

## Diversity and Evolutionary Histories of Human Coronaviruses NL63 and 229E Associated with Acute Upper Respiratory Tract Symptoms in Kuala Lumpur, Malaysia

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**Abstract.** The human alphacoronaviruses HCoV-NL63 and HCoV-229E are commonly associated with upper respiratory tract infections (URTI). Information on their molecular epidemiology and evolutionary dynamics in the tropical region of southeast Asia however is limited. Here, we analyzed the phylogenetic, temporal distribution, population history, and clinical manifestations among patients infected with HCoV-NL63 and HCoV-229E. Nasopharyngeal swabs were collected from 2,060 consenting adults presented with acute URTI symptoms in Kuala Lumpur, Malaysia, between 2012 and 2013. The presence of HCoV-NL63 and HCoV-229E was detected using multiplex polymerase chain reaction (PCR). The spike glycoprotein, nucleocapsid, and *Ia* genes were sequenced for phylogenetic reconstruction and Bayesian coalescent inference. A total of 68/2,060 (3.3%) subjects were positive for human alphacoronavirus; HCoV-NL63 and HCoV-229E were detected in 45 (2.2%) and 23 (1.1%) patients, respectively. A peak in the number of HCoV-NL63 infections was recorded between June and October 2012. Phylogenetic inference revealed that 62.8% of HCoV-NL63 infections belonged to genotype B, 37.2% was genotype C, while all HCoV-229E sequences were clustered within group 4. Molecular dating analysis indicated that the origin of HCoV-NL63 was dated to 1921, before it diverged into genotype A (1975), genotype B (1996), and genotype C (2003). The root of the HCoV-229E tree was dated to 1955, before it diverged into groups 1–4 between the 1970s and 1990s. The study described the seasonality, molecular diversity, and evolutionary dynamics of human alphacoronavirus infections in a tropical region.

### INTRODUCTION

Human coronaviruses were first reported in the mid-1960s and are known to be associated with acute upper respiratory tract infections (URTI) or the common cold.<sup>1–3</sup> According to the International Committee for Taxonomy of Viruses, human coronavirus NL63 (HCoV-NL63) and 229E (HCoV-229E) belong to the alphacoronavirus genus, a member of the *Coronaviridae* family. Coronaviruses are positive-strand RNA viruses with the largest genome of approximately 27–31 kb in size.<sup>4</sup> In previous studies, analysis of the spike (*S*) glycoprotein, nucleocapsid (*N*), and *Ia* genes of HCoV-NL63 and HCoV-229E revealed evidence of genetic recombination, genetic drift, and positive selection events as part of the evolution of the virus.<sup>5,6</sup> Phylogenetically, HCoV-NL63 and HCoV-229E are more closely related to each other than to any other human coronavirus.<sup>7</sup>

HCoV-NL63 and HCoV-229E account for about 5% of all acute URTI,<sup>7–9</sup> and in some cases, a small proportion of infections are associated with hospital admission.<sup>10,11</sup> URTI symptoms such as cough and sore throat are often observed in patients infected with either HCoV-NL63 or HCoV-229E.<sup>12,13</sup> The prevalence of HCoV-NL63 varies from one study to another; however, in most temperate and tropical countries, it appears to peak around September–April, whereas HCoV-229E is usually detected at low rates throughout the year.<sup>14–16</sup> In spite of the clinical importance of HCoV infections,<sup>17</sup> the prevalence, seasonality, clinical, and phylogenetic characteristics of HCoVs remain mostly unreported from the tropical region of southeast Asia. On the basis of the *S*, *N*, and *Ia* genes of the HCoV-NL63 and HCoV-229E sequences from

Malaysia and also worldwide, we describe the genetic history and phylodynamic profiles of both human alphacoronaviruses using a set of phylogenetic tools.

### MATERIALS AND METHODS

**Ethics statement.** The study was approved by the University of Malaya Medical Ethics Committee (MEC890.1). Standard, multilingual consent forms permitted by the Medical Ethics Committee were used. Written consent was obtained from all study participants.

**Clinical specimens.** A total of 2,060 consenting outpatients who presented with acute URTI symptoms were recruited at the Primary Care Clinic of University Malaya Medical Center in Kuala Lumpur, Malaysia, between March 2012 and February 2013. Demographic data such as age, gender, and ethnicity were acquired before the collection of nasopharyngeal swabs. The severity of the URTI symptoms (sneezing, nasal discharge, nasal congestion, headache, sore throat, voice hoarseness, muscle ache, and cough) was graded according to criteria described earlier.<sup>18–21</sup> The nasopharyngeal swabs were transferred to the laboratory in universal transport media and stored at  $-80^{\circ}\text{C}$ .

