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<td>Author(s)</td>
<td>Woo, PCY; Lau, SKP; Yip, CCY; Huang, Y; Tsoi, HW; Chan, KH; Yuen, KY</td>
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Comparative analysis of 22 coronavirus HKU1 genomes reveals a novel genotype and evidence of natural recombination in coronavirus HKU1

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Running title: Novel genotype and recombination in coronavirus HKU1

†PCY Woo and SKP Lau contributed the same to the manuscript.

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ABSTRACT

We sequenced and compared the complete genomes of 22 strains of coronavirus HKU1 (CoV-HKU1) obtained from nasopharyngeal aspirates of patients with respiratory tract infections over a two-year period. Phylogenetic analysis of 24 putative proteins and polypeptides showed that the 22 CoV-HKU1 strains fell into three clusters (genotype A, 13 strains; genotype B, three strains and genotype C, six strains). However, different phylogenetic relationships among the three clusters were observed in different regions of their genomes. From nsp4 to nsp6, the genotype A strains were clustered with the genotype B strains. For nsp7 and nsp8, and from nsp10 to nsp16, the genotype A strains were clustered with the genotype C strains. From hemagglutinin esterase (HE) to nucleocapsid (N), the genotype B strains were clustered closely with the genotype C strains. Bootscan analysis showed possible recombination between genotypes B and C from nucleotide positions 11500 to 13000, corresponding to the nsp6/nsp7 junction, giving rise to genotype A; and between genotypes A and B from nucleotide positions 21500 to 22500, corresponding to the nsp16/HE junction, giving rise to genotype C. Multiple alignments further narrowed the sites of cross-over to a 143-bp region between nucleotide positions 11750 and 11892, and a 29-bp region between nucleotide positions 21502 and 21530. Genome analysis also revealed variable numbers of tandem copies of a perfect 30-base acidic tandem repeat (ATR) which encodes NDDEDVVTGD and variable numbers and sequences of imperfect repeats in the N-terminal of nsp3 inside the acidic domain upstream of papain-like protease 1 among the 22 genomes. All 10 CoV-HKU1 with incomplete imperfect repeats (1.4 and 4.4) belonged to genotype A. The present study represents the first evidence for natural recombination in coronavirus associated with human infection. Analysis of a single gene is not sufficient for genotyping of CoV-
HKU1, but would require amplification and sequencing of at least two gene loci, one from nsp10 to nsp16 (e.g. pol or helicase) and another from HE to N (e.g. spike or N). Further studies will delineate whether the ATR is useful for molecular typing of CoV-HKU1.
INTRODUCTION

The recent severe acute respiratory syndrome (SARS) epidemic, the discovery of SARS coronavirus (SARS-CoV) and the identification of SARS-CoV-like viruses from Himalayan palm civets and a raccoon dog from wild live markets in mainland China have led to a boost in interests in the discovery of novel coronaviruses in both humans and animals (8, 23, 26, 28, 40, 42). In 2004, a novel group 1 human coronavirus, human coronavirus NL63 (HCoV-NL63) was reported independently by two groups (6, 34). In 2005, we described the discovery, complete genome sequence, clinical features and molecular epidemiology of a novel group 2 human coronavirus, coronavirus HKU1 (CoV-HKU1, genotype A) (17, 37-39, 41). This virus has also been subsequently found in patients with respiratory tract infections in other countries (1, 30, 35). Recently, we have also identified a SARS-CoV-like virus in Chinese horseshoe bats and a novel group 1 coronavirus in large bent-winged bats, lesser bent-winged bats and Japanese long-winged bats in Hong Kong Special Administrative Region (HKSAR) (16, 27). The discovery of SARS-CoV-like viruses in horseshoe bats was confirmed by another group in other provinces in China (19).

