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# Coexistence of Different Genotypes in the Same Bat and Serological Characterization of *Rousettus* Bat Coronavirus HKU9 Belonging to a Novel *Betacoronavirus* Subgroup▽

Susanna K. P. Lau,[1,2,3,4]† Rosana W. S. Poon,[1]† Beatrice H. L. Wong,[1] Ming Wang,[5] Yi Huang,[1] Huifang Xu,[5] Rongtong Guo,[5] Kenneth S. M. Li,[1] Kai Gao,[5] Kwok-Hung Chan,[1] Bo-Jian Zheng,[1,2,3,4] Patrick C. Y. Woo,[1,2,3,4]* and Kwok-Yung Yuen[1,2,3,4]*

*Department of Microbiology,[1] Research Centre of Infection and Immunology,[2] State Key Laboratory of Emerging Infectious Diseases,[3] and Carol Yu Centre for Infection,[4] the University of Hong Kong, Hong Kong, China, and Guangzhou Center for Disease Control and Prevention, Guangzhou, China[5]*

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***Rousettus* bat coronavirus HKU9 (Ro-BatCoV HKU9), a recently identified coronavirus of novel *Betacoronavirus* subgroup D, from Leschenault's rousette, was previously found to display marked sequence polymorphism among genomes of four strains. Among 10 bats with complete RNA-dependent RNA polymerase (RdRp), spike (S), and nucleocapsid (N) genes sequenced, three and two sequence clades for all three genes were codetected in two and five bats, respectively, suggesting the coexistence of two or three distinct genotypes of Ro-BatCoV HKU9 in the same bat. Complete genome sequencing of the distinct genotypes from two bats, using degenerate/genome-specific primers with overlapping sequences confirmed by specific PCR, supported the coexistence of at least two distinct genomes in each bat. Recombination analysis using eight Ro-BatCoV HKU9 genomes showed possible recombination events between strains from different bat individuals, which may have allowed for the generation of different genotypes. Western blot assays using recombinant N proteins of Ro-BatCoV HKU9, *Betacoronavirus* subgroup A (HCoV-HKU1), subgroup B (SARSr-Rh-BatCoV), and subgroup C (Ty-BatCoV HKU4 and Pi-BatCoV HKU5) coronaviruses were subgroup specific, supporting their classification as separate subgroups under *Betacoronavirus*. Antibodies were detected in 75 (43%) of 175 and 224 (64%) of 350 tested serum samples from Leschenault's rousette bats by Ro-BatCoV HKU9 N-protein-based Western blot and enzyme immunoassays, respectively. This is the first report describing coinfection of different coronavirus genotypes in bats and coronavirus genotypes of diverse nucleotide variation in the same host. Such unique phenomena, and the unusual instability of ORF7a, are likely due to recombination which may have been facilitated by the dense roosting behavior and long foraging range of Leschenault's rousette.**

Coronaviruses infect a wide variety of animals in which they can cause respiratory, enteric, hepatic, and neurological diseases of various severities. Based on genotypic and serological characterization, coronaviruses were traditionally classified into three distinct groups, groups 1, 2, and 3 (3, 27, 59). Recently, the Coronavirus Study Group of the International Committee for Taxonomy of Viruses has renamed the traditional group 1, 2, and 3 coronaviruses as *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus*, respectively (http://talk.ictvonline.org/media/p/1230.aspx). Coronaviruses are known to have a high frequency of recombination as a result of their unique mechanism of viral replication (27). Such tendency for recombination and high mutation rates may allow them to adapt to new hosts and ecological niches (24, 47, 52).

The severe acute respiratory syndrome (SARS) epidemic has boosted interest in the study of coronaviruses in humans and animals (21, 34, 38, 41, 54). In the past few years, there has been a dramatic increase in the number of newly described human and animal coronaviruses (2, 4, 5, 8–10, 15–20, 23, 25, 28, 30, 32, 35, 36, 39, 43, 45, 50, 51, 53, 56, 58). Two novel human coronaviruses, human coronavirus NL63 (HCoV-NL63) and human coronavirus HKU1 (HCoV-HKU1), belonging to *Alphacoronavirus* and *Betacoronavirus*, respectively, have been discovered, in addition to the human coronavirus OC43 (HCoV-OC43), human coronavirus 229E (HCoV-229E), and SARS coronavirus (SARS-CoV) (17, 29, 45, 53, 55). We have also previously described the discovery of a diversity of novel coronaviruses in wild bats and birds in China, including SARSr-Rh-BatCoV, belonging to *Betacoronavirus* subgroup B, from Chinese horseshoe bats (30, 48, 56). Among these novel coronaviruses, three avian coronaviruses were found to belong to a novel subgroup of *Gammacoronavirus* (*Gammacoronavirus* subgroup C), while three bat coronaviruses were found to belong to two novel subgroups of *Betacoronavirus* (*Betacoronavirus* subgroups C and D) (48, 50). Based on the presence of the huge diversity of coronaviruses in *Alphacoronavirus* and *Betacoronavirus* among various bat species, bats are likely the reservoir for the ancestor of these two coronavirus genera (47).

During our genome analysis of these novel coronaviruses, one of them, *Rousettus* bat coronavirus HKU9 (Ro-BatCoV HKU9), belonging to *Betacoronavirus* subgroup D, which was

* Corresponding author. Mailing address: State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Hong Kong, China. Phone: (852) 22554892. Fax: (852) 28551241. E-mail for P. C. Y. Woo: pcywoo@hkucc.hku.hk. E-mail for K.-Y. Yuen: kyyuen@hkucc.hku.hk.
† These authors contributed equally to the manuscript.
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identified in Leschenault's rousette bats, was found to display marked nucleotide and amino acid sequence polymorphism among the four strains with complete genome sequences (50). In our study on HCoV-HKU1, it has been shown that such sequence polymorphisms may indicate the presence of different genotypes (52). By complete genome sequence analysis of the potentially different genotypes of HCoV-HKU1, we have demonstrated for the first time natural recombination in a human coronavirus, resulting in the generation of at least three genotypes (52). We have also recently shown that recombination between different strains of SARSr-Rh-BatCoV from different regions of China may have given rise to the emergence of civet SARSr-CoV (31). To investigate the presence of different genotypes of Ro-BatCoV HKU9, the complete RNA-dependent RNA polymerase (RdRp) (corresponding to nsp12), spike (S), and nucleocapsid (N) gene sequences of Ro-BatCoV HKU9 from 10 additional bats were determined. Since sequence analysis showed the possible coexistence of different genotypes in seven bat individuals, complete genome sequencing of these distinct genotypes from two bats was carried out to investigate for possible recombination events among the different genotypes. In addition, serological characterization of Ro-BatCoV HKU9 was also performed by Western blot and enzyme immunoassays using recombinant Ro-BatCoV HKU9 nucleocapsid proteins and recombinant nucleocapsid proteins of *Betacoronavirus* subgroup A, B, and C coronaviruses to determine possible cross-reactivity among the different *Betacoronavirus* subgroups and the seroepidemiology of Ro-BatCoV HKU9 in Leschenault's rousette bats.

