PREVALENCE AND GENETIC CHARACTERIZATION OF HUMAN CORONAVIRUSES IN SOUTHERN THAILAND FROM JULY 2009 TO JANUARY 2011

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Abstract. Emergence of viruses belonging to the coronavirus family has been widespread in the past, causing respiratory infections in humans, such as severe acute respiratory syndrome (SARS). This study investigated the prevalence of human coronavirus (HCoV) and characterized the molecular viral genetics. We collected 1,254 samples from patients diagnosed with respiratory infection in southern Thailand from July 2009 to January 2011 and screened for HCoV by RT-PCR and genotyped by BLAST analysis of nsp12 gene. Phylogenetic analysis was performed based on S gene sequences. Thirty-five of 1,254 samples were positive for HCoV. Viral genotyping revealed 4 genotypes with HCoV-OC43 being the predominant genotype. Viral prevalence and genotype distribution were not in accordance with seasonal distribution. Phylogenetic analysis and deduced amino acid sequences of the S gene showed amino acid variations in each genotype. The S gene sequence of HCoV-OC43 genotype indicated that it resulted from recombination between subgenotypes B and C. Viral genetics analysis disclosed genetic variations of HCoV and additionally, it can provide information suitable for monitoring and prevention of the emergence and re-emergence of various types of coronavirus.

Keywords: human coronavirus, phylogenetics analysis, prevalence, S gene

INTRODUCTION

Human coronaviruses (HCoV) have been recognized as an important cause of respiratory disease (Nokso-Koivisto *et al*, 2000). Coronaviruses are enveloped RNA viruses widely distributed among mammals and birds (Nokso-Koivisto *et al*, 2000; Lai and Holmes, 2001). Taxonomi-

cally, coronaviruses have been classified as members of the genus *Coronavirus* of the Coronaviridae family (Lai and Cavanagh, 1997), which has classified into four genera, namely, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (Adams and Carstens, 2012).

The genomes of these viruses comprise a positive-stranded RNA of approximately 27 to 32 kb in length, which contains 7 to 10 open reading frames (ORFs) and untranslated regions (UTR) at the 5' and 3' ends of the RNA (Lai and Cavanagh, 1997). The order of the genes

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encoding the polymerase (Pol) and the four structural proteins that are present in all coronaviruses is 5'-Pol-S-E-M-N-3' (S: spike protein; E: envelope protein; M: membrane glycoprotein; N: nucleocapsid phosphoprotein) (Lai and Cavanagh, 1997). The S1 region of S gene comprising the receptor binding site is one of the two regions in the spike protein, with the other region, S2, being essential for membrane fusion (Zheng *et al*, 2006). These genes are interspersed with several ORFs encoding various nonstructural proteins and the hemagglutinin-esterase (HE) glycoprotein, all of which differ markedly with respect to nucleotide sequence, gene order and method of expression.

Previously, five genotypes of coronaviruses are known to infect humans (Lai and Cavanagh, 1997; Lau *et al*, 2006; Eickmann *et al*, 2003). HCoVs were categorized based on genetic and serological characterization into two genera, *Alphacoronavirus* (HCoV-229E) and *Betacoronavirus* (HCoV-OC43) (Lai and Cavanagh, 1997). In 2003, the discovery of severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) added yet another virus member to the list of HCoVs. Based on genome analysis, SARS-CoV is a distant relative of *Betacoronavirus* (Eickmann *et al*, 2003; Kim *et al*, 2006). A fourth genotype of HCoV, named NL63 (HCoV-NL63), belongs to *Alphacoronavirus* (van der Hoek *et al*, 2004). In early 2005 another HCoV type was reported and named HKU1 (HCoV-HKU1) (Woo *et al*, 2005a). However, in 2012 a sixth genotype has been discovered in Qatar and Saudi Arabia and was identified as HCoV-EMC/2012, closely related to bat coronavirus *Betacoronavirus* (Zaki *et al*, 2012).

HCoVs are associated with a range of respiratory symptoms with high morbidity, including pneumonia and bronchiolitis (Pene *et al*, 2003; Vabret *et al*, 2003; Woo *et al*, 2005b). The contributions of HCoV-229E and HCoV-OC43 account for 5% to 30% of human respiratory tract infections, while HCoV-NL63 and HCoVHKU1 are recovered from < 5% of respiratory tract infection samples (McIntosh *et al*, 1970; Chiu *et al*, 2005; Ebihara *et al*, 2005; Moës *et al*, 2005; Lau *et al*, 2006). However, different HCoVs often co-circulate, with one or two genotypes, the predominance of which depends on geographical area and year of infection (Esposito *et al*, 2006; Gerna *et al*, 2006; Lau *et al*, 2006; Kuypers *et al*, 2007).

Pandemic influenza H1N1 virus, wide-spread in 2009, has caused outbreaks in several countries including Thailand (Prachayangprecha *et al*, 2011). Surveillance studies conducted in many parts of Thailand has discovered patients infected with respiratory agents, with 25% positive for influenza viruses (Prachayangprecha *et al*, 2011). However, the other respiratory tract viruses were not identified.

