

Original Article

## Evidence of transmission of influenza A and influenza B co-infection in healthcare workers

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### Abstract

**Introduction:** Co-infection of influenza A and B has been reported, especially in outbreak situations, but epidemiological and clinical information is limited. We aimed to investigate an outbreak of influenza among health care workers in which the index case suffered from influenza A and B co-infection.

**Methodology:** We investigated the outbreak setting through the utilization of structural questionnaires, molecular methods, and serological tests.

**Results:** Among 13 persons, one index case and five confirmed secondary cases were confirmed. The overall influenza infection rate was 46.2% (6/13), with infection rates for influenza A and B at 38.5% (5/13) and 23.1% (3/13), respectively. Interestingly, one of the secondary cases had influenza A and B co-infection identical to the index case. There was no significant association between vaccination status and influenza infection.

**Conclusions:** This study unveils the demonstration of human-to-human influenza A and B co-infection transmission for the first time. Surveillance systems, combined with epidemiological case investigation comprising molecular diagnosis, should be strengthened for future influenza outbreak preparedness.

**Key words:** Influenza outbreak; co-infection; influenza A and B co-infection; human-to-human transmission; healthcare workers.

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### Introduction

Respiratory viral infection remains a global health concern. Several emerging respiratory disease-causing viruses had been considered life-threatening, including severe acute respiratory syndrome (SARS, 2002) [1], Middle East respiratory syndrome (MERS, 2012) [2], and coronavirus disease 2019 (COVID-19, 2019) [3]. Influenza virus was and remains a seasonal epidemic despite vaccine availability and antiviral treatments. Two types of influenza virus, type A and B, are the main causes of disease in humans. In the past century, several influenza pandemics have been documented: in 1918 (caused by influenza A (subtype H1N1), 1957 (A, subtype H2N2), 1968 (A, H3N2), and 2009 (A, H1N1). Besides the influenza pandemic, approximately 3 to 5 million influenza infections have been reported annually [4].

Both influenza A and B viruses circulate worldwide and cause respiratory diseases in humans. The co-infection of influenza A and B was sporadically

reported with concerns about the occurrence of a new genetic reassortment strain [5] and a more severe illness [6]. However, there was no relationship or association among individual cases. Despite increasing recognition and epidemiological studies, the pathogenesis and the infectivity are still unclear [7]. To date, there is no evidence of transmission of co-infection of influenza A and B in humans.

In February 2013, a cluster of influenza cases among healthcare workers (HCW) in the Hospital for Tropical Diseases, Bangkok, Thailand was noticed. An outbreak in health care facilities warrants a thorough investigation and contact tracing to avoid further spread, especially to high-risk individuals. The cases occurred after a two-day trip during which close contact with the index case transpired. Interestingly, further investigation revealed a co-infection of influenza A and B in the index case and confirmed infection of influenza A, B, or both in five out of 12 people being in close contact with the index case. In this retrospective cohort

study, we aimed to report the transmission of influenza A and B co-infection and to determine the characteristics of cases, infection rate, and protection granted by influenza vaccination during the outbreak. The related literature was reviewed.

## Methodology

### *Outbreak setting and case finding*

On 26 February 2013, an influenza outbreak was suspected. An outbreak investigation was initiated when three out of 11 HCWs who recently returned from their two-day trip on 23–24 February 2013 were diagnosed with influenza. All HCWs who participated in this trip were called for an investigation. The completed physical examination was performed and a structural questionnaire was obtained. A suspected influenza case was defined as an attendee of the trip who had a fever (temperature 37.8 °C or higher) and cough and/or a sore throat without a known cause other than influenza (influenza-like illness (ILI) [8]. A confirmed case was defined as the suspected case with any positive influenza test results. A retrospective cohort study design was conducted to determine the influenza infection rates and the protective effect of the influenza vaccine. Data were collected by using a structured questionnaire. The overall infection rate was determined by the total number of new cases divided by the total population.