As a result of the unique mechanism of viral replication, coronaviruses have a high frequency of recombination (15). Their tendency for recombination and high mutation rates may allow them to adapt to new hosts and ecological niches. However, no convincing evidence of genetic recombination has been documented among human coronaviruses which may have contributed to their ability of human re-infection. In our study on phylogeny of the RNA-dependent RNA polymerase (pol), spike (S) and nucleocapsid (N) genes of nine isolates of CoV-HKU1 recovered from patients with pneumonia, it was discovered that the sequences of the S and N genes fell into two distinct genotypes, with seven strains belonging to genotype A and two belonging to
genotype B (41). On the other hand, for the *pol* gene, one of the two “genotype B” strains by the S and N sequences (from patient 8) was clustered with the other seven “genotype A” strains (41). Furthermore, the same phenomenon was also observed in our subsequent prospective study on CoV-HKU1 associated respiratory tract infections (17). Based on these observations, we suspected that there is an additional CoV-HKU1 genotype which has arisen from recombination between genotypes A and B of CoV-HKU1.

To test this hypothesis, we performed complete genome sequencing on 21 additional strains of CoV-HKU1 and compared their genomes to the CoV-HKU1 genotype A strain (38). The sites of recombination were identified and a novel CoV-HKU1 genotype, genotype C, was defined.
MATERIALS AND METHODS

CoV-HKU1 strains. All 22 CoV-HKU1 strains were isolated from patients with respiratory tract infections in Hong Kong in a two-year period (March 2003 – February 2005) (Table 1) (17, 38, 41).

RNA extraction. Viral RNA was extracted from the nasopharyngeal aspirates of the patients using QIAamp Viral RNA Mini Kit (QIAgen, Hilden, Germany). The RNA pellet was resuspended in 10 μl of DNase-free, RNase-free double-distilled water and was used as the template for RT-PCR.

Complete genome sequencing and genome analysis. The complete genome sequence of the CoV-HKU1 genotype A strain was described previously (GenBank accession no. NC_006577) (38). The complete genomes of the other 21 CoV-HKU1 strains were amplified and sequenced using the RNA extracted from the nasopharyngeal aspirates of the corresponding patients as template, using a strategy described previously (38). The RNA was converted to cDNA by a combined random-priming and oligo(dT) priming strategy. The 5’ ends of the viral genomes were confirmed by rapid amplification of cDNA ends using the 5'/3' RACE kit (Roche, Germany). Sequences were assembled and manually edited to produce final sequences of the viral genomes. The 21 genomes were compared to that of the CoV-HKU1 genotype A strain and were manually annotated.

Phylogenetic tree construction. The nucleotide sequences of nsp1, nsp2, conserved portions of nsp3 [including papain-like protease 1 (PL1^pro), Appr-1-p processing enzyme family (A1pp), papain-like protease 2 (PL2^pro) and hydrophobic domain (HD)], nsp4-nsp10, nsp12-nsp16, hemagglutinin esterase (HE), S, ORF4, envelope (E), membrane (M) and N were extracted from the 22 CoV-HKU1 genomes. Phylogenetic tree construction was performed using neighbor joining method with
ClustalX 1.83. The corresponding nucleotide sequences of human coronavirus OC43 (HCoV-OC43) (GenBank accession no. AY585229) were used as outgroups.

**Bootscan analysis.** To perform bootscan analysis, a nucleotide alignment of the genome sequences of one genotype A (38), one genotype B (Patient 1 of Ref 41) and one genotype C (Patient 8 of Ref 41) strain of CoV-HKU1 and one HCoV-OC43 (GenBank accession no. AY585229) was generated by ClustalX version 1.83 and edited manually. Bootscan analysis was performed using Simplot version 3.5.1 (20) (F84 model; window size, 1000 bp; step, 200 bp) with the genome sequence of HCoV-OC43 as a query.

**Nucleotide sequence accession number.** The nucleotide sequences of the 21 additional genomes of CoV-HKU1 have been lodged within the GenBank sequence database under accession no. AY884001, DQ339101, DQ415896, DQ415897, DQ415898, DQ415899, DQ415900, DQ415901, DQ415902, DQ415903, DQ415904, DQ415905, DQ415906, DQ415907, DQ415908, DQ415909, DQ415910, DQ415911, DQ415912, DQ415913 and DQ415914.
RESULTS

Complete genome sequence, genome organization, phylogenetic analysis and genotypes. The sizes of the genomes of the 22 CoV-HKU1 strains ranged from 29295 to 30097 nucleotides. The G + C contents of all 22 genomes are 32%. The overall genome organizations of the 22 CoV-HKU1 strains were the same (Fig. 1A).

