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Research Fund for the Control of Infectious Diseases

Research Dissemination Reports

衛生及醫護服務研究基金
控制傳染病研究基金

研究成果報告

Respiratory infectious diseases
呼吸道傳染病

Antiviral development and therapy
抗病毒藥物的發展和治療

Environment and health
環境與健康

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Health and Health Services Research Fund

Research Fund for the Control of Infectious Diseases

Research Dissemination Reports

Environment and Health

Dissemination reports are concise informative reports of health-related research supported by funds administered by the Food and Health Bureau, namely the *Research Fund for the Control of Infectious Diseases* (RFCID) and the *Health and Health Services Research Fund* (HHSRF). In this edition, 11 dissemination reports of projects related to antiviral development and therapy, environment and health, and respiratory infectious diseases are presented. In particular, three projects are highlighted due to their potentially significant findings, impact on healthcare delivery and practice, and/or contribution to health policy formulation in Hong Kong.

Highly pathogenic avian influenza H5N1 in wild birds and poultry raises concern about zoonotic transmission and subsequent pandemic. The severity of H5N1-induced lung pathology may be due to increased viral replication and/or inflammatory responses (cytokine cascade). In contrast, deficiency of cyclooxygenase-2 (COX-2) in the infected host results in less severe disease. Lee et al[1] hypothesised that hyperinduction of COX-2 plays an important role in the pathogenesis of H5N1 virus infection. They found that the cytokine cascade was maintained even in the absence of significant virus infection in the lung. This has potential health implications as, in addition to antiviral therapy, interventions to selectively modulate the cytokine cascade (eg with COX-2 pathway inhibitors) may be helpful. Such an approach may be more beneficial than attenuating the action of a single cytokine such as TNF-α using direct antagonists. COX-2 inhibitors are either already registered for clinical use or undergoing late phase clinical trials, and may also have the added benefit of inhibiting viral replication.

The number of people aged 65 years or older in Hong Kong is growing rapidly. Regular engagement in physical activity contributes to healthy ageing. Walking is recommended for seniors because it is versatile, affordable, and relatively safe. The accrual of 30 minutes of walking per day has significant health benefits. However, the neighbourhood environment often plays a decisive role in facilitating residents' walking habits. Knowledge of built environmental characteristics conducive to an active lifestyle can inform policies on public health, land use, and transportation. Cerin et al[2] aimed to develop and validate instruments for investigating the associations between the built environment and walking among senior residents of Hong Kong and to provide effects of perceived attributes of the neighbourhood on walking for different purposes. A total of 484 Hong Kong seniors participated in validation of an instrument designed to evaluate the neighbourhood environment. Although most correlates of walking in older adults were similar to those observed elsewhere and in younger cohorts, neighbourhood attributes that were peculiar to Hong Kong (ie access to residential entrances, lifts in high-rise buildings) and seniors (facilities for sitting and rest) were identified.

Asthma is the most common chronic respiratory disorder in childhood, affecting about 10% of Hong Kong children. Asthma exacerbations commonly result in hospitalisation, which accounts for a major fraction of the total cost of asthma care. Increasing evidence supports the importance of respiratory infections in asthma exacerbations. Leung et al[3] conducted a case control study in 209 children with asthma exacerbations in order to understand the roles of respiratory pathogens in precipitating asthmatic attacks. They found that respiratory viral infections were significantly associated with asthma exacerbations in Hong Kong children, particularly human rhinovirus (HRV). This observation was consistent with other studies that identified HRV as the most important viral aetiology of childhood asthma. Previous studies have shown that *M pneumoniae* and *C pneumoniae* are common pathogens associated with asthma exacerbations and that telithromycin is beneficial for treatment. However, in this study, *M pneumoniae* and *C pneumoniae* were rarely detected in respiratory secretions from children with asthma exacerbations or patients with chronic stable asthma. These results do not support the usefulness of macrolide treatment for asthma exacerbations. Despite the suggestive results, the study was somewhat underpowered and larger studies are required to delineate the relationship between asthma exacerbations and other respiratory pathogens.

We hope you enjoy this selection of research dissemination reports. Electronic copies of these dissemination reports and the corresponding full reports can be downloaded individually from the Research Fund Secretariat website (http://www.fhb.gov.hk/grants). Researchers interested in funds administered by the Food and Health Bureau may also visit the website for detailed information about application procedures.

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Statistical algorithms for early detection of the annual influenza peak season in Hong Kong using sentinel surveil ance datal

Key Message

In Hong Kong, influenza sentinel surveillance systems have been recently established. Methods that compare current data to data from recent weeks may be appropriate to indicate the start of peak influenza activity. These methods can produce reliable and timely alerts at the start of the annual influenza peak season.

Introduction

Influenza surveillance has been conducted in many countries. In Hong Kong, a network of sentinel general practitioners provides weekly data on community influenza-like illness (ILI) rates. The second source of surveillance data is from influenza virus isolations in samples submitted to the local public health laboratory.

Given their timeliness, sentinel surveillance data facilitate early warning at the start of the annual period of peak influenza activity ('peak season'). However, given strong year-to-year variations, a simple fixed threshold is unlikely to produce useful alarms. In countries with long-standing sentinel surveillance networks, alarms are typically generated when current ILI rates surpass the 'normal' range of ILI rates in past years prior to peak seasons. In Hong Kong, sentinel surveillance has just been established, and thus historical methods are less applicable. We therefore evaluated three alternative statistical methods for generating alarms. Each method involves comparison of current rates to the 'normal' range of recently encountered rates.

Methods

This study was conducted from January to December 2006. In Hong Kong, a network of sentinel general practitioners provides influenza surveillance data on a weekly basis. At the end of each week, the sentinel practitioners report the number of consultations of patients complaining of ILI symptoms (fever plus cough or sore throat) out of the total number of consultations. Data are collated and analysed by the following Wednesday or Thursday, thus the reporting delay is approximately one week. The data are retrieved from Hong Kong island, Kowloon, New Territories East, and New Territories West, and then aggregated. Influenza virus isolated in samples obtained primarily from hospitals and sent to the Government Virus Unit of the Department of Health serves as a complementary source of surveillance data.

Three statistical algorithms (a time series method, a Shewhart control chart, and a cumulative sum method) on the aggregated sentinel surveillance data were compared. The performance of the time series on sentinel data from the separate geographical areas was then evaluated. Three algorithms were compared using the metrics of sensitivity, specificity, and timeliness, as well as a composite metric (range, 0-1) based on all three metrics, known as the volume under the time–receiver operating characteristic surface (VUTROCS).[1] The laboratory data were used to provide a 'gold standard' estimate of the start of the peak season each year.

Results

In the Hong Kong setting, the time series method was found to be optimal (best VUTROCS = 0.82 and 0.90 for general practitioner and general out-patient clinic

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data, respectively), whereas the control chart method had slightly inferior performance. In the analysis of sentinel reports from separate geographical areas, as a means of detecting peak influenza activity, data from a particular geographical area could be even more useful than those from the whole territory. Moreover, combining data streams from different areas may further improve performance.[2]

Conclusions

For influenza surveillance, methods that compare current data to data from recent weeks can generate sensitive, specific, and timely alerts at the start of peak influenza activity, and may be an alternative to comparing current data to historical data. These methods could be useful in Hong Kong, as there is not a long historical series of sentinel surveillance data to implement the historical method.

Acknowledgements

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Viral evolution from one generation of human influenza infection to the next

Key Messages

1. In a sub-tropical epidemic, most of the apparent household secondary cases are actually secondary infections.
2. The consensus sequence for the entire influenza virus genome is not usually identical within the same household sample. Rather, there are commonly one or two nucleotide changes.
3. These results hint at an obvious generational threshold for adaptation at the level of the consensus sequence.

Introduction

Phylodynamics describes the complex interaction between the evolution of a pathogen and its transmission dynamics between hosts as a single system.[1] Influenza presents unique patterns of evolution and infection. Explicit theoretical models have been used to explain the two time scales of antigenic drift and antigenic shift of human influenza infection.[2,3] In addition, implicit phylodynamic models are often used to interpret the results of phylogenetic analysis of influenza. For example, the apparent exchange of internal genes in human influenza A samples taken from New York between 1999 and 2004 suggests that co-infection and subsequent recombination in humans is more common than previously thought.[4] The scales at which phylodynamic models (both implicit and explicit) can be applied to uncover fundamental interactions between a pathogen and its host depend on the resolution of available data. Sequence data from only the neuraminidase gene for many samples over 30 years were used to infer the presence of a short-term, non-specific immune response generated by one influenza strain against all influenza strains.[3] The entire genomes of 156 samples taken over 2 years were used to describe frequent recombination.[4] The robust statistical incorporation of pathogen evolution dynamics into more detailed transmission models requires more finely resolved genetic data than has been used to date.

We suggest that there exists an intuitive fundamental unit for phylodynamic analysis of human influenza infection, namely, the expected evolutionary distance between virions isolated from a typical infector and infectee. This generational unit of viral evolution can be used in academic and public health investigations of infectious disease outbreaks. Laboratory techniques for isolation and sequencing of viruses continue to improve. Therefore, evolutionary data on viral infections may be incorporated into routine epidemiological analysis in the same way as for bacterial infections. However, strains captured by global surveillance systems are not useful for the study of smaller-scale patterns of influenza transmission and evolution. Most strains captured by surveillance systems come from clinical settings. Because many influenza infections are either mild or entirely asymptomatic, very few sequential infections are captured. However, viral samples obtained from intensive household-based transmission studies enable investigation of smaller-scale evolutionary patterns of influenza in humans. We present the full genome sequences of viral samples from such a study. We compare the distribution of genetic distances between isolates from the same household and isolates from different households. Also, we use the dates of isolation of samples to construct a timed evolutionary tree, with which the 2007 influenza A H3N2 season was compared with a set of global samples.

Methods

This study was conducted from January to December 2009. Viral samples from households with apparent transmission during a trial of non-pharmaceutical interventions were used.[5] For the intervention study, individuals who were at least 2 years old, exhibited two or more symptoms of influenza-like illness (ILI), and were living in a household with at least two other individuals who had not reported ILI symptoms in the previous 2 weeks were recruited at clinics.

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Participants were tested with the QuickVue Influenza A + B rapid test. If the test was positive, they and their household members were followed up immediately (same or next day) and then on the 3rd, 6th, and 9th days. Nose and throat swabs were taken at each household visit, and each swab was cultured for influenza A and B. Standard cultures were obtained from all swabs.

Viral RNA was extracted directly from cell culture using the QIAamp viral RNA minikit (Qiagen Inc). Complementary DNA was synthesised by reverse transcription reaction with gene amplification performed by polymerase chain reaction (PCR) using specific primers for each gene segment. The PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc) and sequenced by synthetic oligonucleotides. Following the manufacturer's instructions, reactions were performed with a Big Dye-Terminator v3.1 Cycle Sequencing Reaction Kit on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems). All sequences were assembled and edited with Lasergene version 8.0 (DNASTAR). Phylogenetic trees were inferred using the neighbour-joining distance method, with genetic distances calculated by total distance. Temporal phylogenies and rates of evolution were inferred using a relaxed molecular clock model that allows rates to vary among lineages within a Bayesian Markov chain Monte Carlo framework.[6] This was used to sample phylogenies and the dates of divergences between viruses from their joint posterior distribution, in which the sequences were constrained by their known date of sampling and a codon-position-specific HKY1C substitution model was used.

Results

A total of 79 samples from 38 individuals in 17 households were positive for either influenza A or B based on the rapid test (Table). Influenza B samples and samples from which successful cultures could not be obtained were excluded, as were all samples from household in which secondary transmission of influenza A H3N2 was not confirmed by culture. Therefore, the current study was based on the full genome sequences of 31 samples of influenza A H3N2 obtained from 23 individuals in 11 households during 2007.

To visualise total nucleotide differences between 13 apparent transmission events in 10 households, a total distance neighbour-joining tree was constructed (Fig 1a). The households from the Hong Kong non-pharmaceutical intervention study did not form a single monophyletic group. However, in all but one case, viruses sequenced from the same household clustered together. Household clusters were distributed throughout the tree, with most closely related isolates from North America and, in one instance, Taiwan.

The samples from the intervention study were grouped into three clades. Out of 35 possible pair-wise comparisons of full viral genomes within study households, 13 pairs were identical, 14 pairs differed by a single nucleotide, five pairs by two nucleotides, one pair by three nucleotides, and two pairs by 39 nucleotides (Fig 1b). The latter arose from household 111. Two samples from the index case were identical: one was taken during the baseline visit and the second during the first follow-up visit. However, a third virus isolated from a second household member during visit 2 differed by 39 nucleotides. In contrast, the most similar pair of viral isolates from different households in the intervention study differed by 30 nucleotides.

To make full use of dates of isolation and sequence data, a temporal phylogeny of the full genomes of all viruses in our sample is shown (Fig 2). The grouping of the Hong Kong samples into three clades was preserved. The most recent common ancestor for samples in household 111 was estimated with narrow confidence bounds to be ~1 month prior to the recruitment of the household into the study. The phylogenetic relationship between full genome sequences from a study of seasonality in Managua, Equador[5] contrast sharply with the samples from the Hong Kong intervention study; the Managua samples form a monophyletic group.