In this study epidemiological and prevalent studies of HCoVs were performed in patients diagnosed with influenza-like illness in southern Thailand during 2009 and 2010. Molecular characterization also was conducted. These results will help contribute to the understanding of the circulation of HCoVs and their genetic make-up.

MATERIALS AND METHODS

Samples

A total of 1,254 nasopharyngeal aspirates and throat swab samples were collected from non-hospitalized patients who were under-going routine examination or treatment for influenza-like illness at Nakhon Si Thammarat, southern Thailand between July 2009 and January 2011. All

samples were collected in viral transport media with additional of antibiotics ($2x10^6$ U/l of penicillin G and 200 mg/l of streptomycin) and sent within 48 hours to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok and stored at -70°C until further analyzed.

Patients' identifiers were removed from these samples to protect patients' confidentiality. Permission to include these samples in the study was granted by the Director of the Hospital. Sample collection data were recorded but clinical data and patients' profiles information were not obtained. The protocol of this study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University and followed the international guidelines for human research protection established by the Declaration of Helsinki (certification no. COA No. 768/2011 and IRB No. 490/54).

Viral RNA extraction and HCoV screening

Viral RNA was extracted from 200 l of samples using Real Genomics Viral Nucleic Acid Kit (RBC Bioscience, New Taipei City, Taiwan) and cDNA subsequently synthesized at 37°C for 3 hours using M-MLV reverse-transcription system (Promega, Madison, WI) and 1 M random hexamer primer. Samples were screened for HCoV nsp12 gene using nested reverse transcription (RT)-PCR using primers listed in Table 1. In brief, in the first round RT-PCR, 1 l of cDNA was added to the reaction mixture containing 12.5 l of PerfectTaq Plus Mastermix (5Prime, Hamburg, Germany), 0.5 M each forward (HCoV_Pol_F1) and reverse (HCoV_Pol_R1) primers and nuclease-free water to a final volume of 25 l. Thermocycling (Eppendorf, Hamburg, Germany) was performed as

follows: 94°C for 3 minutes; 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 90 seconds; with a final heating at 72°C for 7 minutes. The amplicon was subjected to the second round of PCR by adding 1 l of first PCR product to 25 l reaction volume containing 0.5 M each forward (HCoV-Pol_F2) and reverse (HCoV-Pol_R2) primers, PerfectTaq Plus Mastermix (5Prime, Hamburg, Germany) and nuclease-free water. Thermocycling conditions were as follows: 94°C for 3 minutes; 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 90 seconds; with a final heating at 72°C for 7 minutes. The amplicon was separated by 2% agarose gel-electrophoresis contained ethidium bromide. The 588 bp amplicon was gel-purified using HiYield Gel DNA Fragment Extraction kit (RBC Bioscience, New Taipei City, Taiwan) and sequenced by First BASE Laboratories (Selangor Darul Ehsan, Malaysia) and analyzed by BLAST.

S gene amplification

In order to investigate the spike (S) gene of HCoV, PCR using S gene genotype specific primers (Table 1) was performed using samples of known HCoV nsp12 genotype as described above. Positive samples were amplified by nested RT-PCR with specific genotype primers. The reaction mixtures of both rounds of nested PCR were the same as described above except for the primers. Thermocycling conditions for both rounds of PCR were as follows: 94°C for 3 minutes; 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 90 seconds; with a final heating at 72°C for 7 minutes. The final amplicons were isolated and sequenced as described above. The HCoV sequences have been deposited in GenBank, accession nos. JX513211-JX513268.

Table 1
Primers for HCoV screening (nsP12) and Spike gene amplification.

Gene	Primer	Genotype	Sequence (5′ to 3′)	Position*
nsp12	HCoV_Pol_F1		ATGGGTTGGGATTATCCYAARTGTGA	14324-14349
	HCoV_Pol_R1		GTRTGYTGTGAACARAAYTCATGAGG	14906-14931
	HCoV_Pol_F2		TCCTAARTGTGATMGWGCTATGCC	14338-14361
	HCoV_Pol_R2		TGYTGTGAACARAAYTCATGAGGTCC	14903-14925
Spike	HCoV_S229E_F1	229E	GTGGGTGCACTACCTAAGAC	21116-21135
	HCoV_S229E_R1	229E	CGTGGTTGAACAGCAATTATAGAACC	22244-22269
	HCoV_S229E_F2	229E	GAGTTTGTTATTTCACGCACAGGAC	21143-21167
	HCoV_S229E_R2	229E	CCATCTGCACAAACGCCAAAAC	22224-22245
	HCoV_SHKU1_F1	HKU1	TCACCTCTTAATTGGGAACGTA	21925-21904
	HCoV_SHKU1_R1	HKU1	CATTAGAACAAGTGGTGCCAC	21141-21121
	HCoV_SHKU1_F2	HKU1	GATTTGCAGTTGGGCAGTTCTGG	21745-21723
	HCoV_SHKU1_R2	HKU1	AAAGGCATCAGGACTACAAA	21240-21221
	HCoV_SNL63_F1	NL63	GACACCACAATACCTTTTGG	19464-19445
	HCoV_SNL63_R1	NL63	CTGGTTGGTTACATGGTGTCAC	18450-18429
	HCoV_SNL63_F2	NL63	CATGTTAGCACTTTTGTGGGT	19392-19372
	HCoV_SNL63_R2	NL63	CCACCAGCAAGTGACTGGTTTG	18542-18521
	HCoV_SOC43_F1	OC43	GTCGGTGCCCTCTCCATTAAATT	22595-22573
	HCoV_SOC43_R1	OC43	GGCCGCAGAAACACGAC	21591-21575
	HCoV_SOC43_F2	OC43	AATATGAGCAGCCTGATGTC	22540-22521
	HCoV_SOC43_R2	OC43	CCGAAATAGCAATGCTGGTTC	21556-21536