## MATERIALS AND METHODS

**Sample collection.** The 350 bats, of the species Leschaenault's rousette (*Rousettus leschenaulti*), were captured from various locations in the Guangdong province of Southern China over a 7-month period (October 2005 to April 2006). Their respiratory and alimentary specimens were collected using procedures described previously (30, 50, 57).

**RNA extraction.** Viral RNA was extracted from the respiratory and alimentary specimens using the Qiaamp viral RNA minikit (Qiagen, Hilden, Germany). The RNA was eluted in 50 μl of AVE buffer and was used as the template for reverse transcription (RT)-PCR.

**Sequencing of the complete RdRp, S, and N genes of Ro-BatCoV HKU9 strains.** The complete RdRp, S, and N genes of Ro-BatCoV HKU9 strains from 10 bats were amplified and sequenced using primers designed by multiple alignments of available nucleotide sequences of Ro-BatCoV HKU9 (50). Reverse transcription was performed using the SuperScript III kit (Invitrogen, San Diego, CA). The PCR mixture (25 μl) contained cDNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM $MgCl_2$, and 0.01% gelatin), 200 μM each deoxynucleoside triphosphates (dNTPs), and 1.0 U *Taq* polymerase (Applied Biosystems, Foster City, CA). The mixtures were amplified in 40 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystem, Foster City, CA). Standard precautions were taken to avoid PCR contamination, and no false-positive results were observed in negative controls.

The PCR products were gel purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Both strands of the PCR products were sequenced twice with an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA), using the PCR primers. For PCR products that contained more than one sequence type (multiple signals at certain bases), specific primers were designed over the ambiguous positions based on the previously published genome sequences of the four Ro-BatCoV HKU9 strains, HKU9-1, HKU9-2, HKU9-3, and HKU9-4, since the sequences of different sequence types were often closely related to at least one of the four previous strains. PCR was performed using these specific primers to obtain the individual sequences of the different sequence types. The sequences of the PCR products were assembled manually, and all assembled sequences were confirmed by independent PCR using specific primers across overlapping regions to ensure accuracy of the assembled sequences before comparison with known sequences of the RdRp, S, and N genes of coronaviruses from the GenBank database.

**Complete genome sequencing of different Ro-BatCoV HKU9 strains from the same bat individual.** The complete genomes of two Ro-BatCoV HKU9 strains each from two bats were amplified and sequenced using the RNA extracted from the corresponding alimentary specimens as templates using degenerate primers designed by multiple alignment of available Ro-BatCoV HKU9 genome sequences (50) and/or genome-specific primers designed from genome sequences generated from previous rounds of sequencing. 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. All overlapping sequences were confirmed by independent PCR and sequencing using genome-specific primers to ensure that the flanking sequences were from the same viral genome. These genome-specific primers are shown in Tables 1 to 3 in the data posted at http://covdb.microbiology.hku.hk:8080/COV-newpages/file/supple_t1.pdf.

**Genome analysis.** The nucleotide sequences of the genomes and the deduced amino acid sequences of the open reading frames (ORFs) were compared to those of other coronaviruses. Phylogenetic tree construction was performed using the neighbor-joining method with ClustalX 1.83. Synonymous and nonsynonymous substitution rates were calculated using the Nei-Gojobori method (Jukes-Cantor) in MEGA 3.1 (26). Sliding window analysis was used to detect possible recombination, using nucleotide alignment of the available genome sequences of different Ro-BatCoV HKU9 strains generated by ClustalX version 1.83 and edited manually. Bootscan analysis was performed using Simplot version 3.5.1 as described previously (31, 33, 52) with selected strains, including HKU9-4 and HKU9-5-2, as the query strains.

**Cloning and purification of $His_6$-tagged recombinant nucleocapsid protein of Ty-BatCoV HKU4, Pi-BatCoV HKU5, and Ro-BatCoV HKU9.** Cloning and purification of $His_6$-tagged recombinant N protein of *Tylonycteris* bat coronavirus HKU4 (Ty-BatCoV HKU4), *Pipistrellus* bat coronavirus HKU5 (Pi-BatCoV HKU5), and Ro-BatCoV HKU9 were performed as described previously (30, 53, 54). Primers—5′-CTAGCTAGCATGGCTACTCCTGCTCC-3′ and 5′-CTAGCTAGCTTATGCCTCTGAATCGGG-3′, corresponding to amino acid residues 1 to 428 of Ty-BatCoV HKU4, 5′-CTAGCTAGCATGGCTACTCCTGCTCC-3′ and 5′-CTAGCTAGCTTATGCCTCTGAATCGGG-3′, corresponding to amino acid residues 1 to 428 of Pi-BatCoV HKU5, and 5′-CTAGCTAGCATGTCTGGACGGAATAAG-3′ and 5′-CTAGCTAGCTTAAGACCTTTCAGCTGCC-3′, corresponding to amino acid residues 1 to 471 of Ro-BatCoV HKU9—were used to amplify the N genes by RT-PCR. The PCR products were cloned into the NheI site of expression vector pET-28b(+) (Novagen, Madison, WI) in frame and downstream of the series of six histidine residues. The $His_6$-tagged recombinant nucleocapsid proteins were expressed and purified using the $Ni^{2+}$-loaded HiTrap chelating system (Amersham Pharmacia) according to the manufacturer's instructions. Approximately 1 mg of purified protein was routinely obtained from 1 liter of *Escherichia coli* carrying the fusion plasmid.