Conclusions

By obtaining full genetic sequences for 31 samples from 17 household outbreaks, the degree of adaptation that occurs in that setting was quantified. Most pair-wise comparisons between consensus sequences showed three or fewer nucleotide changes. These results extend an earlier study in which the haemagglutinin genes were identical in all household outbreaks.[6]

The 17 household outbreaks appeared to be a result of between-household transmission, rather than within-household transmission. A single seed did not initiate the 2007 Hong Kong transmission season of H3N2 influenza. In contrast, the 2007 outbreak of influenza in Managua, Equador was very likely initiated by a single introduction. These patterns are consistent with Hong Kong acting as

Table. Properties of samples included

Properties of samples	Households	Individuals	Samples
Laboratory confirmed apparent secondary cases in the Hong Kong non-pharmaceutical intervention study[5]	17	38	79
Influenza B on culture	-3	-7	-13
All apparent secondary infections negative on culture	-3	-8	-20
Other negatives on culture	-0	-0	-13
Included	11	23	31

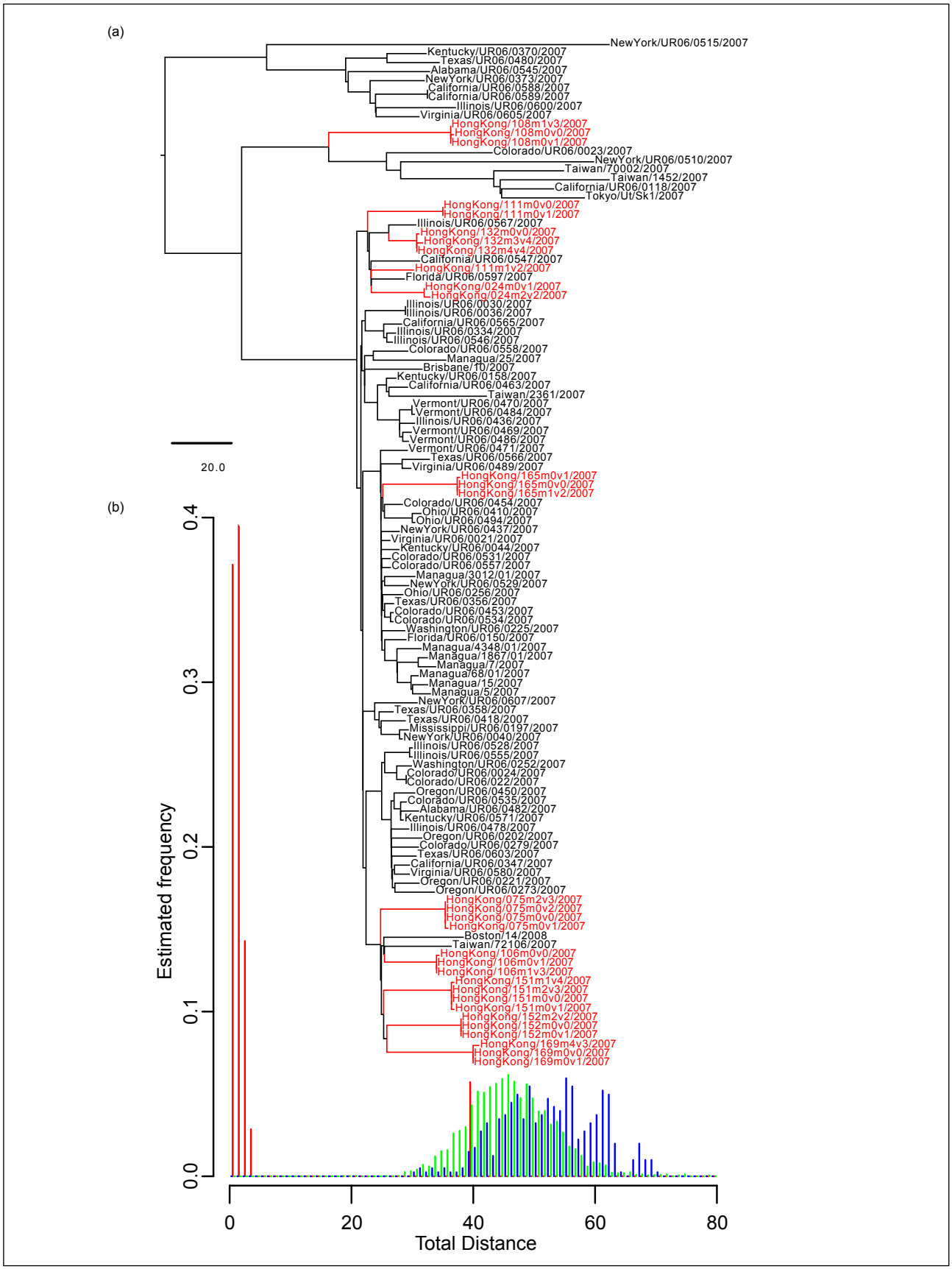

Fig 1. (a) Neighbour-joining total distance tree for 120 of the 153 genomes: the number of taxa are reduced to aid presentation, and all sequences from the Hong Kong study are included. (b) Distribution of pair-wise distances for three groups of samples: between isolates from the same household in the intervention study (red), between isolates from different households in the intervention study (blue), and between samples from the other 2007 samples in Genbank with full sequence and date of isolation (green).

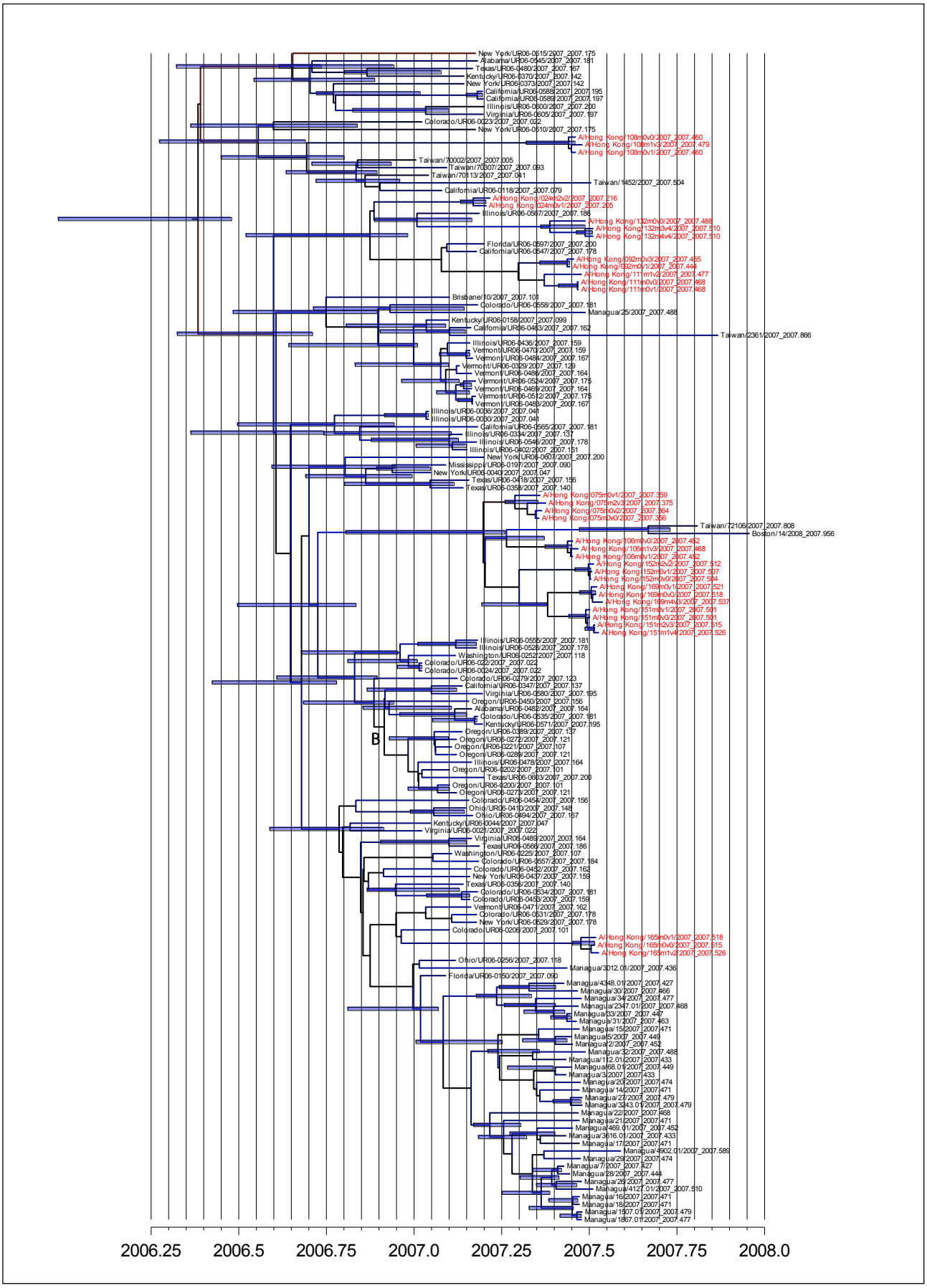

Fig 2. Dated phylogenetic tree of the full genome sequences for this study and all those full genomes for samples collected during 2007 (as available from NCBI).

a highly connected international hub, whereas the study population in Managua was far less connected. Notably, the study in Managua was not in any way intended to be representative of the entire city and was largely restricted to a number of smaller local communities. Thus, the phylogenetic patterns for full genome sequences from Hong Kong and Managua during 2007 are strikingly different, despite differences in recruitment strategies.

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Respiratory viruses and atypical bacteria triggering severe asthma exacerbation in childrenl

Key Messages

1. Respiratory viruses and atypical bacteria were detected in 51.0% of Hong Kong children with asthma exacerbations, which was significantly higher than the detection rate of 27.3% in children with chronic stable asthma.
2. Co-infections of two or more respiratory pathogens were more commonly found in children with asthma exacerbations (10.7%) than in patients with stable asthma (2.6%).
3. Human rhinovirus infection was a significant risk factor for asthma exacerbations.
4. There was no significant association between the severity of asthma exacerbations and respiratory viral or atypical bacterial infections.
5. Routine use of macrolide antibiotics in the treatment of childhood asthma exacerbations should be discouraged.

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Introduction

Asthma is the most common chronic respiratory disorder in childhood, affecting about 10% of Hong Kong children. Asthma exacerbations in children commonly result in hospitalisation, which accounts for a major fraction of the total cost of asthma care. Increasing evidence supports the importance of respiratory infections in asthma exacerbations. Prospective epidemiologic studies show that up to 80% of childhood asthma attacks are associated with viral upper respiratory infections of human rhinovirus (HRV), respiratory syncytial virus (RSV), adenovirus, human metapneumovirus (HMPV), and influenza viruses.[1] Beside respiratory viruses, atypical bacteria such as *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* have also been linked to asthma exacerbations in children. In 46 asthmatic patients seropositive for *C pneumoniae* treated with oral macrolides, asthma-related symptoms resolved or significantly improved in about half of them. *M pneumoniae* was found to be present in >50% of asthmatics. Therefore, understanding the roles of respiratory pathogens in precipitating asthmatic attacks may provide ways to reduce the health care burden associated with asthma-related hospitalisation.

Methods

This case-control study was conducted from November 2006 to April 2008. We investigated the epidemiology of respiratory viruses and atypical bacteria in respiratory secretions collected from children with asthma exacerbations (as cases) and those with chronic stable asthma (as controls). Associations between asthma exacerbations and presence of these organisms were analysed.

Patients aged 3 to 18 years with physician-diagnosed asthma and having exacerbations were recruited. Most of them were hospitalised for severe exacerbations, and assessed within 48 hours of hospitalisation. Asthmatic children with stable disease (controls) were recruited from our paediatric clinics. They had been free of symptoms of respiratory infection for ≥4 weeks. Patients who received antimicrobial agents in the 2 weeks before assessment were excluded. Patients' parents gave informed written consent, and the Joint CUHK-NTEC Clinical Research Ethics Committee approved this study.

The baseline characteristics, asthma status, and treatments of patients were recorded. The severity of asthma exacerbations was evaluated according to the Global Initiative for Asthma guideline. Patients in both groups underwent exhaled nitric oxide measurements followed by spirometry. Each patient with an acute asthma was reassessed 3 weeks after the episode to collect samples for microbiological studies.

In accordance with the Hospital Authority policy on Infection Control, nasopharyngeal aspirates were collected from each subject in negative-pressure isolation rooms. Deep nasal swabs were obtained in situations where such infection control facility was not available. These specimens were put in viral transport medium at 4 to 10ºC, and nucleic acids were extracted for molecular studies on the same day. In patients with asthma exacerbations, paired serum samples were also obtained, if clinically indicated, to determine antibody titres

to respiratory pathogens.

Both RNA and DNA in respiratory samples were extracted by PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Extracted RNA was converted to cDNA by reverse transcriptase, and all DNA and cDNA were used immediately for the five groups of multiplex nested PCR assays as described previously.[2] Twenty respiratory pathogens could be simultaneously detected by these PCR assays. Group 1 comprised influenza A and B group-specific and subtypes H1N1, H3N2, H5N1-specific primers; group 2 comprised parainfluenza viruses (PIV-1, PIV-2, PIV-3, PIV-4a and PIV-4b); group 3 comprised RSV A and B, HRV and enterovirus (EV); group 4 comprised human coronavirus (HCoV)-OC43, -229E and SARS-CoV and HMPV; and group 5 comprised *M pneumoniae, C pneumoniae*, human bocavirus, and adenovirus. Both the first and second rounds of PCR were conducted in 20-μl reaction mixtures using 'fast' thermal cycler (Applied Biosystems, Foster City [CA], USA). For the first round of PCR, 2 μL of the cDNA preparation was used as the template for groups 1 to 4, whereas 8 μL of the extracted preparation was used for group 5. In the second round of PCR, a 0.2-μL aliquot of the first-round PCR product was used as a template. Following PCR reactions, the products were prestained by SYBR Safe and visualised by electrophoresis in 1.5% agarose gels. Four corresponding positive controls and one negative control (sterile water) samples were included for each group simultaneously. The preparation of reagents, processing of samples, and nested PCR assays were performed in separate rooms away from the area where amplified products were analysed in order to prevent PCR contamination. Aerosol-resistant pipette tips were used throughout the experiments.

For serologic detection of respiratory infections, the titres of IgG antibodies to common respiratory viruses, *M pneumoniae* and *C pneumoniae* in paired serum samples were measured by complement fixation tests. Significant seroconversion was defined as having ≥4-fold change in antibody titre to the organism.

Results

A total of 209 children with asthma exacerbations (cases) and 77 children with stable asthma (controls) were recruited (Table 1). The patient age in the controls was significantly older (7.6±4.1 vs 11.1±4.5 years, P<0.001). This was mainly due to our inability to recruit one age-matched control for each participant with asthma exacerbation.