### *Laboratory investigation*

We used multiple diagnostic methods for diagnosis; reversed transcriptase polymerase chain reaction (RT-PCR) was the gold standard diagnosis method while both the rapid diagnosis test (RDT) and serology were optional tests conducted at the discretion of the attending physician. A nasopharyngeal swab was performed for all HCWs and acquired specimens were subsequently tested by RT-PCR for seasonal influenza A (H3N2 and H1N1), H1N1 2009, and influenza B. The procedure was conducted as described previously by Mizuike *et al.* [9]. The RDT for Influenza A, B, and RSV (QuickNavi™-Flu + RSV, Denka Seiken, Tokyo, Japan) was optionally applied depending on the decision of the attending physician [10]. The serum of the index case was tested for influenza immunoglobulin M (IgM) antibodies (Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand).

### *Sequencing and phylogenetic analysis*

To confirm the diagnosis, the influenza viruses from leftover specimens from the RT-PCR technique of all subjects were isolated by cell culture technique using

the MDCK cell line [11]. Specimens from all cases that either met the ILI definition or had a positive result from RT-PCR, were sent for DNA sequencing. The amplicon of genes encoding matrix protein (M) or nucleoprotein (NP) was targeted for influenza A and B, respectively [9,12]. The resulting nucleotide sequences from infected individuals were aligned with the corresponding influenza virus gene retrieved from National Center for Biotechnology Information (NCBI) database using ClustalW [13]. Phylogenetic analysis of each data set was performed to identify the evolutionary relationship among the isolates using the Maximum Likelihood (ML) method with 1,000 replicate bootstraps, Molecular Evolutionary Genetics Analysis (MEGA) software, version 7.0 [14].

### *Statistical analysis*

Data were analyzed by Epi-Info V.3.4.1 (US CDC). Fisher's exact test was used to determine associations between selected variables and influenza illness.

### *Ethical approval*

This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTU 2016-057-01). Informed consents were waived due to the nature of the outbreak investigation.

## Results

### *Case history and demographics*

On 23 February 2013, 11 HCW and two of their friends participated in a two-day group trip. The majority of HCW worked in the out-patient department (OPD) and had no known underlying disease. This small group, consisting of 8 females and 5 males with a median age of 26 (21–52) years, shared a six-hour drive in an air-conditioned van. They also stayed the night in one shared bedroom. A day before (22 February 2013) and during the trip, one female HCW (the index case) showed symptoms of ILI, myalgia, and diarrhea. Upon returning on 24 February, one HCW developed ILI and was followed by two other cases on 25 February, and one case each on 26 and 28 February, respectively, accounting for a total of six symptomatic individuals out of 13 persons joining the trip (Table 1, Figure 1). RDT for influenza A, B, and RSV was performed on five symptomatic individuals and showed positive results for influenza A in three cases. All nasopharyngeal swab samples from symptomatic individuals were subjected to RT-PCR for seasonal influenza A and B, to which three of them displayed positive results for influenza A (H3N2). All six samples

were sent for DNA sequencing. Two samples, one of which was the index case, displayed influenza A and B co-infection, three samples were positive for influenza A, and one sample was influenza B.

The index case was a 26-year-old female practical nurse in an out-patient care department (OPD). She developed fever, cough, sore throat, and myalgia on the 22nd of February before the trip from 23-24 February 2016. Her nasopharyngeal swab was conducted on day 4 after the onset of illness and revealed negative results for rapid influenza test and RT-PCR for seasonal influenza A (H3N2 and H1N1), H1N1 2009, and influenza B. However, the sequencing confirmed dual

infection (Table 1, Figure 1). The acute serum of the index case taken six days after onset was positive for both anti-Influenza A-IgM and anti-Influenza B-IgM.