Phylogenetic trees using the nucleotide sequences of putative proteins and polypeptides [nsp1, nsp2, conserved portions of nsp3 (PL1\textsuperscript{pro}, A1pp, PL2\textsuperscript{pro} and HD), nsp4-nsp10, nsp12-nsp16, HE, S, ORF4, E, M and N] of the 22 CoV-HKU1 were constructed and shown in Fig. 1B. In 18 of the 24 trees, the 22 CoV-HKU1 strains fell clearly into three clusters, named as genotype A (13 strains), genotype B (three strains) and genotype C (six strains). The exceptions are the five trees constructed using nsp1, nsp2, PL1\textsuperscript{pro}, PL2\textsuperscript{pro} and HD in which the differences among the sequences were too small and the nsp10 tree in which two genotype A strains, N1 and N3, were clustered with genotype C strains.

The three genotypes exhibited different relationships to each other in different regions of their genomes. From nsp4 to nsp6, the genotype A strains were clustered with the genotype B strains, but for nsp7 and nsp8, the genotype A strains were clustered with the genotype C strains. From nsp10 to nsp16, the genotype A strains were clustered closely with the genotype C strains with high bootstrap values, but from HE to N, the genotype B strains were clustered closely with the genotype C strains, with bootstrap values of 1000 in all cases. No association was observed between the genotypes and the time of detection or the age, sex, clinical disease, presence of underlying disease and outcome of the patients (Table 1).

The putative transcription regulatory sequence (TRS) motif, 5'-AAUCUAAAC-3' [as in mouse hepatitis virus (MHV) and bovine coronavirus] (22),
or alternatively, 5’-UAAAUCUAAAC-3’, that was found at the 3’ end of the leader sequence and precedes each translated ORF except ORF5 described in the genome of the CoV-HKU1 genotype A strain, was also present in all the other 21 CoV-HKU1 genomes. On the other hand, the sequence of the putative internal ribosomal entry site (IRES) (32) for the ORF of the envelope protein in the genomes of all three CoV-HKU1 genotype B strains and all six genotype C CoV-HKU1 strains were UUUUAUCGCUUGG, instead of AUUUAUUGUUUGG in all 13 CoV-HKU1 genotype A strains, although both sequences were similar to the IRES element, UUUUAUUCUUUUU, in MHV (10).

The 22 genomes differed in the number of tandem copies of the 30-base acidic tandem repeat (ATR) in the N-terminal of nsp3 inside the acidic domain upstream of PL1pro (Tables 1 and 2). All 22 genomes had tandem copies of a perfect 30-base repeat which encodes NDDEDVVTGD and variable numbers of imperfect repeats. The median number of tandem copies of the perfect 30-base repeat was 11.5 (range 2-17) and the median number of imperfect repeat was 2 (range 1-4). All the 10 CoV-HKU1 with incomplete imperfect repeats (1.4 and 4.4) belonged to genotype A.

**Bootscan analysis.** Bootscan analysis showed that from the 5’ end of the genome to position 12000, there could be a number of possible recombination sites in the genomes of the three genotypes (Fig. 2). Right upstream to position 11500, high bootstrap support for clustering between the CoV-HKU1 genotype A strain and the CoV-HKU1 genotype B strain was observed. From positions 13000 to 21500, high bootstrap support for clustering between the CoV-HKU1 genotype A strain and the CoV-HKU1 genotype C strain was observed. From position 22500 to the 3’ end of the genome, high bootstrap support for clustering between the CoV-HKU1 genotype B strain and CoV-HKU1 genotype C strain was observed. These indicate that
recombination has possibly taken place between nucleotide positions 11500 and 13000, corresponding to the nsp6/nsp7 junction, and between nucleotide positions 21500 and 22500, corresponding to the nsp16/HE junction.

**Comparative sequence analysis of the nsp6/nsp7 junction and nsp13/HE gene junction.** Since both phylogenetic trees and bootscan analysis showed that there was a possible recombination site at the nsp6/nsp7 junction and the nsp16/HE gene junction, multiple alignments among the nucleotide sequences of the 22 genomes were performed to ascertain the exact sites of recombination.

