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Journal of Microbiology, Immunology and Infection (2017) 50, 763—770

Available online at www.sciencedirect.com

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ORIGINAL ARTICLE

Epidemiology of human coronavirus NL63 infection among hospitalized patients with pneumonia in Taiwan

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Received 28 July 2015; received in revised form 18 September 2015; accepted 22 October 2015
Available online 19 November 2015

KEYWORDS
age distribution;
human coronavirus NL63;
phylogenetic analysis;
pneumonia;
seasonality

Abstract *Background/Purpose:* Human coronavirus (HCoV) NL63 is recognized in association with upper or lower respiratory tract illnesses in children. This study surveyed the prevalence of HCoV-NL63 and influenza viruses in patients with influenza-like illness in Taiwan during 2010—2011.
Methods: Throat samples from 107 hospitalized patients with pneumonia and 175 outpatients with influenza-like illness were examined using real-time polymerase chain reaction assays with virus-specific primers, and then virus-positive specimens were confirmed by sequencing the polymerase chain reaction products.
Results: HCoV-NL63 infection was identified in 8.4% (9/107) of hospitalized patients with pneumonia, but not found in outpatients with influenza-like illness. Age distribution of HCoV-NL63 infection in hospitalized patients with pneumonia indicated that the group aged 16—25 years (20%) had the highest positive rate compared with the other groups, and exhibited a similar age-specific pattern to influenza A/H1N1 infection, but not influenza A/H3N2 and B infections in hospitalized patients. Seasonal prevalence of HCoV-NL63 infection was late winter, overlapping the highest peak of the influenza A/H1N1 epidemic during December 2010 to March 2011

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http://dx.doi.org/10.1016/j.jmii.2015.10.008

in Taiwan. Co-infection of HCoV-NL63 and influenza A/H1N1 was detected in three hospitalized patients. Clinical manifestation analysis indicated that the main symptoms for HCoV-NL63 infection included fever (88.9%), cough (77.8%), and pneumonia (100%). Co-infection caused significantly higher rates of breathing difficulties, cough, and sore throat than those of single infection with HCoV-NL63 and influenza A/H1N1. Phylogenetic analysis indicated a low level of heterogeneity between Taiwan and global HCoV-NL63 strains.
Conclusion: Understanding epidemiology of HCoV-NL63 in Taiwan provides an insight for worldwide surveillance of HCoV-NL63 infection.

Introduction

Human coronavirus (HCoV) NL63 was identified from the nasopharyngeal aspirate specimen of a 7-month-old child with coryza, conjunctivitis, fever, and bronchiolitis in 2004, as a member of the Coronaviridae family, like HCoV-229E, HCoV-OC43, severe acute respiratory syndrome CoV, HCoV-HKU1, and Middle East respiratory syndrome CoV.[1,2] CoV genome is a near 30-kb positive-strand RNA with a 5′ cap and 3′ poly (A) tract that contains 14 open reading frames (ORFs) encoding for nonstructural proteins and structural proteins [conserved spike (S), envelope (E), membrane (M), and nucleocapsid].[1,2] The 5′ proximal and largest of these ORFs encodes two large overlapping polyproteins replicase 1a and 1ab (~450 kDa and ~750 kDa, respectively) processed to produce nonstructural proteins (nsps) primarily involved in RNA replication. Two specific embedded proteases, papain-like (PLpro) and 3C-like (3CLpro), mediate processing of 1a and 1ab precursors into 16 nsps (termed nsp1—nsp16). Phylogenetic tree analysis of CoV genomes indicates HCoV-NL63 forming a subcluster with HCoV-229E, porcine epidemic diarrhea virus, and Bat-CoV, assigned to the alphacoronaviruses.[3] Among all genes, HCoV-NL63 nucleocapsid (N) shows a low percentage of nucleotide and amino acid identity compared to other CoVs.[2]