**Western blot analysis.** To evaluate the specificity of the recombinant Ro-BatCoV HKU9 N protein-based assay, purified $His_6$-tagged recombinant N proteins of HCoV-HKU1 (a *Betacoronavirus* subgroup A coronavirus) and SARSr-Rh-BatCoV (a *Betacoronavirus* subgroup B coronavirus), prepared as described previously, were also subjected to Western blot analysis, in addition to those of Ty-BatCoV HKU4 and Pi-BatCoV HKU5 (both *Betacoronavirus* subgroup C coronaviruses) (30, 53, 54). Briefly, 600 ng of purified $His_6$-tagged recombinant nucleocapsid protein was loaded into each well of a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and subsequently electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The blot was cut into strips, and the strips were incubated separately with a 1:500 dilution of bat serum samples. Antigen-antibody interaction was detected with an ECL fluorescence system (Amersham Life Science, Buckinghamshire, United Kingdom). Convalescent-phase serum from a patient with recent infection by HCoV-HKU1 and serum from a Chinese horseshoe bat infected by SARSr-Rh-BatCoV were used as positive controls for the HCoV-HKU1 and SARSr-Rh-BatCoV protein-based assays, respectively. As serum was not available from lesser bamboo bats infected with Ty-BatCoV HKU4 and Japanese pipistrelles bats infected with Pi-BatCoV HKU5, only anti-His antibody was used as a positive control for Ty-BatCoV HKU4 and Pi-BatCoV HKU5 protein-based assays. Serum from a great round-leaf bat (*Hipposideros armiger*), negative for *Betacoronavirus*, was used as a negative control.

**Enzyme-linked immunosorbent assay (ELISA) for seroprevalence study of Ro-BatCoV HKU9 among Leschenault's rousette bats.** The presence of IgG antibodies against Ro-BatCoV HKU9 in serum samples from Leschenault's

rousette bats was tested by an ELISA using recombinant N protein of Ro-BatCoV HKU9. Sera from 100 Leschenault's rousette bats, negative for antibody against Ro-BatCoV HKU9 recombinant N protein by Western blot assay, were used to set up the baseline for the ELISA. The test was performed as described previously with modifications (30, 54). Briefly, MaxiSorp Nunc-immuno 96-microwell plates (Roskilde, Denmark) coated with 20 ng purified $His_6$-tagged recombinant Ro-BatCoV HKU9 N protein per well were used. Diluted bat serum (100 μl of a 1:100 dilution) was added to each well. Detection was performed using 1:4,000 horseradish peroxidase-conjugated goat anti-bat IgG (Zymed, South San Francisco, CA) and 3,3′,5,5′-tetramethylbenzidine (Zymed). Each sample was tested in duplicate, and the mean absorbance for each serum was calculated.

**Statistical analysis.** Comparison of serological results between groups was performed using a chi-square test for categorical variables and Student's *t* test for continuous variables (SPSS version 11.5). *P* values of $<0.05$ were regarded as statistically significant.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the four genomes of Ro-BatCoV HKU9 have been added to the GenBank sequence database under accession no. HM211098 to HM211101.

## RESULTS

**Complete RdRp, S, and N gene sequence analysis of Ro-BatCoV HKU9 from Leschenault's rousette bats.** Among the 700 respiratory and alimentary specimens from 350 Leschenault's rousette bats, RT-PCR and sequencing of a 440-bp fragment in the RdRp genes of coronaviruses showed the presence of Ro-BatCoV HKU9 in alimentary specimens from 42 (12%) bats in our previous study (50). The complete RdRp, S, and N gene sequences of Ro-BatCoV HKU9 from 10 bats (HKU9-5 to HKU9-14) that showed apparent sequence polymorphisms within the 440-bp fragment of the RdRp gene were determined, in addition to the four strains (HKU9-1 to HKU9-4) with complete genome sequences available from our previous study (50). In all three genes, significant nucleotide and amino acid sequence polymorphism was observed, with more divergence in S and N genes than in the RdRp gene (Fig. 1A). Moreover, 7 of the 10 samples were found to contain at least two different gene sequences in all three genes, suggesting the presence of more than one distinct genome sequence of Ro-BatCoV HKU9 in the same bat individual (Table 1; Fig. 1A). The number and distribution of different gene sequence clades were highly similar between samples HKU9-5 and HKU9-6, among HKU9-7 to HKU9-11, and between HKU9-12 and HKU9-13 (Table 1).

For the RdRp gene, at least four distinct sequence clades (P1 to P4) were identified (Table 1; Fig. 1A). For the seven samples (HKU9-5 to HKU9-11) that contained at least two different RdRp gene sequences, they formed two separate clades, one closely related to the RdRp gene sequence of Ro-BatCoV HKU9-2 (P1) and the other closely related to that of Ro-BatCoV HKU9-3 (P2). Two samples, HKU9-5 and HKU9-6, each contained three different RdRp gene sequences, two belonging to P1 and one belonging to the P2 sequence clade. One sample, HKU9-14, possessed a sequence closely related to that of Ro-BatCoV HKU9-1, forming a third clade (P3), while Ro-BatCoV HKU9-4 possessed a sequence distantly related to the other three clades (P4).

For the S gene, at least six distinct sequence clades (S1 to S6) were identified (Table 1; Fig. 1A). For the seven samples (HKU9-5 to HKU9-11) that contained at least two different S gene sequences, they also formed two separate clades, one closely related to the S gene sequence of Ro-BatCoV HKU9-3 (S1) and the other only distantly related to that of the four previously sequenced strains (S2). Again, samples HKU9-5 and HKU9-6 each contained three different S gene sequences, one belonging to the S1 and two belonging to the S2 sequence clade. HKU9-2 (S3) and strains Ro-BatCoV HKU9-4 (S4), Ro-BatCoV HKU9-14 (S5), and Ro-BatCoV HKU9-1 (S6) possessed a sequence only distantly related to the two clades and to that of each of the others.

For the N gene, at least seven distinct sequence clades (N1 to N7) were identified (Table 1; Fig. 1A). For the seven samples (HKU9-5 to HKU9-11) that contained at least two different N gene sequences, they formed three separate clades. One clade of sequences was closely related to that of Ro-BatCoV HKU9-3 (N1), while the other two clades were only distantly related to that of the four previously sequenced strains (N2 and N3). The two samples, HKU9-5 and HKU9-6, contained three different N sequences each belonging to one of the three clades (N1 to N3). Similar to the result of S gene analysis, HKU9-2 (N4) and strains Ro-BatCoV HKU9-4 (N5), Ro-BatCoV HKU9-14 (N6), and Ro-BatCoV HKU9-1 (N7) possessed an N sequence only distantly related to the three clades and to that of each of the others.