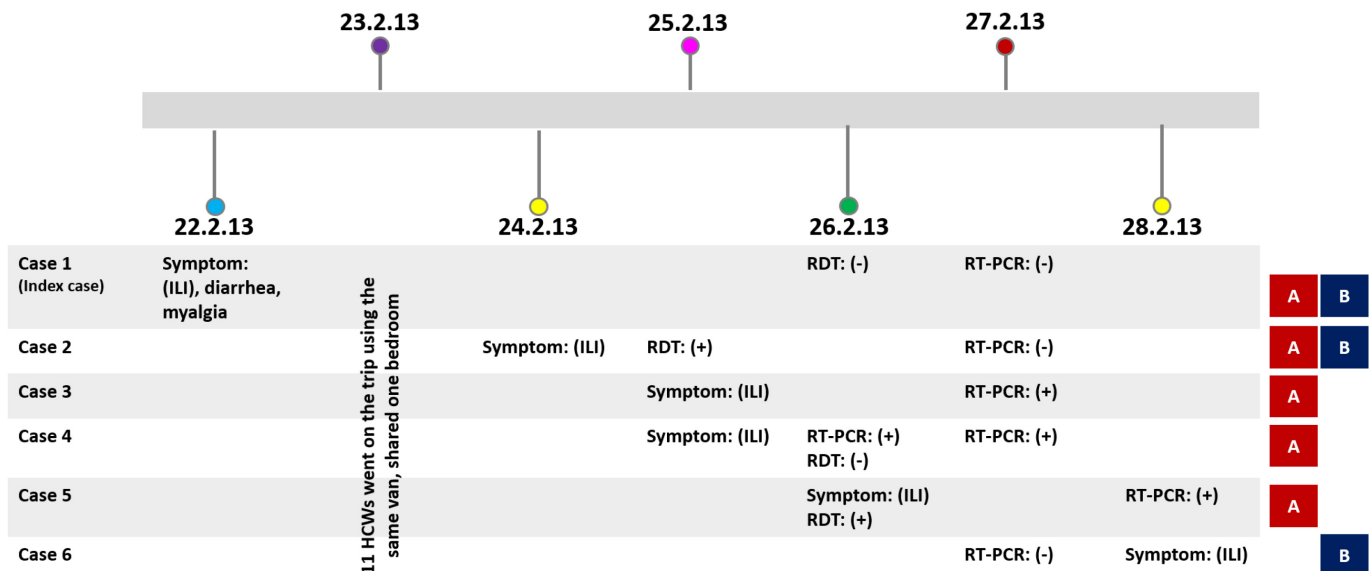
Some symptomatic individuals received oseltamivir, an antiviral treatment, at the discretion of the physician in charge. They were isolated for five days in a hospital room or at their homes. Asymptomatic contact persons were put under close observation. Strict contact, droplet precaution, and vaccination were advised to all contact persons. All symptomatic individuals completely recovered and returned to work within five days with no complications.

**Table 1.** Clinical presentations and laboratory investigations of health care workers in the outbreak.

Case	Gender, age	Influenza vaccine	Onset	Symptoms	RDT** (date)	RT-PCR (date)	ACCESSION NO.	Sequencing results	Antiviral treatment
1*	F, 26	No	22-Feb	ILI, Diarrhea, Myalgia	Negative (26-Feb)	Negative (27-Feb)	MH279480.1 MH371287.1	Influenza A virus (A/Thailand/CU-B8364/2013(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds. (KP336191.1) Influenza B virus (B/Thailand/CU-H3052/2011) segment 5 nucleoprotein (NP) gene, complete cds. (JX513207.1)	Yes
2	F, 24	No	24-Feb	ILI	Influenza A (25-Feb)	Negative (27-Feb)	MH279481.1 MH371288.1	Influenza A virus (A/Thailand/CU-B8364/2013(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds. (KP336191.1) Influenza B virus (B/Thailand/CU-H3052/2011) segment 5 nucleoprotein (NP) gene, complete cds. (JX513207.1)	Yes
3	F, 26	No	25-Feb	Cough, runny nose	Influenza A (26-Feb)	Positive (H3N2) (27-Feb)	MH279482.1	Influenza A virus (A/Thailand/CU-B8364/2013(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds. (KP336191.1)	Yes
4	F, 21	Yes	25-Feb	ILI	Negative (26-Feb)	Positive (H3N2) (26-Feb)	MH279484.1	Influenza A virus (A/Thailand/CU-B8364/2013(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds. (KP336191.1)	No
5	M, 52	Yes	26-Feb	ILI	Influenza A (26-Feb)	Positive (H3N2) (28-Feb)	MH279483.1	Influenza A virus (A/Thailand/CU-B8364/2013(H3N2)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds. (KP336191.1)	Yes
6	M, 25	Yes	28-Feb	ILI	Not done	Negative (27-Feb)	MH371289.1	Influenza B virus (B/Thailand/CU-H3052/2011) segment 5 nucleoprotein (NP) gene, complete cds. (JX513207.1)	Yes

\*Index case; ILI: influenza like illness; \*\* QuickNavi™-Flu + RSV, Denka Seiken, Tokyo, Japan [10].

