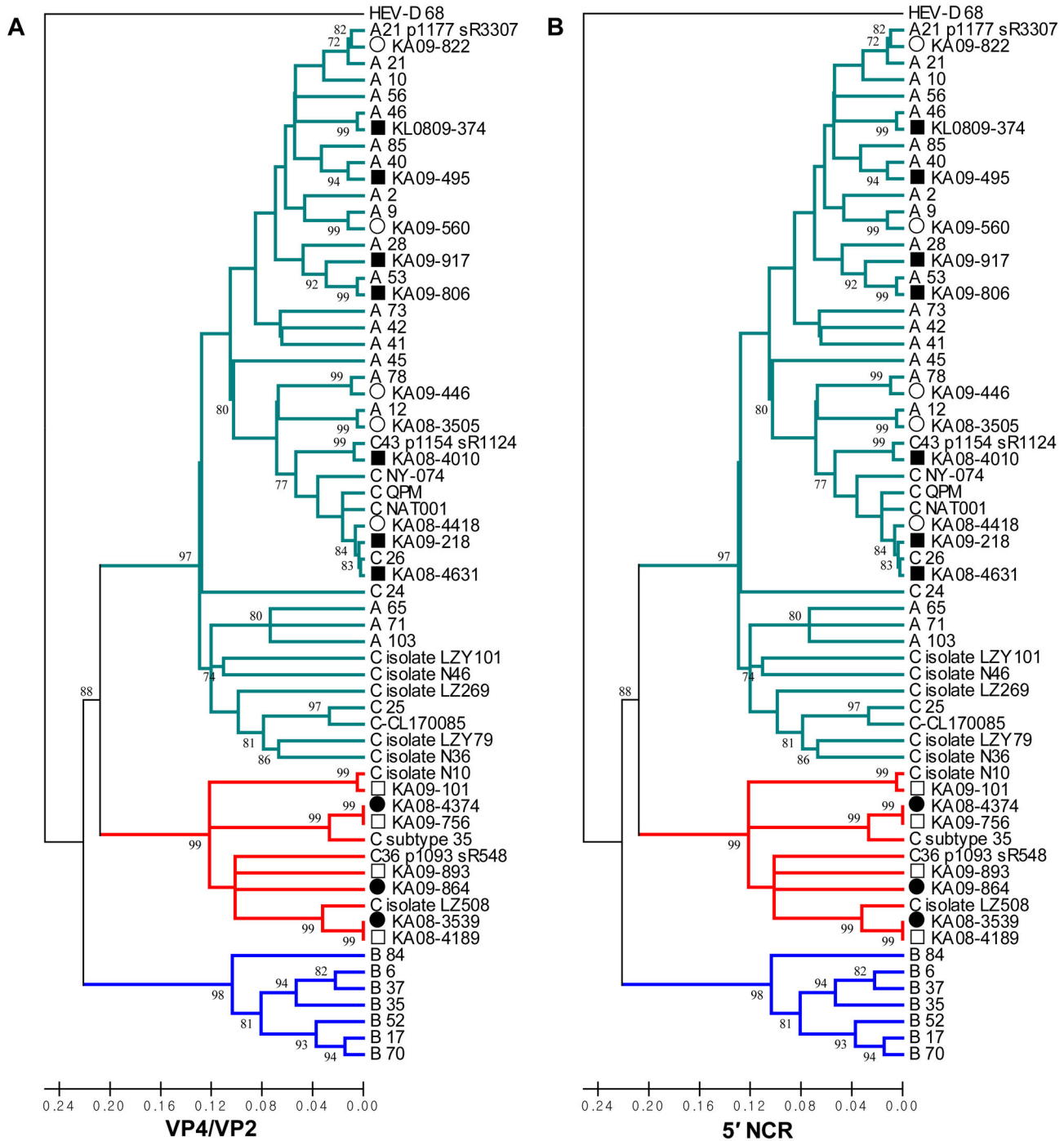


Figure 1. Phylogenetic analysis of field HRVs of ARINET and SLRI based on the VP4/VP2 and the 5' NCR sequences. The phylogenetic trees were constructed using the Neighbor-Joining method in the program MEGA 4 and HEV-D 68 (accession no. AY426531) was designated as outgroup [35]. The distances of trees were computed using the Maximum Composite Likelihood method [42], and the units are the number of base substitutions per site. The intraspecies recombination strains of HRV-A, interspecies recombination strains of HRV-A, intraspecies recombination strains of HRV-C and interspecies recombination strains of HRV-C are labeled using white circles, black circles, white squares and black squares, respectively.

