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Research article

# Etiology of febrile respiratory infections in the general adult population in Singapore, 2007–2013

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ABSTRACT

Pathogens that cause upper respiratory infections are numerous and specific preventive and therapeutic strategies are scarce. In order to ascertain the etiological agents resulting in upper respiratory tract infections (URTI) in adults in Singapore, nasal swab samples were collected from 2057 patients presenting with fever at primary healthcare clinics in Singapore from December 2007 to February 2013. Samples were tested using the Luminex NxTAG Respiratory Pathogen Panel that includes 22 respiratory pathogen targets. Patient-reported symptoms and vital signs were recorded and full blood and differential counts taken. Pathogens were detected in the following order of frequency: influenza viruses, rhino-/enteroviruses, coronaviruses, parainfluenza viruses, pneumoviruses, adenovirus, bocavirus and *C. pneumoniae*. Fifteen virus species were detected as part of coinfections, in which rhinoviruses were the most commonly observed pathogen. Our results suggest that influenza viruses are the main etiological agents, but multiple other respiratory viruses contribute to the total burden of URTI in adults in Singapore.

## 1. Introduction

Upper respiratory tract infections (URTI) are commonly diagnosed and exert a large health and economic burden globally (Keech et al., 1998; Nichol et al., 2005). The major etiological agents causing URTI are respiratory viruses and their distribution and frequency varies with age and geographic location (Cooney et al., 1975; Hodinka, 2016; Silva et al., 2015). The focus of prior work describing the epidemiology of respiratory viruses is on pediatric populations with rather few reports of infections in adults.

In Singapore, acute respiratory infections have been described among children as well as adults. Hong et al. identified a viral etiology in 25% of adults with URTI presenting to family care physicians, with rhinovirus detected most frequently (Hong et al., 2004). Descriptions of epidemic outbreaks in Singapore by other respiratory viruses among hospitalized children include metapneumovirus (Loo et al., 2007; Ong et al., 2007), bocavirus (Tan et al., 2009a), rhinovirus group C (Tan et al., 2009b), and parainfluenza virus 3 (Teo et al., 2010). Respiratory syncytial virus (RSV), influenza A, and parainfluenza 3 displayed annual periodicity among hospitalized patients in Singapore, despite comparatively little variation in temperature and humidity throughout the year (Chew et al., 1998). URTI is the most common acute diagnosis in government primary healthcare clinics, with 594,000 diagnoses in 2018 (Ministry of Health Singapore, 2018) and a variable percentage of these being influenza-like illness (ILI), defined as elevated body temperature of $\geq$38.0 °C and cough with an onset of symptoms within the last 10 days (World Health Organization, 2014).

In spite of the high burden of URTI and ILI disease in Singapore, an etiological diagnosis is not established for the vast majority of outpatient visits and laboratory diagnosis is reserved for severe cases that require hospitalization. Apart from influenza viruses and RSV, specific preventive or therapeutic strategies to ameliorate the disease burden caused by respiratory viruses are currently not available. In this study, we identify respiratory pathogens in nasal swab samples collected from a community

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cohort of febrile adults. Elucidating the etiology of URTI will help to focus existing preventive strategies and prioritize the development of novel interventions to fight common respiratory pathogens.

## 2. Materials and methods

### 2.1. Study design

Participants were recruited between December 2007 and February 2013 at five primary healthcare clinics throughout Singapore as part of the Early DENgue infection and outcome study (EDEN) (Low et al., 2006, 2011) and were enrolled after obtaining informed consent. Inclusion criteria for the study were age ≥18 years old and body temperature at time of recruitment of ≥37.5 °C. Nasal swab samples were collected during two subsequent clinic visits at 1–3 and 4–7 days post fever onset. Whole blood was collected from participants and sent for blood count and differential laboratory testing. Baseline socio-demographic characteristics, patient-reported symptoms and vital signs were recorded.

