

Research Note

Phylogenetic analysis of human metapneumovirus among children with acute respiratory infections in Kuala Lumpur, Malaysia

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Abstract. Human metapneumovirus (HMPV) is a recently discovered cause of viral respiratory infections. We describe clinical and molecular epidemiology of HMPV cases diagnosed in children with respiratory infection at University of Malaya Medical Centre, Kuala Lumpur, Malaysia. The prevalence rate of HMPV between 2010 and 2012 was 1.1%, and HMPV contributed 6.5% of confirmed viral respiratory infections. The HMPV patients had a median age of 1.6 years, and a median hospital admission of 4 days. The most common clinical presentations were fever, rhinitis, pneumonia, vomiting/diarrhoea, and bronchiolitis. Based on the partial sequences of F fusion gene from 26 HMPV strains, 14 (54%) were subgenotype A2b, which was predominant in 2010; 11 (42%) were subgenotype B1, which was predominant in 2012; and 1 (4%) was subgenotype A2a. Knowledge of the circulating subgenotypes in Malaysia, and the displacement of predominant subgenotypes within 3 years, is useful data for future vaccine planning.

Human metapneumovirus (HMPV), a newly discovered paramyxovirus, was first identified in 2001 from nasopharyngeal aspirates obtained from young children in the Netherlands (van den Hoogen *et al.*, 2001). Since then, infections have been reported worldwide (Garcia *et al.*, 2012; Lamson *et al.*, 2012). It causes a wide range of clinical manifestations ranging from coryza, and bronchiolitis to pneumonia. The virus is approximately 13 kb and divided into eight gene segments, namely N, P, M, F, M2, SH, G and L. There are two major lineages of HMPV, genotypes A and B, based on sequence variations of the fusion (F) and glycoprotein (G) genes. Each genotype has two distinct subgenotypes, A1 and A2, and B1 and B2, and

subgenotype A2 can be further divided into A2a and A2b (Yang *et al.*, 2009; Papenburg *et al.*, 2013). Understanding of the clinical characteristics and epidemiology of HMPV is important for the development of HMPV interventions such as vaccines. No data about HMPV infection is available in Malaysia. In this study, we characterized the clinical manifestations and genotypic variation of HMPV detected at University Malaya Medical Centre, a tertiary hospital located in Kuala Lumpur, Malaysia.

All nasopharyngeal aspirates and bronchial washings received from children presenting with acute respiratory infection are routinely screened for respiratory syncytial virus (RSV), adenovirus, influenza

virus, and parainfluenza virus by direct fluorescent-antibody staining (Light Diagnostics, Germany) and viral culture. Between April 2010 to December 2012 (except April and May 2011), samples were also screened for HMPV by direct fluorescent-antibody staining (Light Diagnostics or Diagnostic Hybrids, USA), and cultured in LLC-MK2 cells (ATCC CCL-7) and R-Mix ReadyCells (Diagnostic Hybrids, USA). Nucleic acid was extracted from 140 μ L of the HMPV-positive respiratory specimens or virus cultures with the QIAamp viral RNA kit (Qiagen, Germany). RT-PCR was performed using SuperScript III One-Step RT-PCR System (Invitrogen, USA) using primers for the F gene (nucleotide positions 3691-4435 of isolate NL/1/94, GenBank accession no. FJ168778), BF100 5'-CAATGCAGGTATAACACCAGC AATATC-3' and BF101 5'-GCAACAATTGAA CTGATCTTCAGGAAAC-3' (van den Hoogen *et al.*, 2004). Thermocycling was performed under the following conditions: 50°C for 30 min, 95°C for 3 min, 38 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 2 min, and finally 72°C for 7 min. Amplified gene fragments were purified and sequenced in both directions using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA) with a 3730xl DNA Analyzer (Applied Biosystems). Sequences were edited and aligned using Geneious v6 (Biomatters, New Zealand). Sequences were deposited into GenBank with accession numbers KJ196300-KJ1963025. These F gene sequences of 563 bp were compared to those available complete genomes of HMPV and also those from neighbouring Asian countries including Singapore, Thailand, Vietnam, Cambodia, Taiwan, South Korea, Japan and China.