HCoV-NL63 infection is usually surveyed in children with upper or lower respiratory tract illnesses. HCoV-NL63 infection is found worldwide, but is rarely positive by reverse transcriptase polymerase chain reaction (RT-PCR) assays.[4–7] The positive rate of HCoV-NL63 infection in children ranges from 1.2% in Japan,[7] 1.3% in Taiwan,[8] 2.1% in Australia,[4] 2.3% in Belgium,[9] 2.5% in Canada,[10] to 7% in Switzerland.[11] For adults, HCoV-NL63 infection is identified in 9.3% of respiratory tract illness patients under the age of 20 years in France.[12] HCoV-NL63 infection is predominant in the winter season in Australia, Belgium, Canada, France, Germany and Japan,[4,6,9,10,12,13] but in spring and summer in Hong Kong,[5] and in autumn and winter in Taiwan.[8] This study analyzed 2010—2011 surveillance data for HCoV-NL63 and influenza virus infection in hospitalized patients with pneumonia and outpatients with influenza-like illness in Taiwan, indicating the prevalence and phylogenetic analysis of HCoV-NL63 infection. Our results demonstrate a comprehensive comparison between HCoV-NL63 and influenza virus infection in hospitalized patients and outpatients.

Methods

Study design

The study recruited 107 hospitalized patients with pneumonia and 175 outpatients with influenza-like symptoms in China Medical University Hospital (Taichung, Taiwan) during 2010—2011. One throat swab was taken from each indicated patient, and then examined using RT-PCR and real-time RT-PCR for detection of HCoV-NL63, influenza viruses A/H1N1, A/H3N2, and B. We followed guidelines established by the China Medical University Hospital Institutional Review Board (Taichung, Taiwan).

RT-PCR, real-time RT-PCR and sequencing

Human coronavirus NL63 provided by Dr. Lia van der Hoek (Academic Medical Center, University of Amsterdam, Meibergdreef, Amsterdam, The Netherlands) propagated in LLC-MK2 cells that grow in Modified Eagle's Medium supplemented with 2 mM L-glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, and 10% fetal bovine serum. A QIAamp Virus RNA Mini Kit (Qiagen, Hilden, Germany) was used to extract viral RNA from clinical samples and supernatant of infected cells with HCoV-NL63, with influenza A and B viruses as the positive controls. For detection of HCoV-NL63 infection, a two-step RT-PCR using SYBR Green I was used. The specific primer pair for HCoV-NL63 N gene (nucleotides 26,416—26,666) was forward primer 5′-CTGATGGTGTTGTTTGGGTTGC-3′ and reverse primer 5′-AGAATCAGAACGAGTGCGAGAC-3′. Real-time RT-PCR mixture contained 2.5 μL cDNA (reverse transcription mixture), 200nM each primer in SYBR Green I master mix (LightCycler TaqMAn Master; Roche Diagnostics, Indianapolis, Indiana, United States). PCR was performed with amplification protocol consisting of one cycle at 50°C for 2 minutes, one cycle at 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Amplification and detection of specific products were conducted in ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, California, United States). For typing of

influenza A and B viruses as well as subtyping of H1 and H3, RT-PCR and real-time RT-PCR assays were performed, as described previously.[14]

Phylogenetic analysis

To confirm the real-time RT-PCR assays, the products of nested RT-PCR for HCoV-NL63 1a gene were further sequenced. The primers for nested RT-PCR were 5′-CTTTTGATAACGGTCACTATG-3' (SS 5852-5P) and 5′-CTCATTACATAAAACATCAAA CGG-3′ (P4G1M-5-3P) in the first PCR; and 5′-GGTCACTATGTAGTTTATGATG-3′ (P3E2-5P) and 5′-GGATTTTTCATAACCACTTAC-3′ (SS 6375-3P; coordinate 6313) in the nested PCR, as described previously.[15] Nucleotide sequences of 1a gene from the product of nested RT-PCR were sequenced and used for phylogenetic tree analysis. Reference sequences were chosen from GenBank (www.ncbi.nlm.nih.gov/genbank). Genotypes and genotypic relationships for HCoV-NL63 1a genes were identified by BioEdit 7.0.8 program (North Carolina State University at Raleigh, NC, USA, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to align sequences with reference sequences. Resulting datasets constructed phylogenetic trees for HCoV-NL63 1a genes, using MEGA version 5.2 software (http://www.megasoftware.net/). After maximum-likelihood phylogenetic analyses in 1000 bootstrap replicates, branch bootstrap values $>$ 60% or p values $<$ 0.05 clustering with specific genotype strains were determined. Cluster robustness could not all be statistically rated at 75% bootstrap due to the large size and high genetic similarity of the data sets, thus, we used 60% to identify epidemic clusters. For further support of lower bootstrap values in cluster node, the maximum likelihood (ML) tree confirmed statistical significance ($p < 0.05$) in each cluster node.