doi: 10.1371/journal.pone.0068081.g001

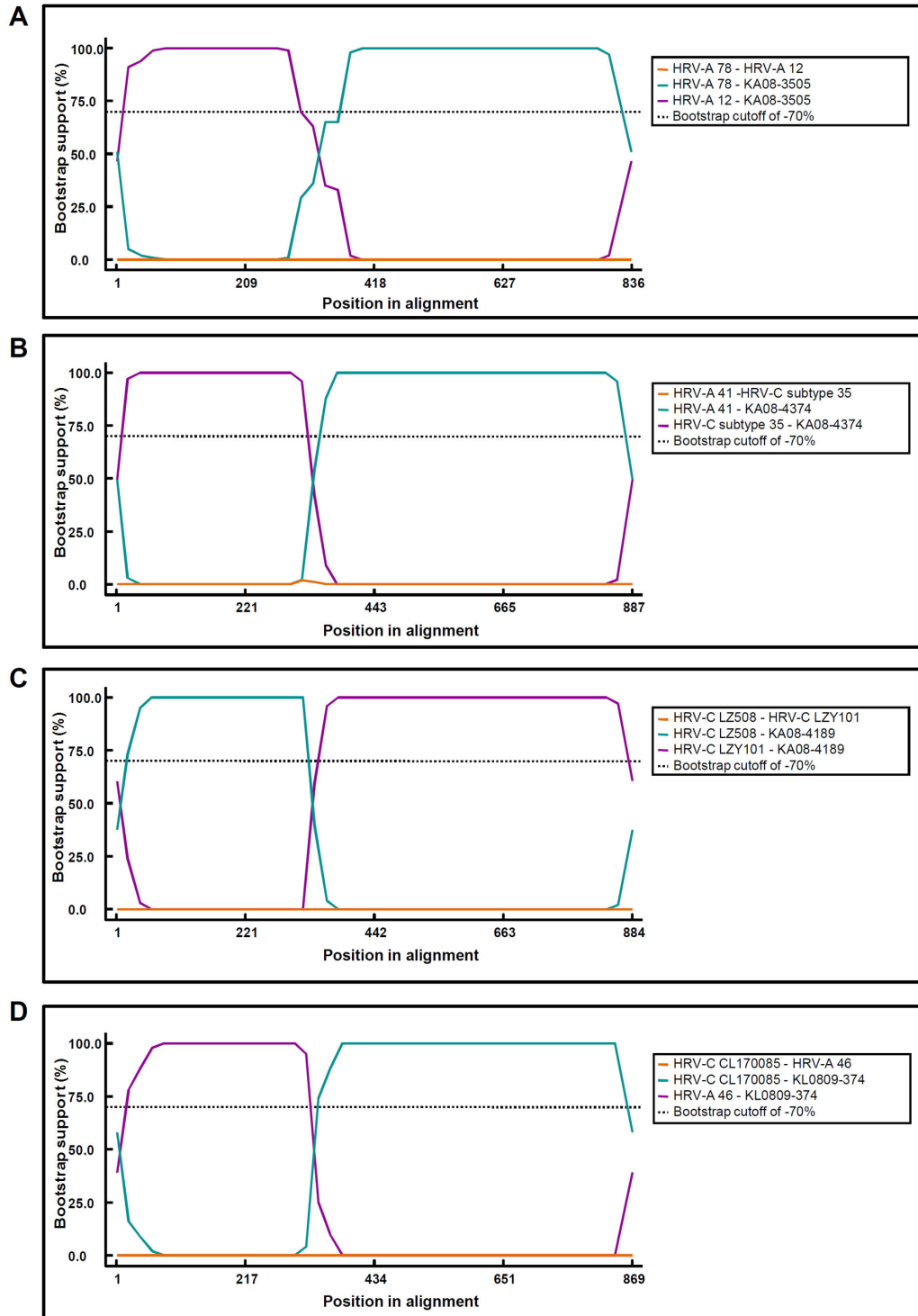


Figure 2. Analysis of recombination events in the 5' NCR-VP2 sequences of HRV field isolates. The diagrams show the results of bootscan analysis in RDP. **(A)** KA08-3505 is a representative intraspecies recombinant of HRV-A which consists of the 5' NCR sequence of HRV-A 78 and the VP sequence of HRV-A 12. **(B)** KA08-4374 is a representative interspecies recombination strain of HRV-A. The recombination event occurred between two different parent viruses: the 5' NCR sequence of HRV-C subtype 35 and the VP sequence of HRV-A 41. **(C)** KL0809-374 was identified as an intraspecies recombination strain of HRV-C combining HRV-A 46 and HRV-C strain CL-170085. **(D)** KA08-4189 has undergone an interspecies recombination between two HRV-C viruses: the 5' NCR sequence of HRV-C isolate LZ508 and the VP sequence of HRV-C isolate LZY101.