Sufficient respiratory samples were collected from 206 (98.6%) of the cases and all the controls. The samples consisted of 236 nasopharyngeal aspirates and 47 nasal

Table 1. Comparison of patients with asthma exacerbations and stable asthmatic controls

Clinical feature	Asthma exacerbation (n=209)	Stable asthma (n=77)
Mean±SD age (years)	7.6±4.1†	11.1±4.5
Male (% of patients)	68.4	75.3
Mean±SD hospitalisation (days)	3.6±1.9	-
Domestic tobacco smoke exposure (% of patients)	22.3†	13.0
Clinical status (% of patients)		
Fever	33.0	-
Shortness of breath on talking or at rest	8.7	-
Only able to talk in phrases or words	11.2	-
Altered consciousness (agitation or drowsiness)	0	-
Mean±SD duration of fever (days)	0.49±0.86	-
Received supplemental oxygen	23.0	-
Severity of exacerbations (No. [%] of patients)		
Mild	5 (2.4)	-
Moderate	101 (48.3)	-
Severe	103 (49.3)	-
Imminent respiratory arrest	0	-
Vital signs (mean±SD)		
Minimum SaO_{2v}(%)	94.1±2.4	-
Maximum pulse rate (/min)	135±22	-
Maximum respiratory rate (/min)	34±9	-
Systolic blood pressure (mm Hg)	110±15	-
Diastolic blood pressure (mm Hg)	69±10	-
Laboratory results (mean±SD)		
Fractional exhaled nitric oxide concentration (ppb)	57.1±43.0	77.2±59.6
Forced expiratory volume in 1 second (FEV_1) predicted (%)	73.2±21.7†	95.8±15.2
Forced vital capacity (FVC) predicted (%)	81.6±32.5*	94.2±18.8
FEV_{1v} to FVC ratio	0.81±0.25	0.86±0.10
Peak expiratory flow (l/min)	194±79†	344±132
Treatment and outcome (% of patients)		
Received systemic corticosteroid	75.4	-
Intensive care unit care	3.5	-
Death	0	-

* P<0.05
† P<0.001

swabs. Respiratory pathogens were identified in samples of 105 (51.0%) cases and 21 (27.3%) controls (P<0.001, Table 2). The presence of any virus with or without atypical bacteria was significantly associated with asthma exacerbations (P<0.001 for both). Specifically, HRV infection was significantly more common among cases than controls (26.2% vs 13.0%, P=0.018), whereas other pathogens were not related to asthma exacerbations. In addition, co-infections of ≥2 pathogens was associated with asthma exacerbations (10.7% vs 2.6%, P=0.030). Nonetheless, none of the respiratory pathogens, or their co-infections, was associated with the severity of asthma exacerbations as defined by the Global Initiative for Asthma guideline (P>0.15 for all).

Patients with asthma exacerbations caused by respiratory viruses were significantly younger than those without identifiable viral infections (P<0.05, Table 3). This was mainly attributed to RSV, influenza A, and HMPV infections (P<0.05).

Regarding the seasonality of respiratory infections in 2007, patients with asthma exacerbations were infected with HRV throughout the whole period, but the peak seasons appeared to be spring (March to June) and autumn to winter (September to December). The low detection rates for all other organisms precluded analysis of their seasonality.

Only 14 (6.7%) of our 209 recruited patients with asthma exacerbations had blood checking for detection of respiratory pathogens. Paired blood samples were not collected from the remaining patients because their parents did not give consent or the tests were not clinically indicated. Two of these 14 patients in whom paired serum samples were collected had HRV infection, and one each had HMPV and influenza A. With this small number of samples, further evaluation was not feasible.

Discussion

Respiratory tract infections can be caused by a

Table 2. Detection of different viral and bacterial pathogens in respiratory samples

Parameter	(%) of patients		P value (Chi-squared or Fisher exact test)
	Asthma exacerbation (n=206*)	Stable asthma (n=77)	
Organism			
Rhinovirus	54 (26.2)	10 (13.0)	0.018
Human metapneumovirus	12 (5.8)	2 (2.6)	0.265
Influenza A virus	16 (7.8)	4 (5.2)	0.452
Influenza B virus	3 (1.5)	0	0.287
Parainfluenza viruses types 1-4	14 (6.8)	2 (2.6)	0.173
Respiratory syncytial virus	8 (3.9)	1 (1.3)	0.270
Bocavirus	5 (2.4)	2 (2.6)	0.935
Adenovirus	5 (2.4)	0	0.168
Human coronaviruses OC43 or 229E	5 (2.4)	0	0.168
Enterovirus	2 (1.0)	0	0.386
Mycoplasma pneumonia	2 (1.0)	2 (2.6)	0.302
Chlamydia pneumonia	4 (1.9)	1 (1.3)	0.715
Presence of any virus	103 (50.0)	20 (26.0)	<0.001
Presence of *M pneumoniae* or *C pneumoniae*	5 (2.4)	1 (1.3)	0.558
Presence of any pathogen	105 (51.0)	21 (27.3)	<0.001
Co-infection by ≥2 pathogens	22 (10.7)	2 (2.6)	0.030

* Insufficient respiratory specimens in three patients

Table 3. Relationship between different respiratory pathogens and patient age in 206 evaluable patients with asthma exacerbations

Parameter	Mean±SD patient age (years)	
	Infection	No infection
Pathogen		
Rhinovirus	7.3±3.8	7.7±4.1
Human metapneumovirus	5.8±2.8*	7.7±4.1
Influenza A virus	6.0±3.0*	7.7±4.1
Parainfluenza viruses types 1-4	7.7±4.9	7.6±4.0
Respiratory syncytial virus	4.5±2.1‡	7.7±4.1
Bocavirus	7.4±2.8	7.6±4.1
Adenovirus	6.2±3.8	7.6±4.1
Human coronaviruses OC43 or 229E	8.3±4.3	7.6±4.1
Presence of any virus	6.9±3.5*	8.3±4.4
Presence of *Mycoplasma pneumoniae* or *Chlamydia pneumoniae*	6.3±4.0	7.6±4.1
Presence of any pathogen	6.9±3.5†	8.4±4.4
Co-infection by ≥2 pathogens	6.5±4.1	7.7±4.0

* P<0.05
† P<0.01
‡ P<0.005

heterogeneous group of viruses and bacteria that produce similar clinical presentations. The present study made use of our published multiplex nested PCR assays that could simultaneously detect 20 different respiratory pathogens.[2] Respiratory viral infections were significantly associated with asthma exacerbations in Hong Kong children, particularly HRV. This observation was consistent with studies that identified HRV as the most important viral aetiology of childhood asthma.[3,4] More studies are necessary to delineate the link between HRV infection and worsening of asthma status.

M pneumoniae and *C pneumoniae* are common pathogens associated with asthma exacerbations; 61% of adults with asthma attacks have evidence of infection with *C pneumoniae, M pneumoniae*, or both.[5] Telithromycin is beneficial for treatment. However, in the current study, *M pneumoniae* and *C pneumoniae* were only detected in respiratory secretions from 2.4% of children with asthma exacerbations and 1.3% of patients with chronic stable asthma. These results do not support the usefulness of macrolide treatment for asthma exacerbations.

A limitation of the current study related to its power. The number of recruited controls was much lower than the target of 180, as case-control matching was not feasible on many occasions when relatively 'stable' asthmatics also had non-specific upper respiratory symptoms. Thus, our sample size had a power of 97% for detecting any difference in the detection of any virus between cases and controls, but had a marginal power of 70% for HRV infections. Besides, the study was underpowered (<50%) for the detection of other respiratory pathogens. Thus, larger studies are needed to delineate the relationship between asthma exacerbations and other respiratory pathogens.

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Influenza virus oad in hospitalised patientsl

Key Messages

1. Hospitalised patients with severe influenza have persistently high viral loads, for whom a different therapeutic approach may be needed.
2. Active screening of influenza infection should be performed in all high-risk patients hospitalised with febrile respiratory illness. Early diagnosis and treatment to suppress the high viral load may maximise clinical benefit.
3. For late presenting high-risk patients with severe symptoms, their viral load may remain high, and initiation of antiviral treatment may still be worthwhile.
4. More stringent infection control measures, including strict droplet precautions and preferably isolation for an extended period of time may be necessary owing to prolonged viral shedding.
5. Randomised, controlled trials are indicated to address timing and dosage of treatment for severe influenza infection.

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Introduction

Influenza is estimated to be responsible for 4.1 to 4.5 million excess illnesses in individuals aged 20 years and older. Its symptoms are debilitating, and lower respiratory (eg bronchitis, pneumonia) and non-respiratory (eg cardiovascular, cerebrovascular) complications are common. Influenza-related complications result to approximately 150 000 hospitalisations and 20 000 deaths annually in the United States.

Viral kinetics and its clinical correlation with human influenza infections are not well known, especially in sicker, hospitalised patients who are usually elderly and have co-existing medical illnesses. In young children and immunocompromised hosts, influenza-virus titres correlated with symptom severity, and extensive virus shedding can persist for prolonged periods. We hypothesised that patients with severe, complicated influenza infection may have higher viral loads and more prolonged viral shedding. If confirmed, such patients should be subject to more aggressive antiviral treatment and more stringent infection control measures.

Methods

This prospective, observational study was conducted from January to December 2007. Ethics approval was obtained from the Institutional Review Boards of the Hospital Authority of Hong Kong and The Chinese University of Hong Kong. Consecutive laboratory-confirmed patients aged ≥16 years with influenza who were admitted to the medical department of the Prince of Wales Hospital (PWH) were recruited. All patients presenting with acute febrile respiratory illness needing hospitalisation were admitted to designated medical wards and managed according to a standard protocol. Nasopharyngeal aspiration and immunofluorescence assay was used for case identification. Patients might be prescribed a standard course of oseltamivir (Tamiflu) 75 mg twice daily for 5 days, based on existing recommendations. Patients were managed and discharged according to usual clinical practice.

Patients were recruited regardless of disease severity and treatment status after informed consent. A pair of combined nasal and throat swabs was collected from each patient starting from the day of recruitment (baseline) and then serially until week 1. All specimens were sent for viral RNA quantification (viral load) and virus isolation. Clinical data was recorded including demographics, medical comorbidities (eg congestive heart failure, cerebrovascular, neoplastic, chronic liver and renal diseases, chronic pulmonary diseases, use of immunosuppressants, and chronic respiratory conditions), influenza vaccination status, symptom onset time, symptom severity score, complications, antiviral treatment, and clinical outcomes.

Controls were patients aged 16 to 65 years presented to the emergency department of PWH with influenza but not hospitalised and without any comorbidity/complication.

All serially collected nasal/throat swabs were subjected to influenza viral RNA quantification and virus isolation. Viral RNA quantification was performed using a real-time RT-PCR technique, targeting the M-gene of influenza A and

B. Virus isolation was performed using MDCK cells. Any cytopathic effect on the cell monolayer was observed and confirmed by immunofluoresent testing. All influenza A virus isolates were sent for sub-typing (eg H3, H1).

All viral RNA concentrations were log-transformed for statistical analysis. Linear and logistic regression analyses and multilevel (mixed-effect) models were used to assess independent factors affecting viral loads.

Results

A total of 147 hosptalised influenza A cases, 29 influenza B cases, and 19 non-hospitalised influenza A controls were recruited.

Influenza A

Influenza A virus was isolated in 128 (87.1%) of the 147 cases, and the remaining cases were both IFA and RT-PCR positive. Sub-typing was performed for 126 isolates, and all were confirmed to be the H3N2 virus (Table).

Baseline samples were collected within the first week of symptom onset; 81.6% were collected on or after day 3. A median of four serial specimens was collected from each patient. Most patients were of advanced age, had comorbid illnesses, and developed influenza-related complications necessitating prolonged hospitalisation.

For the baseline samples, 88 were collected pre-treatment and 59 after one to two doses of oseltamivir. Of which, 120 (81.6%) were detectable or tested positive for influenza A virus by RT-PCR. Viral concentrations correlated positively with the four-point symptom score (Spearman's ϱ=0.219, P=0.010), and were 1.5 logs higher in hospitalised patients than in controls (5.96±1.19 vs 4.41±1.91 $\log_{10}$copies/mL, P=0.003).

The mean viral concentration of all pre-treatment samples was 4.68 (standard deviation [SD], 2.06) $\log_{10}$copies/mL. Viral concentrations correlated negatively with days from symptom onset, indicating natural viral clearance (Spearman's ϱ=-0.388, P<0.0001). Univariate analyses showed that patients with major systemic comorbidities had significantly higher baseline viral concentrations. Viral concentrations on/beyond day 3 of the illness were 1.4 logs higher in these patients than in controls (5.06±1.85 vs 3.62±2.13, P=0.005, Fig 1). Multiple linear regression analyses showed that presence of major systemic comorbidities (β=0.765, standard error [SE]=0.354, 95% confidence interval [CI]=0.065-1.465, P=0.032), longer time elapsed from symptom onset (β=-0.457, SE=0.107, 95% CI=-0.668 to -0.246, P<0.0001), and antiviral initiation (β=-0.899, SE=0.351, 95% CI=-1.592 to -0.206, P=0.011) were independent factors associated with virus concentrations, after adjusting for age and gender. Factors associated with an undetectable viral load included antiviral initiation (odds ratio [OR]=2.94, 95% CI=1.20-7.25, P=0.019) and time elapsed from symptom onset (OR=1.48, 95% CI=1.13-1.94, P=0.004), after adjusting for baseline characteristics.

The mean±SD virus concentrations on days 1, 2, 3, 4, 5, 6, and 7 of symptom onset were 6.30±1.38, 5.77±1.06, 4.30±1.87, 3.50±2.11, 2.98±2.31, 2.39±2.08, and 1.76±2.25 $\log_{10}$copies/mL, respectively. By days 4, 5, 6, and 7 of symptom onset, 78.6%, 68.5%, 52.6%, and 32.7% of these hospitalised patients still had detectable influenza viral RNA, respectively. Viral concentrations declined non-linearly with time elapsed from symptom onset (P<0.001, linear and quadratic trend likelihood ratio tests). In a final multilevel model, patients with major systemic comorbidities had persistently higher viral concentrations (mean=0.854, SE=0.266, P<0.0001, likelihood ratio test), whereas those starting antiviral treatment on day 1 (mean=

Table. Clinical characteristics and outcomes of patients with influenza A infection (n=147)

Patient characteristics	No. (%) of patients
Age >65 years	111 (75.5)
Male	77 (52.4)
Elderly home residents	22 (15.0)
Influenza vaccination*	32 (21.8)
Comorbidity†	
Any	94 (63.9)
Major systemic (congestive heart failure, cerebrovascular, neoplastic, chronic liver and renal diseases, and immunosuppression)	53 (36.1)
Influenza-related complication‡	
Any	118 (80.3)
Cardiorespiratory	104 (70.7)
Use of supplemental oxygen	88 (59.9)
Use of antiviral treatment§	110 (74.8)
Death	2 (1.4)
Transferred to convalescent care facilities	34 (23.1)
Total length of hospital stay >7 days	62 (42.2)

* Vaccination status is unknown in 14 patients

† Classification is based on the Pneumonia PORT Severity Index scoring system. 'Any' referred to the presence of major or other significant medical illnesses, including diabetes, ischemic heart disease, chronic pulmonary (asthma, chronic obstructive pulmonary disease, bronchiectasis), and neurological diseases

‡ 'Any' was defined as new or exacerbation of underlying medical problems, whereas 'cardiorespiratory' referred to pneumonia, bronchitis, exacerbation of chronic pulmonary diseases, and acute cardio-/cerebro-vascular events. Patients might have >1 complications

§ Oseltamivir is prescribed on days 1-3 (n=85) or days 4-6 (n=25). The median (interquartile range) was day 3 (2-3). No antiviral treatment was given to 37 patients

-1.301, SE=0.459, P<0.001) and on days 2-3 (mean= -0.743, SE=0.341, P=0.030) had faster declines in viral concentrations, after adjusting for baseline characteristics (Fig 2). In patients with major systemic comorbidities, virus was persistently detectable by the end of the first week in 87.5% of patients not given oseltamivir treatment, 27.3% of those received it on days 1-3, and 60.0% of those received it on days 4-6 (chi-square, P=0.011).