**Molecular detection of HCoV-NL63 and HCoV-229E.** Extraction of total nucleic acids from the nasopharyngeal swabs was carried out using the magnetic bead-based protocols applied in the NucliSENS easyMAG automated nucleic acid extraction system (BioMérieux, Marcy l’Etoile, France).<sup>22,23</sup> The presence of respiratory viruses in specimens was examined using the xTAG Respiratory Virus Panel FAST multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay (Luminex Molecular Diagnostics, Toronto, Canada), which can identify HCoV-NL63, HCoV-229E, HCoV-OC43, HCoV-HKU1, and other respiratory viruses and subtypes.<sup>24</sup>

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**Genetic analysis of HCoV-NL63 and HCoV-229E.** Gene fragment sequencing of the *S* (S1 domain), complete *N*, and partial *Ia* (nsp3) genes was performed for HCoV-NL63 and HCoV-229E specimens. The S1 is a highly variable receptor-binding domain, whereas the *N* and nsp3 are conserved regions within the coronavirus genome, and these three regions are therefore efficiently used for genotyping.<sup>5,6</sup> Viral RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript III kit (Invitrogen, Carlsbad, CA) with random hexamers (Applied Biosystems, Carlsbad, CA). The partial *S* gene (S1 domain) (HCoV-NL63: 1,383 nt [20,413–21,796] and HCoV-229E: 855 nt [20,819–21,674]), complete *N* gene (HCoV-NL63: 1,133 nt [26,133–27,266] and HCoV-229E: 1,330 nt [25,673–27,003]), and partial *Ia* (nsp3) gene (HCoV-NL63: 781 nt [5,811–6,592] and HCoV-229E: 766 nt [5,898–6,664]) were amplified through PCR using 10  $\mu$ M of newly designed or previously published primers listed in Table 1. The PCR mixture (25  $\mu$ L) contained cDNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM MgCl<sub>2</sub>, and 0.01% gelatin), 100  $\mu$ M (each) deoxynucleoside triphosphates, Hi-Spec additive and 4 U/ $\mu$ L BIO-X-ACT Short DNA polymerase (BioLine, London, United Kingdom). The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes followed by 40 cycles of 94°C for 1 minute, 54.5°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR reactions were performed in a C1000 Touch automated thermal cycler (Bio-Rad, Hercules, CA). Nested/semi-nested PCR was performed if necessary, under the same cycling conditions at 30 cycles. Purified PCR products were sequenced using the ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). The nucleotide sequences were codon aligned with relevant complete and partial HCoV-NL63 and HCoV-229E reference sequences retrieved from the GenBank.<sup>5,6,28–31</sup>

Maximum clade credibility (MCC) trees for the partial *S* (S1 domain), complete *N*, and partial *Ia* (nsp3) genes were reconstructed in BEAST (version 1.7).<sup>32</sup> MCC trees were produced using a relaxed molecular clock, assuming uncorrelated lognormal distribution under the general time-reversible

nucleotide substitution model with a proportion of invariant sites (GTR+I) and a constant coalescent/exponential tree model. The Markov chain Monte Carlo run was set at  $6 \times 10^6$  steps long sampled every 10,000 state. The trees were annotated using Tree Annotator program included in the BEAST package, after a 10% burn-in, and visualized in FigTree (version 1.3.1).<sup>33</sup> The evolutionary history and divergence time (in calendar year) for the HCoV-NL63 and HCoV-229E genotypes were also assessed. The mean divergence time and the 95% highest posterior density regions were evaluated. The best-fitting model was determined by the Bayes factor using marginal likelihood analysis implemented in Tracer (version 1.5).<sup>32</sup> The substitution rate of  $3.3 \times 10^{-4}$  substitutions/site/year for the *S* gene of human alphacoronavirus estimated previously was used for the divergence time inference.<sup>5</sup>

Maximum likelihood (ML) phylogenetic trees were also reconstructed for the three regions in the phylogenetic analysis using parsimony (PAUP 4.0) software,<sup>34</sup> with a Hasegawa–Kishino–Yano nucleotide substitution model plus discrete gamma categories. The statistical robustness and reliability of the branching orders were evaluated by a bootstrap analysis of 1,000 replicates. To investigate the genetic relatedness among the HCoV-NL63 and HCoV-229E genotypes, inter-genotype pairwise nucleotide distances were estimated for the *S* gene using MEGA 5.1.<sup>35</sup> Such analysis was not implemented for the *N* and *Ia* genes due to their high genetic invariability across HCoV-NL63 and HCoV-229E genotypes.<sup>5,6</sup>

**Statistical analysis.** All categorical variables were analyzed using the two-tailed Fisher's exact test/ $\chi^2$  test by the Statistical Package for the Social Sciences (release 16.0; IBM Corp., Chicago, IL). *P* values < 0.05 were considered significant.