Statistical analysis

SPSS version 12.0 software, two-tailed test, χ^2 test, and Fisher's exact test were used to analyze all data. Statistical significance between both groups was noted at $p < 0.05$.

Results

Sensitivity of two-step real-time RT-PCR with SYBR Green I

To examine the sensitivity and specificity of HCoV-NL63 detection, viral genomes were extracted from 200 μL diluted supernatant containing 10—1000 pfu/mL HCoV-NL63, and then quantitated using two-step real-time RT-PCR assays. The C_t values were 30 for two copies, 27 for 20 copies, and 24 for 200 copies of HCoV-NL63. Melting curve analysis revealed HCoV-NL63 N-specific amplicon melting at 81°C. The PCR products were separated using 2% agarose gel electrophoresis, where a 251-bp band was clearly observed in the PCRs with 20 copies and 200 copies of HCoV-NL63 post gels stained with ethidium bromide. The results indicated that the detection limit of the two-step real-time RT-PCR assay was around 20 copies of HCoV-NL63. In addition, cultured supernatants of coxsackie virus 16, enterovirus 71, and influenza viruses were not detectable using two-step real-time RT-PCR assay with HCoV N-specific primers. Therefore, the two-step real-time RT-PCR assay with HCoV N-specific primers had high sensitivity and specificity, as applicable for high-throughput detection of HCoV-NL63 infection.

Table 1 Clinical data of hospitalized patients with pneumonia in this study

	Hospitalized patients with pneumonia					
	Negative[a]	HCoV NL63 positive	Flu A/H1N1 positive	Flu A/H3N2 positive	Flu B positive	HCoV NL63 positive/Flu A/H1N1 positive[b]
Cases	70	9	17	9	5	3
Average age (y)	37.3	41.7	42.4	56.1	45.6	58.7
Clinical symptom (%)						
Breathing difficulties	48.6	55.6	52.9	33.3	60.0	66.7[c]
Cough	60.0	77.8	82.4	88.9	100.0	100[d]
Fever	88.6	88.9	82.4	77.8	80.0	66.7
Pneumonia (chest X-ray)	100.0	100.00	100.0	100.0	100.0	100
Myalgia	20.0	44.4	41.2	11.1	20.0	33.3
Sore throat	2.9	11.1	11.8	22.2	20.0	33.3[d]
Vomiting	0.0	0.0	0.0	0.0	0.0	0.0
Muscle spasm	0.0	0.0	0.0	0.0	0.0	0.0
Herpes angina	0.0	0.0	0.0	0.0	0.0	0.0
Headache	4.3	0.0	0.0	0.0	0.0	0.0
Encephalitis	0.0	0.0	0.0	0.0	0.0	0.0

[a] Negative for real-time reverse transcriptase polymerase chain reaction detection of HCoV NL63, influenza A/H1N1, influenza A/H3N2, and influenza B viruses.
[b] Patients co-infected with HCoV NL63 and influenza A/H1N1.
[c] $p = 0.037$ (negative or Flu A/H1N1 positive vs. co-infection).
[d] $p < 0.02$ (negative, HCoV NL63 positive or Flu A/H1N1 positive vs. co-infection).
Flu = influenza; HCoV = human coronavirus.