### 2.2. Sample analysis

The NxTAG Respiratory Pathogen Panel (RPP) (Luminex, Texas, USA) can detect 19 common respiratory viruses and three bacteria based on multiplex PCR bead technology. Pathogen testing was performed according to manufacturer's instructions. Briefly, nucleic acid was extracted from 200 μl of nasal swab sample using the QIAcube HT kit and the QIAxtractor (Qiagen, Hilden, Germany). Samples were spiked with 20 μl of MS2 bacteriophage internal control prior to nucleic acid extraction, and 35 μl of eluate was added to each well containing reagents that are required for PCR amplification and bead-hybridization. After PCR and hybridization, the test plate was transferred to a MAGPIX instrument (Luminex, Texas, USA) for data acquisition. Raw data was processed in the SYNCT software (Luminex, Texas, USA) and positive calls were determined based on multi-dimensional detection (MDD) values above the validated threshold for each target. Appropriate negative and positive controls, e.g. viral nucleic acid or plasmid-encoded viral sequences for each viral target in the panel, were used during individual runs. Ambiguous results (low-positive or high-negative MDD values) as well as detections of more than one target were verified by qPCR assays as described previously (Hoek et al., 2013).

### 2.3. Statistical analysis and software

Visualization of coinfections was produced using the Gephi 0.9.2 software (Bastian et al., 2009). Statistical analyses were performed using R version 3.5.2 (R Development Core Team, 2018). Continuous variables were summarized as mean and range; categorical variables were expressed as count and percentage of total. Differences in mean values of two variables were compared using Student's t-test for parametric continuous variables or Wilcoxon rank sum test for non-parametric continuous variables. Associations between categorical variables were evaluated using the chi-squared test of independence. In order to explore possible associations between pathogens and vital signs or biochemical measurements, the parametric analysis of variance (ANOVA) was performed. Kruskal-Wallis one-way analysis of variance was used for variables with values that were not normally distributed. Student's t-test or Wilcoxon rank sum test was applied post-hoc to compare each pathogen group against all positive detections. Non-influenza pathogens were grouped into their respective taxonomic families to achieve reasonable sample sizes. Groups of pathogens that had fewer than ten positive detections (sH1N1, human bocavirus, *C. pneumoniae*) and variables that had fewer than ten measurements for a particular pathogen group due to missing data (neutrophil and mixed cell counts) were excluded from the analyses. Statistical significance was defined as a p-value of $<0.05$.

### 2.4. Ethical approval

This work has been conducted within current guidelines for ethical approval. Approvals for this study were obtained from the National Healthcare Group Institutional Review Board (reference code DSRB B/05/013) and the Institutional Review Board of the National University of Singapore (reference code B-14-209E).

## 3. Results

Nasal swab samples obtained from 2057 study participants with febrile illness at their initial clinic visit were analyzed for the presence of 22 respiratory pathogens. A total of 976 (47.4%) samples tested positive (Figure 1), of which 22 (2.2%) samples contained two pathogens (Figure 2) resulting in a total of 998 detections. All viral pathogens in the NxTAG RPP panel were detected at least once. Influenza A (pandemic influenza A virus subtype H1N1, pdmH1N1; seasonal influenza A viruses subtypes H3N2 and sH1N1) and influenza B viruses were detected most frequently (n = 627, 62.8%), followed by rhinoviruses/enteroviruses (RV/EV, n = 183, 18.3%), coronaviruses 229E, NL63, OC43, and HKU1 (n = 67, 6.7%), parainfluenza viruses (PIV 1–4, n = 50, 5.0%), pneumoviruses (RSV; human metapneumovirus, HMPV, n = 47, 4.7%), adenovirus (AdV, n = 20, 2.0%), bocavirus (BoV, n = 3, 0.30%) and *C. pneumoniae* (n = 1, 0.10%) (Figure 1A). Eight sH1N1 cases were detected between December 2008 and May 2009, coinciding with the onset of the pdmH1N1 epidemic and the first detection of pdmH1N1 in our dataset in June 2009 (Figure 1B). Subsequently, pdmH1N1 became the most prevalent influenza subtype from July 2009 to April 2010. Between June 2011 and August 2012, H3N2 and influenza B viruses were dominant. RV/EV was detected at varying frequencies throughout the study period (Figure 1C). The order of frequency of detection of coronaviruses was OC43 > 229E > NL63 > HKU1 (Figure 1D), while that of PIV types was PIV3 > PIV1 > PIV2 > PIV4 (Figure 1E). Interestingly, HMPV (n = 28, 2.8%) was detected more often than RSVA (n = 9, 0.90%) and RSVB (n = 10, 1.0%) combined (Figure 1F), while AdV, BoV and *C. pneumoniae* were only detected sporadically (Figure 1G). Of the 22 coinfections, RV/EV (n = 11, 50.0%) and pdmH1N1 (n = 9, 40.9%) were the most frequently co-detected viruses (Figure 2). Four combinations of two pathogens occurred more than once, and RV/EV was found in three out of these four combinations (RV/EV-pdmH1N1, n = 3; RV/EV-H3N2, n = 2; RV/EV-PIV3, n = 2; and pdmH1N1-OC43, n = 2). All pathogens except HKU1, PIV4, sH1N1 and *C. pneumoniae* were detected as part of a coinfection and two out of three BoV detections occurred as part of a coinfection (BoV-PIV2 and BoV-pdmH1N1).