Overall, by days 4, 5, 6, and 7 of symptom onset, 17.2%, 8.9%, 4.2%, and 2.1% of the patients remained culture positive, respectively, as did 38.5% and 11.1% of 'untreated' and 'treated' patients, and 23.7% and 5.0% of patients with and without comorbidity. The presence of comorbidities (OR=6.98, 95% CI=1.44-33.78, P=0.016) and lack of antiviral treatment (OR=0.17, 95% CI=0.06-0.52, P=0.002) were independent factors associated with persistence of virus. Patients with major systemic comorbidities had a more prolonged (>7 days) illness and hospitalisation (56.6% vs 34.0%, P=0.008).

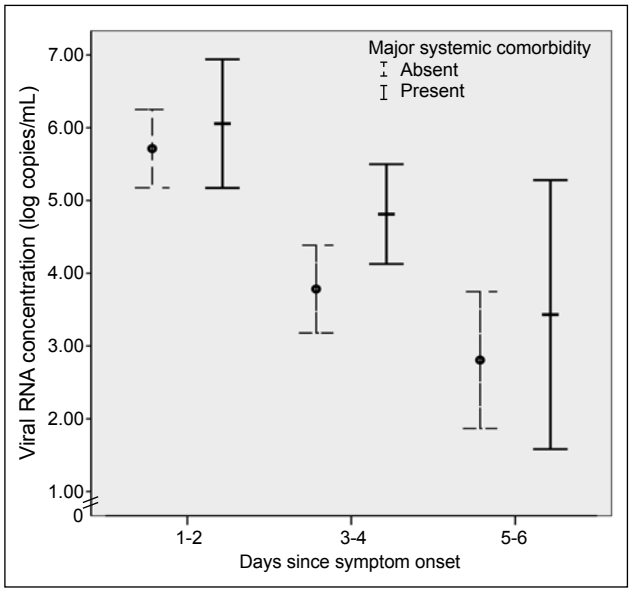

Fig 1. Baseline influenza A viral RNA concentrations according to day of symptom, in the presence or absence of major systemic comorbidities (congestive heart failure, cerebrovascular, neoplastic, chronic liver and renal diseases, and immunosuppression)

Influenza B

In 29 influenza B patients, multiple linear regression analyses of baseline samples showed that presence of comorbidities (β=2.291, SE=0.927, 95% CI=0.386-4.196, P=0.020) and longer time elapsing from symptom onset (β=-0.886, SE=0.275, 95% CI=-1.451 to -0.321, P=0.003) were independently associated with increased and decreased virus concentrations, respectively. In 69.6%, viral RNA remained detectable by the end of the first week of illness. This was associated with older age (>65 years) [92.3% vs 40.0%, P=0.019], presence of comorbidities (75.0% vs 33.3%, P>0.05), and lack of timely antiviral treatment (78.6% vs 55.6%, P>0.05). Viral cultures remained positive by days 4 and 6 of symptom onset in 56.0% and 18.5% of the patients, respectively.

Factors affecting viral clearance and outcomes

Among all cases, influenza B (OR=4.72, 95% CI=1.74-12.84, P=0.002), major systemic comorbidity (OR=2.30, 95% CI=0.95-5.56, P=0.065), and no early antiviral treatment (OR=4.95, 95% CI=2.02-12.16, P<0.001) were associated with persistence of virus. Influenza B (OR=4.45, 95% CI=1.28-15.50, P=0.019), age older than 65 years (OR=13.89, 95% CI=2.70-71.36, P=0.002), and persistently detectable viral RNA by the end of the first

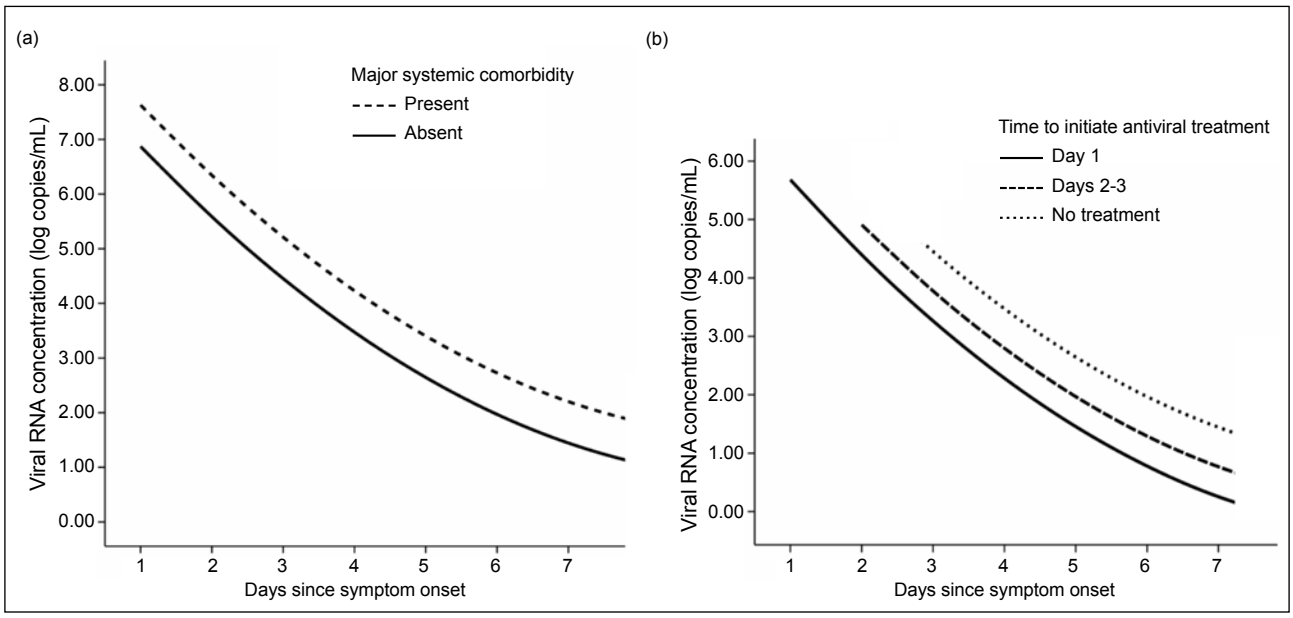

Fig 2. (a) Effects of comorbidity and (b) effects of time of treatment initiation on serial influenza A viral loads in a final multilevel model

week of illness (OR=3.42, 95% CI=1.35-8.66, P=0.009) were associated with prolonged hospitalisation and needing of convalescence care.

Discussion

In our hospitalised patients, higher viral loads correlated with more severe symptoms. Immunosuppressed patients may develop very severe influenza infection, have high viral loads and prolonged viral shedding. Elderly with underlying major systemic medical conditions (congestive heart failure, cerebrovascular, neoplastic, chronic liver and renal diseases) have more active viral replication and thus more severe illness. In these patients, natural viral clearance is slow, and the high viral load persists beyond the first 2 to 3 days of the illness. They also have significantly higher viral loads even following a standard course of antiviral treatment.

Our findings have several important implications. Previous clinical trials on neuraminidase inhibitors for seasonal influenza mostly involved young patients without underlying medical conditions. Benefit of antiviral treatment initiated after beyond 2 days from symptom onset was not demonstrated, as the viral load already declined significantly during recovery. Our results suggest that in high-risk patients with severe symptoms, the viral load may remain high, and late initiation of antiviral treatment may still be worth considering. Higher dosage of oseltamivir (eg 150 mg twice daily) may be necessary to achieve a faster viral load reduction and to prevent drug resistance. In H5N1-infected patients with very active and persistent viral replication, late initiation (on days 4-5) and higher dosage of antiviral treatment may suppress viral replication. In severe seasonal influenza, survival benefits from delayed antiviral treatment have also been reported. Randomised, controlled trials are needed to resolve these issues. Early diagnosis and treatment is important for high-risk patients. RT-PCR assay is more sensitive than culture and can be considered for rapid diagnosis. Early antiviral treatment enables more rapid reduction in viral load. Over 80% of untreated patients remain PCR positive one week after illness onset, and almost 40% remain culture positive on day 4. Therefore, strict droplet precaution coupled with isolation for an extended period is recommended. Early detection, treatment, and isolation of hospitalised influenza patients can effectively prevent nosocomial spread. In contrast to influenza A patients, influenza B patients tend to have more prolonged viral shedding and clinical symptoms, despite oseltamivir treatment. This is consistent with the recent observations that oseltamivir is less effective against influenza B, possibly related to a structural difference in its viral neuraminidase. Thus, a different treatment regimen may be needed when treating influenza B patients.

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Viral shedding, clinical history and transmission of influenzal

Key Messages

1. During influenza infections, most viral shedding occurs within a few days of illness onset.
2. Children may be more infectious than adults because they shed more virus.
3. The degree of viral shedding (infectiousness) correlates with symptoms and tympanic temperature.

Introduction

Influenza is associated with significant morbidity and mortality through seasonal epidemics and occasional pandemics.[1] An accurate understanding of the effectiveness of intervention strategies through experimental studies and mathematical models often involves profiling of infectiousness.[2]

Data on viral shedding from the respiratory tract, corresponding time lines, and duration of clinical illness are available from volunteer challenge studies.[3] Nonetheless, whether these results can be generalised to natural infections is uncertain, because participants are usually young adults with low levels of pre-existing antibody to the influenza strain. Although there are studies on viral shedding in hospitalised patients,[4,5] studies on the patterns of viral shedding after naturally acquired infection in out-patients or the community are limited. We analysed the dynamics of clinical illness and viral shedding in subjects that acquired influenza virus infections in a community setting, and inferred infectiousness profiles.

Methods

This study was conducted from September 2008 to August 2009. In 2008, we conducted a cluster-randomised controlled trial to study the efficacy of hand hygiene and face masks to prevent the transmission of influenza in households.[6] Subjects presented to out-patient clinics and private hospital emergency rooms with at least two symptoms (fever ≥37.8ºC, cough, sore throat, headache, runny nose, phlegm, muscle pain) were recruited. They also had to be (1) a Hong Kong resident, (2) with symptoms started in the preceding 48 hours, and (3) with two or more household members free of influenza-like illness in the preceding 2 weeks. If this index patient was found to be positive for influenza A or B virus infection following a rapid antigen test, every household member was followed up with a series of three home visits (each of which involved responses to a questionnaire) and nasal and throat swabs (NTS). Symptoms were self-recorded daily, and the body temperature was recorded using a digital tympanic thermometer.

A total of 3868 NTS specimens were collected over the course of the study. Specimens were tested by quantitative reverse transcription polymerase chain reaction (RT-PCR) to detect molecular viral loads and determine influenza A or B virus infection.[7] A subset of specimens were further tested to detect tissue culture infectious doses ($TCID_{50}$) and determine replicating viral loads by quantitative viral dilutions.[7]

There were three groups of symptoms: systemic symptoms (fever ≥37.8ºC, headache, myalgia), lower respiratory symptoms (cough, phlegm), and upper respiratory symptoms (sore throat, runny nose). Daily scores were tabulated by presence versus absence of each symptom and divided by the total number of symptoms in each group.[3] Trends in symptom scores and quantitative viral loads were plotted since the day of self-reported illness onset for index cases and since the day of onset of acute respiratory illness (ARI) for secondary cases.[8]

Viral shedding in secondary cases was considered representative of natural infections. We used a modelling approach to infer infectiousness from viral shedding. We used a Bayesian approach to fit lognormal, Weibull, and gamma-

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form parametric forms to the viral shedding trajectories and selected between models using the Bayesian information criterion.[9] All statistical analyses were conducted using R version 2.7.1 (R Development Core Team, Vienna, Austria)[10] and WinBUGS version 1.4.[11]

Results

A total of 1015 household members from 322 households were followed up. All index subjects and 135 (13%) household members were confirmed by RT-PCR to have influenza virus infection. Among index cases, molecular viral shedding was highest on the day of symptom onset and gradually declined to undetectable levels within approximately 10 days (data not shown). The mean duration of shedding was 6 days. The dynamics of molecular viral shedding for influenza A and B virus infections were similar. Viral shedding was significantly higher in children than in adults with influenza A virus infections (data not shown).

Among secondary cases, 59 household members for whom the first NTS specimens collected were RT-PCR positive for influenza virus were excluded from analysis. An additional 17 household members were also excluded owing to the presence of ARI on the first home visit and RT-PCR confirmed influenza infection subsequently. Of the 59 secondary cases analysed, 16 were influenza A/H1N1 virus infections, 17 were influenza A/H3N2 infections, one was an influenza A/H1N1 and A/H3N2 coinfection, and the remaining 25 were influenza B virus infections. On the day of ARI onset, the most frequently reported symptoms were cough, nasal congestion/runny nose, and sore throat (Table). Thirty (51%) of them reported seeking medical care for their illness.

In secondary cases, peak molecular viral shedding for influenza A virus infections occurred on the day of ARI onset. Viral shedding declined steadily during the subsequent 7 days (Fig 1). The viral shedding patterns for influenza A/H1N1 and influenza A/H3N2 were similar (data not shown). Pre-ARI onset viral shedding was detected in four of the 15 subjects (27%; 95% confidence interval [CI], 8-55%). Viral shedding of influenza B virus was more variable over time and a clear peak was not recorded; pre-ARI shedding was detected on days 1 and 2 before ARI onset in four of the 14 subjects (29%; 95% CI, 8-58%). Influenza B viral shedding continued for about 6 days before declining to undetectable levels.

Table. Symptoms of naturally acquired influenza A and B virus infections reported at the onset of acute respiratory illness

Symptom	No. (%) of patients	
	Influenza A (n=26)	Influenza B (n=18)
Runny nose or nasal congestion	19 (73)	11 (61)
Cough	18 (69)	14 (78)
Sore throat	14 (54)	7 (39)
Headache	14 (54)	5 (28)
Phlegm	12 (46)	5 (28)
Myalgia	9 (35)	6 (33)
Fever ≥37.8°C	8 (31)	8 (44)

For influenza A virus infections, the peak replicating viral load (assessed by viral culture) was on the day of ARI onset and declined steadily over the subsequent 5 days (Fig 1). For influenza B virus infections, $TCID_{50}$ levels initially peaked on the day of ARI onset and were more variable over time. Pre-ARI onset replicating viral load was detected in one adult with influenza A infection, and two adults and three children with influenza B virus infection. For both influenza A and B virus infections, the average symptom score and mean tympanic temperature peaked on the day of ARI onset, and steadily declined to nil after 3 to 5 days (Fig 1). Systemic symptoms resolved at a faster rate than respiratory symptoms (Fig 1).