Surveillance of HCoV-NL63 infection in Taiwan

A total of 282 throat swabs were taken from 107 hospitalized patients and 175 outpatients at the University Hospital in Central Taiwan from April 2010 to December 2011. All swabs were screened, using rapid diagnostic tests to detect HCoV-NL63 and influenza A/H1N1, A/H3N2, and B viruses. In hospitalized patients, positive rates of real-time RT-PCR detection were 8.4% (9/107) for HCoV-NL63, 15.9% (17/107) for influenza A/H1N1, 8.4% (9/107) for influenza A/H3N2, and 4.7% (5/107) for influenza B (Table 1, Figure 1A). Importantly, co-infection of HCoV-NL63 and influenza A/H1N1 was identified in three hospitalized patients. However, HCoV-NL63 infection was not found in outpatients with influenza-like illness (Table 2, Figure 1B). Lower positive rates of influenza A/H1N1 (13.1%), A/H3N2 (2.9%), and B (0.6%) were discovered in outpatients. Age distribution of HCoV-NL63 positive cases ranged from 3 years to 79 years (Figure 1A). Interestingly, the prevalence of HCoV-NL63 was the second highest among adults aged 76—85 years (1/7, 14.7%), and the highest among young people aged 16—25 years (1/5, 20.0%; Figure 1A). In contrast to HCoV-NL63, the positive rates in hospitalized patients were the highest among adults aged 66—75 years (3/10, 30.0%), and the lowest among groups aged $<$ 5 years (0/16, 0%) for influenza A/H3N2 infection, as well as the highest among adults aged 36—45 years (4/14, 28.6%), second highest among adults aged 56—65 years (3/13, 23.1%), and lowest among the group aged 16—25 years (0/14, 0%) for influenza A/H1N1 infection (Figure 1C). In addition, positive rates of influenza A/H1N1 in outpatients ranked the highest among adults aged 26—35 years (1/5, 20.0%), second highest among children aged 2—5 years (17/92, 18.5%), and lowest among the group aged $>$ 36 years (0/18, 0%; Figure 1D). Results indicated that age-specific