* nsp12 gene primers correspond to positions from GenBank accession number NC_002645; primers for S gene genotype 229E, HKU1, NL63 and OC43 correspond to positions from GenBank accession number NC_002645, NC_006577, NC_005831 and NC_005147, respectively.

Phylogenetic analysis

Nucleotide sequences of the HCoV S gene from this study and those reported in GenBank were aligned using MUSCLE (Edgar, 2004). Phylogenetic trees were constructed using the Neighbor-joining (NJ) approach implemented in MEGA 5 (Tamura *et al*, 2011). Bootstrap support for tree topologies was accomplished using the NJ method implemented in MEGA with 1,000 replicates. Genetic distances based on NJ phylogenetic trees were calculated by applying Kimura's two-parameter method using MEGA 5.

Boot-scanning analysis

In order to prove intra-subgenotype recombination in HCoV-OC43, boot-scanning analysis was performed using nucleotide sequences of spike gene and comparing between each subgenotype. Boot-scanning analysis was accomplished using SimPlot version 3.5.1 (Lole *et al*, 1999) with Kimura-2 parameter, windows size 300 bp and step on 10 bp.

RESULTS

The prevalence results were based on 1,254 samples collected between July 2009
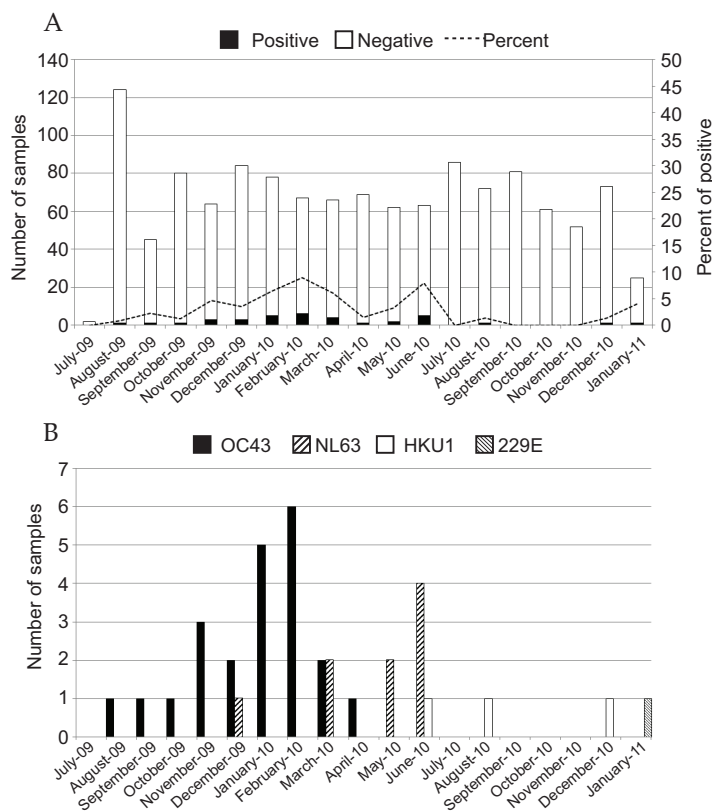
A

B

Fig 1–Distribution of HCoV during July 2009 to January 2011. (A) Total number of cases (white bar) and positive cases (black bar) in each month. Line graph shows percent positive cases for HCoV. (B) Distribution of HCoV genotypes in each month.

and January 2011 at Nakhon Si Thammarat, southern Thailand from patients who were diagnosed with influenza-like illness, of whom 35 were positive for HCoV (Fig 1A). During the study period (1 year and 7 months), the highest percent positive cases were in January 2010. The positive frequency period could be divided into 2 peak periods: the first between August 2009 and March 2010 rising to a maximum in February 2010 (8.9% positive), and the second period between April to July 2010 with a maximum in June 2010 (7.9% positive). Between September and November 2010 there were no positive cases of HCoV. Positive cases slightly increased in December 2010 and January 2011.

Based on the nucleotide sequences of nsp12 gene, the 35 HCoV samples were classified as HCoV-OC43 (22 specimens, 63%), HCoV-HKU1 (9, 26%), HCoV-NL63 (3, 8%) and HCoV-229E (1, 3%). The distribution of positive cases in each month showed that HCoV-OC43 was the predominant genotype from August 2009 to April 2010 (Fig 1B). Subsequently, HCoV-NL63 was found between March and June 2010. In the second half of 2010, the positive cases decreased and all positive samples during this period were HCoV-HKU1. One sample detected in January 2011 was classified as HCoV-229E.