Upstream to nucleotide position 11750 of the CoV-HKU1 genotype A genome (227 bases before the end of nsp6), there was high nucleotide identity between the sequences of the CoV-HKU1 genotype A and genotype B strains, whereas downstream to nucleotide position 11892 of the CoV-HKU1 genotype A genome (85 bases before the end of nsp6), there was high nucleotide identity between the sequences of the CoV-HKU1 genotype A and genotype C strains (Fig. 3). This indicates that the site of cross-over was probably within a 143-bp region between nucleotide positions 11750 and 11892.

Upstream to nucleotide position 21502 of the CoV-HKU1 genotype A genome (249 bases before the stop codon of ORF1ab), there was high nucleotide identity between the sequences of the CoV-HKU1 genotype A and genotype C strains, whereas downstream to nucleotide position 21530 of the CoV-HKU1 genotype A genome (221 bases before the stop codon of ORF1ab), there was high nucleotide identity between the sequences of the CoV-HKU1 genotype B and genotype C strains, including a 13-bp insertion just downstream to the stop codon of ORF1ab (Fig. 4). This indicates that the site of cross-over was probably within a 29-bp region between nucleotide positions 21502 and 21530.
DISCUSSION

This is the first time that evidence for natural coronavirus recombination is documented in coronavirus associated with human infection. Coronaviruses are unique in having high frequency of homologous RNA recombination, as a result of random template switching during RNA replication, thought to be mediated by a “copy-choice” mechanism (2, 4, 13, 14, 21, 33). In feline coronavirus (FCoV), it has been documented that FCoV type II strains originated from a double recombination between FCoV type I and canine coronavirus, and the site of recombination has been pinpointed to a region of about 50 nucleotides in the M gene by multiple alignment (9). As for recombination between different strains of MHV, in vitro studies have shown variations in both sites and rates of recombination, with the S gene having a frequency three fold that of the polymerase gene (7, 21). In the present study, by comparing the sequences of 22 complete genomes of CoV-HKU1, we documented that major recombination has occurred among the three CoV-HKU1 genotypes. Both phylogenetic and bootscan analysis showed that the nucleotide sequences of the six genotype C strains were almost identical to those of the 13 genotype A strains from nsp10 to nsp16 (Fig. 1B and 4). Interestingly, the topologies of the phylogenetic trees changed dramatically starting from the HE gene. From HE to N, the nucleotide sequences of the six genotype C strains were almost identical to those of the three genotype B strains (Fig. 1B). This is also in line with results of bootscan analysis, suggesting recombination between genotypes A and B, giving rise to genotype C (Fig. 2). Multiple alignments of the nucleotide sequences of the nsp16/HE regions of the three genotypes confirmed our suspicion and results localized the site of recombination to a stretch of 29 nucleotides in nsp16, just upstream to the stop codon of ORF1ab (Fig. 4). This is in keeping with the finding that the putative IRES of all
the genotype B and genotype C strains were the same, but different from those of the
geno
type A strains, as it is located downstream to HE. In addition to the
recombination site in nsp16, there was another one at the end of nsp6, also evidenced
by a shift in clustering in the phylogenetic trees, bootscan analysis and multiple
alignments results (Fig. 1B, 2 and 3). In contrast to the nsp16 recombination site,
recombination has occurred between genotypes B and C in this region, giving rise to
genotype A. Furthermore, as shown in bootscan and phylogenetic analysis, additional
recombination events might have occurred in ORF1ab upstream to nsp5 (Fig. 1B and
2). However, due to the relatively small variations in the sequence among the three
genotypes, these putative recombination sites were difficult to ascertain by multiple
alignments.