doi: 10.1371/journal.pone.0068081.g002

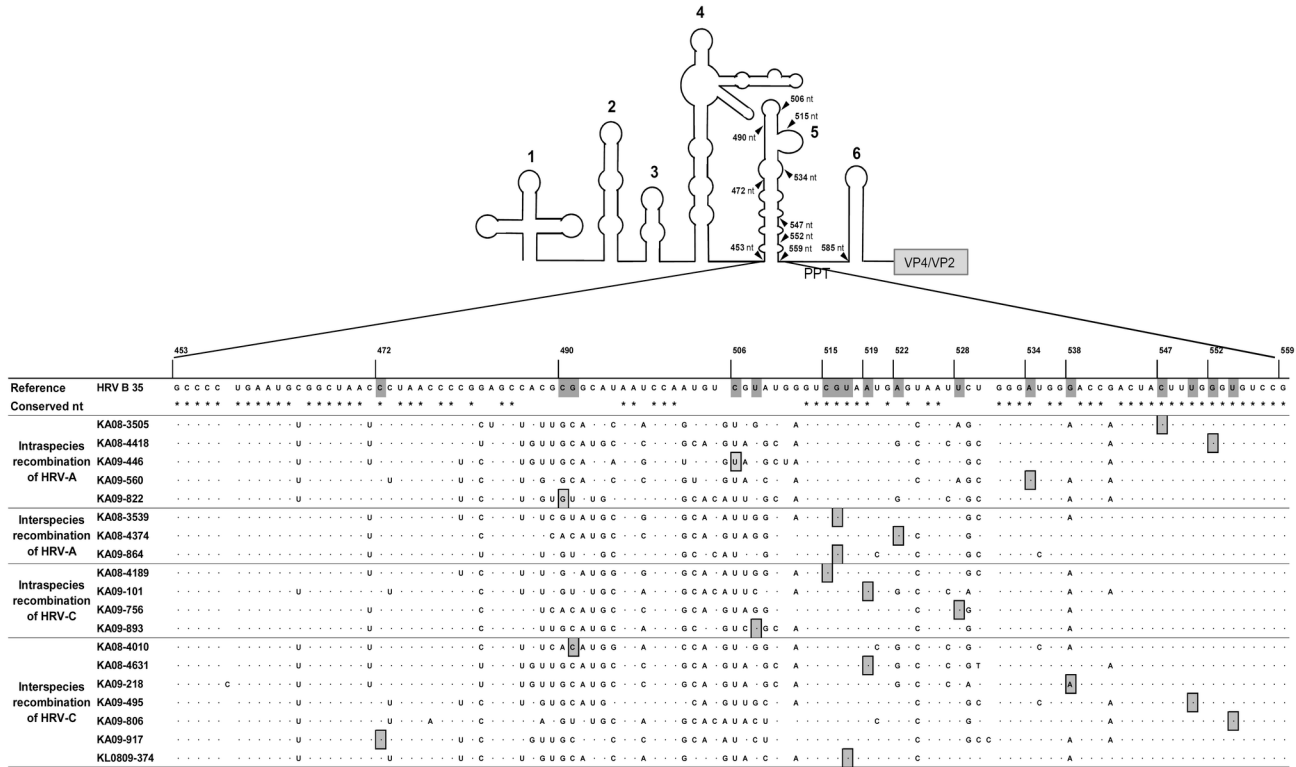


Figure 3. Prediction of the recombination breakpoints. Recombination breakpoints located in SL5 of the IRES (nt 472–554) were identified using RDP. The conserved nucleotides of each field strain are indicated by stars, and gray boxes show the recombination sites of each strain. The breakpoints were mainly scattered in a conserved region of SL5 (nt 515–554). doi: 10.1371/journal.pone.0068081.g003

it is known that the efficiency of PTB in stimulating IRES activity is affected by variations in IRES structure in polioviruses [40].