The S gene was sequenced in order to elucidate the genetic characteristics of the surface protein, the antigenic determinant of HCoV. HCoV subtype HCoV-OC43 can be divided into 4 subgenotypes, namely, A, B, C and D (Lau *et al*, 2011). Phylogenetic analysis of the S gene of HCoV-OC43 from this study showed that the samples were closely related to HK04-01 and Belgium2004 strains, subgenotype C (Fig 2). However, the study samples formed a unique cluster in the subgenotype C lineage.

As representative sequences are not available at the GenBank database, subgenotype D has not been shown. However, subgenotype D is believed to constitute a recombinant strain between subgenotype B and C (Lau *et al*, 2011). In order to prove this notion, boot-scanning
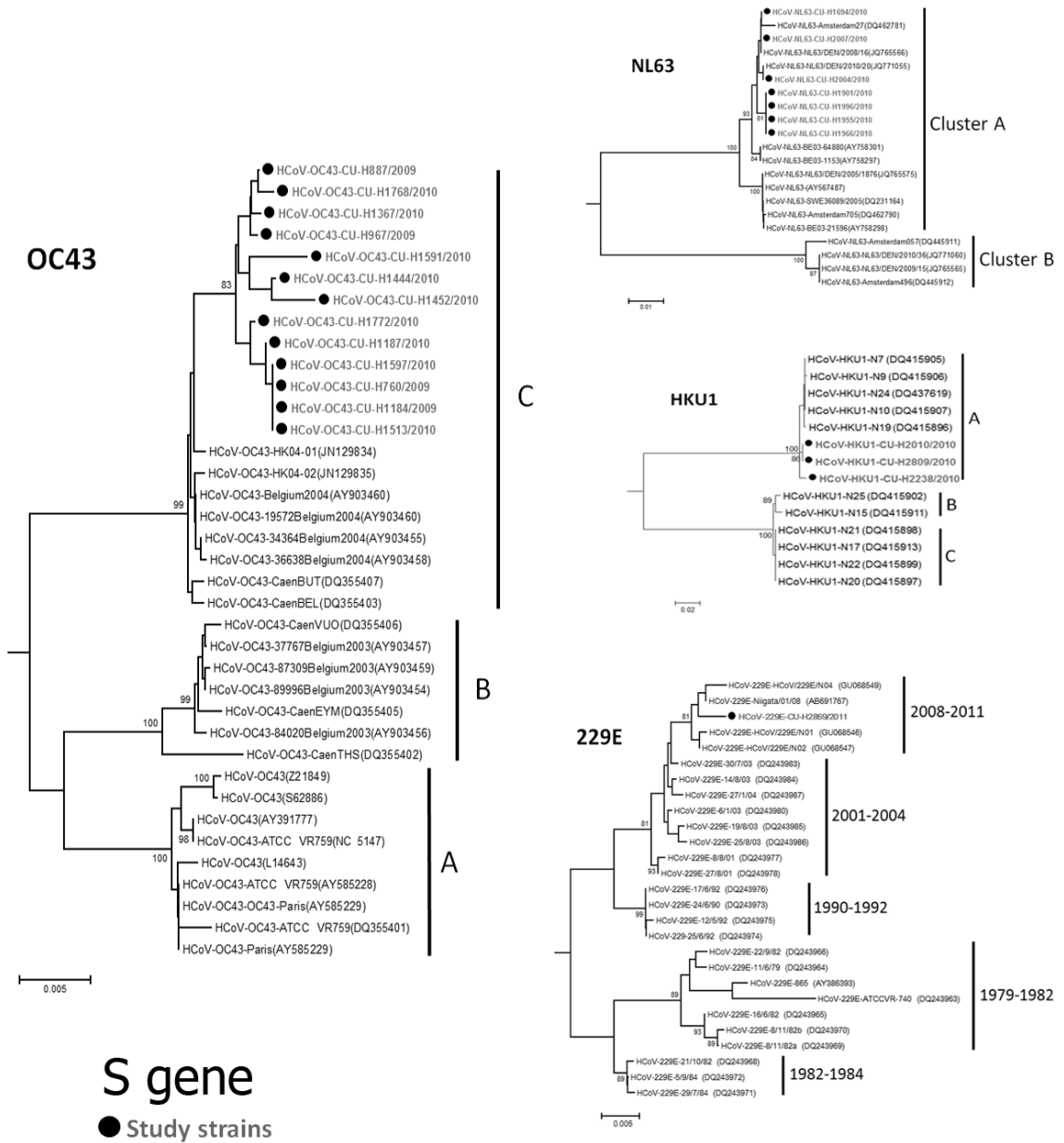
Fig 2–Phylogenetic tree of 4 S gene genotypes. Letters with a black dot indicate the study strains. The node clusters were supported by bootstrap values > 90%.

analysis of the HCoV-OC43 S genes was performed, which showed that S genes may have resulted from a combination between subgenotypes B and C (Fig 3). The break points of the recombination event are at nucleotide position 700 and 1100. These sequences were similar to subgenotype B in the up-stream region of the S gene, while displaying profound similarity to subgenotype C in the down-stream region relative to the break point region.