**Figure 1.** A timeline describing the onset and course of infection in six confirmed influenza cases in Bangkok. The confirmed index case (case 1) harbored influenza A and B, which was transmitted to five confirmed secondary cases (case 2 – 6) through close contact during a two-day trip. ILI: influenza-like illness, HCW: health care worker, RDT: rapid diagnostic test, RT-PCR: reverse transcriptase polymerase chain reaction.



*Influenza infection rate, infectivity of the index case, and vaccine protection*

The first case, which presented symptoms a day before the trip, was identified as the confirmed index case which harbored both influenza A and B co-infection. There were five confirmed secondary cases in this setting; none of which showed any symptoms prior to the trip but contracted the infection one to five days after the trip. The overall infection rate of laboratory-confirmed influenza was 46.2% (6/13). Of those, the

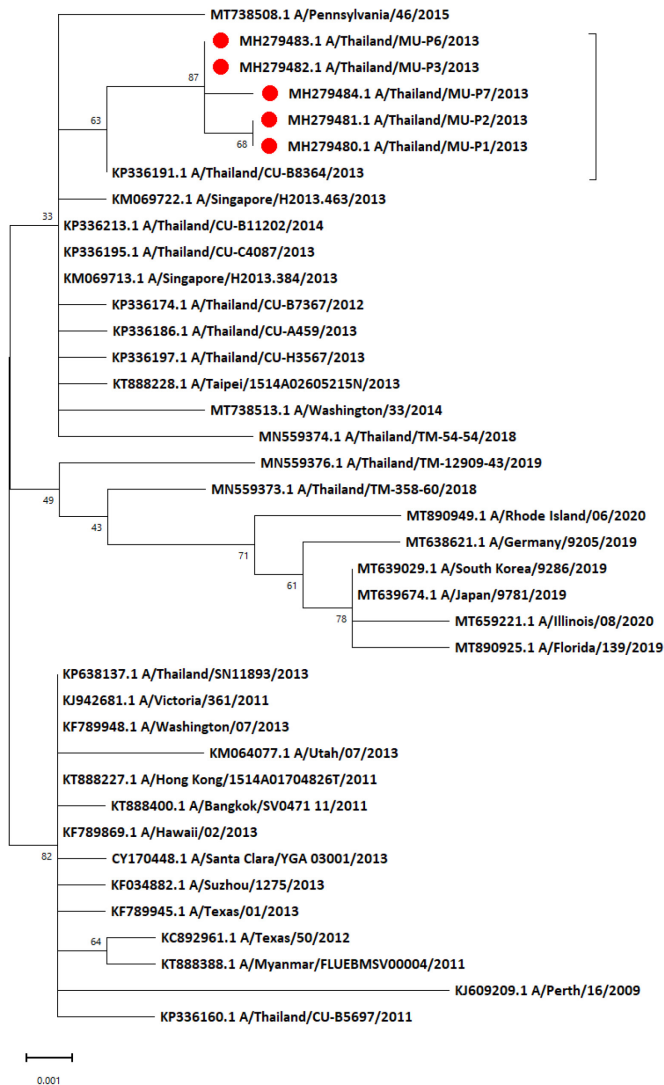
influenza A and B infection rates were 38.5% (5/13) and 23.1% (3/13), respectively.

The data from the structured questionnaire was analyzed. There was no statistically significant difference in the infection rate between vaccinated and unvaccinated groups (50% vs. 42.9%,  $p = 0.616$ ). (Table 1) Moreover, other behavioral factors, including sharing food and hand hygiene practices, were not associated with influenza illness (data not shown).