**Complete genome analysis of multiple Ro-BatCoV HKU9 strains from the same bat individual.** To confirm the presence of distinct genome sequences from the same bat and examine for possible recombination events among the different genotypes, two samples (HKU9-5 and HKU9-10), each containing at least two different sequences in RdRp, S, and N genes, were subject to complete genome sequencing. The sizes of the two genomes of sample HKU9-5 (HKU9-5-1 and HKU9-5-2) were 29,136 and 29,112 bases, with G+C contents of 43% and 41%, respectively. They possessed 77% nucleotide identity to each other. The size of the two genomes of Ro-BatCoV HKU9-10 (HKU9-10-1 and HKU9-10-2) were 29,136 and 29,122 bases, with G+C contents of 43% and 41%, respectively. They possessed 77% nucleotide identity to each other. The genome organizations of HKU9-5-1, HKU9-5-2, HKU9-10-1, and HKU9-10-2 are identical to those of the four previously sequenced strains. The putative TRS motif, 5′-ACGAAC-3′, is observed in all four genomes. They also exhibited other unique genome features similar to the four previously described strains, supporting that they belong to the same coronavirus species, Ro-BatCoV HKU9. First, the histidine at the P1 position of the putative cleavage site at the nsp9/nsp10 junction is conserved among all genomes. This position was occupied by glutamine in other coronaviruses except Ro-BatCoV HKU9 (50). Second, the 3′ ends of all genomes contain the longest stretch of nucleotides after the N gene among all known coronaviruses with complete genomes available, with two unique ORFs encoding NS7a and NS7b. A putative bulged stem-loop at the putative coding region of NS7b, followed by a pseudoknot structure >1 kb downstream of N, was also identified, which is different from the classical structures observed immediately downstream of N in other betacoronaviruses (47, 50).

The $K_a/K_s$ ratio for the various coding regions in Ro-BatCoV HKU9 is shown at http://covdb.microbiology.hku.hk:8080/COV-newpages/file/supple_t1.pdf. Similar to results previously obtained using four genome sequences, the highest mean $K_a/K_s$ ratio was observed at NS7a (0.983, comparable to 0.961

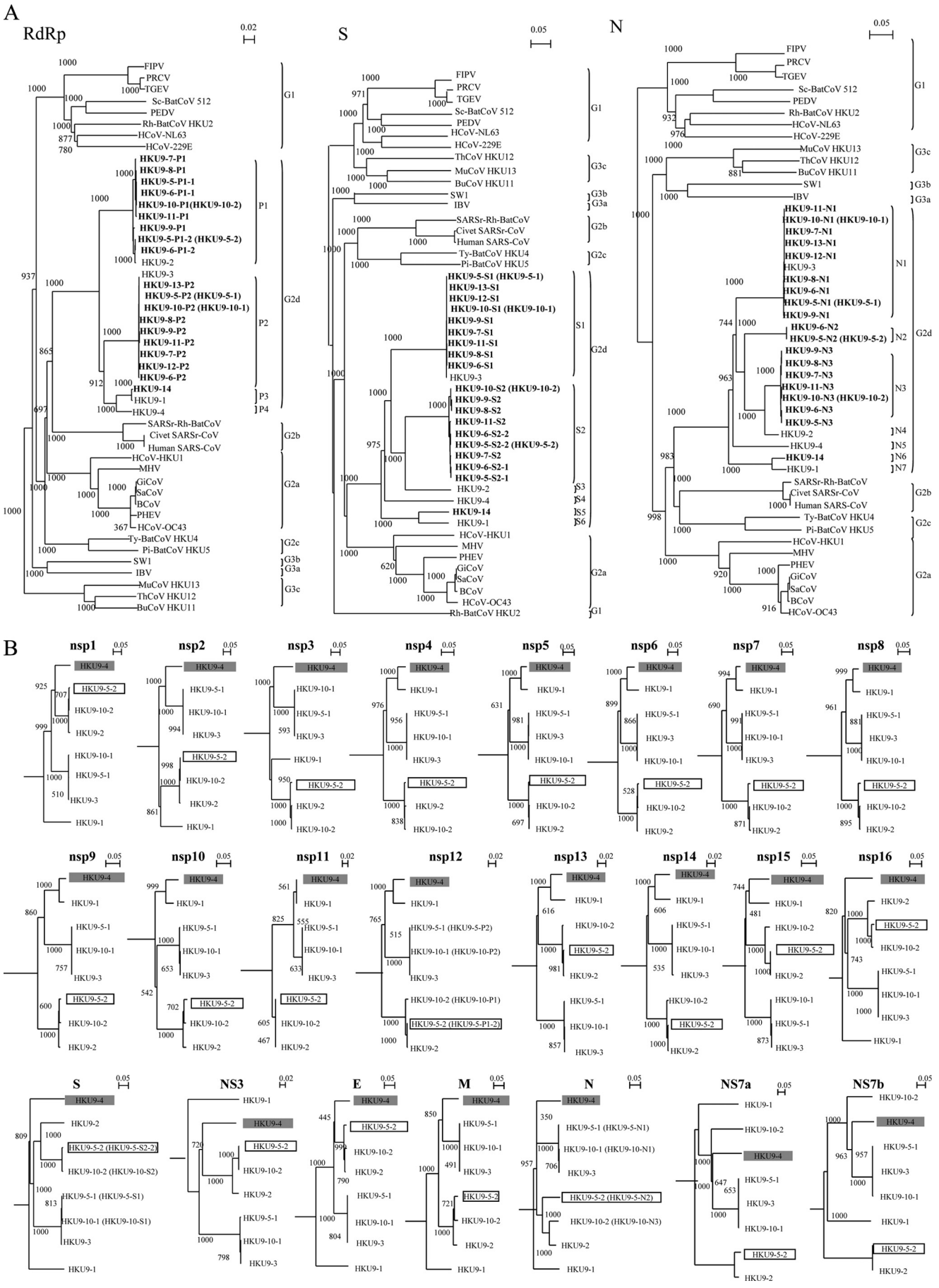

TABLE 1. Distribution of different sequence clades of Ro-BatCoV HKU9 among the 14 Lechenault's rousette bat individuals

| Sample no. | No. of sequence clades | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RdRp gene | | | | Spike gene | | | | | | Nucleocapsid gene | | | | | | |
| | P1 | P2 | P3 | P4 | S1 | S2 | S3 | S4 | S5 | S6 | N1 | N2 | N3 | N4 | N5 | N6 | N7 |
| HKU9-1 | | | 1 | | | | | | | 1 | | | | | | | 1 |
| HKU9-2 | 1 | | | | | | 1 | | | | | | | 1 | | | |
| HKU9-3 | | 1 | | | 1 | | | | | | 1 | | | | | | |
| HKU9-4 | | | | 1 | | | | 1 | | | | | | | 1 | | |
| HKU9-5 | 2 | 1 | | | 1 | 2 | | | | | 1 | 1 | 1 | | | | |
| HKU9-6 | 2 | 1 | | | 1 | 2 | | | | | 1 | 1 | 1 | | | | |
| HKU9-7 | 1 | 1 | | | 1 | 1 | | | | | 1 | | 1 | | | | |
| HKU9-8 | 1 | 1 | | | 1 | 1 | | | | | 1 | | 1 | | | | |
| HKU9-9 | 1 | 1 | | | 1 | 1 | | | | | 1 | | 1 | | | | |
| HKU9-10 | 1 | 1 | | | 1 | 1 | | | | | 1 | | 1 | | | | |
| HKU9-11 | 1 | 1 | | | 1 | 1 | | | | | 1 | | 1 | | | | |
| HKU9-12 | | 1 | | | 1 | | | | | | 1 | | | | | | |
| HKU9-13 | | 1 | | | 1 | | | | | | 1 | | | | | | |
| HKU9-14 | | | 1 | | | | | | 1 | | | | | | | 1 | |

in a previous study [50]), supporting that this gene is rapidly evolving. A high mean $K_a/K_s$ ratio was also observed at nsp11 (0.814 compared to 0.283 in a previous study [50]) which is a short 15-amino-acid peptide of unknown function at the end of ORF1a, suggesting that nsp11 may be nonfunctional.