The general demographics and clinical parameters of this cohort were described previously (Tun et al., 2016) and hence we report pathogen-specific clinical and demographic characteristics here. Participants reported the following symptoms in addition to fever: ILI symptoms (74.4%), headache (70.3%), myalgia (64.2%), loss of appetite (56.1%), drowsiness (44.2%), nausea (25.4%), red eyes (23.5%), vomiting (7.8%), diarrhea (6.8%), and rash (3.5%). Patients with a positive nasal swab sample were more likely to report ILI symptoms ($p < 0.001$), whereas rash was associated with sample negativity ($p < 0.001$, Table 1). Infection with influenza viruses was associated with a significantly higher frequency of headache ($p = 0.019$), loss of appetite ($p = 0.025$), nausea ($p < 0.001$) and vomiting ($p < 0.031$) in patients. There was no significant difference between virus groups in terms of individuals living in the same household that recently fell ill (household sick contacts) ($p = 0.058$) or frequency of ILI symptoms ($p = 0.96$).

Participants infected with pdmH1N1 or AdV were relatively younger with a mean age of 34.5 (range 17–80) years and 29.4 (range 18–66) years respectively, while individuals with H3N2 or RSV/HMPV detections were relatively older with a mean age of 42.1 (17–83) years and 41.4 (19–74) years respectively. The mean age in the entire cohort was 36.6 years (range 17–84, Figure 3A). Participants testing positive had a higher mean body temperature and pulse rate compared to participants