**Nucleotide sequences.** HCoV-NL63 and HCoV-229E nucleotide sequences produced in the study have been deposited in GenBank under the accession nos. KT359730–KT359913.

## RESULTS AND DISCUSSION

**Detection of HCoV-NL63 and HCoV-229E in nasopharyngeal swabs.** In the current cross-sectional study, a total of

TABLE 1  
Polymerase chain reaction primers for HCoV-NL63 and HCoV-229E

| Target gene               | HCoV          | Primer        | Location*                                    | Sequence (5'–3')                             | Reference  |
|---------------------------|---------------|---------------|--|--|------------|
| Spike ( <i>S</i> )        | NL63          | SP1F          | 20,390–20,412                                | Forward: TGAGTTTGATTAAGAGTGGTAGG             | 25         |
|                           |               | SP2F          | 20,397–20,418                                | Forward (nested): GATTAAGAGTGGTAGGTTGTTG     | 25         |
|                           |               | SP1R          | 21,809–21,828                                | Reverse: CAAACTGCAAGTGCTCACAC                | 25         |
|                           | 229E          | SP2R          | 21,797–21,816                                | Reverse (nested): GCTCACACTGCAACTTTTCA       | 25         |
|                           |               | LPS1          | 20,732–20,751                                | Forward: AATAATTGGTTCCITCTAAC                | 26         |
|                           |               | JH1           | 20,797–20,818                                | Forward (nested): TTTGTTGCTTAATTGCTTATGG     | 26         |
|                           |               | LPR           | 21,710–21,728                                | Reverse: AACATACACTGCCAAATTT                 | This study |
|                           |               | JH2           | 21,675–21,694                                | Reverse (nested): TTTGCCAAAAGAAAAAGGGC       | 26         |
|                           |               | $\alpha$ N-F  | 26,102–26,127 (NL63)<br>25,652–25,672 (229E) | Forward: ARRTTGCTTCATTTWWTCTAA               | This study |
| Nucleocapsid ( <i>N</i> ) | NL63 and 229E | $\alpha$ N-Fn | 26,112–26,132                                | Forward (nested): ATTTWWTCTAAACTAAACRAA      | This study |
|                           |               | NL-NR         | 27,278–27,299                                | Reverse: ATAATAAACAKTCAACTGGAAT              | This study |
|                           | 229E          | NL-NRn        | 27,267–27,287                                | Reverse (nested): CAACTGGAATTACAAAACAAT      | This study |
|                           |               | E-NR          | 27,046–27,063                                | Reverse: GATCCTTGTCAGCCAAA                   | This study |
|                           |               | E-NRn         | 26,882–26,900                                | Reverse (nested): AAAATTCCAACTAAAGCCT        | This study |
| <i>Ia</i>                 | NL63          | SS5852-5Pf    | 5,778–5,798                                  | Forward: CTTTTGATAACGGTCACTATG               | 27         |
|                           |               | P3E2-5Pf      | 5,789–5,810                                  | Forward (semi-nested): GGTCACATGTAGTTTATGATG | 27         |
|                           |               | NL-1aR        | 6,593–6,616                                  | Reverse: CTCATTACATAAAAACATCRAACGG           | This study |
|                           | 229E          | E-1aF         | 5,865–5,885                                  | Forward: CTGTTGAYAAAGGTCATTATA               | This study |
|                           |               | E-1aFn        | 5,876–5,897                                  | Forward (semi-nested): GGTCATTACTGTTTATGAYA  | This study |
|                           |               | E-1aR         | 6,665–6,688                                  | Reverse: TTCATCACAAAATAACATCAAATGG           | This study |

\*Nucleotide location was determined based on the HCoV-NL63 (NC\_005831) and HCoV-229E (NC\_002645) reference sequences.