Asymptomatic viral shedding was detected by RT-PCR in eight of the 59 secondary cases (14%; 95% CI, 6-25%); five and three cases were positive for influenza A and B viruses, respectively. In two of these eight, only the NTS specimen collected on the final home visit was positive. These may be cases of pre-ARI onset shedding, as subjects could develop symptoms after our follow ups. Viral shedding was detected in a further seven of 59 subjects that were subclinical, or reported just one symptom over the course of illness.

Infectiousness profiles

A modified lognormal form provided a good fit to influenza A virus infection molecular viral shedding patterns. Assuming infectiousness as proportional to molecular viral shedding determined by RT-PCR, infectiousness was maximal within 2 to 3 days of the ARI onset. If infectiousness was assumed to be proportional to $\log_{10}$ molecular viral shedding or presence of detectable viral RNA, a longer duration could be inferred (Fig 2). Parametric forms did not offer any good fits to the influenza B molecular viral shedding patterns, or to replicating viral loads of influenza A or B assessed by viral culture.

Discussion

Three different models have been used to describe infectiousness in influenza A virus infections over time. Assuming infectiousness is proportional to molecular viral shedding, most of the infectiousness is within 1 to 2 days (or 3 to 4 days) of the day of ARI onset (Fig 2). Considering the more rapid decline in replicating viral load compared to molecular viral shedding (Fig 1), the true duration of infectiousness may be overestimated by this method.

The mean serial interval for influenza infections is estimated to be 3.6 days,[8] whereas the incubation period is 1.5 to 2 days.[12] These estimates imply that the average time between ARI onset and transmission to a household contact is about 2 days, which is in line with the trend of

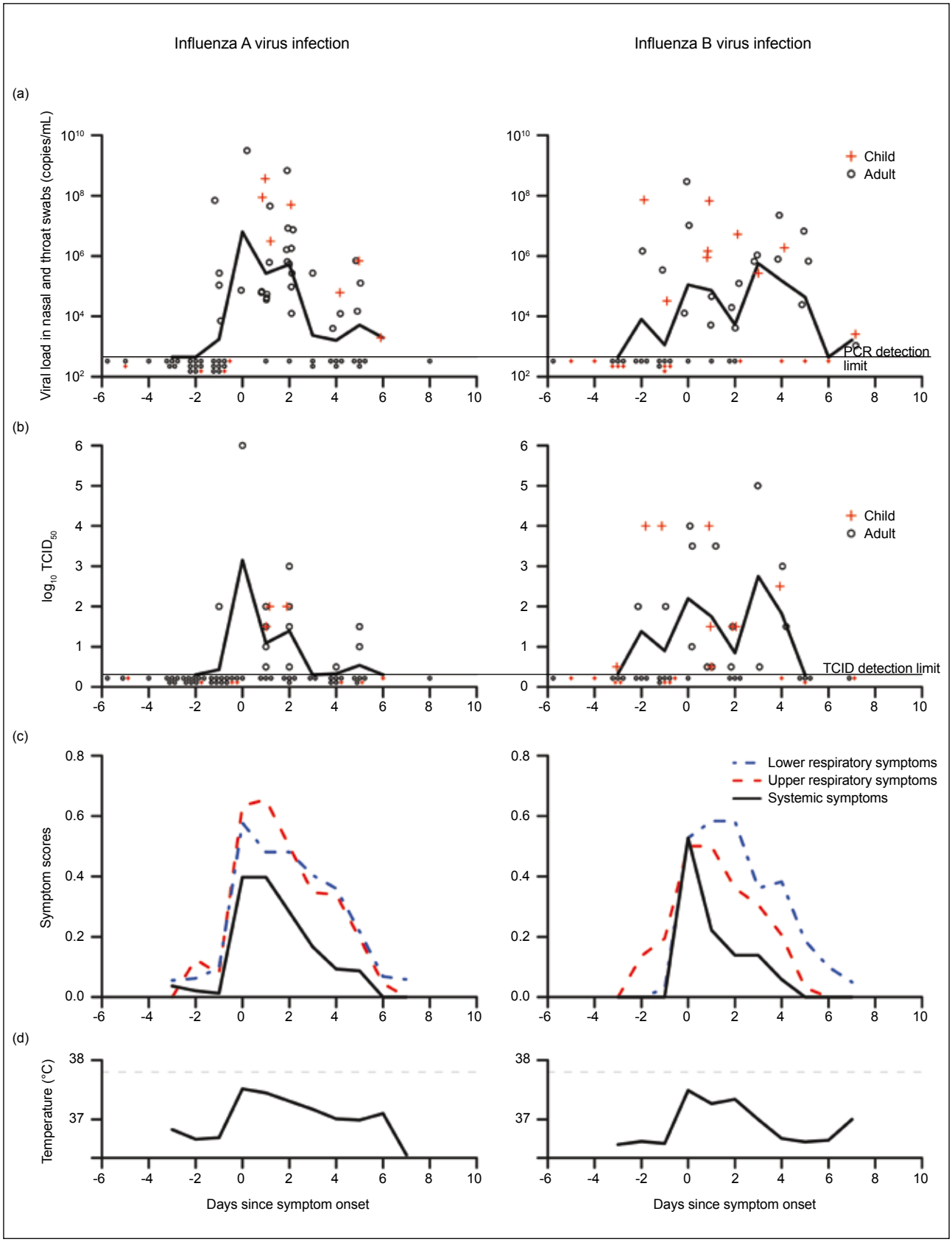

Fig 1. Patterns of viral shedding and symptom scores in naturally acquired influenza A (n=26) and B (n=18) virus infections
(a) The geometric mean viral shedding (the lower limit of detection of the RT-PCR assay is approximately 900 copies/mL), (b) the geometric mean tissue culture infectious dose ($TCID_{50}$), (c) symptom scores, and (d) the mean tympanic temperature

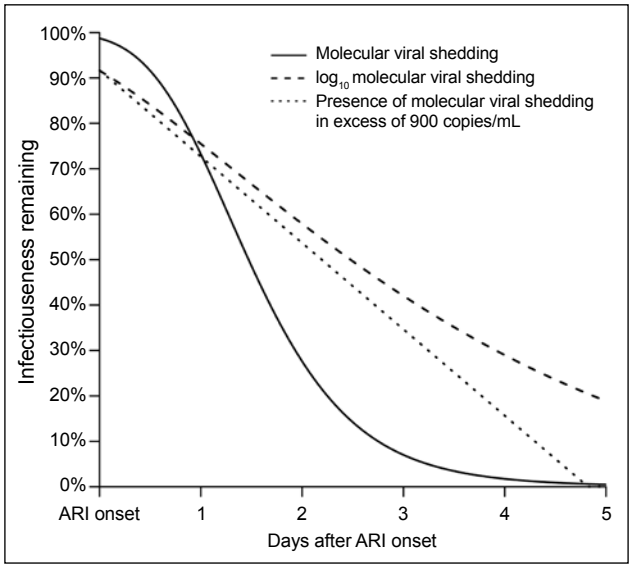

Fig 2. Infectiousness since the onset of acute respiratory illness (ARI) in the course of naturally acquired influenza A virus infection

Infectiousness is assumed to be proportional to molecular viral shedding, $\log_{10}$ molecular viral shedding, and presence of molecular viral shedding in excess of 900 copies/mL under the fitted curve

infectiousness over time based on molecular viral shedding and $\log_{10}$ viral shedding.

The trends in viral shedding and average symptom scores in secondary cases were consistent with findings in the volunteer study.[3] Viral shedding for influenza A virus infections peaked at approximately the same time as ARI onset before subsiding. In a small proportion of cases, viral shedding was detected prior to ARI onset. The daily replicating viral load (assessed by $TCID_{50}$) subsided at a faster rate than viral shedding (measured by RT-PCR). For influenza B infections, the patterns of viral shedding over the course of illness was more variable but consistent with data from the volunteer study, showing sustained shedding from the time of ARI onset for approximately 5 days.[3]

In secondary cases, systemic symptoms decline more quickly than respiratory symptoms for both influenza A and B virus infections.[3] The more rapid decline of systemic symptoms (specifically fever, its profile is similar to viral shedding) can be attributed to the controlled decrease in immune response, as the virus is gradually cleared from the body.[13]

Among the 59 household contacts with RT-PCR–confirmed infection, eight (14%; 95% CI, 6-25%) did not report any symptoms, and 15 (25%; 95% CI, 15-38%) were either asymptomatic or subclinical. Previous experimental infectiousness studies found a frequency of asymptomatic infection higher than the upper bound of our results[3] and similarly in longitudinal studies that examined paired pre- and post-season serology in household contacts.[14] Our study might have failed to detect infected subjects that were either shedding lower quantities of virus or shedding virus for a very short duration, and proportionally more of these subjects may be asymptomatic or subclinical.[3,15] It is unclear whether asymptomatic individuals have the potential to transmit influenza virus.[16] However, mathematical models typically assume that 33% to 50% of all infections are asymptomatic or subclinical, and the transmission potential of these subjects is half of that of symptomatic individuals.[2,17] Our results suggest that asymptomatic infections may be less important epidemiologically than previously thought.

The small sample size limited the ability to analyse the association between viral shedding and age or other characteristics, to characterise the patterns of viral shedding in secondary cases, and to ascertain an accurate proportion of asymptomatic and subclinical cases. In addition, our recruitment criteria and study design restricted recruitment to households without any illness during recruitment of the index subjects, possibly biasing the recruited households to those with a lower risk of infection or illness.[6] Besides, this study was not designed to address the degree to which asymptomatic or subclinical cases are responsible for transmission in the community. Addition of serological evidence to our findings would have been valuable, and further studies should consider the inclusion of such testing.

Conclusions

Viral shedding determined by RT-PCR and $TCID_{50}$ in natural community influenza virus infections peaks around the day of symptom onset. The trend of viral shedding closely matches the trends of the average symptom score and mean tympanic temperature suggesting that infectiousness is likely to be correlated with illness severity, and that asymptomatic persons may be less important in influenza transmission than previously thought. The greatest infectiousness of influenza A virus is within 1 to 2 days following ARI onset. Individuals should take protective measures against transmission while they have febrile illness, and if possible while any symptoms persist.

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Replication and pathogenesis of avian influenza A (H5N1) virus infection in polarised human bronchial and alveolar epitheliuml

Key Messages

1. In vitro models of polarised human respiratory epithelial cells were established to investigate the tropism and innate host responses of influenza A (H5N1 and H1N1) viruses.
2. Both viruses efficiently infected alveolar epithelial cells of both the apical and basolateral surfaces of the epithelium, whereas release of newly formed virus was mainly from the apical surface of the epithelium.
3. H5N1 virus was a more potent inducer of cytokines and chemokines in alveolar epithelial cells than H1N1 virus. Such chemokines were secreted onto both the apical and basolateral surfaces of the polarised alveolar epithelium.
4. In bronchial epithelium, the H5N1 virus replicated more efficiently and induced a stronger type I interferon response in the undifferentiated NHBE cells than did H1N1 virus. In contrast, in well-differentiated cultures, H5N1 virus replication was less efficient and elicited a lower interferon-beta response than did H1N1 virus.
5. Recombinant virus with vRNPs of a mammalian PB2 and an avian PB1 had the strongest polymerase activities, and replicated better in human cell cultures, especially at a high incubation temperature. These viruses were potent inducers of cytokines and chemokines in primary human alveolar epithelial cells.

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Introduction

Avian influenza A (subtype H5N1) transmitted from poultry to humans in Hong Kong in 1997 and caused a potentially fatal human respiratory disease.[1] So far, human-to-human transmission of the H5N1 subtype influenza virus appears to be inefficient, although it can cause exceptionally severe disease in 60% of humans it infects. Most such patients have a primary viral pneumonia complicated by acute respiratory distress and multiple organ dysfunction, and notably there may be lymphopaenia and haemophagocytosis.[2,3] These haematological abnormalities have been ascribed to cytokine dysregulation.[3-5]

The respiratory epithelial cells are the primary targets for influenza A virus replication.[6] In primates experimentally infected with H5N1/97 virus, types I and II pneumocytes and alveolar macrophages were found to contain viral antigen.[7] Virus infection of alveolar pneumocytes was also demonstrated in the lung of a patient with fatal H5N1 disease.[8] Human alveolar epithelium is vital for the maintenance of lung function and the pulmonary air-blood barrier. Human respiratory epithelial cells respond to viral infections by mounting a cytokine response that contributes to innate and adaptive host defences.[9] In our previous studies, we investigated the proinflammatory cytokine profile associated with influenza A (H5N1) virus infection of primary monocyte-derived macrophages and non-polarised respiratory epithelial cells. We hypothesised that the hyper-induction of cytokines may contribute to the unusual severity of human H5N1 disease.

However, the physiological relevance of findings from non-polarised and undifferentiated cells, as well as transformed cells lines is uncertain. It is therefore important to study the influenza A (H5N1) virus–cell interactions using polarised and differentiated respiratory epithelial cells. We aimed to (1) establish a well-differentiated and polarised primary human respiratory epithelial cell culture model in vitro to study the replication and pathogenesis of avian influenza A (H5N1) virus disease in humans, (2) identify differences in the virus replication kinetics of H5N1 and H1N1 viruses in polarised human alveolar and bronchial epithelial cells in vitro, (3) define the contribution of different viral genes to the viral replication kinetics and polarisation using recombinant influenza viruses containing different gene combinations between human H1N1 and avian H5N1 viruses, and (4) define the contribution of different viral genes to the cytokine induction profile of H1N1 and H5N1 viruses.