The best substitution model was determined using jModelTest v0.1.1 (Posada, 2008) as the transitional model 2 with equal base frequencies and rate variation among sites (TIM2ef+G). Phylogenetic trees were constructed using the Bayesian Markov Chain Monte Carlo method implemented in BEAST 1.7.4 (Drummond & Rambaut, 2007), run for 30 million generations with a 10% burn-in. All runs reached convergence with estimated sample sizes of >200. The tree prior was coalescent GMRF Bayesian

Skyride and the clock model was uncorrelated lognormal relaxed. The maximum clade credibility tree (Figure 1) was viewed using FigTree 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Throughout the study, 3258 non-duplicate samples were tested and 565 (17.3%) were positive for respiratory viruses by immunofluorescence and/or culture. There were 37 (1.1%) samples positive for HMPV. The HMPV patients were aged between 4 months and 15 years, with a median of 1.6 years and a male:female ratio of 1.8:1. Case notes were available for 31 (83.8%) patients, and reviewed following approval by the hospital's Medical Ethics Committee (reference no. 788.3). All 31 cases were community-acquired. Eight (25.8%) children had underlying medical conditions, including five with prematurity (including two with chronic lung disease, and one each with cerebral palsy and Down's syndrome), and three with asthma. The median length of stay for the 28 admitted to hospital was four days (range, 1 to 64 days). The most common presentations were fever (96.8%), rhinitis (58.1%), pneumonia (51.6%), vomiting/diarrhoea (45.2%), bronchiolitis (32.3%), and pharyngitis (19.4%). A total of 24 (77.4%) patients were treated with antibiotics. None of the patients required intensive care or died.

A total of 26 HMPV samples were successfully sequenced. Phylogenetic analysis of the partial F gene showed that the Malaysian isolates clustered mainly into the two subgenotypes, A2b (54%) and B1 (42%) (Figure 1). A2b was strongly predominant in 2010, making up 11/14 (78.6%) of the sequenced strains, while B1 predominated in 2012, comprising 9/11 (81.8%) of strains. Only one isolate from 2011 was clustered in subgenotype A2a. No HMPV-A1 and HMPV-B2 subtypes were detected in this study. Malaysian isolates in the B1 subgenotype were clustered with isolates from Thailand, Cambodia and Vietnam. Malaysian isolates from the A2b subgenotype were clustered with China, Cambodia and Vietnam isolates. Our finding of co-circulation of subgenotypes in the population, with replacement of predominant subgenotypes within 3 years, has also been noted in other studies

(Schildgen *et al.*, 2011). In a Singapore study, only subgenotype A2 was found (Loo *et al.*, 2007), while in Kolkata, India from 2006-2009, subgenotype A2 predominated with low circulation of subgenotype B1 (Agrawal *et al.*, 2011). A2b was also dominant in southwest China in 2008-2011 (Zhang *et al.*, 2012); however, in Cambodia from 2007-2009, subgenotype B2 predominated with low circulation of subgenotype A2b (Arnott *et al.*, 2013). These studies showed that the circulation of specific subgenotypes was geographically restricted, and was not similar over the Asian region.

The nucleotide similarity amongst our Malaysian A2b and B1 isolates were between 96-100% and 98-100%, respectively. The nucleotide similarity between Malaysian A2b and B1 isolates was only 83 to 84%. Five amino acid substitutions were observed in the sequenced region (Table 1). These amino acid substitutions were genotype-specific mutations also found in other isolates of the same genotype (Yang *et al.*, 2009).

Our study highlights HMPV as an important although relatively infrequent cause of respiratory virus infection in hospitalised children in Malaysia, with prematurity and asthma as the commonest predisposing conditions. HMPV made up 6.5% of the 565 confirmed respiratory virus cases during the study period, compared to RSV (58.2%), adenovirus (12.2%), influenza A and B (11.3%), and parainfluenza viruses (9.4%). The overall HMPV detection rate in children with respiratory infections at our hospital was 1.1%, while the HMPV prevalence rates reported by other countries vary from 0.4-12.7% (Garcia *et al.*, 2012; Qaisy *et al.*, 2012). However, as this study used relatively

insensitive conventional detection methods, the numbers of HMPV infections will likely increase if more sensitive molecular methods are used. The differences in circulating subgenotypes from those in neighbouring countries, and the displacement of different genotypes every few years show some similarities to RSV, a related pneumovirus (Khor *et al.*, 2013). Understanding the clinical and molecular epidemiology of HMPV in different countries will be important for the development and subsequent deployment of vaccines. Further studies of emerging respiratory viruses in Malaysia, including HMPV, are currently underway at our centre.

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Table 1. Comparison of amino acid substitutions in Malaysian human metapneumoviruses

Amino acid position in F protein	Subgenotype		
	A2a	A2b	B1
287	V	V	I
297	K	K	N
313	Q	Q	K
349	K	K	R
405	N	N	P

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