TABLE 2

Demographic data of 68 adult outpatients infected with human alphacoronavirus in Kuala Lumpur, Malaysia, 2012–2013

| Factor              | HCoV-NL63 (N = 45) | HCoV-229E (N = 23) | P value |
|---------------------|--------------------|--------------------|---------|
| Gender              |                    |                    | 0.80    |
| Male                | 25 (55.6%)         | 12 (52.2%)         |         |
| Female              | 20 (44.4%)         | 11 (47.8%)         |         |
| Age                 |                    |                    | 0.45    |
| < 40                | 13 (28.9%)         | 7 (30.4%)          |         |
| 40–60               | 10 (22.2%)         | 8 (34.8%)          |         |
| > 60                | 22 (48.9%)         | 8 (34.8%)          |         |
| Symptoms            |                    |                    | 0.99    |
| Sneezing            | 42 (93.3%)         | 20 (87.0%)         |         |
| Nasal discharge     | 38 (84.4%)         | 19 (82.6%)         |         |
| Nasal congestion    | 29 (64.4%)         | 15 (65.2%)         |         |
| Headache            | 23 (51.1%)         | 13 (56.5%)         |         |
| Sore throat         | 32 (68.9%)         | 14 (60.9%)         |         |
| Hoarseness of voice | 35 (77.8%)         | 15 (65.2%)         |         |
| Muscle ache         | 27 (60.0%)         | 16 (69.6%)         |         |
| Cough               | 43 (95.6%)         | 20 (87.0%)         |         |
| Ethnicity           |                    |                    | 0.08    |
| Malay               | 11 (24.5%)         | 5 (21.8%)          |         |
| Chinese             | 24 (53.3%)         | 7 (30.4%)          |         |
| Indian              | 10 (22.2%)         | 11 (47.8%)         |         |

2,060 nasopharyngeal swab specimens collected from Kuala Lumpur, Malaysia, throughout a 12-month study period (March 2012 to February 2013), were screened for the presence of HCoV-NL63 and HCoV-229E using the multiplex RT-PCR method, as an alternative approach to other detection methods such as cell culture.<sup>36</sup> Human alphacoronavirus was identified in 68 (3.3%) subjects; HCoV-NL63 and HCoV-229E were detected in 45/2,060 (2.2%) and 23/2,060 (1.1%) patients, respectively. These findings are consistent with the global average prevalence of human alphacoronavirus, which ranges between 1% and 10%, with HCoV-229E generally detected at lower rates than HCoV-NL63.<sup>8–10,27,37–40</sup> In contrast to an earlier study,<sup>41</sup> no coinfection of alpha- and betacoronavirus (HCoV-OC43 and HCoV-HKU1) was observed within an individual. Age, gender, and ethnicity of the patients were summarized in Table 2. A peak in the number of HCoV-NL63 infections was recorded for the period between June and October 2012, although the number of patients with URTI symptoms screened during those months

was relatively low (Figure 1). This pattern of virus prevalence corroborates with that observed in neighboring country Thailand, in which a peak of HCoV-NL63 incidence was recorded in September.<sup>14</sup> In contrast, studies from temperate regions commonly reported a higher prevalence of HCoV-NL63 during winter seasons.<sup>7–9,42</sup> However, the number of HCoV-229E infections detected in Malaysia was low, with no significant peak observed throughout the year, similar to other studies reported worldwide.<sup>14,38,43</sup> It is important to note that the study was performed in a relatively short duration, therefore limiting the epidemiological and disease trend comparison with reports from other countries.

**Phylogenetic analysis of the *S*, *N*, and *1a* genes.** A total of 42/45 (93.3%) partial *S* (S1 domain) and 43/45 (95.6%) of each complete *N* and partial *1a* (nsp3) genes were successfully sequenced from HCoV-NL63 specimens. Amplification of these genes was difficult for two xTAG-positive HCoV-NL63 specimens, possibly due to their low viral copy number. Phylogenetic analysis of HCoV-NL63 (Figure 2 and Supplemental Figure 1) showed that 27 subjects (27/43, 62.8%) in the study belonged to genotype B (supported by a posterior probability of 1.0 and bootstrap value of 100% at the internal nodes of the MCC and ML trees of the *S* gene, respectively, with an intra-group pairwise genetic distance of  $0.6\% \pm 0.1\%$ ) together with previously reported sequences from the United States, Europe, and Asia.<sup>5,25,28,29</sup> Another 16 subjects (16/43, 37.2%) were found to be grouped under genotype C (supported by a posterior probability of 1.0 and bootstrap value of 67% at the internal nodes of the MCC and ML trees of the *S* gene, respectively, with an intra-group pairwise genetic distance of  $0.2\% \pm 0.1\%$ ) with recently described global sequences.<sup>25,28,29</sup> Discordance in phylogenetic clustering among the *S*, *N*, and *1a* genes of the HCoV-NL63 Malaysian sequences had been observed (Supplemental Figure 1). On the basis of the *S* (S1 domain) gene analysis, 26 Malaysian strains (26/42; 61.9%) belong to genotype B while another 16 Malaysian strains (16/42; 38.1%) were classified within genotype C. In contrast, sequences of the three HCoV-NL63 genotypes (A, B, and C) appear to be intermingled in the *N* and *1a* phylogenetic trees. Such discordance was similarly reported in earlier studies where it was