**Recombination analysis.** To detect recombination between genomes of different strains of Ro-BatCoV HKU9, sliding window analysis was conducted using the eight available genome sequences (HKU9-1, HKU9-2, HKU9-3, HKU9-4, HKU9-5-1, HKU-9-5-2, HKU9-10-1, and HKU9-10-2). Results showed possible recombination events between strains from different bat individuals but not between strains from the same bat individual. When strain HKU9-4 was used as the query with other strains as potential parents, a recombination breakpoint at nsp3 was identified (Fig. 2A). Upstream of this breakpoint before position 6100, high bootstrap support for clustering of strain HKU9-4 with HKU9-3 was observed. However, an abrupt change in clustering occurred after position 7700, with high bootstrap support for clustering of strain HKU9-4 with HKU9-1 up to position 18700 (at nsp15), where a fall in bootstrap support was observed. This is in line with results from phylogenetic analysis, where strain HKU9-4 was more closely related to strain HKU9-3, HKU9-5-1, and HKU9-10-1 in nsp2 and nsp3 but more to HKU9-1 from nsp4 to nsp15 (Fig. 1B).

Another potential recombination event was also observed when strain HKU9-5-2 was used as a query, with a breakpoint identified at the nsp15/16 junction (Fig. 2B). From positions 13100 to 19900, high bootstrap support for clustering of strain HKU9-5-2 with HKU9-2 was observed. However, a change in clustering occurred after position 20300, with high bootstrap support for clustering of strain HKU9-5-2 with HKU9-10-2. This is also in keeping with results from phylogenetic analysis, where strain HKU9-5-2 was more closely related to strain HKU9-2 from nsp12 to nsp15 but more to HKU9-10-2 from nsp16 to M protein (Fig. 1B). Interestingly, sample HKU9-5 (and similarly HKU9-6) contained three different sequences at the RdRp, S, and N genes, with two sequence clades at RdRp

FIG. 1. (A) Phylogenetic analysis of RNA-dependent RNA polymerase (RdRp), spike (S), and nucleocapsid (N) of Ro-BatCoV HKU9 strains from different samples. The different RdRp, S, and N sequence types were marked by P1 to P4, S1 to S6, and N1 to N7, respectively. The trees were constructed by the neighbor-joining method using Kimura's two-parameter correction and bootstrap values calculated from 1,000 trees. Totals of 2,845, 3,778, and 1,234 nucleotides in RdRp, S, and N, respectively, were included in the analysis. The scale bars indicate the estimated numbers of substitutions per 50 bases for RdRp and per 20 bases for S and N. Sequences obtained in the present study are shown in bold. The following strains were used: HCoV-229E, human coronavirus 229E (NC002645); PEDV, porcine epidemic diarrhea virus (NC003436); PRCV, porcine respiratory coronavirus (DQ811787); TGEV, porcine transmissible gastroenteritis virus (NC002306); FIPV, feline infectious peritonitis virus (AY994055); HCoV-NL63, human coronavirus NL63 (NC005831); Rh-BatCoV HKU2 (DQ249235); Sc-BatCoV 512 (NC009657); HCoV-HKU1, human coronavirus HKU1 (NC006577); HCoV-OC43, human coronavirus OC43 (NC005147); MHV, murine hepatitis virus (NC006852); BCoV, bovine coronavirus (NC003045); PHEV, porcine hemagglutinating encephalomyelitis virus (NC007732); SaCoV, sable antelope coronavirus (EF424621); GiCoV, giraffe coronavirus (EF424622); SARS-CoV (human), human SARS coronavirus (NC004718); SARSr-CoV (Civet), civet SARS-related coronavirus (AY304488); SARSr-Rh-BatCoV HKU3, SARS-related *Rhinolophus* bat coronavirus HKU3 (DQ022305); Ty-BatCoV HKU4 (DQ074652); Pi-BatCoV HKU5 (DQ249219); Ro-BatCoV HKU9-1 (EF065513); Ro-BatCoV HKU9-2 (EF065514); Ro-BatCoV HKU9-3 (EF065515); Ro-BatCoV HKU9-4 (EF065516); IBV-like, IBV isolated from peafowl (AY641576); SW1, Beluga whale coronavirus (NC010646); BulCoV HKU11, Bulbul coronavirus HKU11 (FJ376620); ThCoV, thrush coronavirus HKU12 (FJ376621); MuCoV, Munia coronavirus HKU13 (FJ376622). (B) Phylogenetic analysis of nsp1 to nsp16, S, NS3, E, M, N, NS7a, and NS7b of the eight complete Ro-BatCoV HKU9 genomes. The two genomes, HKU9-4 and HKU9-5-2, used as queries for the bootscan analysis shown in Fig. 2 are marked in gray and open boxes, respectively. The trees were constructed by the neighbor-joining method using Kimura's two-parameter correction and bootstrap values calculated from 1,000 trees. Totals of 561, 1,811, 5,844, 1,502, 918, 878, 267, 595, 336, 414, 24, 2,772, 1,803, 1,585, 1,123, 892, 4,054, 695, 235, 670, 1,385, 594, and 445 nucleotide positions in nsp1 to nsp16, S, NS3, E, M, N, NS7a, and NS7b, respectively, were included in the analysis. The scale bar indicates the estimated number of substitutions per 20 or 50 nucleotides as indicated. The corresponding nucleotide sequences of HCoV-HKU1 were used as the outgroups.