Amino acid alignments of the S1 protein of subgenotypes A, B and C have
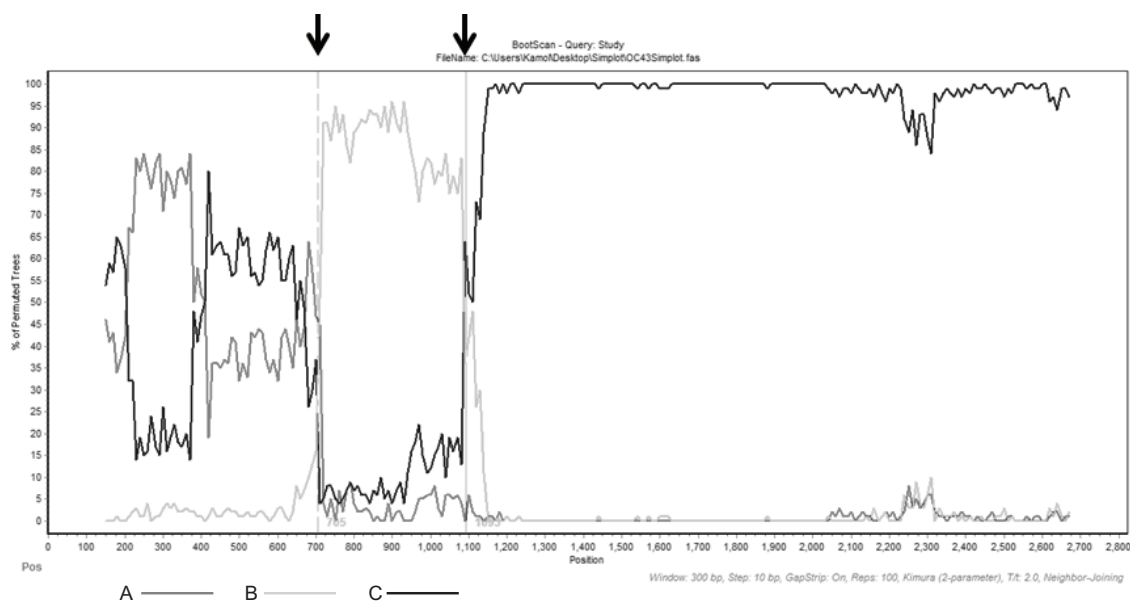
Fig 3–Boot-scanning analysis of HCoV-OC43. Study strains show 2 breaking points at nucleotide position 705 and 1093. Gray line, subgenotype A; light gray line, subgenotype B; Black line, subgenotype C.

showed that the N-terminal regions are highly variable (data not shown). For example, subgenotypes B and C have 5 amino acid (RLKGS) insertions at position 25 (NC_005147 numbering of S1 protein). The central region comprising the receptor binding domain was unique in each subgenotype. For instance, a deletion between position 495 and 501 represents a different genotype pattern. The study samples have 7 amino acids (LNGSCVG) deletions, whereas the Belgium2004 strain (subgenotype B) has 4 deletions (GSCV).

Classification of variants based on the HCoV S gene genotype NL63 (HCoV-NL63) can be divided into 2 clusters, A and B, employing subtyping of polymerase 1a (Leung *et al*, 2009). HCoV-NL63 in this study belonged to cluster A (Fig 2). Amino acid alignments of the S1 protein of this cluster displayed high diversity in the N-terminal region, whereas both clusters show conserved amino acids in the central and C-terminal regions, which

serve as a receptor binding site domain.

Based on S gene classification, genotype of HCoV-HKU1 can be divided into sub-genotypes A, B and C (Fig 2). Phylogenetic analysis of HCoV-HKU1 samples from this study revealed they belong to subgenotype A. Amino acid alignments of the receptor binding domain have showed high diversity among the three subgenotypes (Fig 4). The receptor binding domains of subgenotypes B and C are similar whereas that of subgenotype A is different from either subgenotype.