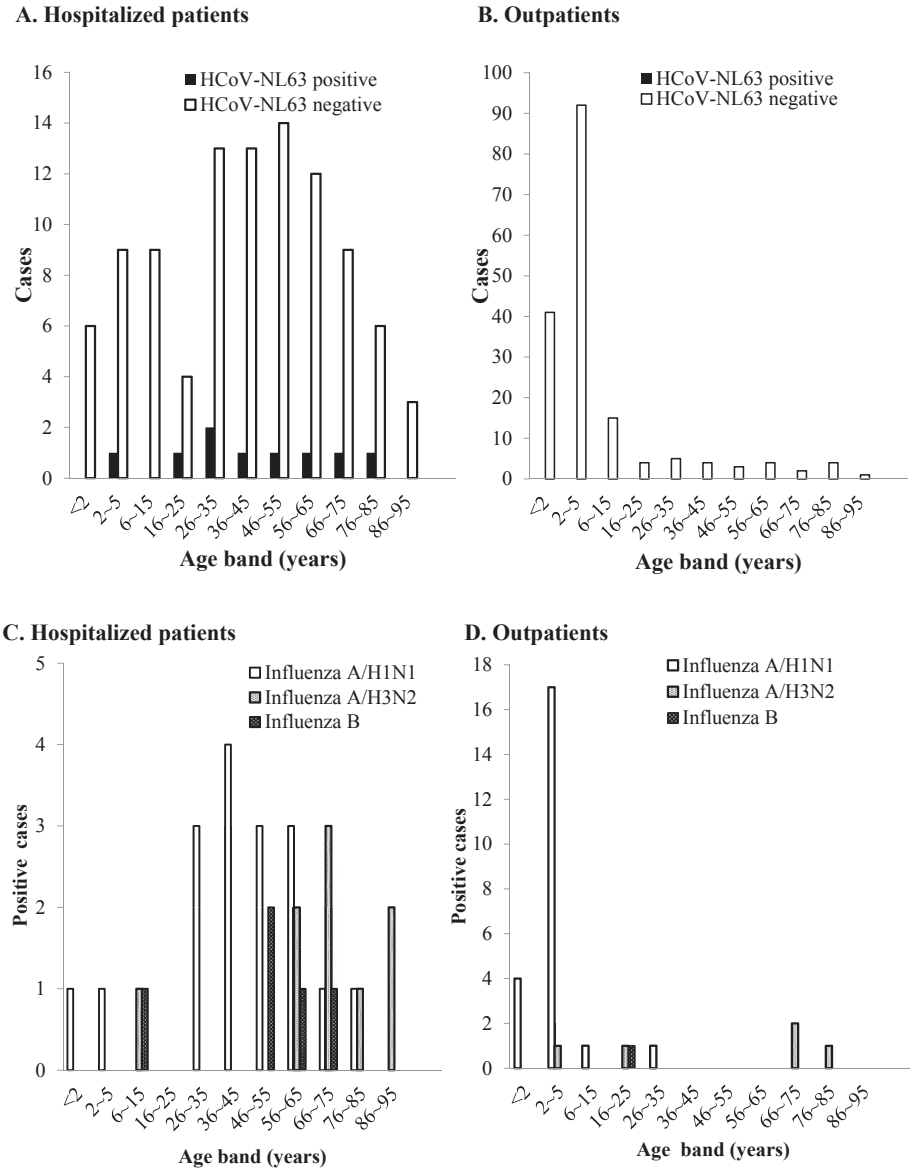

Figure 1. Age distribution of human coronavirus NL63 (A, B) and influenza virus (C, D) infection in hospitalized patients with pneumonia (A, C) and outpatients with influenza-like illness (B, D).

distribution of HCoV-NL63 and influenza AH1N1, A/H3N2, and B-positive cases had different patterns.

Seasonality of HCoV-NL63 infection in Taiwan

HCoV-NL63 infection was identified in late winter in Taiwan between January 2011 and February 2011 (Figure 2A). In contrast to HCoV-NL63, influenza A/H3N2 infection was predominant between August 2010 and December 2010, whereas a positive rate of influenza A/H1N1 proved second highest in November 2010 and highest in January 2011, and declined after February 2011 (Figure 2B and C). No significant difference between hospitalized patients and outpatients was observed in the seasonality of influenza A/H1N1 and A/H3N2. The results revealed that seasonality of HCoV-NL63 infection overlapped the periods of influenza A/H1N1 circulation in Taiwan. Importantly, co-infection of HCoV-NL63 and influenza A/H1N1 appeared in three hospitalized patients >40 years of age in February 2011.

Clinical association of HCoV-NL63 infection

Patients were divided into six groups including negative, HCoV-NL63 positive, influenza A/H1N1 positive, influenza A/H3N2 positive, influenza B positive, and co-infection. The clinical data of each group among hospitalized patient and outpatients are shown in Tables 1 and 2. Among hospitalized patients, the average age (41.7 years) of the HCoV-NL63 positive group was higher than the negative group, but lower than the influenza A/H1N1 positive and influenza B positive groups. Clinical symptoms of the HCoV-NL63 positive group were breathing difficulties (55.6%), cough (77.8%), fever (88.9%), pneumonia (100%), myalgia (44.44%), and sore throat (11.8%), which were similar to those of influenza A/H1N1 infection. However, co-infected patients presented with a significantly higher incidence of breathing difficulties (66.7%), cough (100%), and sore throat (33.3%). By contrast, the average ages of the influenza A/H1N1 (3.2 years) and B (23 years) positive groups among outpatients were lower than those among hospitalized patients (Tables 1 and 2). In addition, signs and symptoms were infrequently observed in outpatients. The results indicated no significant differences in clinical features between HCoV-NL63 and influenza infections, but co-infection of HCoV-NL63 and influenza A/H1N1 could cause more severe symptoms than single viral infection.

Phylogenetic tree analysis of HCoV-NL63 Taiwan isolates

For confirming HCoV-NL63 infection, the nucleotide sequences of 1a genes from Taiwan isolates were amplified by RT-PCR, sequenced, and then used to analyze a phylogenetic relationship with worldwide strains (Figure 3). ML analysis constructed a phylogenetic tree based on 1a gene nucleotide sequences of 39 global strains as references and four Taiwan isolates identified in the study. ML analysis of HCoV-NL63 viruses distinguished two clusters, and indicated that Taiwan isolates had a genetically similarity to Cluster I, and formed a monophyletic clade with statistical significance (bootstrap > 60%).