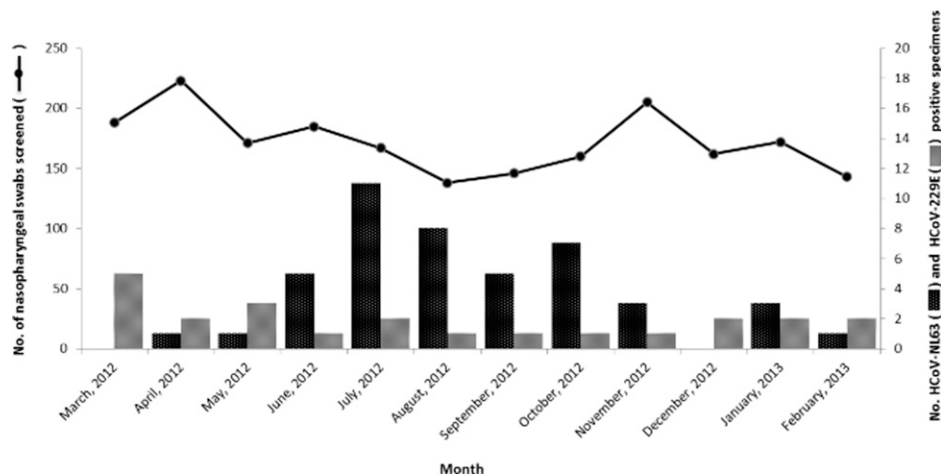


FIGURE 1. Annual distribution of HCoV-NL63 and HCoV-229E among adults with acute upper respiratory tract infections in Kuala Lumpur, Malaysia. The total number of nasopharyngeal swabs screened and the monthly distribution of HCoV-NL63 and HCoV-229E between March 2012 and February 2013 were presented.