Phylogenetic tree topology of HCoV-229E from other strains reported in Gen-Bank shows that genetic drift progresses over time (Fig 2). The only sample of HCoV-229E S gene in this study is closely related to samples isolated in 2008 (Fig 2). The amino acid changes in viral surface protein of HCoV-229E of this study are at positions 192-555 (NC_002645 numbering of S1 protein), which include the receptor binding site.
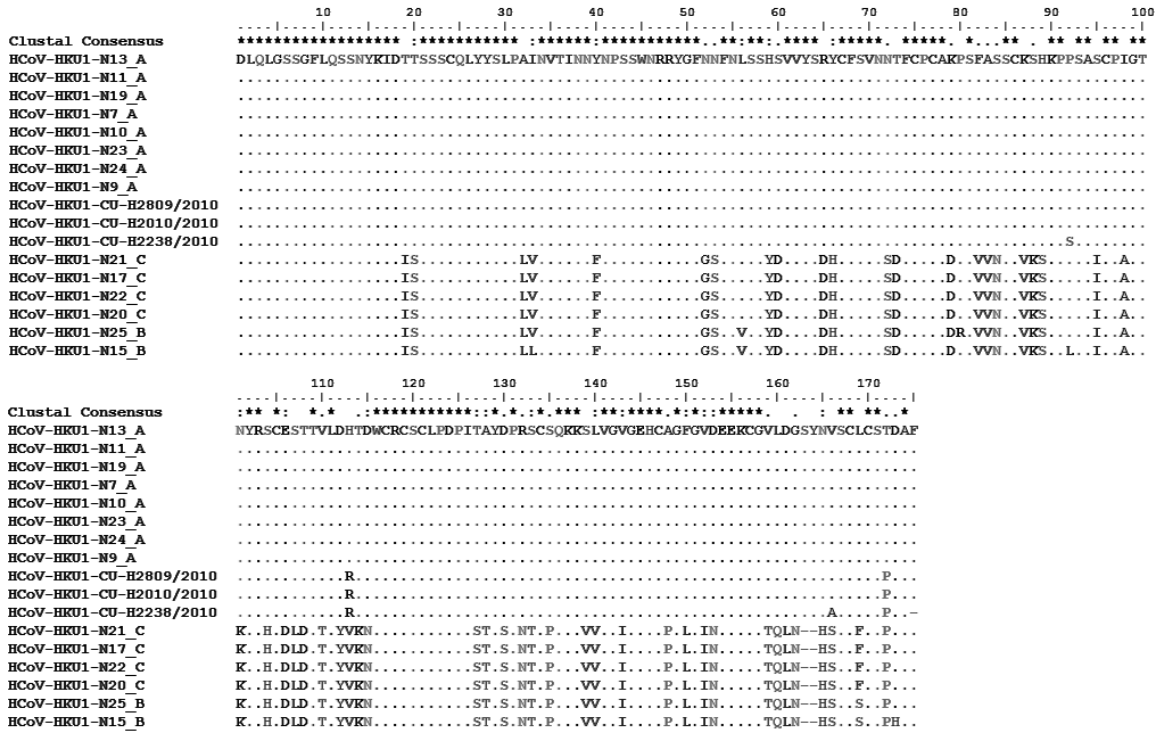
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                    10        20        30        40        50        60        70        80        90        100
                 ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
Clustal Consensus ******************* :*********** :******:***********..**:**:.**** :*****. *****. *...** . ** ** ** **
HCoV-HKU1-N13_A   DLQLGSSGFLQSSNYKIDTTSSSCQLYYSLPAINVTINNYNPSSWNRRYGFNNFNLSSHSVVYSRYCFSVNNTFCPCAKPSFASSCKSHKPPSASCPIGT
HCoV-HKU1-N11_A   ....................................................................................................
HCoV-HKU1-N19_A   ....................................................................................................
HCoV-HKU1-N7_A    ....................................................................................................
HCoV-HKU1-N10_A   ....................................................................................................
HCoV-HKU1-N23_A   ....................................................................................................
HCoV-HKU1-N24_A   ....................................................................................................
HCoV-HKU1-N9_A    ....................................................................................................
HCoV-HKU1-CU-H2809/2010 ..............................................................................................
HCoV-HKU1-CU-H2010/2010 ..............................................................................................
HCoV-HKU1-CU-H2238/2010 ...........................................................................................S..
HCoV-HKU1-N21_C   ..................IS..........LV.....F..........GS....YD...DH.....SD.....D..VVN..VKS.....I..A..
HCoV-HKU1-N17_C   ..................IS..........LV.....F..........GS....YD...DH.....SD.....D..VVN..VKS.....I..A..
HCoV-HKU1-N22_C   ..................IS..........LV.....F..........GS....YD...DH.....SD.....D..VVN..VKS.....I..A..
HCoV-HKU1-N20_C   ..................IS..........LV.....F..........GS....YD...DH.....SD.....D..VVN..VKS.....I..A..
HCoV-HKU1-N25_B   ..................IS..........LV.....F..........GS..V..YD...DH.....SD.....DR.VVN..VKS.....I..A..
HCoV-HKU1-N15_B   ..................IS..........LL.....F..........GS..V..YD...DH.....SD.....D..VVN..VKS..L..I..A..

                    110       120       130       140       150       160       170
                 ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
Clustal Consensus :** *:  *.*  .:***********::*.*.:*.*** :**:****.*:*::*****.  .  : ** **..*
HCoV-HKU1-N13_A   NYRSCESTTVLDHTDWCRCSCLPDPITAYDPRSCSQKKSLVGVGEHCAGFGVDEEKCGVLDGSYNVSCLCSTDAF
HCoV-HKU1-N11_A   ...........................................................................
HCoV-HKU1-N19_A   ...........................................................................
HCoV-HKU1-N7_A    ...........................................................................
HCoV-HKU1-N10_A   ...........................................................................
HCoV-HKU1-N23_A   ...........................................................................
HCoV-HKU1-N24_A   ...........................................................................
HCoV-HKU1-N9_A    ...........................................................................
HCoV-HKU1-CU-H2809/2010 ............R.........................................................P...
HCoV-HKU1-CU-H2010/2010 ............R.........................................................P...
HCoV-HKU1-CU-H2238/2010 ............R...................................................A.....P..-
HCoV-HKU1-N21_C   K..H.DLD.T.YVKN...........ST.S.NT.P...VV..I....P.L.IN.....TQLN--HS..F..P...
HCoV-HKU1-N17_C   K..H.DLD.T.YVKN...........ST.S.NT.P...VV..I....P.L.IN.....TQLN--HS..F..P...
HCoV-HKU1-N22_C   K..H.DLD.T.YVKN...........ST.S.NT.P...VV..I....P.L.IN.....TQLN--HS..F..P...
HCoV-HKU1-N20_C   K..H.DLD.T.YVKN...........ST.S.NT.P...VV..I....P.L.IN.....TQLN--HS..F..P...
HCoV-HKU1-N25_B   K..H.DLD.T.YVKN...........ST.S.NT.P...VV..I....P.L.IN.....TQLN--HS..S..P...
HCoV-HKU1-N15_B   K..H.DLD.T.YVKN...........ST.S.NT.P...VV..I....P.L.IN.....TQLN--HS..S..PH..
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Fig 4–Amino acid alignments of the receptor binding domain of HCoV-HKU1.