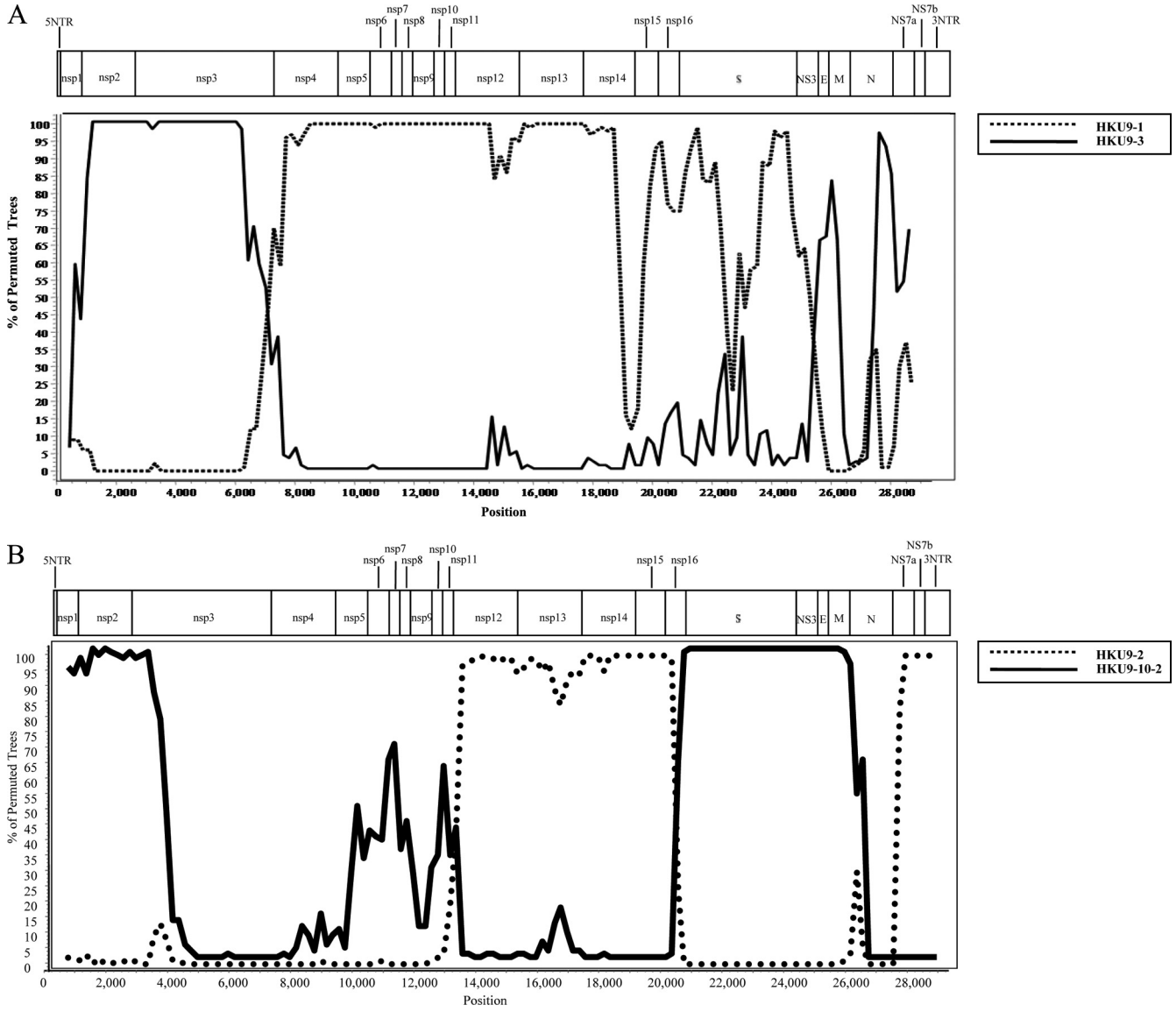


FIG. 2. (A) Bootscan analysis using the genome sequence of HKU9-4 as the query sequence. The dotted line denotes strain HKU9-1, and the solid line denotes strain HKU9-3. (B) Bootscan analysis using the genome sequence of HKU9-5-2 as the query sequence. The dotted line denotes strain HKU9-2, and the solid line denotes strain HKU9-10-2. Bootscanning was conducted with Simplot version 3.5.1 (F84 model; window size, 1,000 bp; step, 200 bp) on a gapless nucleotide alignment, generated with ClustalX.

and S but three sequence clades at N (Fig. 1A). The complete genome sequences of the two different strains from sample HKU9-5 also showed that strain HKU9-5-2 possessed a different N gene sequence (HKU9-5-N2) compared to other regions of the genome upon phylogenetic analysis. Since no further sequence clades at the RdRp and S regions could be identified from sample HKU9-5 by cloning experiments (data not shown), the distinct N gene sequence (HKU9-5-N2) probably was acquired from recombination with other Ro-BatCoV HKU9 strains.

**Serological studies.** The recombinant N-protein-based Western blot assays were found to be subgroup specific among strains of *Betacoronavirus* (Fig. 3A). Human serum positive for HCoV-HKU1 antibody was negative by Western blot assays based on recombinant SARSr-Rh-BatCoV, Ty-BatCoV HKU4, Pi-BatCoV HKU5, and Ro-BatCoV HKU9 N protein. Serum from Chinese horseshoe bats positive for SARSr-Rh-BatCoV antibody was negative by Western blot assays based on recombinant HCoV-HKU1, Ty-BatCoV HKU4, Pi-BatCoV HKU5, and Ro-BatCoV HKU9 N protein. Serum from a Leschenault's rousette bat positive for antibody against Ro-BatCoV HKU9 N protein was negative by Western blot assays based on recombinant HCoV-HKU1, SARSr-Rh-BatCoV, Ty-BatCoV HKU4, and Pi-BatCoV HKU5 protein.

Prominent immunoreactive protein bands of about 60 kDa were observed in 75 (43%) of 175 tested serum samples from Leschenault's rousette bats in the recombinant Ro-BatCoV HKU9 N-protein-based Western blot assay. An ELISA-based Ro-BatCoV HKU9 antibody test was developed for seroprevalence study. Box titration with different dilutions of $His_6$-