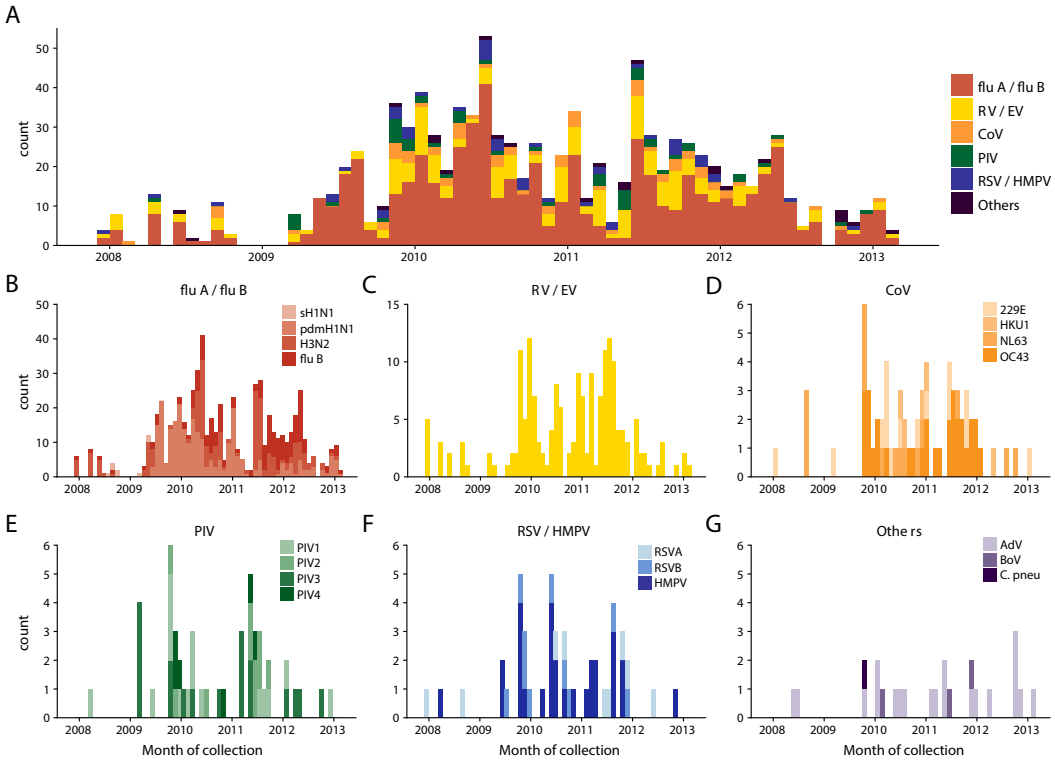


**Figure 1.** Overall detections of respiratory pathogens in febrile participants in Singapore from December 2007 to February 2013. Overview of all respiratory pathogens detected (A); influenza A viruses sH1N1, pdmH1N1, H3N2 and influenza B virus (B); rhino/enteroviruses (C); seasonal coronaviruses 229E, HKU1, NL63, OC43 (D); parainfluenza viruses 1, 2, 3, 4 (E); RSVA, RSVB and HMPV (F); adenovirus, bocavirus and *C. pneumoniae* (G).

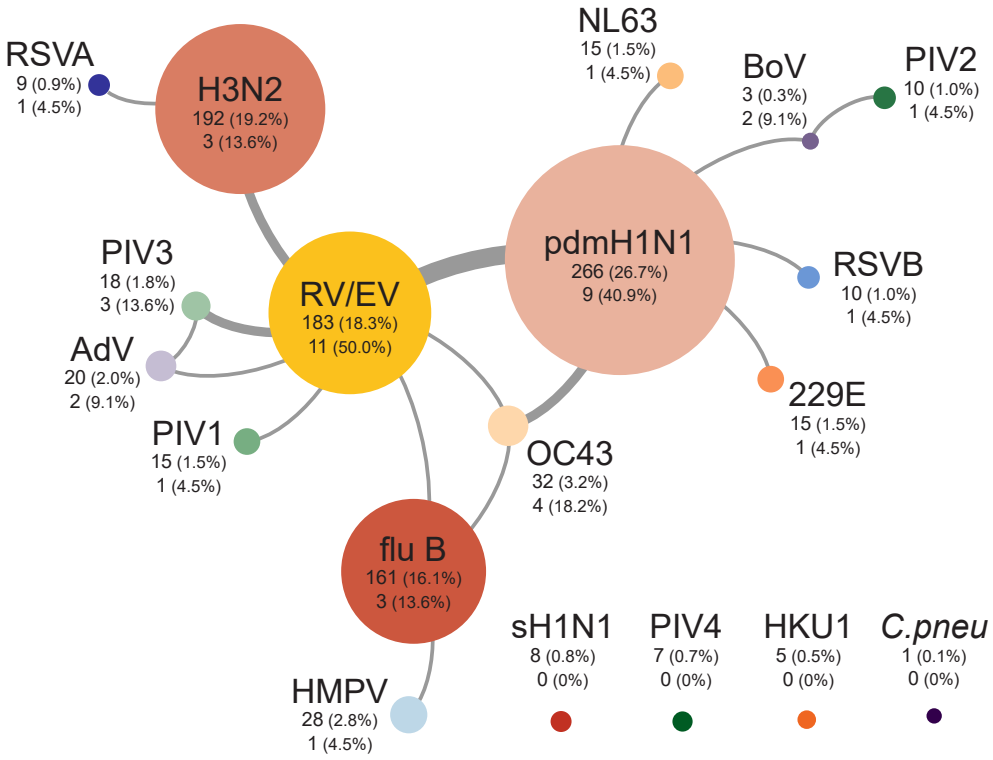


**Figure 2.** Schematic representation of coinfections. Numbers indicated denote the pathogen detection count and their percentage of total positives as well as the number of coinfections comprising two pathogens and their percentage of all coinfections. The size of the circles proportionally represents the total number of detections for each pathogen. The weight of the connecting lines indicates the frequency of detection of a particular pathogen combination (fine, n = 1; intermediate, n = 2; bold, n = 3).

who tested negative (Figure 3B and C). Participants with pdmH1N1 and AdV infection recorded the highest mean body temperatures (38.6 °C), followed by individuals infected with H3N2 and influenza B virus (38.4 °C) (Figure 3B). RV/EV positive cases displayed an increase in platelet counts, while participants with no evidence of a respiratory infection had higher total white blood cell and lymphocyte counts (Figure 3D–F). Cases of AdV and RV/EV infection were associated with significantly higher white blood cell and lymphocyte counts compared to participants testing