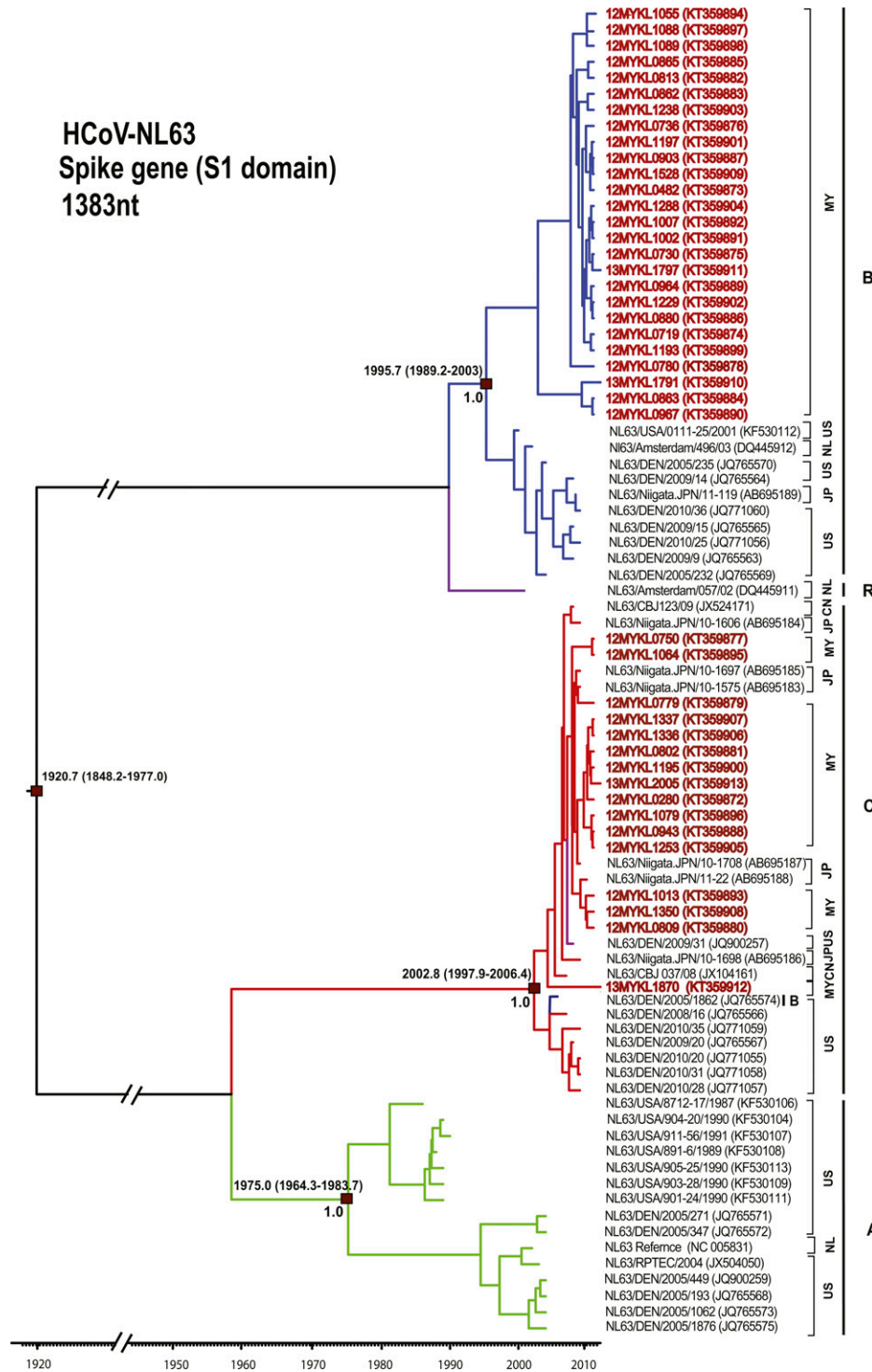


FIGURE 2. Maximum clade credibility tree of HCoV-NL63. Spike gene (S1 domain) sequences (1,383 nucleotides) were analyzed under the relaxed molecular clock with a GTR+I substitution model and a constant size coalescent model implemented in BEAST. Posterior probability values and the estimation of the time of the most recent common ancestors with 95% highest posterior density were indicated on major nodes. The HCoV-NL63 sequences obtained in this study were color coded and HCoV-NL63 genotypes A–C were indicated; green = genotype A, blue = genotype B, and red = genotype C. The recombinant genotype is indicated by purple color. The sampling site for each sequence was indicated by codes for the representation of countries. Country codes are as follows; MY = Malaysia; US = United States; JP = Japan; NL = Netherlands; CN = China.

confirmed that such phylogenetic pattern was resulted from multiple recombination events along the HCoV-NL63 genome, in addition to the fact that the S1 region sequenced in this study is considered the most variable along the genome, while

the *N* and *Ia* (nsp3) genes are too conserved.<sup>5</sup> To estimate the genetic diversity between HCoV-NL63 genotypes A, B, and C, inter-genotype pairwise genetic distance was assessed for the *S* gene (Table 3). Genetic distances between genotypes A versus B

TABLE 3

The genetic diversity among alphacoronavirus genotypes in the spike gene

| HCoV | Genetic distance |            |            |         |     |
|------|------------------|------------|------------|---------|-----|
|      | genotype A       | genotype B | genotype C |         |     |
| NL63 | genotype A       | 0.8†       | 0.5        |         |     |
|      | genotype B       | 7.6*       | -          | 0.6     |     |
|      | genotype C       | 2.1        | 6.7        | -       |     |
|      |                  |            |            |         |     |
| 229E | group 1          | group 2    | group 3    | group 4 |     |
|      | group 1          | -          | 0.4        | 0.6     | 0.7 |
|      | group 2          | 1.5        | -          | 0.3     | 0.4 |
|      | group 3          | 2.5        | 1.2        | -       | 0.3 |
|      | group 4          | 3.5        | 2.6        | 1.5     | -   |

\*Pairwise genetic distances are expressed in percentage (%) of nucleotide difference.  
†Standard error estimates of the mean genetic distances are shown in the upper diagonal.

and B versus C were high (more than 5.0%), compared with that between genotypes A versus C, which was at 2.1%. This is consistent with the phylogenetic tree topology in which genotypes A and C were more closely related and probably shared a common ancestor.