Methods

This study was conducted from March 2007 to December 2009. Primary human in vitro well-differentiated alveolar and bronchial epithelial cells were infected with influenza A (H5N1 and H1N1) viruses in vitro. Virus replication was monitored by measuring the levels of the influenza matrix gene, by immune-fluorescence detection of the influenza matrix and nucleoprotein, and by titration of the infectious virus in MDCK cells. Using reverse genetics derived

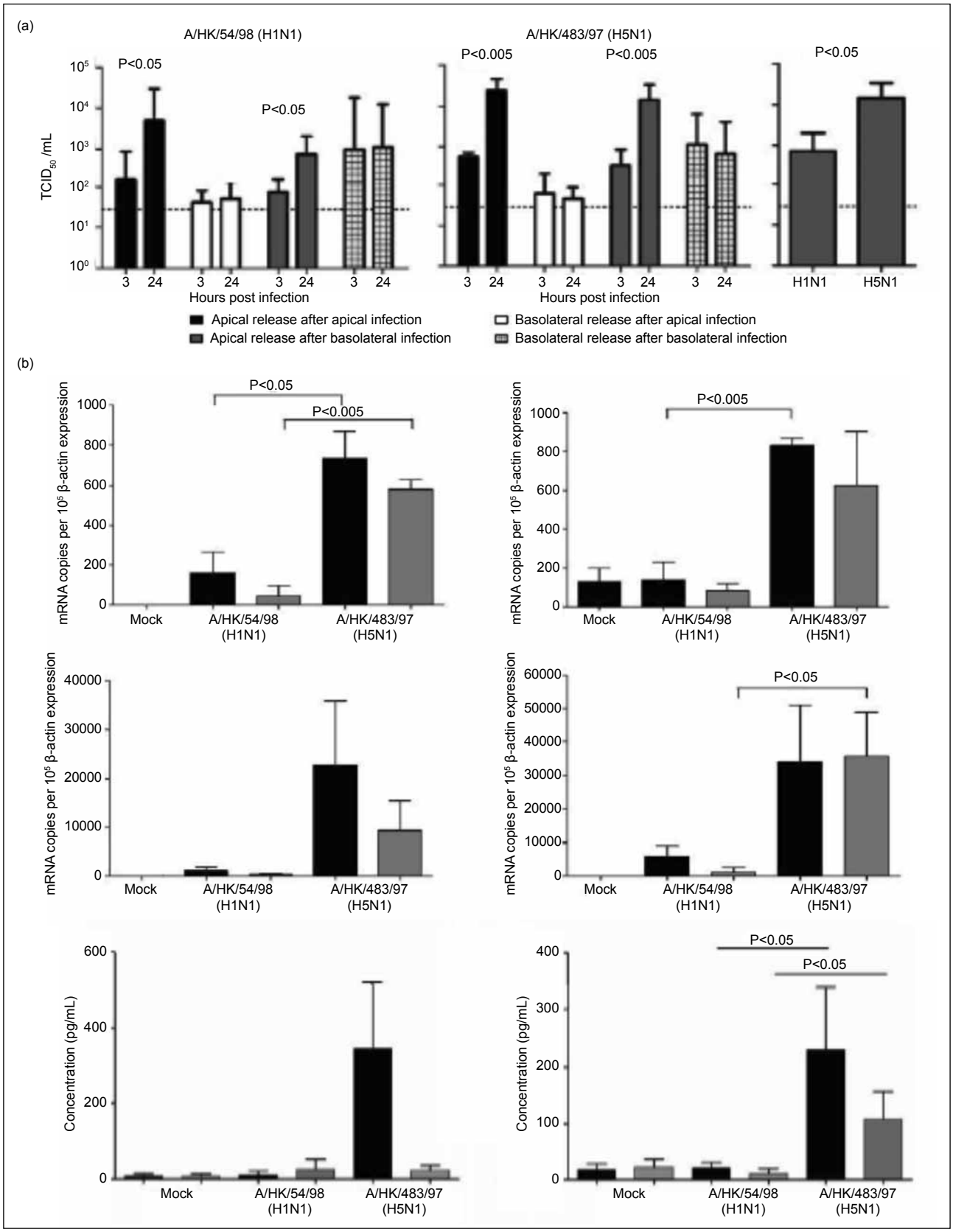

Fig 1. Replication kinetics and innate host response of influenza A virus–infected polarised alveolar epithelial cells

(a) Virus titres of the A/HK/54/98 (H1N1) and A/HK/483/97 (H5N1) viruses are determined after apical and basolateral infections of the type I-like alveolar epithelial cells at and 24 hours. At 24 hours following basolateral infection, the titre of influenza H5N1 virus at the apical surface of the cells is significantly more than that of H1N1 infected cells. (b) The cytokine (IFN-β and IL-6) and chemokine (RANTES and IP-10) gene expression from type I-like alveolar epithelial cells as well as release of RANTES and IP-10 proteins from alveolar epithelial cells after 24 hours of apical and basolateral infections of H1N1 and H5N1 viruses. The means and the standard errors from three representative experiments are shown. The dotted line represents the lowest detection limit of the $TCID_{50}$ assay.

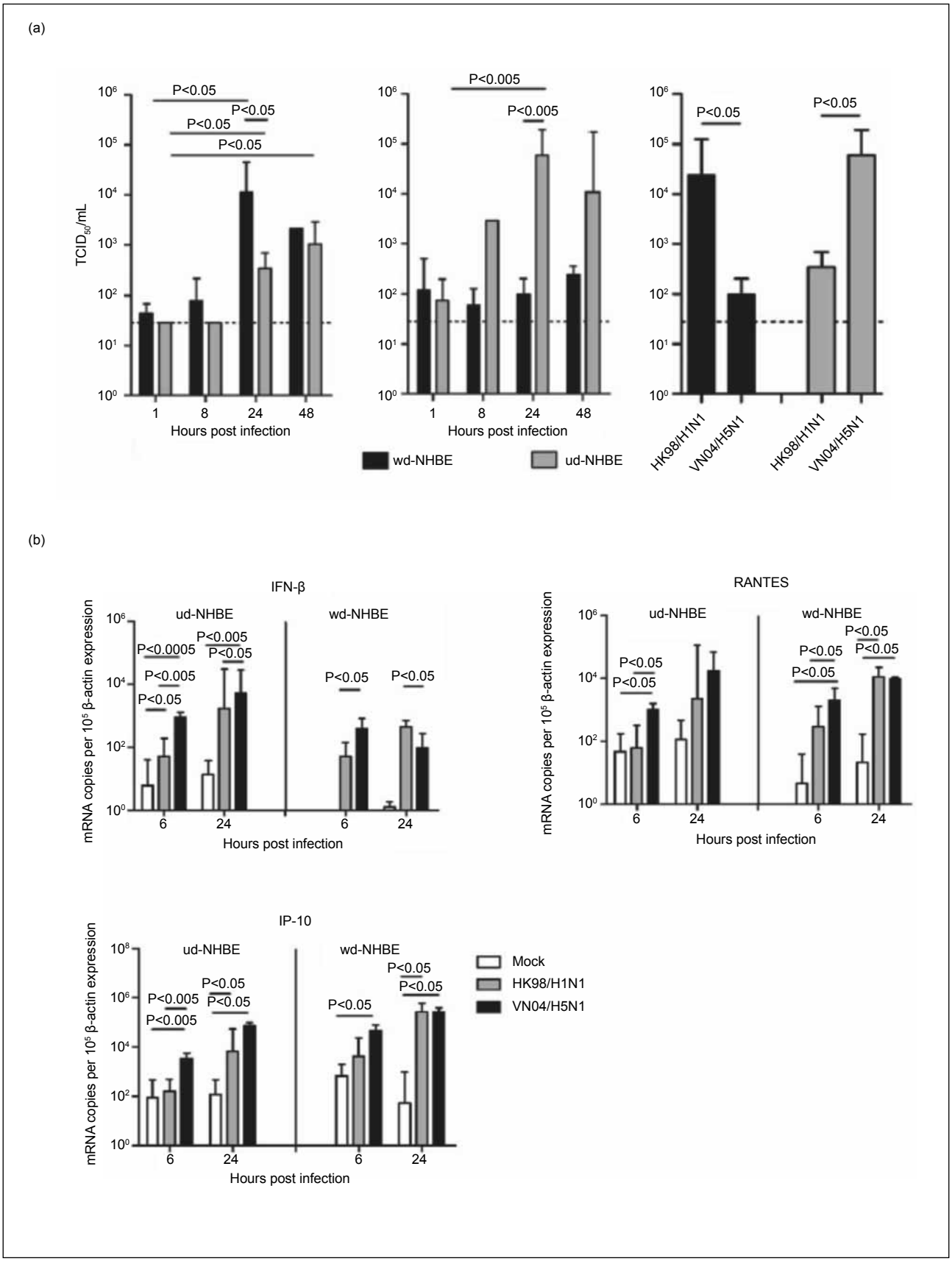

Fig 2. Replication kinetics and innate host response of influenza A virus–infected well-differentiated bronchial epithelial cells
(a) Virus titres of the H1N1 and H5N1 viruses are determined after infection in the ud- and wd-NHBE cells for 1 to 48 hours at MOI of 2. At 24 hours post infection, viral replication kinetics of the 2 viruses are compared. (b) The IFN-β, RANTES, and IP-10 gene expression of the ud- and wd-NHBE cells at 6 and 24 hours post infection of H1N1 and H5N1 viruses. The means and standard errors from three independent experiments are shown. The dotted line represents the detection limit of the $TCID_{50}$ assay.

recombinant viruses, the role of viral PB1 and PB2 segments of H5N1 virus on viral replication and cytokine induction in the human respiratory tract was demonstrated. Gene and protein expression profiles (cytokine and chemokine) of respiratory epithelial cells infected with various influenza A viruses and recombinant viruses were compared by using real-time quantitative reverse transcriptase PCR.

Results

Both influenza H1N1 and H5N1 viruses efficiently infected alveolar epithelial cells of both the apical and basolateral surfaces of the epithelium, whereas release of newly formed virus was mainly from the apical surface of the epithelium (Fig 1a). H5N1 virus was a more potent inducer of cytokines and chemokines in alveolar epithelial cells than H1N1 virus. Such chemokines were secreted onto both the apical and basolateral surfaces of the polarised alveolar epithelium (Fig 1b). The H5N1 virus replicated more efficiently and induced a stronger type I interferon response in the undifferentiated NHBE cells than did H1N1 virus (Fig 2a). For other cytokines (eg RANTES and IP-10), H5N1 virus led to significantly higher induction of IP-10 and RANTES mRNA expression in ud-NHBE cells than did H1N1 or mock infection (Fig 2b). In contrast, in well-differentiated cultures, H5N1 virus replication was less efficient and elicited a lower interferon-beta response than did H1N1 virus. Recombinant vRNPs with a mammalian PB2 and an avian PB1 had the strongest polymerase activities in human cells, and replicated better in cell cultures, especially at a high incubation temperature (Fig 3a). These viruses were potent inducers of cytokines and chemokines (eg RANTES and IP-10) in primary human alveolar epithelial cells (Fig 3b).

Discussion

Influenza viral infections are major causes of economic loss and of morbidity and mortality worldwide, and threaten economies and social stability as well as human health. These in vitro models of human respiratory epithelial cells enables better understanding of the transmission, prevention, and control of influenza A virus infections (especially the highly pathogenic H5N1 virus) and other emerging respiratory infections (such as SARS-CoV), as well as the underlying mechanism of inter-species transmission of animal pathogens to humans. These models have also been used to investigate the tropism and host responses of the swine-origin pandemic (H1N1pdm) virus. This is especially important when a good small animal model is lacking and in adherence to the 3R principles pertaining to the use of animals in research. Nonetheless, further investigations with relevant animal models of influenza virus infection and the potential use of immunomodulators in addition to antivirals may be considered. Cytokine dysregulation may play an important role in severe human influenza disease. The use of immunomodulators in combination

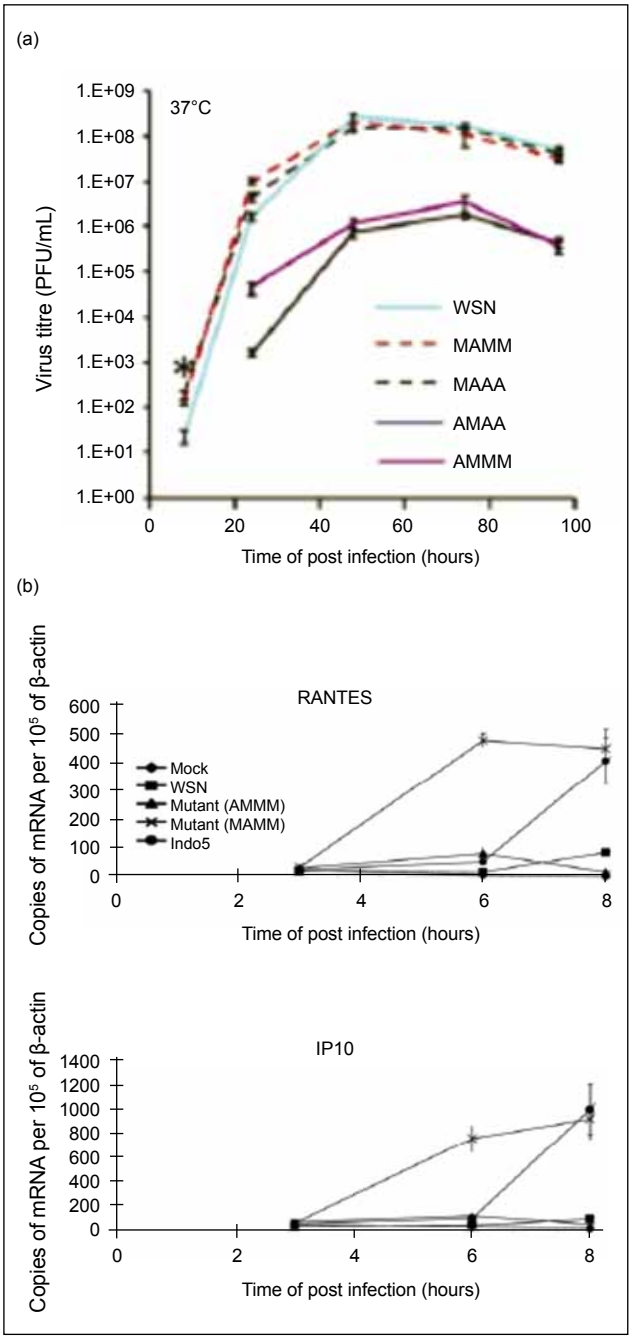

Fig 3. Characterisation of viral replication and cytokine and chemokine responses of recombinant viruses with chimeric polymerase complexes

The origins of PB2, PB1, PA, and NP in each recombinant virus are shown (A=avian, M=mammalian). (a) Growth properties of the WSN (MMMM) and recombinant viruses in MDCK cells: the number of infectious progeny viral particles generated from MDCK cells infected with the corresponding virus at a MOI of 0.01 is determined by standard plaque assay. Mutant AMAA and AMMM are significantly attenuated. At 8 hours post infection, the amounts of infectious progeny of MAAA and MAMM are significantly higher than the wild type controls. (b) Cytokine and chemokine (RANTES and IP-10) gene expression profiles from primary human alveolar epithelial cells: total RNA from cells infected at a MOI of 2 is harvested at the indicated time points and tested by the corresponding quantitative RT-PCR. The means of triplicate assays and the recombinant viruses used in the experiments are shown.

with antivirals may have clinical benefits. Our results may provide a biological basis for the observed therapeutic impact of immunomodulators for treating severe human influenza disease.