**Table 1.** Summary of demographics and presenting symptoms of enrolled participants.

| | negative, n (%) (N = 1081) | positive, n (%) (N = 976) | p-value |
|---|---|---|---|
| **Demographics** | | | |
| Gender, male | 718 (66.4%) | 616 (63.1%) | |
| Household sick contacts | 156 (14.4%) | 165 (16.9%) | |
| **Presenting symptom** | | | |
| Drowsiness | 486 (45.0%) | 424 (43.4%) | |
| Headache | 761 (70.5%) | 684 (70.1%) | |
| Myalgia | 705 (65.2%) | 616 (63.1%) | |
| Loss of appetite | 615 (56.9%) | 539 (55.2%) | |
| Diarrhea | 87 (8.0%) | 52 (5.3%) | 0.018 |
| Nausea | 295 (27.3%) | 248 (25.4%) | |
| Red eyes | 233 (21.6%) | 250 (25.6%) | 0.034 |
| Vomiting | 92 (8.5%) | 69 (7.1%) | |
| Rash | 65 (6.0%) | 7 (0.7%) | <0.001 |
| ILI symptoms | 659 (61.0%) | 872 (89.3%) | <0.001 |

P-values denote statistically significant differences ($p < 0.05$) for a particular parameter between participants testing either positive or negative.

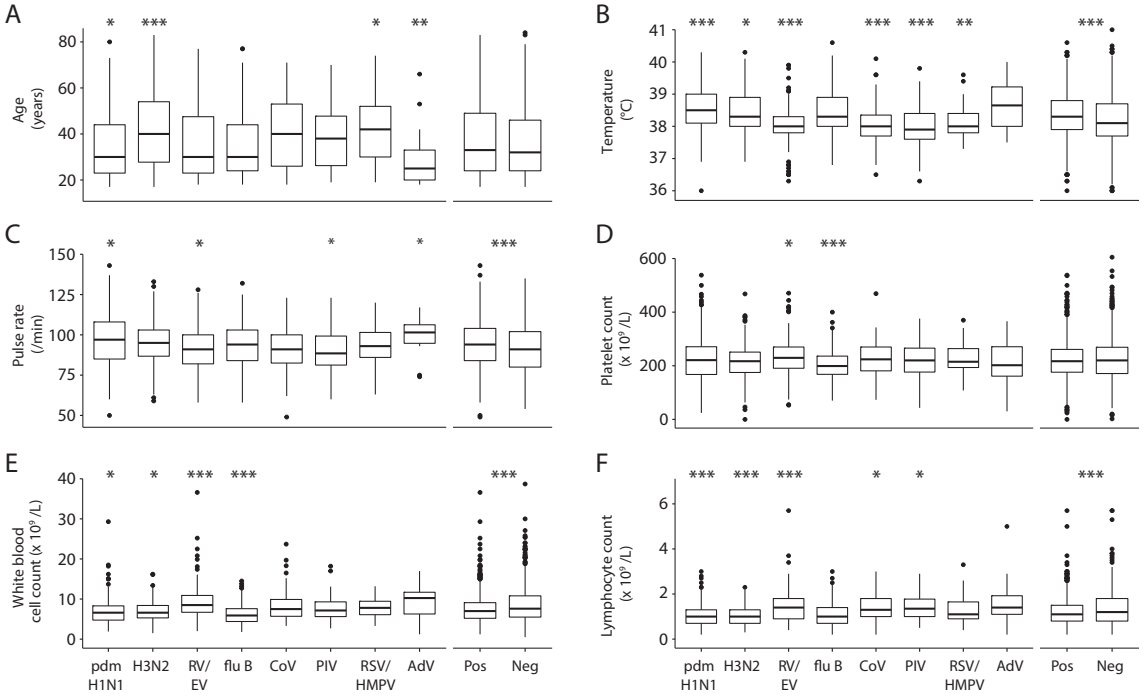


**Figure 3.** Age, vital signs and biochemical blood parameters of participants testing positive for respiratory viruses. Parameters with mean values that differ significantly ($p < 0.05$) between virus groups are summarized; age (A), body temperature (B), pulse rate (C), platelet count (D), white blood cell count (E) and lymphocyte count (F). Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) denote significant differences between participants testing positive and negative as well as virus groups that differ significantly in their mean value compared to all positive samples.