At least one gene (*S*, *N*, and/or *Ia*) was successfully sequenced from 23 positively tested HCoV-229E specimens (16, 18, and 22 of HCoV-229E *S*, *N*, and *Ia* genes, respectively). Phylogenetic analysis revealed that all of the HCoV-229E sequences obtained in this study were classified with group 4, which includes isolates that have been globally circulating since 2001 (Figure 3 and Supplemental Figure 2).<sup>6,30,31</sup> The group was supported by a posterior probability of 1.0 and

bootstrap value of 100% at the internal nodes of the MCC and ML trees of the *S* gene, respectively, with an intra-group pairwise genetic distance of 0.3% ± 0.1%. Such phylogenetic data were comparable to those obtained from the *N* tree, resulted from the hot substitution spots in the S1 and N regions of the HCoV-229E genome.<sup>30</sup> The four HCoV-229E groups could not be clearly defined within the *Ia* gene tree because of the limited number of reference sequences available in the public database (Supplemental Figure 2). Inter-genotype pairwise genetic distance was generally low (below 5.0%) in the *S* gene among groups 1–4 (Table 3).

**Estimation of divergence times.** The molecular clock analysis of HCoV-NL63 and HCoV-229E was performed using the coalescent-based Bayesian relaxed molecular clock under the constant and exponential tree models (Figures 2 and 3). The mean evolutionary rates for the *S* gene of HCoV-NL63 and HCoV-229E were newly estimated based on the constant tree model at  $4.3 \times 10^{-4}$  ( $2.3\text{--}6.7 \times 10^{-4}$ ) and  $3.9 \times 10^{-4}$  ( $1.3\text{--}6.4 \times 10^{-4}$ ) substitutions/site/year, respectively. These results were similar to the previously reported substitution rate of the alphacoronavirus *S* gene ( $3.3 \times 10^{-4}$  substitutions/site/year).<sup>5</sup> The evolutionary analysis indicated that the time of the most recent common ancestor (tMRCA) of HCoV-NL63 was dated back to the 1920s, while the estimated divergence time of genotype A was dated to 1975, followed by genotype B around 1996 and genotype C in 2003 (Figure 2). Furthermore, the divergence time of HCoV-229E