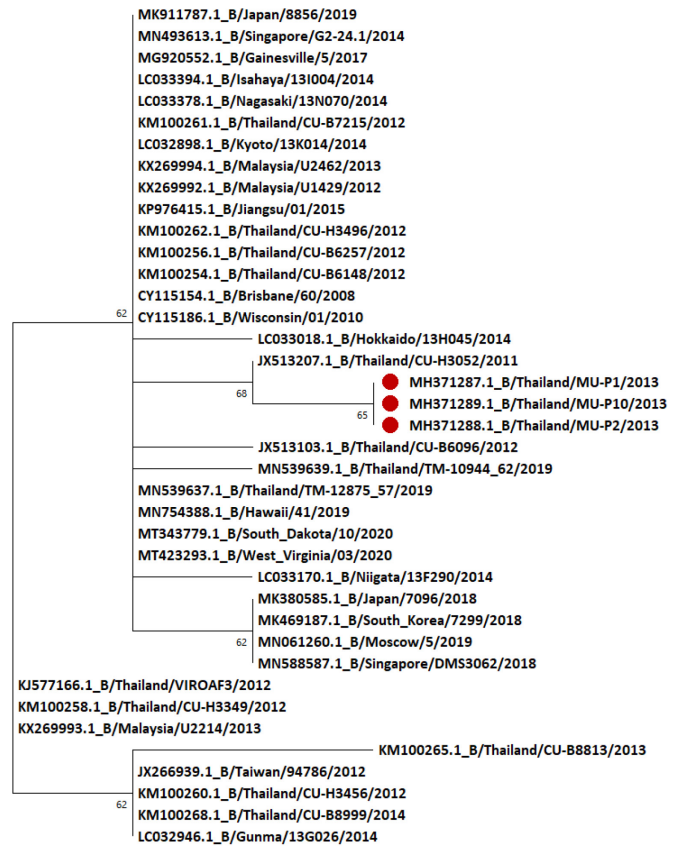
*Phylogenetic analysis*

The amplicons from six samples were subjected to nucleotide sequencing. To determine the evolution in the obtained samples, the Maximum Likelihood (ML) method was applied to generate the phylogenetic tree. Two sets of ML trees were generated; the first set was

**Figure 2.** Phylogeny of influenza virus type A. The Maximum Likelihood (ML) method with 1,000 replicate bootstraps was performed using Molecular Evolutionary Genetic Analysis (MEGA) software, version 7.0 [14]. The matrix sequences included in the present study are indicated by the red spheres, along with sequences obtained from GenBank database collected during 2009-2020. The strain names are shown as accession number, type/country/strain/and reported year of each sequence. Numbers on branches represent bootstrap support values.



**Figure 3.** Phylogeny of influenza virus type B. The Maximum Likelihood (ML) method with 1,000 replicate bootstraps was performed using Molecular Evolutionary Genetics Analysis (MEGA) software, version 7.0 [14]. The nucleoprotein sequences included in the present study are indicated by red spheres and sequences obtained from the GenBank data collected during 2008-2020. The strain names are shown as the accession number, type/country/strain/and reported year of each sequence. Numbers on branches represent bootstrap support values.





generated based on the matrix gene segment of influenza A (H2N3), while the other was generated using the nucleoprotein gene of influenza B. For influenza A, out of 34 selected strains circulating during 2009-2020, we noticed five samples (A/Thailand/MU-P1/2013, A/Thailand/MU-P2/2013, A/Thailand/MU-P3/2013, A/Thailand/MU-P6/2013, and A/Thailand/MU-P7/2013) clustered together with the influenza A that was also circulating in Thailand in 2013 (KP336191.1 strain A/Thailand/CU-B8364/2013) (Figure 2). From the nucleoprotein gene segment of influenza B, the ML tree revealed that our three samples (B/Thailand/MU-P1, B/Thailand/MU-P2, and B/Thailand/MU-P10) were associated with the influenza strain B that was also circulating in Thailand in 2011 (JX513207.1\_B/Thailand/CU-H3052/2011) (Figure 3).