Recently, artificial 5' NCR interspecies strains produced by recombination between enteroviruses and rhinoviruses were investigated for studying the efficiency of the 5' NCR in translation and replication *in vitro*. This study showed that the genome of the field virus was more efficient with translation and replication than the artificial recombination genomes [41]. Accordingly, we assume that the translation and replication efficiencies are generally decreased by recombination events during evolution, except for a few favorable combinations and the recombinant viruses may have a different optimal temperature resulting to upper and lower respiratory tract infections. Although genetic and immunological predispositions of patients are primary contributor for determination of disease severity, current results presented here also support that hypothesis, with 18 cases of recombinant viruses in the ARINET isolates but only one case in the SLRI isolates. Another suggestion from the current hypothesis is that sequence information of 5' NCR and recombination characteristics may lead us to identify a closer relationship between viral diversity and disease severity rather than single criteria of HRVs classification based on VP region sequences.

In conclusion, this study is the first report describing intra- and interspecies genomic recombination in circulating HRV-A and -C isolated from patients with acute or severe respiratory illness and these results will assist in investigating the causes of the diversity and evolution of HRVs arising through recombination events. Further study should be required on the correlation between recombination at SL5 and the assignment of virulence factor(s) in recombinant viruses to elucidate the public health impact of HRV diversity.

Supporting Information

Figure S1. Phylogenetic tree with 53 reference HRVs and 14 previously isolated HRV strains NY-074 from New York (USA), CL170085 from Geneva (Switzerland), QPM from Australia, C subtype 35 from Sweden, N10, N36, and N46 from Shanghai, China, LZ268, LZ79, LZ508, and LZ101 from Beijing, China and A21_p1177_sR3307, C36_p1075_s3911, and C43_p1154_sR1124 from Wisconsin (USA) based on VP4/VP2 regions (A) and 5' NCR regions (B) of human rhinovirus. The trees were constructed by 'Neighbor-Joining' method in the MEGA 4 program and HEV-D 68 strain was used as outgroup. The distances of trees were computed using the Maximum Composite Likelihood method and were the units of the number of base substitutions per site. The intraspecies recombination

Table 5. Analysis results of recombination break points in recombinant viruses.