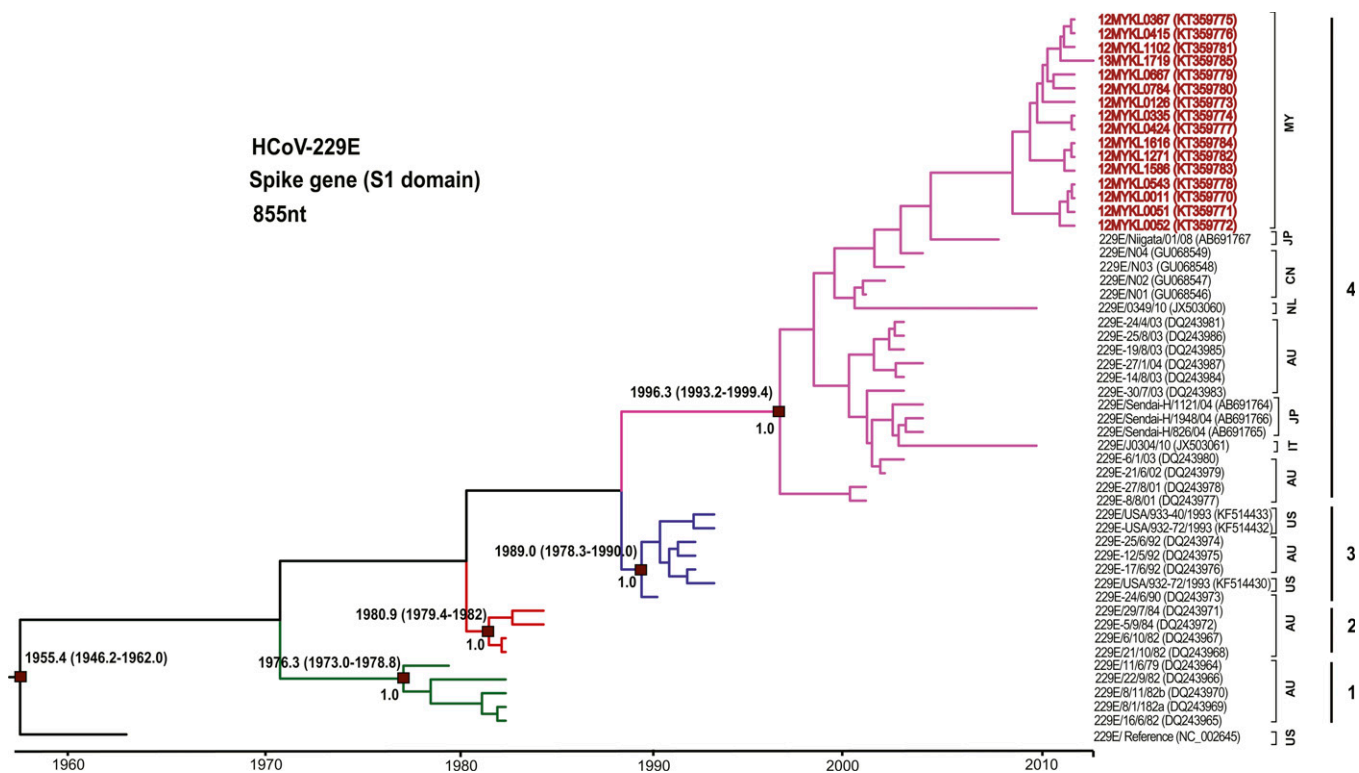


FIGURE 3. Maximum clade credibility tree of HCoV-229E. Spike gene (S1 domain) sequences (855 nucleotides) were analyzed under the relaxed molecular clock with a GTR+I substitution model and a constant size coalescent model implemented in BEAST. Posterior probability values and the estimation of the time to the most recent common ancestors with 95% highest posterior density were indicated on the major nodes. The HCoV-229E sequences obtained in this study were color coded and the HCoV-229E groups 1–4 were indicated green = genotype 1, red = genotype 2, blue = genotype 3, and purple = genotype 4. The sampling site for each sequence was indicated by codes for the representation of countries. Country codes are as follows; MY = Malaysia; US = United States; JP = Japan; NL = Netherlands; CN = China; AU = Australia; IT = Italy.

(Figure 3) was estimated around 1955 while the tMRCA of group 1 diverged in 1976, followed by that of group 2 in 1981, group 3 in 1989, and group 4 in 1996. The appearance of groups 1–4 in a timely ordered manner would give strength to the earlier reported hypothesis that positive selection and genetic drift play a major role in the evolution of HCoV-229E.<sup>6,30</sup> To the best of our knowledge, this is the first study that reported the divergence times of human alphacoronavirus genotypes. In addition, the most recently reported HCoV-229E strains (between 2001 and 2013) from major parts of the world belong to group 4. In accordance with earlier studies, genotype replacement is evident within HCoV-229E, although sampling bias may also influence the results.<sup>6,30</sup> Bayes factor analysis showed insignificant differences (Bayes factor less than 3.0) between the constant and exponential coalescent models of demographic analysis, in which the divergence times estimated using the constant coalescent tree model were similar to those calculated using the exponential model (Supplemental Table 1).

**Clinical symptoms assessment.** Clinical findings of the URTI symptoms (sneezing, nasal discharge, nasal congestion, headache, sore throat, hoarseness of voice, muscle ache, and cough) and their severity levels (none, moderate, and severe) were analyzed using the two-tailed Fisher's exact test. The association between symptom severity and HCoV-NL63/HCoV-229E infection was insignificant ( $P$  values  $> 0.05$ ) (Supplemental Table 2). In line with previous clinical studies,<sup>10,44,45</sup> the majority of patients infected with HCoV-NL63 and HCoV-229E presented with at least one respiratory symptom that was moderately severe.

In summary, this study provides insight into the phylogeny and evolution of the HCoV-NL63 and HCoV-229E genotypes. Genetic characterization of human alphacoronavirus isolates currently circulating in Malaysia indicates the circulation of globally prevalent genotypes in the tropical region of southeast Asia. This study has detailed the genetic history of HCoV-NL63 and HCoV-229E genotypes. Since alphacoronavirus evolve through recombination, positive selection, and genetic drift events, continuous molecular surveillance of human alphacoronavirus is warranted to keep track on the evolution of the virus in southeast Asia.

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**Disclaimer:** The outcomes and conclusions that this study has reported were drawn by the authors and do not necessarily represent the views or policies of the institutions of affiliation.

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