A novel genotype, genotype C, of CoV-HKU1 is defined. It has been well
known that recombination is an important mechanism for the generation and
evolution of virus genotypes (12, 29, 31). In our previous study, we showed that seven
of the nine CoV-HKU1 strains were of genotype A and one of the nine strains were of
genotype B by pol, S and N gene sequence analysis (41). In the present study, we
showed that the latter half of the genomes of the six genotype C strains probably
represent a result of recombination between genotypes A and B. Analysis of the
complete genomes of more CoV-HKU1 strains from other countries will reveal the
relative prevalence of the different genotypes in different localities. From the results
of the present study, no association was observed between the genotypes and clinical
characteristics of the patients. Furthermore, amplification and sequencing of a single
gene is not sufficient to define the genotype of CoV-HKU1. It would require
amplification and sequencing of at least two gene loci, one from nsp10 to nsp16 (e.g
pol or helicase) and another from HE to N (e.g S or N).
The origin and function of the ATR located inside the acidic domain upstream of PL1<sup>pro</sup>, unique to CoV-HKU1, remain enigmatic. Significant variations were observed among the ATR of CoV-HKU1 strains. Only two pairs of CoV-HKU1 strains (N1 and N3, N7 and N9) possessed the same nucleotide sequence in the ATR. No relationship was found between the number of repeats and the genotype or virulence of the strains. We speculate that this “independent evolution” of the number of repeats was due to the random expansion or deletion of part of the repeat region during the process of viral replication as a result of inaccurate replication by the viral polymerase or recombination between the repeat regions of different CoV-HKU1, a phenomenon widely observed in tandem repeats of genomes in all domains of life (3, 24). On the other hand, the sequence of the imperfect repeats seemed to co-evolve with the rest of the genomes, most notably that all 10 CoV-HKU1 strains with incomplete imperfect repeats (NDDD) were of genotype A (Tables 1 and 2). This could be due to the deletion of part of a repeat in one genotype A strain, and subsequent expansion or deletion of whole repeats in its descendents. Further studies will delineate whether this ATR is useful for molecular typing of CoV-HKU1.

This high frequency of recombination has resulted in the generation of a high diversity of coronaviruses in different animals. Before the SARS epidemic in 2003, a total of 19 coronaviruses were known, including two human, 13 mammalian and four avian coronaviruses. After the SARS epidemic, within a short period of three years, 20 additional novel coronaviruses were described (5, 6, 11, 16, 19, 25, 26, 34, 36, 38, 43). These include three human coronaviruses, 11 mammalian coronaviruses and six avian coronaviruses. Notably the recent discovery of at least eight different species of coronaviruses in bats in Hong Kong, including SARS-CoV-like viruses and a probable novel subgroup, group 2c, of coronavirus (16, 43). The high frequency of
recombination in such a high diversity of coronaviruses may easily result in the
generation of novel coronavirus species or genotypes that can cross host species
barriers, leading to major zoonotic outbreaks with disastrous consequences. The
potential of generation of novel species leading to zoonotic outbreaks and major
consequences is analogous to the situation of avian and human influenza
epidemiology, although the mechanism of generation of novel types and variants is by
reassortment, which is different from recombination in coronaviruses (18, 44).
Amplification of conserved regions in coronaviruses using RNA extracted from
various animal specimens will lead to the discovery of more coronaviruses and
subsequent complete genome sequencing and comparative genome analysis will
reveal the intricate relationships among the various coronaviruses.
ACKNOWLEDGEMENTS

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REFERENCES


LEGENDS TO FIGURES

FIG. 1. Genome organization and phylogenetic analysis of CoV-HKU1. (A) Genome organization of CoV-HKU1. The regions of the genome used for phylogenetic tree construction are labeled. (B) Phylogenetic analysis of nsp1, nsp2, conserved portions of nsp3 (PL1\textsuperscript{pro}, A1pp, PL2\textsuperscript{pro} and HD), nsp4-10, nsp12-16, HE, S, ORF4, E, M and N of the 22 CoV-HKU1 genomes. The trees were constructed by neighbor joining method using Jukes-Cantor correction and bootstrap values calculated from 1000 trees. 740, 1821, 655, 333, 895, 1263, 1488, 909, 861, 276, 582, 330, 411, 2783, 1809, 1563, 1125, 900, 1276, 4126, 330, 253, 687, 1358 nucleotide positions in nsp1, nsp2, PL1\textsuperscript{pro}, A1pp, PL2\textsuperscript{pro}, HD, nsp4, nsp5, nsp6, nsp7, nsp8, nsp9, nsp10, nsp12, nsp13, nsp14, nsp15, nsp16, HE, S, ORF4, E, M and N respectively were included in the analysis. The scale bar indicates the estimated number of substitutions per 50 or 100 nucleotides as indicated. The corresponding nucleotide sequences of HCoV-OC43 were used as the outgroups. A, genotype A; B, genotype B and C, genotype C.