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Role of cyclooxygenase-2 in H5N1 viral pathogenesis and the potential use of its inhibitorsl

Key Messages

1. Cyclooxygenase-2 (COX-2), along with TNF-α and other proinflammatory cytokines, was hyperinduced in H5N1-infected macrophages in vitro and in epithelial cells of autopsied lung tissues of infected patients.
2. The COX-2 mediated amplification of the proinflammatory response is rapid, and the effects elicited by the H5N1-triggered proinflammatory cascade are broader than those arising from direct viral infection.
3. Selective COX-2 inhibitors suppress the H5N1-hyperinduced cytokines in the proinflammatory cascade.

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Introduction

The highly pathogenic avian influenza viruses (H5N1) in poultry and wild birds and their repeated zoonotic transmission to humans has raised concerns about pandemics. The severity of H5N1-induced lung pathology may be due to increased viral replication and/or inflammatory responses.[1] In vitro, infection of macrophages and alveolar epithelial cells by H5N1 virus hyperinduces proinflammatory cytokines, compared to infection by H1N1 virus.[2,3] Deficiency of cyclooxygenase-2 (COX-2) in the host response to influenza results in less severe disease.[4] Moreover, COX-2 is hyperinduced in response to H5N1 infection, in addition to the proinflammatory cytokine cascade. Therefore, we aimed to investigate the role of COX-2 in H5N1 pathogenesis. We hypothesised that hyperinduction of COX-2 may play an important role in the pathogenesis of H5N1 virus infection.

Methods

This study was conducted from January 2007 to December 2008. The viruses used were A/Hong Kong/483/97 (H5N1 virus), a virus isolated from a patient with fatal H5N1 disease in Hong Kong in 1997, A/Vietnam/3212/04 (H5N1 virus), a virus isolated from a patient with H5N1 disease in Vietnam during 2004, and A/Hong Kong/54/98 (H1N1 virus).

Differentiated human macrophages or A549 cells were infected at an MOI=2. After 30 minutes of virus absorption, the virus inoculum was removed, and the cells were washed and then incubated in the corresponding medium.

To investigate the paracrine effects of virus-infected macrophages, supernatants of H5N1-, H1N1-, and mock-infected macrophages were collected at 6 hours post infection. The supernatants were filtered using a 100kDa filter (Millipore) to remove any virus and added to fresh (uninfected) macrophages or A549 cells. The macrophages or A549 cells were harvested for RNA to study COX-2 and cytokine gene expression.

To investigate the effects of selective COX-2 inhibitors on cytokine induction in macrophages or epithelial cells, the cells were pre-treated for 1 hour with nimesulide or NS-398 (Cayman) dissolved in a vehicle consisting of 0.1% DMSO prior to virus infection or application of filtered culture supernatant from infected macrophages. Cells were incubated in the presence of the selective COX-2 inhibitor or drug vehicle (as control) throughout the experiment.

Gene expression of COX-2 and cytokine was performed using real-time PCR. Total RNA was isolated and reverse transcripted. Transcript expression was monitored using a Power SYBR Green PCR master mix kit (Applied Biosystems) with specific primers. The fluorescence signals were measured in real-time during the extension step with MX3000P QPCR System (Stratagene). The ratio change in target gene relative to the β-actin control gene was determined by the 2-ΔΔCt method.

The presence of COX-2 in autopsy lung tissues of H5N1-infected patients

was detected by specific COX-2 antibody (Cayman) using the immunoperoxidase technique. For the co-localisation of COX-2 with macrophage and epithelial cell markers, the sections were incubated with CD68 (Dako) and AE1/AE3 (Dako) respectively, followed by incubation with FITC-conjugated donkey anti-mouse (Jackson Lab).

Results

As early as 3 hours after infection, COX-2 was markedly up-regulated in H5N1-infected macrophages (Fig 1a). The less virulent H1N1 virus induced COX-2 mRNA with similar kinetics as H5N1 virus but at much lower magnitude. As the induction of COX-2 in virus-infected cells is abolished by pre-treatment of cells with cycloheximide (data not shown), its induction is likely to be due to cellular mediators rather than direct stimulation by the virus. In contrast to virus-infected macrophages, COX-2 mRNA was not up-regulated in H5N1- or H1N1-infected human alveolar epithelial (A549) cells (Fig 1b).

To determine whether COX-2 was induced in patients with H5N1 infection, immunohistochemical study for COX-2 protein was performed on autopsy specimens from three H5N1-infected patients. In all three specimens, there was evidence of cytoplasmic immunostaining for COX-2 in the respiratory bronchial epithelial cells and pneumocytes (Figs 1c and 1d). The COX-2 expression co-localised with cytokeratin antigen demonstrated expression of COX-2 in basal cells and ciliated cells of the respiratory epithelium (Fig 1e). COX-2 was identified in cytokeratin positive alveolar epithelial cells (Fig 1f) but was not co-expressed with macrophages as identified by the macrophage marker, CD68 (Fig 1g). In contrast, normal lung controls showed negative or very weak cytoplasmic staining for COX-2 (Fig 1h).

An in vitro model was developed to investigate the proinflammatory cascade mimicking the macrophage-epithelial cell interaction. Culture supernatants of influenza A virus–infected human macrophages were collected at 6 hours after infection, filtered to remove any infectious virus, and added to uninfected A549 cells. COX-2 expression was markedly up-regulated in epithelial cells challenged with supernatant from H5N1-infected macrophages; more so than in those challenged with supernatants from H1N1-infected cells (Fig 2a). Comparable induction of a number of cytokines, including TNF-α (Fig 2b to 2g) was also observed.

Selective COX-2 inhibitor (nimesulide) was used to further investigate the role of COX-2 in the proinflammatory response induced by H5N1 virus. Expression of all tested cytokine mRNAs were significantly suppressed by nimesulide at 600 μM in both H5N1- and H1N1-infected macrophages (Fig 3a).

As the expression of viral M gene was also suppressed by nimesulide in a dose-dependent manner (Fig 3b), it is uncertain whether the observed reduction in the induction of cytokines was partly or wholly due to suppression in viral replication. To further investigate the role of COX-2 within the proinflammatory cascade (distinct from any confounding effect of viral replication), virus-filtered supernatants from human macrophages infected with influenza A virus with or without nimesulide treatment were added to uninfected epithelial cells and the consequential effect on cytokine expression was determined. Supernatant from influenza A virus–infected macrophages which had been pre-treated with nimesulide induced lower levels of cytokine expression in epithelial cells than did supernatant from nimesulide untreated macrophages (Fig 3c). Furthermore, treatment of uninfected epithelial cells with nimesulide (Fig 3d) led to a suppression of the cytokines induced by treating these cells with supernatants from influenza A virus–infected macrophages.

Discussion

In the current study, we demonstrated that effects of the proinflammatory cascade was rapid, and the proinflammatory mediators induced were more diverse than those induced by direct influenza virus infection. Moreover, lethal H5N1 viruses induced the proinflammatory cascade to levels that were much higher than those induced by a seasonal H1N1 virus. This may explain, to certain extent, why H5N1 virus is so pathogenic in humans. Autopsy tissue from patients with H5N1 disease showed an extensive induction of COX-2 and TNF-α in epithelial cells. As virus antigen was scarce in these lung tissues showing COX-2 and TNF-α expression, our findings support the hypothesis that the cytokine cascade sustains itself even when viral replication has been largely controlled.[5] As influenza virus infection of epithelial cells does not directly trigger COX-2 or TNF-α induction, the expression of these proinflammatory genes in the lung in the absence of significant viral replication is more likely due to a self-sustaining cytokine cascade rather than ongoing viral replication, at least in the later stages of the illness. Taken together, these findings support the hypothesis that excessively induced host inflammatory responses play a major role in contributing to the severity of human H5N1 disease.

Selective COX-2 inhibitors were able to attenuate the expression of a number of influenza A virus induced proinflammatory cytokines. Nimesulide was able to decrease the transcription of viral M gene, suggesting that nimesulide may also suppress viral replication. As the decrease in cytokine induction could be at least in part attributed to decrease in viral replication, to further demonstrate that COX-2 was indeed involved in mediating the induction of proinflammatory cytokines, we designed experiments to show that nimesulide reduced the induction of proinflammatory cytokines elicited by soluble factors derived from H5N1-infected macrophages. These experiments avoided the confounding effect of viral

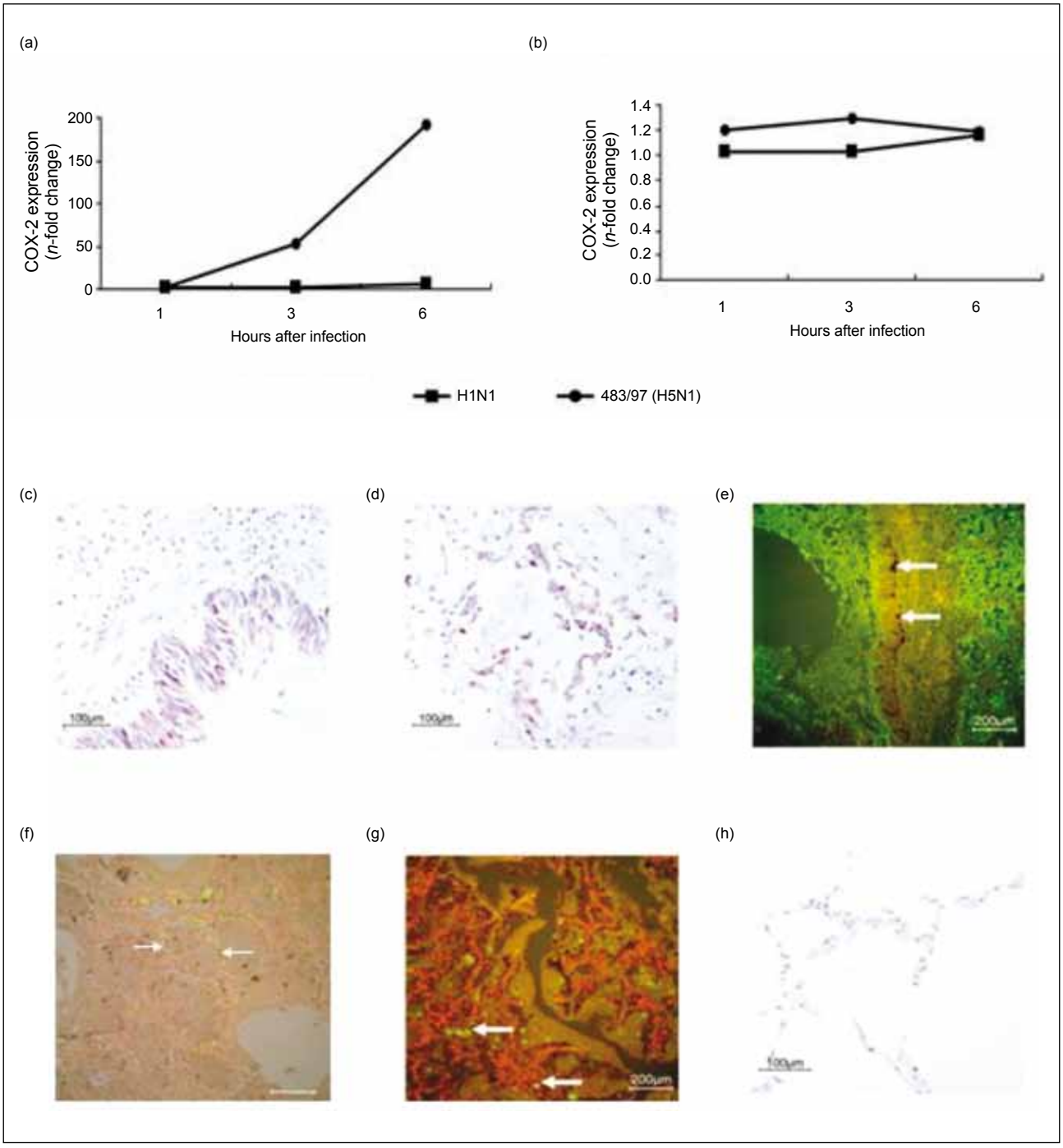


Fig 1. (a) In macrophages, cyclooxygenase-2 (COX-2) is markedly up-regulated by an H5N1 virus within 3 hours and further increased by 6 hours. An H1N1 virus induces a much weaker response. (b) In A549 epithelial cells, there is no induction of COX-2 by either virus. Data shown are fold change of COX-2 expression relative to mock-infected control after normalising to beta-actin in each sample. Representative data of duplicate experiments with means of triplicate assays are shown. COX-2 is expressed extensively in the lung epithelial cells of persons who died of H5N1 infection. Immunohistochemistry for COX-2 protein in H5N1-infected lungs shows strong cytoplasmic staining in (c) bronchial epithelial cells and (d) pneumocytes. (e) Double staining for COX-2 (DAB brown) and cytokeratin antigen (FITC green) in fatal H5N1 pneumonia demonstrates cytoplasmic staining of basal cells and ciliated cells (white arrows). COX-2 is expressed in (f) cytokeratin-positive alveolar epithelial cells (white arrows) but not in (g) macrophages as identified by the CD68 macrophage marker (FITC green) [white arrows]. (h) Representative data of two lung tissues from persons who died of non-respiratory causes show little or no staining for COX-2. Scale bar of double-stained images is 200 μm.