Discussion

This study showed the prevalence of HCoV-NL63 infection in Taiwan during 2010—2011, after the 2009 H1N1 influenza

Table 2 Clinical data of the outpatients with influenza-like illness in this study

	Outpatients with influenza-like illness					
	Negative[a]	HCoV NL63 positive	Flu A/H1N1 positive	Flu A/H3N2 positive	Flu B positive	HCoV NL63 positive/Flu A/H1N1 positive[b]
Cases	146	0	23	5	1	0
Average age (y)	9.0		3.2	52.0	23.0	
Clinical symptom (%)						
Breathing difficulties	5.5		0.0	20.0[c]	0.0	
Cough	14.4		4.3	20.0	0.0	
Fever	30.8		8.7	80.0[c]	0.0	
Pneumonia (chest X-ray)	1.4		4.3	40.0[c]	0.0	
Myalgia	2.7		0.0	40.0[c]	0.0	
Sore throat	0.7		0.	20.0[c]	0.0	
Vomiting	3.4		0.0	0.0	0.0	
Muscle spasm	0.7		0.0	0.0	0.0	
Herpes angina	10.3		0.0	0.0	0.0	
Headache	0.0		0.0	0.0	0.0	
Encephalitis	0.7		0.0	0.0	0.0	

[a] Negative for real-time reverse transcriptase polymerase chain reaction detection of HCoV NL63, influenza A/H1N1, influenza A/H3N2, and influenza B viruses.
[b] Two patients co-infected with HCoV NL63 and influenza A/H1N1 and one co-infected with HCoV NL63 and influenza A/H3N2.
[c] $p < 0.02$ (negative or Flu A/H1N1 positive vs. Flu A/H3N2 positive).
Flu = influenza; HCoV = human coronavirus.