## Discussion

Although co-circulation of multiple influenza strains can occur in an epidemic, co-infection of influenza A and B is not commonly found. However, the real burden of dual infections may be underestimated due to diagnostic investigation limitations [5,15]. The previous cases were documented as case reports [16-18] or found in surveillance studies in epidemics [5,7,15,19-22]. The incidence differed among reports; 0.6% during the 2015-2016 epidemic season in Israel [7], 1.6% during winter 2014-2015 in Spain [19], 2.1% in a UK study [6], 2.2% in 2007 winter epidemic in France [5], and 0.24% in 2008-2016 in Rio Grande do Sul, Brazil [22]. However, no transmission of dual infection among individual patients has previously been reported. This may be explained by the low stability and infectivity of the dual infection. To the best of our knowledge, this is the first documentation of dual infections of influenza A and B transmission. The pathogenesis and virus-virus interactions as well as the transmission rate of dual infection remain a major interest and require further prospective studies.

The clinical presentations of dual infections in our study were not different from single strain infections as described in healthy patients with dual infections in previous studies [7,15,19]. Nevertheless, it was reported that co-infection between seasonal influenza A and B in North West England was significantly associated with the risk of ICU admission and death [6]. It is however important to note that most cases of co-infection in that study were children under five years old with no data of underlying diseases. A single-center case-control study in Brazil also revealed that dual and triple influenza infection was significantly associated

with underlying cardiomyopathy and death [22]. Since the HCWs in our study were healthy adults, there was neither admission nor complication.

The concurrent co-circulation of two influenza subtypes was proposed as the key mechanism of co-infection. Dual infections were mostly reported during epidemic seasons [5,7,15,21,22]. This condition was also reported in small outbreaks such as long-term care facilities that had influenza A and B co-circulating during the same period [23]. In addition, the cases of influenza A (H1N1) pdm09 and B co-infection were also reported to occur during an overlapping period when the circulation of influenza B was high, and the incidences of influenza A started to rise [15]. On an individual level, the dual infection of influenza A and B was reported in a Japanese boy whose mother and sister suffered from influenza A and B, respectively [17]. In our study, the high risk of exposure to multiple strains of influenza might occur in the health-care setting, as the index case, who worked in the OPD, could receive concurrent infection of two different influenza strains. This was supported by a single-center case-control study by Perez-Garcia and group, which revealed that co-infection of influenza A and B co-infection during the winter season in 2014-2015 was significantly associated with a hospital-acquired infection [19]. Whereas, an Israeli epidemiological study revealed contrasting results with co-infection significantly common in community-acquired infection [7]. It is noteworthy that all studies encountered limitations in terms of co-infection case numbers.

The concern arising from dual or multiple infections is the possible occurrence of influenza virus recombination [5,24]. The aforementioned reassortment might lead to the emergence of new strains or the escalation of the anti-viral resistance [23]. Thus, more accurate point of care tests and active surveillance to monitor the dual or multiple infections of influenza viruses should be implemented [5].

The high infection rate in this outbreak could be explained by the close contact in small and confined spaces, including the air-conditioned van and shared bedroom. There was no difference in infection rates between vaccinated and unvaccinated individuals. This may be attributed to the non-coverage of the current strains by the influenza vaccine since the 2012 northern hemisphere winter influenza vaccine contained A/California/7/2009 (H1N1) pdm09, A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010-like viruses [25].

Due to technical limitations, some data and investigations were incomplete. The history taking and

questionnaire might be affected by recall bias while the small sample size limited the statistical analysis. The prior antiviral treatment before the declaration of an outbreak and full investigations in some cases might affect the investigation results. However, advancement in a molecular investigation by applying genetic sequencing to trace transmission chains is an advantage in this study.

## Conclusions

This outbreak report confirmed the infectivity of the co-infection of influenza A and B strains in humans. The co-infection can sporadically occur in nature, which raises concerns about viral recombination and the emergence of new epidemic strains. This discovery urges for the strengthening of surveillance systems and the utilization of more advanced molecular detection methods in assisting outbreak investigations. General influenza prevention measures remain critical in averting the infection expansion in both vaccinated and non-vaccinated populations.

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## Authors' Contributions

Conception and design of the study: VL, WP, PL, SI, data acquisition: VL, NT, PP, data analysis and interpretation: VL, TS, SI, PL, manuscript drafting VL, TS, PL, manuscript revision for important intellectual content: SI, WP, final approval of the version to be submitted: all authors.

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