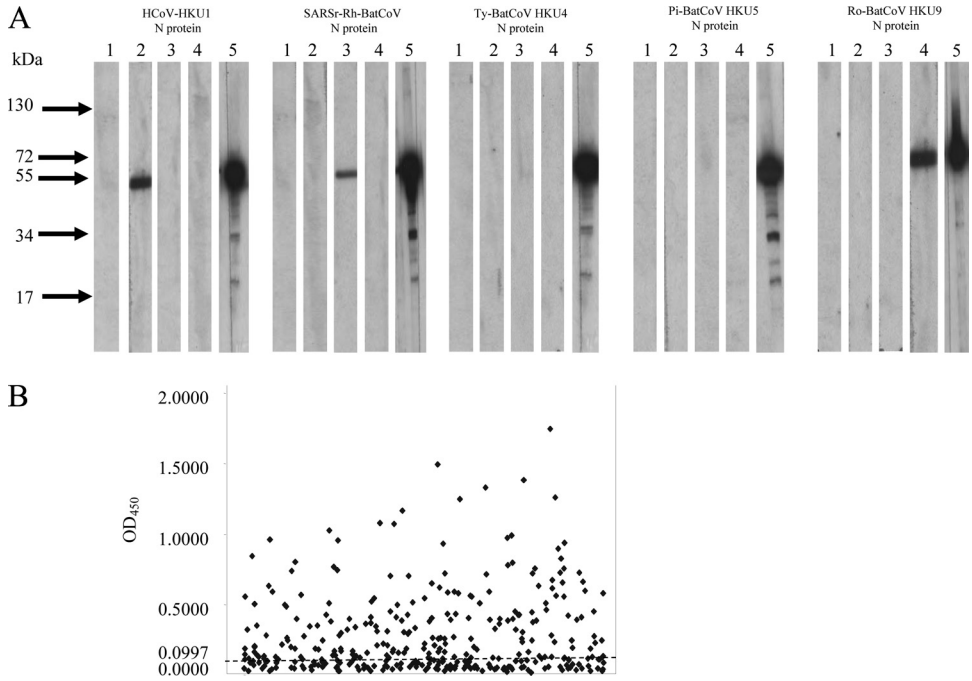


FIG. 3. (A) Western blot analysis against purified $His_6$-tagged recombinant N protein from the different subgroups of *Betacoronavirus*, including HCoV-HKU1 (*Betacoronavirus* subgroup A), SARSr-Rh-BatCoV (*Betacoronavirus* subgroup B), Ty-BatCoV HKU4 (*Betacoronavirus* subgroup C), Pi-BatCoV HKU5 (*Betacoronavirus* subgroup C), and Ro-BatCoV HKU9 (*Betacoronavirus* subgroup D). Western blot analysis was performed using serum from a great roundleaf bat not infected with coronaviruses as a negative control (lane 1), convalescent-phase serum from a patient with recent infection by HCoV-HKU1 (lane 2), serum from a Chinese horseshoe bat infected with SARSr-Rh-BatCoV (lane 3), serum from a Leschenault's rousette bat infected with Ro-BatCoV HKU9 (lane 4), and anti-His antibody as a positive control (lane 5). Prominent immunoreactive protein bands of about 60 kDa, consistent with the expected size of 56.5 kDa of the recombinant Ro-BatCoV HKU9 N protein, were detected with serum from the Leschenault's rousette bat but not with other animal/human serum. (B) Scatter plot of $OD_{450}$ values by ELISA based on recombinant Ro-BatCoV HKU9 nucleocapsid protein using serum samples from Leschenault's rousette bats. The dashed line represents the cutoff $OD_{450}$ value.

tagged recombinant N protein and positive serum by Western blotting identified 20 ng protein per well as the optimal amount for plate coating for IgG detection. Of the 100 serum samples negative by Western blotting used to establish the baseline for the IgG ELISA, the mean optical density at 450 nm ($OD_{450}$) was 0.0534 (standard deviation [SD], 0.0154). An absorbance value of 0.0997 was selected as the cutoff value (mean value of negative serum plus 3 [SD]). Using this cutoff, 224 (64%) of 350 tested sera from Leschenault's rousette bats were positive for IgG against recombinant Ro-BatCoV HKU9 N protein (Fig. 3B). Leschenault's rousette bats that were RT-PCR positive for Ro-BatCoV HKU9 had a higher positive IgG detection rate (78.6% versus 62%, $P = 0.036$ by chi-square test) and a higher mean $OD_{450}$ (0.3617 versus 0.2607, $P = 0.032$ by Student's $t$ test) by ELISA than those that were RT-PCR negative.

## DISCUSSION

This is the first report that describes coinfection of multiple genotypes of the same coronavirus species in the same bat individual. Such a phenomenon, coronavirus genotypes of significant nucleotide variation along the whole viral genome infecting the same animal host, has not been reported previously. Despite the discovery of a large number and huge diversity of novel animal and human coronaviruses since the SARS epidemic in 2003, the coexistence of different genotypes of coronavirus in the same host has been rarely reported. The only coronavirus well reported to cause simultaneous infection by more than one genotype was canine coronavirus (CCoV), which was divided into type I and type II based on M gene analysis (11). However, the two CCoV genotypes share up to 96% nucleotide identity in the viral genome, with sequence divergence mainly observed in the spike protein gene (13). It has been shown that dogs were frequently infected naturally by both genotypes, with viral RNA titers generally higher for type I than type II (14). In another study on raised *Canidae* animals in China, coexistence of the two CCoV genotypes was also detected in 25 of 61 healthy foxes and 16 of 24 raccoon dogs (46). However, the significance of such simultaneous infection by both genotypes, in terms of pathogenesis, remains to be determined (11). Although CCoV has been associated with canine diarrhea, the virus is also frequently detected in healthy animals and shed in feces of naturally infected dogs for up to 6 months (40). In the present study, Leschenault's rousette bats were also found to harbor different genotypes of Ro-BatCoV HKU9 in their alimentary samples. Among 10 bats with complete RdRp, S, and N genes sequenced, three and two sequence clades for all three genes were codetected in two and five bats, respectively, suggesting that these seven bats contained two or three distinct genotypes of Ro-BatCoV HKU9. This was confirmed by complete genome sequencing of two

distinct genomes each from two samples, HKU9-5 and HKU9-10, with the two genomes from the same sample exhibiting >20% nucleotide substitutions rather evenly distributed over the entire genome (Fig. 1B). During our previous studies on bat coronaviruses, a similar phenomenon has not been encountered in other coronavirus species other than Ro-BatCoV HKU9. Although the three different genotypes of HCoV-HKU1 described previously were also detected in different patients with respiratory tract infection, there was no evidence of coinfection by more than one genotype (29, 52, 55). Therefore, the coexistence of different genotypes of coronavirus in the same host is likely to be species or host specific.