DISCUSSION

During the outbreak of pandemic H1N1 influenza virus between mid-2009 and early-2011, this study in southern Thailand demonstrated that 2.8% of patients diagnosed with influenza-like illness were positive for HCoV. Virus distribution was not in accordance with seasonal distribution, but the period from late-2009 to early-2010 and middle-2010 showed high prevalence of HCoV, while it declined thereafter. The positive frequency peaked in February 2010 and June 2010. In Thailand, previous reports have shown the incidence of HCoV infection in patients hospitalized with pneumonia, outpatients with influenza-like illness and children of less than 5 years of age amount for approximately 3 to 5% (Dare *et al*, 2007;

Theamboonlers *et al*, 2007). Unfortunately the clinical data and information regarding patients' profiles were not available, and thus the prevalence results in this study reflect positive cases of anonymous patients diagnosed with influenza-like illness.

Genotype identification of HCoV from sequencing the viral polymerase gene established that the prevailing HCoVs comprised 4 genotypes. The pattern of each genotype was dissimilar to previous reports (Dare *et al*, 2007; Theamboonlers *et al*, 2007). Nevertheless, HCoV-OC43 was the predominant genotype as had been documented in the previous studies.

Characterization of the S gene revealed all three HCoV-NL63 could be classified into cluster A and the nine

HCoV-HKU1 samples into subgenotype A. Interestingly, although the 22 samples of HCoV-OC43 isolated from this study could be identified as subgenotype C, they formed a unique cluster slightly separate from previously reported subgenotype C (Fig 2). Recombination between subgenotype B and subgenotype C to generate a new subgenotype D of HCoV-OC43 has been suggested (Lau *et al*, 2011). Unfortunately, data on subgenoytpe D was not available at the GenBank database and thus, homology comparisons of subgenotype D and HCoV-OC43 samples could not be determined. Additionally, the genetic pattern of HCOV-229E appeared to change over time. A previous study reported a clade of HCoV-229E to be present until 2004 (Chibo and Birch, 2006), while this study showed that the single HCoV-229E sample clustered into the 2008 clade whose sequences are close to the most recent strains in GenBank.

CoV enters host cell through a spike (S) protein on the virus envelope. This envelope protein contains 2 subunits, the receptor-binding subunit S1 and membrane fusion subunit S2 (Raabe *et al*, 1990). The receptor binding domain attaches to the host cell membrane receptor. *Alphacoronavirus* uses the host cell membrane metalloprotease, aminopeptidase-N (APN), whereas HCoV-NL63, although belonging to *Alphacoronavirus*, uses angiotensin-converting enzyme 2 (ACE2) (Yeager *et al*, 1992; Lai and Holmes, 2001). The host cell membrane receptors for HCoV-OC43 and -HKU1 have not been identified (Huang *et al*, 2006), but this study has shown amino acid variations at the receptor binding site of each genotype and each subgenotype.

Interestingly, the receptor binding site in the S1 protein of HCoV-NL63 showed the most conserved amino acids among all 3 subgenotypes, but exhibited high variation in the N-terminal region. HCoV-NL63 and SARS-CoV share the same receptor (Wu *et al*, 2009). The crystal structure of HCoV-NL63 spike protein demonstrates conservation of the core receptor binding domain, which is shared between both strains of CoV, but the receptor-binding motifs (RBMs), which are located in the receptor binding regions are variable (Wu *et al*, 2009), offering an explanation how HCoV-NL63, an *Alphacoronavirus*, recognize host cell receptor different from other homologous genotypes.

Although this study did not explain the factors contributing to the prevalence of HCoV, it demonstrated the distribution of HCoVs in the course of one and a half years. Given that it is difficult to anticipate which HCoV genotype will spread or when, surveillance programs should be established in order to monitor circumstances that may develop into a public health crisis in the future. Essential prevention strategies comprising good personal hygiene practices should be established, as well as further research aimed at vaccine development.

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REFERENCES

Adams MJ, Carstens EB. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2012). *Arch Virol* 2012; 157: 1411-22.

Chibo D, Birch C. Analysis of human coronavirus 229E spike and nucleoprotein genes demonstrates genetic drift between chronologically distinct strains. *J Gen Virol* 2006; 87(Pt 5): 1203-8.

Chiu SS, Chan KH, Chu KW, *et al*. Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. *Clin Infect Dis* 2005; 40: 1721-9.

Dare RK, Fry AM, Chittaganpitch M, Sawanpanyalert P, Olsen SJ, Erdman DD. Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays. *J Infect Dis* 2007; 196: 1321-8.