positive for other pathogens (Figure 3E and F). Lymphocyte counts were significantly lower in patients with dual detections of pathogens compared to those with a single detection (Figure 4A). We observed mean lymphocyte counts of 2.1, 2.1, 1.5, and 1.1 $\times$ $10^9$/L in patients infected with the combinations PIV3-AdV, RV/EV-PIV1, RV/EV-PIV3, and RV/EV-pH1N1 respectively. Patients infected with all other combinations of pathogens detected in our study had mean lymphocyte counts falling below the lower limit of normal (1 $\times$ $10^9$/L) (Figure 4B).

Out of the 2057 individuals initially recruited, 884 patients (43.0%) returned for a second visit and an additional swab sample was analyzed. The median time between first and second visits was 3 days (range 1–9 days) and the same pathogen was detected at both visits in 42.6% (165/387) of participants with a positive first visit test result.

## 4. Discussion

Prior studies on the prevalence of respiratory pathogens have mainly focused on high risk groups such as infants and hospitalized patients or special populations, such as military recruits (Jiang et al., 2017; Lau et al., 2018; Tan et al., 2014; Yeo et al., 2018). This study provides insights into the circulation of respiratory pathogens in febrile adults who sought medical attention at one out of five primary healthcare clinics in Singapore from December 2007 to February 2013. Of all participants, 48.4% (976/2057) tested positive for a respiratory pathogen in the NxTAG respiratory pathogen panel, and the most commonly detected viruses comprised pdmH1N1, H3N2, RV/EV, influenza B virus and CoV.

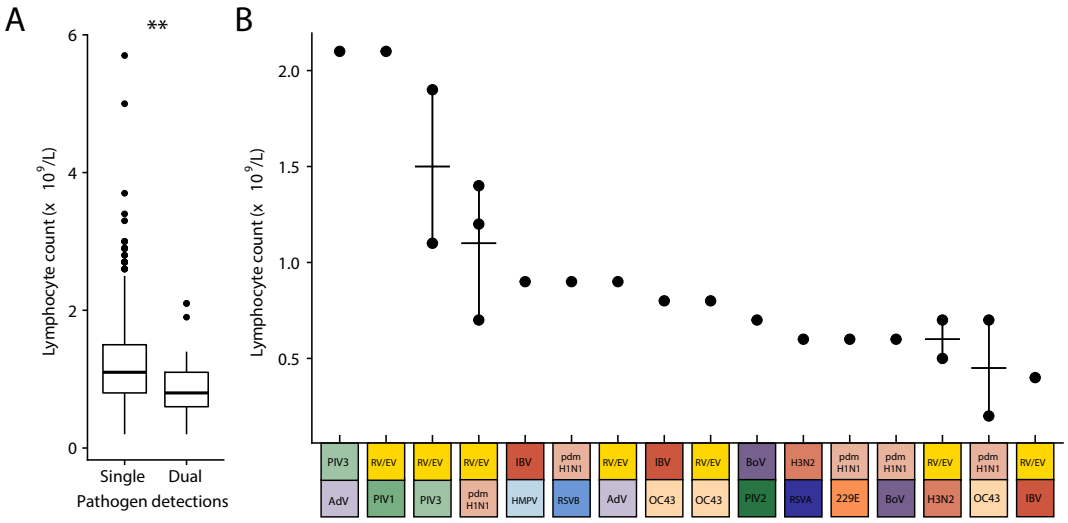


**Figure 4.** Lymphocyte counts of patients with single and dual infections are represented by boxplots and outliers by dots (A). Asterisks indicate a significant difference ($p < 0.01$). Lymphocyte counts of patients and individual pathogen combinations are reported in descending order (B). Where there is more than a single value observed, the mean is indicated by a horizontal line and individual values as dots.