FIG. 2. Bootscan analysis of the CoV-HKU1 genomes. Bootscanning was conducted with Simplot version 3.5.1 (F84 model; window size, 1000 bp; step, 200 bp) on a gapless nucleotide alignment, generated with ClustalX, with the genome sequence of HCoV-OC43 (AY585229) as the query sequence. The dashed line denotes the CoV-HKU1 genotype A (NC_006577), the solid line denotes the CoV-HKU1 genotype B (AY884001) and the dotted line denotes the CoV-HKU1 genotype C (DQ339101).

FIG. 3. Comparative sequence analysis of the nsp6/nsp7 junction. Multiple alignment of the nucleotide sequences of CoV-HKU1 genotypes A, B and C. In CoV-HKU1 genotype B and CoV-HKU1 genotype C, only the nucleotides differing from...
those in CoV-HKU1 genotype A are depicted. The nucleotides in CoV-HKU1
genotype C that are the same as those in CoV-HKU1 genotype A but different from
those in CoV-HKU1 genotype B are highlighted in black, and those in CoV-HKU1
genotype B that are the same as those in CoV-HKU1 genotype A but different from
those in CoV-HKU1 genotype C are highlighted in gray. The putative template
switching region is underlined and bolded. The first (TCA) and last (CAG) codons of
nsp7 are also underlined. The arrows denote positions with nucleotide polymorphism
[at 11414, N16, N17, N20, N21 and N22 (genotype C) were T instead of C; at 11422,
N16 and N20 (genotype C) was C instead of T; at 11449, N25 (genotype B) was C
instead of T; at 11528, N16, N17, N20 and N22 (genotype C) was C instead of T; at
11740, N6, N7, N9, N10, N11, N13, N14, N18, N23 and N24 (genotype A) was T
instead of C; at 12095, N6 and N7 (genotype A) was T instead of C; at 12140, N23
and N24 (genotype A) was C instead of T; and 12367, N15 and N25 (genotype C)
was A instead of G; and at 12400, N6, N7, N9, N10, N11, N13, N14, N18, N23 and
N24 (genotype A) was C instead of T].

FIG. 4. Comparative sequence analysis of the nsp16/HE gene junction.
Multiple alignment of the nucleotide sequences of CoV-HKU1 genotypes A, B and C.
In CoV-HKU1 genotype A and CoV-HKU1 genotype B, only the nucleotides
differing from those in CoV-HKU1 genotype C are depicted. The nucleotides in CoV-
HKU1 genotype C that are the same as those in CoV-HKU1 genotype A but different
from those in CoV-HKU1 genotype B are highlighted in gray, and those in CoV-
HKU1 genotype C that are the same as those in CoV-HKU1 genotype B but different
from those in CoV-HKU1 genotype A are highlighted in black. The putative template
switching region is underlined and bolded. The stop codon of ORF1ab (TAG) and the
start codon of the HE gene (ATG) are also underlined. The arrows denote positions
with nucleotide polymorphism [at 21297, N6, N7, N9, N10, N11, N13, N14, N23 and N24 (genotype A) were T instead of G; at 21429, N6 (genotype A) was C instead of T; at 21576, N15 (genotype B) was C instead of T; at 21908, N15 and N25 (genotype B) was G instead of A; and at 21949, N14 was T instead of C].
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The amino acids underlined denote those that are different from the NDDEDVVTGD tandem repeats.
FIG. 2.

% of Permuted Trees

0 4,000 8,000 12,000 16,000 20,000 24,000 28,000
Position

0 10 20 30 40 50 60 70 80 90 100
% of Permuted Trees

nsp1 nsp2 A1pp HD nsp4 nsp12 nsp13 nsp14 HE S ORF4E M N
PL1 pro PL2 pro nsp5 nsp6 nsp7 nsp8 nsp9 nsp10 nsp11 nsp15 nsp16

--- CoV-HKU1 genotype A --- CoV-HKU1 genotype B --- CoV-HKU1 genotype C