Ebihara T, Endo R, Ma X, Ishiguro N, Kikuta H. Detection of human coronavirus NL63 in young children with bronchiolitis. *J Med Virol* 2005; 75: 463-5.

Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004; 32: 1792-7.

Eickmann M, Becker S, Klenk HD, *et al*. Phylogeny of the SARS coronavirus. *Science* 2003; 302: 1504-5.

Esposito S, Bosis S, Niesters HG, *et al*. Impact of human coronavirus infections in otherwise healthy children who attended an emergency department. *J Med Virol* 2006; 78: 1609-15.

Gerna G, Vitulo P, Rovida F, *et al*. Impact of human metapneumovirus and human cytomegalovirus versus other respiratory viruses on the lower respiratory tract infections of lung transplant recipients. *J Med Virol* 2006; 78: 408-16.

Huang IC, Bosch BJ, Li F, *et al*. SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. *J Biol Chem* 2006; 281: 3198-203.

Kim OJ, Lee DH, Lee CH. Close relationship between SARS-coronavirus and group 2 coronavirus. *J Microbiol* 2006; 44: 83-91.

Kuypers J, Martin ET, Heugel J, Wright N, Morrow R, Englund JA. Clinical disease in children associated with newly described coronavirus subtypes. *Pediatrics* 2007; 119: e70-6.

Lai MM, Cavanagh D. The molecular biology of coronaviruses. *Adv Virus Res* 1997; 48: 1-100.

Lai MMC, Holmes KV. Coronaviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. Fields virology. 4th ed. Philadelphia: Lippincott, Williams & Wilkins, 2001: 1163-85.

Lau SK, Lee P, Tsang AK, *et al*. Molecular epidemiology of human coronavirus OC43 reveals evolution of different genotypes over time and recent emergence of a novel genotype due to natural recombination. *J Virol* 2011; 85: 11325-37.

Lau SK, Woo PC, Yip CC, *et al*. Coronavirus HKU1 and other coronavirus infections in Hong Kong. *J Clin Microbiol* 2006; 44: 2063-71.

Leung TF, Li CY, Lam WY, *et al*. Epidemiology and clinical presentations of human coronavirus NL63 infections in Hong Kong children. *J Clin Microbiol* 2009; 47: 3486-92.

Lole KS, Bollinger RC, Paranjape RS, *et al*. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* 1999; 73: 152-60.

McIntosh K, Kapikian AZ, Turner HC, Hartley JW, Parrott RH, Chanock RM. Seroepidemiologic studies of coronavirus infection in adults and children. *Am J Epidemiol* 1970;

91: 585-92.

Moës E, Vijgen L, Keyaerts E, *et al*. A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. *BMC Infect Dis* 2005; 5: 6.

Nokso-Koivisto J, Pitkäranta A, Blomqvist S, Kilpi T, Hovi T. Respiratory coronavirus infections in children younger than two years of age. *Pediatr Infect Dis J* 2000; 19: 164-6.

Pene F, Merlat A, Vabret A, *et al*. Coronavirus 229E-related pneumonia in immunocompromised patients. *Clin Infect Dis* 2003; 37: 929-32.

Prachayangprecha S, Makkoch J, Vuthitanachot C, *et al*. Epidemiological and serological surveillance of human pandemic influenza A virus infections during 2009-2010 in Thailand. *Jpn J Infect Dis* 2011; 64: 377-81.

Raabe T, Schelle-Prinz B, Siddell SG. Nucleotide sequence of the gene encoding the spike glycoprotein of human coronavirus HCV 229E. *J Gen Virol* 1990; 71: 1065-73.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28: 2731-9.

Theamboonlers A, Samransamruajkit R, Thongme C, Amonsin A, Chongsrisawat V, Poovorawan Y. Human coronavirus infection among children with acute lower respiratory tract infection in Thailand.

Intervirology 2007; 50: 71-7.

Vabret A, Mourez T, Gouarin S, Petitjean J, Freymuth F. An outbreak of coronavirus OC43 respiratory infection in Normandy, France. *Clin Infect Dis* 2003; 36: 985-9.

van der Hoek L, Pyrc K, Jebbink MF, *et al*. Identification of a new human coronavirus. *Nat Med* 2004; 10: 368-73.

Woo PC, Lau SK, Chu CM, *et al*. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* 2005a; 79: 884-959.

Woo PC, Lau SK, Tsoi HW, *et al*. Clinical and molecular epidemiological features of coronavirus HKU1-associated community-acquired pneumonia. *J Infect Dis* 2005b; 192: 1898-907.

Wu K, Li W, Peng G, Li F. Crystal structure of NL63 respiratory coronavirus receptor-binding domain complexed with its human receptor. *Proc Natl Acad Sci USA* 2009; 106: 19970-4.

Yeager CL, Ashmun RA, Williams RK, *et al*. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 1992; 357: 420-2.

Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med*. 2012; 367: 1814-20.

Zheng Q, Deng Y, Liu J, van der Hoek L, Berkhout B, Lu M. Core structure of S2 from the human coronavirus NL63 spike glycoprotein. *Biochemistry* 2006; 45: 15205-15.