Influenza viruses accounted for 62.8% of the total number of positive detections, reflecting the sizeable contribution of influenza viruses to the total number of febrile URTI (Wu et al., 2017). Our study period coincided with the start of the swine-origin H1N1 pandemic, and the first patient testing positive for pdmH1N1 in Singapore reported on May 26$^{th}$ 2009 (Liang et al., 2009). The last case of sH1N1 in our study was detected on May 27$^{th}$ 2009 and the first pdmH1N1 on June 27$^{th}$ 2009. The transition in virus detection from pdmH1N1 to H3N2 and influenza B viruses in the 2011–2012 influenza season likely reflects increased herd immunity to pdmH1N1 as a consequence of infection or immunization (Su et al., 2015). Furthermore, lower H3N2 and influenza B virus vaccine effectiveness relative to that for pdmH1N1 has been reported in these years (Ohmit et al., 2014), while the effect on community-dwelling adults in Singapore may be limited since rates of seasonal influenza vaccine uptake are low (Ang et al., 2017).

Fever and cough were previously described to be strong predictors of infection with influenza viruses (Monto et al., 2000; Vuichard-Gysin et al., 2019). We found similar frequencies of detections of influenza viruses in both the entire study cohort and the subset of ILI patients (62.8% vs 65.0%), indicating that influenza viruses are the main causative agents of ILI, but ILI symptoms are not fully predictive for influenza virus infection. RV/EV was found in 18.3% of the positive detections in our study, but in 50.0% of all coinfections, suggesting that these viruses preferentially infect as a secondary pathogen. Rhinoviruses are the most common etiological agent of the 'common cold' (Waman et al., 2014). We have previously described the serotypes of rhino- and enteroviruses in the same cohort and found a predominance of rhinovirus A, and lower numbers of rhinovirus C and B (Linster et al., 2020). Enteroviruses C and D were only sporadically detected. In another study of multiple respiratory virus infections in hospitalized patients, rhinoviruses were the most common virus seen in coinfections, though coinfections occurred more frequently in children than in adults (Goka et al., 2015). Participants testing positive for RV/EV had increased platelet counts, although not amounting to thrombocytosis. Furthermore, patients whose samples tested negative had higher white blood cell and lymphocyte counts, possibly indicating bacterial infections that were not diagnosed here. The observed lymphocytopenia in the majority of patients with dual infections suggests an additive immunosuppressive effect of the two pathogens.

Our findings affirm that CoV is an important cause of ILI in adults in Singapore, in line with a previous report (Hong et al., 2004). We also detected PIV and pneumoviruses that generally cause mild, self-limiting ILI in otherwise healthy adults, but may cause severe lower respiratory tract illness and pneumonia in infants and the elderly (Shi et al., 2017, 2019). In contrast to earlier findings in a community adult cohort in Singapore (Jiang et al., 2017) we detected more HMPV than RSV infections (28 and 19 detections, respectively). In addition, we report the detection of human bocavirus in community-ambulant adults in Singapore, with earlier accounts exclusively from hospitalized patients (Chan et al., 2018; Tan et al., 2009a).

Potential limitations of our study design include the predetermined set of respiratory pathogens in the test panel and the absence of additional diagnostics for bacterial pathogens that commonly cause URTI such as Group A *Streptococcus*, *S. pneumoniae,* and *H. influenzae* (Dasaraju and Liu, 1996). Furthermore, we exclusively enrolled febrile participants at primary care clinics, while several other forms of general practice are common in Singapore (Ministry of Health Singapore, 2014). Moreover, the number of recruited participants and sample collection fluctuated during the study period and afebrile patients were excluded.

In conclusion, a diverse array of respiratory viruses is associated with URTI symptoms in adults presenting at primary care settings in Singapore. Pandemic H1N1 influenza virus is the most frequently detected respiratory pathogen and thus measures should be taken to increase seasonal influenza vaccine uptake. Several other respiratory viruses, such as RV/EV, CoV, PIV, and RSV/HMPV, are commonly linked to infection of vulnerable populations including children and the elderly. We here demonstrate that these viruses cause a substantial proportion of URTI in the general adult population and thus warrant further research into specific infection control measures.

## Declarations

### *Author contribution statement*

Yihui Chen: Performed the experiments; Wrote the paper.

Marcus G. Mah: Performed the experiments.

Jenny G. H. Low, Eng Eong Ooi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Yvonne C.F. Su: Conceived and designed the experiments; Analyzed and interpreted the data.

Mahesh Moorthy: Performed the experiments; Analyzed and interpreted the data.

Gavin J.D. Smith: Conceived and designed the experiments; Wrote the paper.

Martin Linster: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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*Data availability statement*

Data included in article/supp. material/referenced in article.

*Competing interest statement*

The authors declare no conflict of interest.

*Additional information*

No additional information is available for this paper.

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