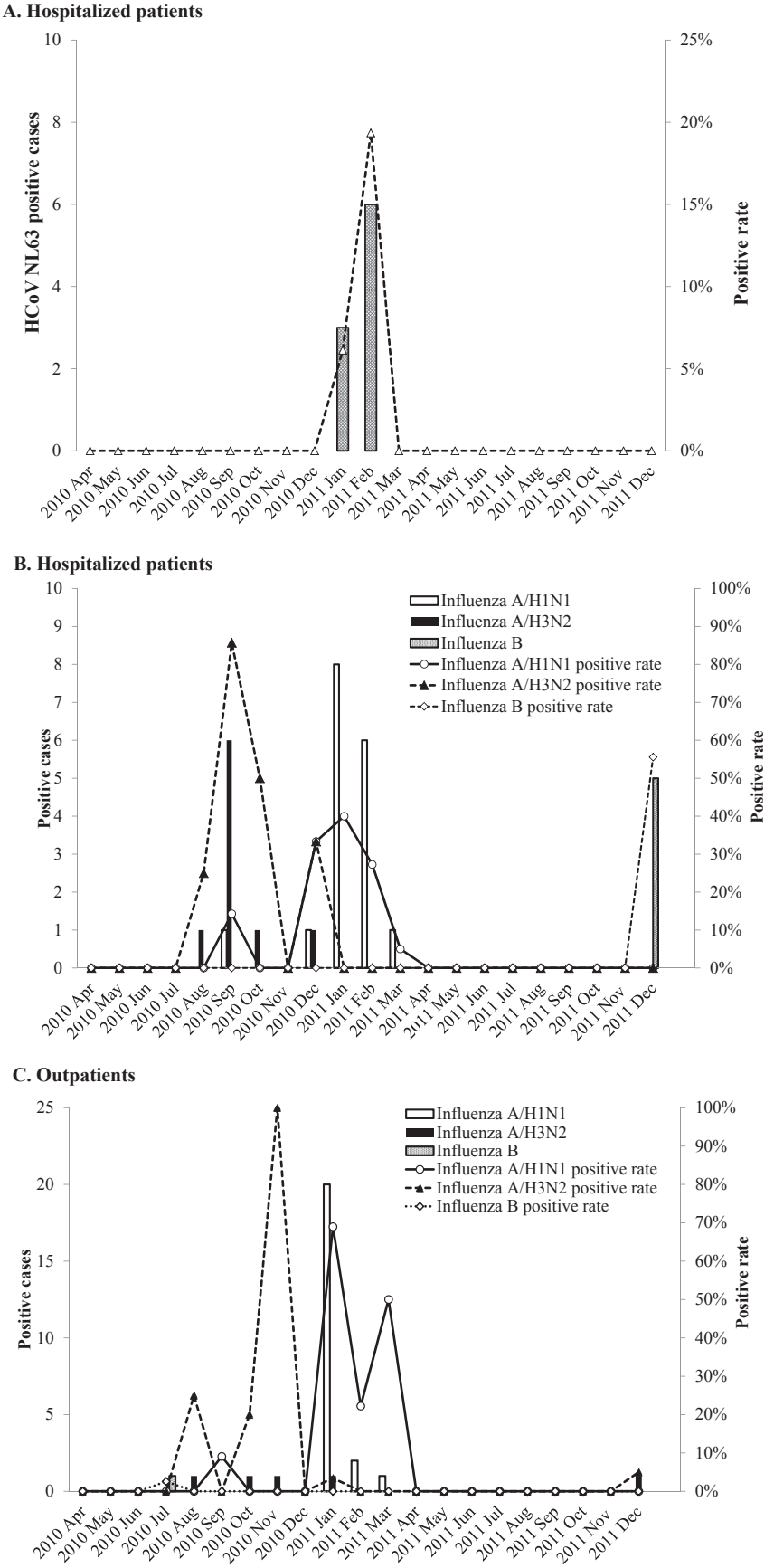

Figure 2. Seasonal prevalence of HCoV-NL63, influenza A/H1N1, A/H3N2, and B viruses during 2010—2011 in Taiwan. Positive numbers (left) and percentage (right) of HCoV-NL63 infection in hospitalized patients (A), as well as influenza A/B infection in hospitalized patients (B) and outpatients (C). HCoV = human coronavirus.

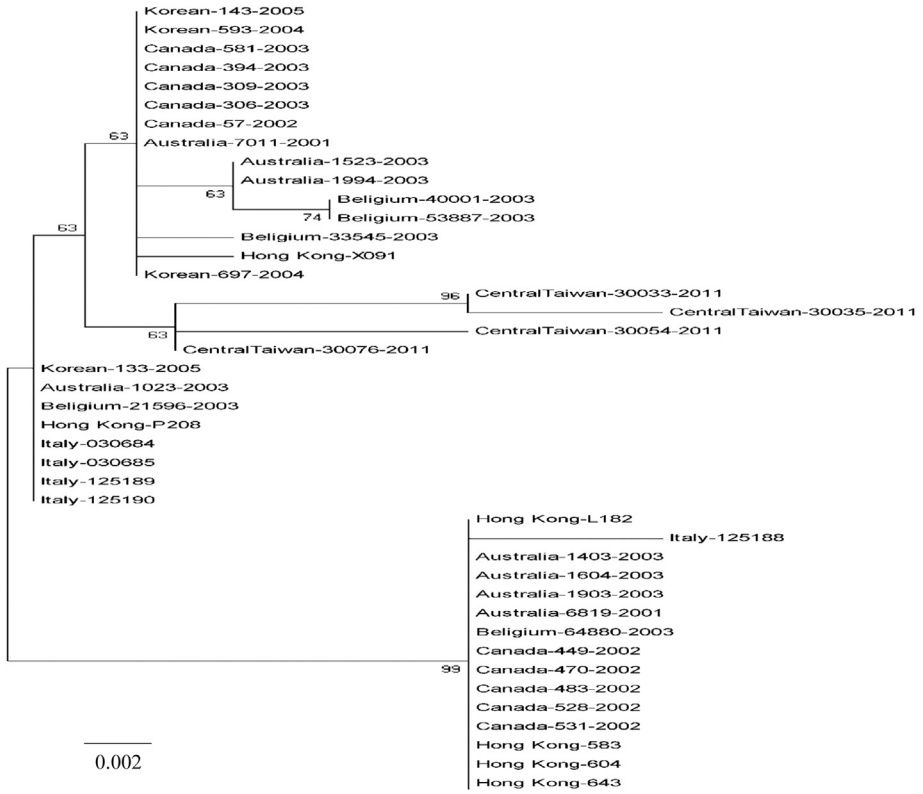

Figure 3. Phylogenetic tree of partial 1a gene sequences from Taiwan and global HCoV-NL63 strains using maximum-likehood method. Phylogenetic tree plots nucleotide sequences of HCoV-NL63 strains in this study and worldwide strains. Sequences were aligned via BioEdit and Clustal_X, phylograms generated by ML methods and MEGA tree-drawing software. Branch labels represent stability of branches >1000 bootstrap replicates, only bootstrap values > 60% are presented. HCoV = human coronavirus.