Samples	RDP		Genconv		Bootscan		MaxChi		Chimaera		3Seq		Classification of recombination
	break point	P-value	break point	P-value	break point	P-value	break point	P-value	break point	P-value	break point	P-value	
KA08-3505	547 ^a	1.746X10 ⁻¹²	547	3.405X10 ⁻¹³	664	4.481X10 ⁻¹⁴	547	6.250X10 ⁻¹¹	547	1.483X10 ⁻¹²	547	2.220X10 ⁻¹⁶	Intraspecies recombination of HRV-A
KA08-4418	552	1.016X10 ⁻²⁸	552	3.904X10 ⁻²⁷	552	3.398X10 ⁻²⁹	552	5.081X10 ⁻¹⁸	552	1.334X10 ⁻¹⁷	552	9.208X10 ⁻³⁵	
KA09-446	506	6.793X10 ⁻¹³	508	4.366X10 ⁻¹¹	606	2.668X10 ⁻¹³	506	4.664X10 ⁻¹²	506	1.724X10 ⁻¹²	506	1.224X10 ⁻¹⁴	
KA09-560	534	7.549X10 ⁻¹⁵	519	1.330X10 ⁻¹³	534	2.177X10 ⁻¹⁴	534	4.617X10 ⁻¹⁴	534	2.416X10 ⁻¹⁵	519	1.994X10 ⁻²³	
KA09-822	490	5.139X10 ⁻¹⁸	490	2.442X10 ⁻¹⁷	498	8.780X10 ⁻¹⁸	490	2.442X10 ⁻¹⁴	490	2.333X10 ⁻¹³	490	5.371X10 ⁻²⁴	
KA08-3539	566	1.701X10 ⁻²³	516	1.216X10 ⁻²²	516	2.547X10 ⁻²⁴	566	4.391X10 ⁻²⁰	516	1.880X10 ⁻²¹	516	5.423X10 ⁻³⁹	Interspecies recombination of HRV-A
KA08-4374	522	5.315X10 ⁻²⁶	522	1.317X10 ⁻²²	522	7.161X10 ⁻²³	534	2.339X10 ⁻¹⁹	526	1.461X10 ⁻²¹	526	1.923X10 ⁻⁴⁶	
KA09-864	507	1.614X10 ⁻²⁰	516	2.666X10 ⁻²⁰	516	3.225X10 ⁻²¹	516	2.470X10 ⁻¹⁵	516	1.568X10 ⁻¹⁸	507	1.641X10 ⁻³²	
KA08-4189	515	1.212X10 ⁻²⁵	515	6.197X10 ⁻²⁵	515	1.574X10 ⁻²⁰	549	8.018X10 ⁻²¹	515	1.039X10 ⁻²²	515	1.451X10 ⁻⁴⁶	Intraspecies recombination of HRV-C
KA09-101	undetermind	undetermind	519	1.010X10 ⁻²⁶	519	3.573X10 ⁻²⁶	519	2.994X10 ⁻²²	519	4.491X10 ⁻²²	519	2.313X10 ⁻⁶⁰	
KA09-756	528	3.465X10 ⁻²²	528	1.170X10 ⁻²¹	506	9.908X10 ⁻¹⁹	550	7.378X10 ⁻²¹	550	4.684X10 ⁻²²	528	1.995X10 ⁻³⁹	
KA09-893	452	1.243X10 ⁻¹⁶	508	1.896X10 ⁻¹⁷	508	7.852X10 ⁻¹⁹	508	8.768X10 ⁻¹³	508	7.926X10 ⁻¹⁵	508	2.160X10 ⁻¹⁸	
KA08-4010	491	2.038X10 ⁻²¹	484	3.907X10 ⁻²²	491	6.837X10 ⁻²²	491	2.019X10 ⁻¹⁷	491	5.843X10 ⁻¹⁸	484	4.683X10 ⁻³²	Interspecies recombination of HRV-C
KA08-4631	519	2.001X10 ⁻³¹	519	1.995X10 ⁻²⁸	519	1.546X10 ⁻³³	519	5.949X10 ⁻²³	519	1.111X10 ⁻²²	519	7.058X10 ⁻⁵⁰	
KA09-218	undetermind	undetermind	538	1.590X10 ⁻³⁰	538	1.318X10 ⁻³⁴	538	1.032X10 ⁻²³	538	5.459X10 ⁻²⁴	538	1.572X10 ⁻⁵⁹	
KA09-495	550	9.932X10 ⁻²⁷	550	3.055X10 ⁻²⁷	563	1.810X10 ⁻²⁶	550	2.229X10 ⁻¹⁸	550	2.837X10 ⁻²⁰	553	4.557X10 ⁻⁴⁰	
KA09-806	554	2.175X10 ⁻²⁶	554	9.415X10 ⁻³¹	554	5.104X10 ⁻³³	554	1.246X10 ⁻²²	588	1.314X10 ⁻²¹	554	7.437X10 ⁻³²	
KA09-917	undetermind	undetermind	472	3.654X10 ⁻¹⁵	472	6.307X10 ⁻¹⁸	515	3.314X10 ⁻¹⁴	515	9.148X10 ⁻¹⁴	472	1.178X10 ⁻²⁰	
KL0809-374	517	7.585X10 ⁻¹⁶	517	9.589X10 ⁻¹³	517	8.460X10 ⁻¹⁵	517	1.822X10 ⁻¹⁷	517	4.989X10 ⁻¹⁹	517	2.532X10 ⁻⁴²	

Results are shown for all recombination events with 6 analysis methods by RDP program.

^a : Position referred by GenBank accession no. FJ445187

strains of HRV-A, interspecies recombination strains of HRV-A, intraspecies recombination strains of HRV-C and interspecies recombination strains of HRV-C are labeled using white circles, black circles, white squares and black squares, respectively. (DOCX)

Figure S2. The splits trees were predicted by decomposition network method in SplitsTree 4 (<http://www.splitstree.org>). 19 recombination strains showed the interconnected relationship in the network and supporting recombination between them. (A) The intraspecies recombinant strains of HRV-A, (B) interspecies recombinant strains of HRV-A, (C) intraspecies

recombinant strains of HRV-C, (D) interspecies recombinant strains of HRV-C. (DOCX)

Author Contributions

Conceived and designed the experiments: HK KK YJK. Performed the experiments: HK KK DWK KHK YJK. Analyzed the data: HK DWK HDJ. Contributed reagents/materials/analysis tools: HMC KHK DSK. Wrote the manuscript: HK KK YJK.

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