The unique presence of diverse genotypes of Ro-BatCoV HKU9 in Leschaenault's rousette is likely the result of a combination of mutation and recombination favored by the biology and behavior of this fruit bat species. Compared to other bat species that were found to harbor coronaviruses in our previous studies (28, 30, 31, 50, 56), Leschaenault's rousette, a cave-dwelling fruit bat species widely distributed in South and Southeast Asia, are found to roost in extremely densely packed colonies of up to 6,800 individuals and have a wide habitat tolerance, living in harsh areas such as sea caves in Hong Kong. Moreover, this common fruit bat, with its large body size and forearm length up to 86 mm, has a particularly long flying distance of 7.5 to 11.7 km to foraging sites and probably can migrate even longer distances (42) (http://www.bio.bris.ac.uk/research/bats/China/bats//rousettusleschenaultii.htm). These special biological features may have facilitated exchange of viruses and recombination among the viruses in the generation of different Ro-BatCoV HKU9 genotypes in this particular bat species. As a result of the infidelity of RNA-dependent RNA polymerase and high frequency of homologous recombination, coronaviruses are able to rapidly evolve to generate a diversity of species and cause cross-species transmission (24, 31, 47, 52). In addition, this has also resulted in the generation of different genotypes in a particular coronavirus species. This has been exemplified by the presence of at least three genotypes in HCoV-HKU1 as a result of natural recombination (52). Novel CCoV type II strains have also been recently suggested to have originated from a double-recombination event with porcine transmissible gastroenteritis virus (TGEV), at the 5′ end of the spike gene (12). As for infectious bronchitis virus (IBV), phylogenetic analysis of partial S1 and N gene sequences of isolates from Italy has also revealed incongruent clustering suggestive of recombination events that could contribute to the genetic diversity (1). In the present study, four, six, and seven different sequence clades were identified in RdRp, S, and N genes of the tested bat samples, respectively. Although the different sequence clades in the three genes mostly fell into the same clusters among the samples, two samples, HKU9-5 and HKU9-10, each containing three different sequences in all three genes, exhibited incongruent tree topology. Complete genome sequencing of two distinct genomes of HKU9-5 confirmed the presence of an evolutionarily distinct N gene (HKU9-5-N2) in one of the genomes (HKU9-5-2), which is likely to have been acquired by recombination. We ruled out the possibility of falsely assembled sequences and the existence of as-yet-undetected RdRp and S sequence clades by confirmation of all assembled sequences using genome-specific primers for PCR across overlapping regions and cloning experiments using conserved primers at RdRp and S regions. Recombination analysis also revealed additional recombination events that may have occurred at the nsp15/16 junction between strains HKU9-2 and HKU9-10-2 in the generation of HKU9-5-2. In addition, potential recombination events were also observed at nsp3 between strain HKU9-1 and HKU9-3 in the generation of HKU9-4. The present findings suggest that, in addition to nucleotide polymorphisms as a result of mutation, recombination between the different genotypes of Ro-BatCoV HKU9 may have occurred frequently in the generation of new genotypes. All Leschenault's rousette bats infected with Ro-BatCoV HKU9 showed no clinical evidence of disease in our present study. Further studies are required to determine if such recombination events confer biological advantage to the virus in terms of immune evasion or persistent infection.

The Ro-BatCoV HKU9 strains of distinct gene sequence clades represent different genotypes of the same coronavirus species rather than different coronavirus species. First, all these viral sequences were found exclusively in Leschenault's rousette bats. Second, despite the nucleotide polymorphisms observed between different sequence clades, the genome sizes and organization of the available eight complete genomes from six bats were highly similar. We have previously reported that Ro-BatCoV HKU9 possessed the longest stretch of nucleotides (>1.2 kb) downstream of the N gene among all known coronaviruses with complete genomes available. It also represented the only betacoronavirus to contain two ORFs, NS7a and 7b, in this region (50). Previously, genes downstream of N have been reported only in feline infectious peritonitis virus (FIPV) and TGEV, both alphacoronaviruses, which are important for virulence and viral replication/assembly, respectively (22, 37, 44). While the presence of the TRS motif supports the idea that NS7a and NS7b of Ro-BatCoV HKU9 are probably expressed, the high $K_a/K_s$ implies that they are rapidly evolving and, therefore, may be recently acquired by recombination (50). In fact, such a high $K_a/K_s$ ratio (0.983) observed in ORF7a of Ro-BatCoV HKU9 has not been seen in any other ORFs of bat coronaviruses in our previous studies (28, 31, 50), suggesting that this part of the genome is unusually unstable. All four complete genomes from the two bat samples, HKU9-5 and HKU9-10, in the present study also contain a long stretch of nucleotides containing NS7a and NS7b downstream of N. Moreover, the phylogenetic analysis of these two ORFs of the eight available Ro-BatCoV HKU9 genomes showed that their tree topology was quite different from those of the rest of the genomes (Fig. 1B), which may reflect the rapid evolution in this region. Furthermore, all these eight genomes possessed a putative bulged stem-loop and pseudoknot structure at a position different from that observed in other betacoronaviruses.

The absence of cross-reactive antibodies between Ro-BatCoV HKU9 N protein and N proteins from HCoV-HKU1 (*Betacoronavirus* subgroup A coronavirus), SARSr-Rh-BatCoV (*Betacoronavirus* subgroup B coronavirus), Ty-BatCoV HKU4, and Pi-BatCoV HKU5 (both *Betacoronavirus* subgroup C coronaviruses) upon Western blot analysis supports their classification as separate subgroups of *Betacoronavirus*. In human coronavirus infections, antigenic cross-reactivity has been commonly observed between SARS-CoV and HCoV-OC43 (*Betacoronavirus* subgroup A coronavirus) by immunofluorescence

assays (6, 7). When a recombinant SARS-CoV N-protein-based ELISA was used, cross-reactivity was observed in only 3 of 21 and 1 of 7 serum samples containing antibodies against HCoV-OC43 and HCoV-229E (49). In another study utilizing N-protein-based line immunoassays, no cross-reactions were found between SARS-CoV and the other four human coronaviruses (HCoV-OC43, HCoV-229E, HCoV-HKU1, and HCoV-NL63), although possible cross-reactions occurred between HCoV-OC43 and HCoV-HKU1 (both *Betacoronavirus* subgroup A) and between HCoV-229E and HCoV-NL63 (both alphacoronaviruses) (49). These suggest that an N-protein-based Western blot assay may be a more specific test for detection of antibody specific to coronavirus groups and subgroups. Since the N protein sequences of Ro-BatCoV HKU9 exhibit <40% amino acid identities to those of other betacoronaviruses, we developed N-protein-based assays for detection of specific antibody in the serum of Leschenault's rousette against Ro-BatCoV HKU9. Western blot assays demonstrated no serological cross-reactions between N proteins of the other three subgroups of *Betacoronavirus*. Moreover, no cross-reaction was identified among the other subgroups, supporting that the assay is subgroup specific, although more serum samples, especially for Ty-BatCoV HKU4 and Pi-BatCoV HKU5, should be tested to confirm the present findings. The higher levels of IgG as detected by ELISA in bats positive for Ro-BatCoV HKU9 by RT-PCR than in negative bats also supported the idea that infection by Ro-BatCoV HKU9 is associated with specific antibody response to Ro-BatCoV HKU9 N-protein.

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