virus pandemic outbreak. Positive rates of real-time RT-PCR for HCoV-NL63 detection were 8.4% in hospitalized patients with pneumonia, but not found in outpatients with influenza-like illness (Tables 1 and 2). Phylogenetic analysis of Taiwan and global strains based on 1a gene revealed two clusters of HCoV-NL63 viruses (Figure 3), with similar patterns to previous studies.[5,8] There was no difference in age-specific distribution between HCoV-NL63 and influenza AH1N1 infections in hospitalized patients (Figure 1). However, the age distribution patterns of HCoV-NL63 infection in hospitalized patients differed from those of influenza A/H3N2 and B infections in hospitalized patients and outpatients (Tables 1 and 2). The results differed from previous reports of low prevalence of HCoV-NL63 infection in children, such as 1.2% in Japan,[7] 1.3% in Taiwan,[8] 2.1% in Australia,[4] 2.3% in Belgium,[9] 2.5% in Canada,[10] and 7% in Switzerland.[11] Recently, the positive rate of HCoV NL63 in a healthy control group (8.5%) was higher than in patients with upper respiratory tract infection (5.1%) in Ghana.[16] The study was the first with a high risk of HCoV-NL63 infection in hospitalized patients with pneumonia. The present study also identified three inpatients aged >40 years co-infected with HCoV-NL63 and influenza A virus. Co-infection caused significantly higher rates of breathing difficulties, cough, and sore throat than single infection with HCoV-NL63 and influenza A/H1N1 caused. Therefore, HCoV-NL63 infection could have a considerable impact on public health.

The study showed the seasonality of HCoV-NL63 infection as late winter, overlapping the second peak of influenza epidemic in Taiwan (Figure 2). The seasonality of HCoV-NL63 infection in Taiwan during 2010—2011 was similar to that in temperate countries,[6,7,9,10,12] but different from some studies in which it was in autumn in Taiwan during 2004—2005,[8] summer and autumn in Chongqing,[17] and summer in Hong Kong.[5] Clinical manifestation analysis indicated fever (88.88%), cough (77.78%), and pneumonia (100%), but no significant association with the group with HCoV-NL63 infection compared to influenza A positive groups among hospitalized patients (Table 1). In France, a survey of patients younger than 20 years indicated that more than one-third of the patients infected by HCoV-NL63 had bronchiolitis and pneumonia.[12] In Brazil, a 46-year-old female patient with HCoV-NL63 infection had hemorrhagic pneumonia, respiratory and renal failure, and died.[18] Thus, our results demonstrated HCoV-NL63 infection correlating with severe lower respiratory tract diseases in adults, implying HCoV-NL63 infection as a higher risk factor of severe respiratory illness for adults than children.

In summary, real-time RT-PCR assay identified the overall positive rate of HCoV-NL63 infection as 8.4% in hospitalized patients with pneumonia in Taiwan during

2010—2011. The proportion of HCoV-NL63 infection in each age-specific group indicated HCoV-NL63 as a high risk factor for pneumonia patients aged 16—25 years and 26—35 years. Prevalence of HCoV-NL63 was predominant in late winter; co-infection with HCoV-NL63 and influenza A/H1N1 was associated with pneumonia in older adults. Phylogenetic analysis indicated HCoV-NL63 strains in Taiwan were in one of two major clusters based on 1a gene sequences of global strains, showing a low level of heterogeneity between Taiwan and global strains. Transmission of HCoV-NL63 in older adults causes severe low respiratory tract diseases. Our results yield a better understanding of the epidemiology of HCoV-NL63 in Taiwan and contribute information necessary for worldwide surveillance of HCoV-NL63 infection.

Conflict of interest

All authors report no conflicts of interest relevant to this article.

Acknowledgments

Funding was provided by China Medical University (CMU103-S-04, CMU102-ASIA-15, and CMU103-ASIA-07), and the Ministry of Science and Technology (MOST 101-2320-B-039-036-MY3).

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