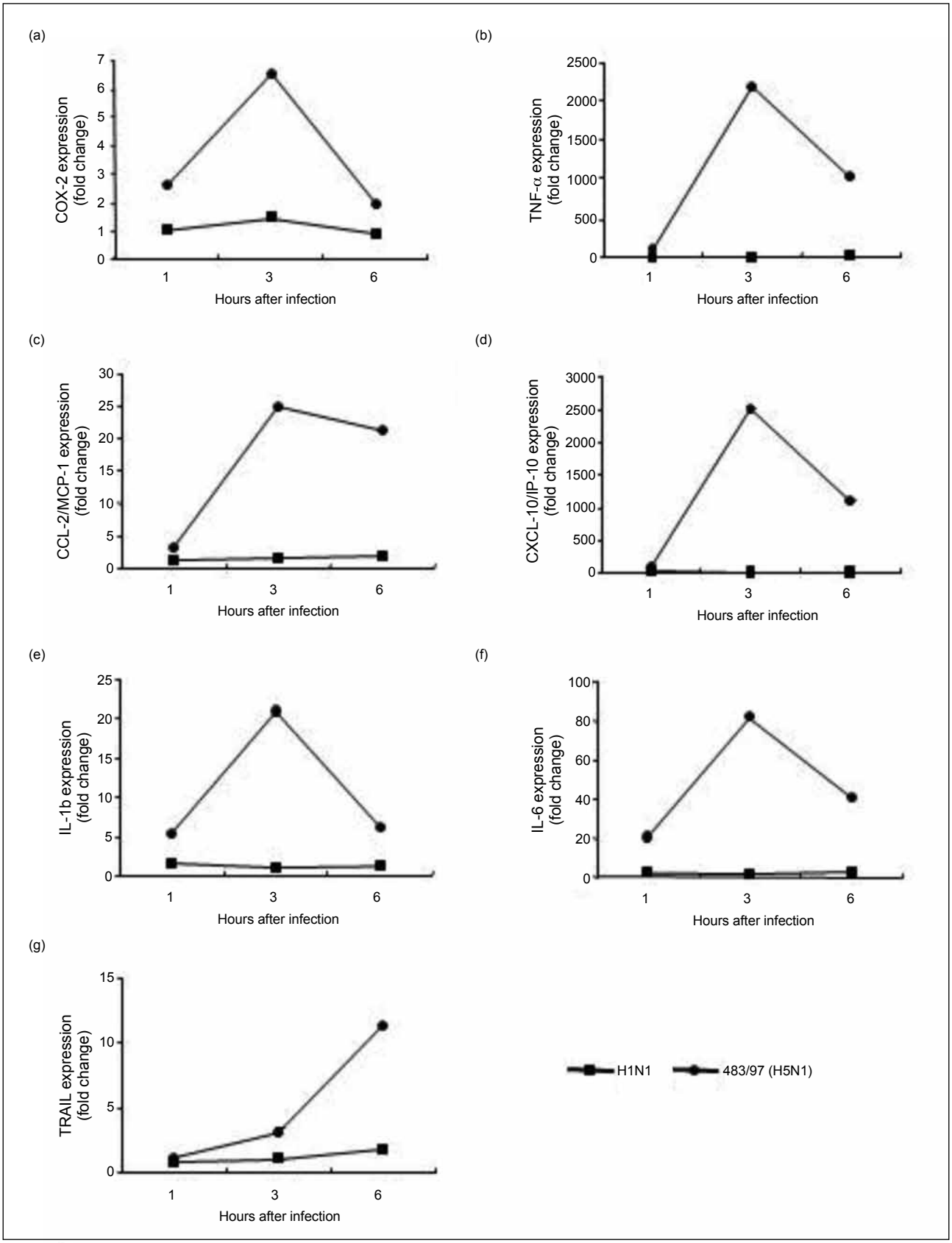
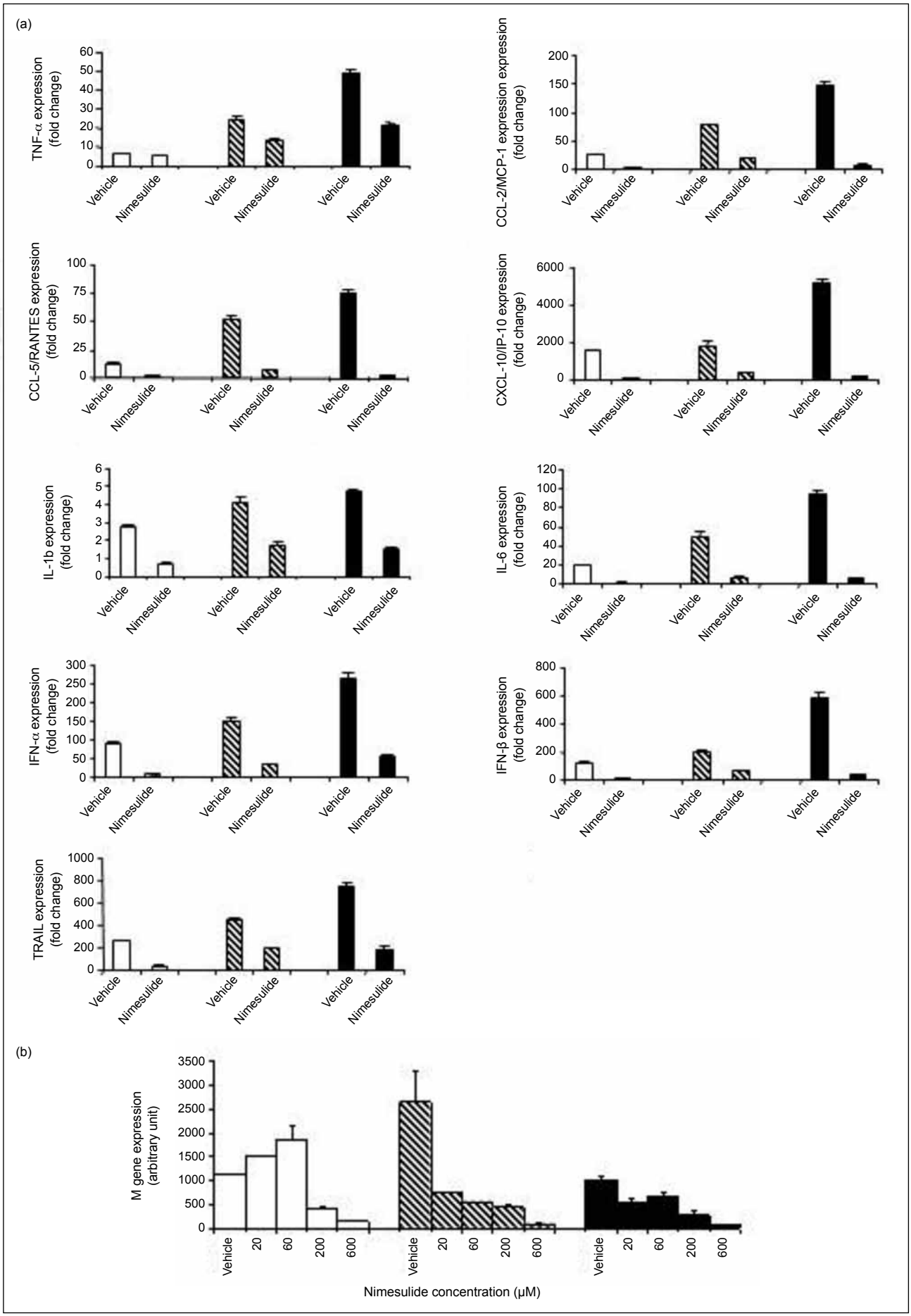


Fig 2. (a) Cyclooxygenase-2 (COX-2) expression is markedly up-regulated by the supernatants collected from H5N1-infected macrophages, peaking at 3 hours post-challenge. Supernatants collected from H1N1-infected macrophages cause only a small rise in COX-2 expression. Comparable findings are observed for other cytokines: (b) TNF-α, (c) CCL-2/MCP-1, (d) CXCL-10/IP-10, (e) IL-1b, (f) IL-6, and (g) TRAIL. Data shown are fold change of gene expression relative to mock-infected control after normalising to beta-actin in each sample. Representative data of duplicate experiments with means of triplicate assays are shown.


(a)
TNF-α expression (fold change)
CCL-2/MCP-1 expression expression (fold change)
CCL-5/RANTES expression (fold change)
CXCL-10/IP-10 expression (fold change)
IL-1b expression (fold change)
IL-6 expression (fold change)
IFN-α expression (fold change)
IFN-β expression (fold change)
TRAIL expression (fold change)
Vehicle
Nimesulide
(b)
M gene expression (arbitrary unit)
Vehicle
20
60
200
600
Nimesulide concentration (μM)

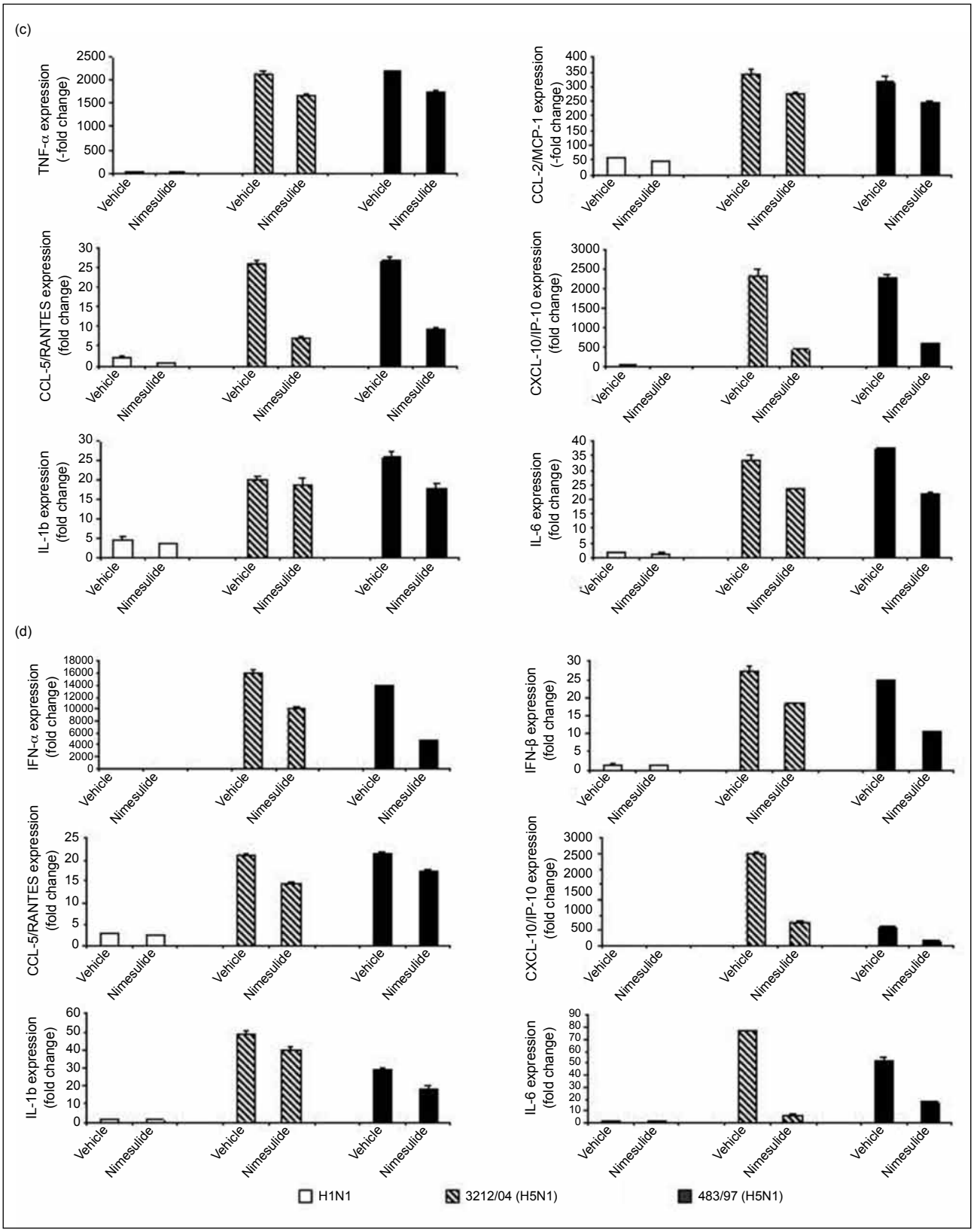


Fig 3. (a) A panel of cytokines tested show attenuation in expression level at 600 μM nimesulide after influenza A infection in macrophages. (b) Viral M gene transcription in influenza A–infected macrophages is attenuated by nimesulide in a dose-dependent manner. Cytokine expression is attenuated within the proinflammatory cascade by nimesulide. (c) Cytokine expression is attenuated in epithelial cells challenged with supernatants from influenza A–infected macrophages, which have been treated with 200 μM nimesulide. (d) Treating epithelial cells with 200 μM nimesulide show similar attenuation in cytokine expression induced by supernatants from influenza A–infected macrophages. Data shown are fold change of gene expression relative to corresponding mock after normalising to beta-actin in each sample. Representative data of duplicate experiments with means of triplicate assays are shown.

infection, as the macrophage supernatants were filtered to remove the virus. The current paradigm is that COX-2 is induced by cytokines.[5] We demonstrated that COX-2 drove and maintained the proinflammatory cascade via a complex positive feedback loop during H5N1 infection.

At present, early antiviral therapy by oseltamivir is the mainstay for managing patients with H5N1 disease. However, the clinical response to antiviral therapy has been variable. This can be attributed to a number of factors, including delayed commencement of therapy, development of antiviral resistance, and poor bio-availability of the oral drug in severely ill patients. The cytokine cascade was maintained even in the absence of significant virus infection in the lung. Thus, in addition to antiviral therapy, interventions to selectively modulate this cascade may be helpful. One such potential intervention is the inhibition of the COX-2 pathway, which may attenuate the proinflammatory cascade and possibly the pathology associated with it. Such an approach may be more beneficial than attenuating the action of a single cytokine such as TNF-α using direct antagonists. COX-2 inhibitors are either already registered for clinical use or undergoing late phase clinical trials, and may also have the added benefit of inhibiting viral replication.

Acknowledgements

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Development of anti-influenza A compounds: a pilot studyl

Key Messages

1. There is no effective anti-H5N1 avian influenza agent.
2. A chemical compound—BFDBSC—can inhibit H5N1 virus infection in cell cultures, and such inhibition might be attributable to its halogenated benzoyl residues.
3. This pilot study assessed anti-H5N1 activity and toxicity of four chemical compounds with halogenated benzoyl residues in cell culture system.
4. Two compounds—FP-BFDBSC and BFB-gallate—showed higher antiviral effects than BFDBSC, whearas the other two—BFB-borneol and BFB-menthol—showed lower antiviral effects. These compounds did not show toxicity.
5. The halogenated benzoyl residues may play a key role in anti-H5N1 effects. However, all these compounds showed poor solubility, which may limit their utility.

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Introduction

A highly pathogenic avian influenza A (H5N1) virus has caused severe disease in humans. The first outbreak was in Hong Kong in 1997 and caused six deaths in 18 infected patients.[1] Subsequently, a re-emergence of human H5N1 disease causing two deaths was reported in a family of five members who visited Fujian, China from Hong Kong in January 2003.[2] Thereafter, human infections with H5N1 viruses continued to be identified in many countries. From January 2004 to October 2006, the virus has afflicted 253 patients and claimed 148 lives, including 18 cases with 13 deaths in China.[3] Most of these cases entailed direct exposure to H5N1 virus-infected poultry; the virus was endemic in poultry in Asia since 2003.[4] The virus has spread along migratory flyways linked to Southeast Asia, Siberia, the Middle East, Africa and Europe.[5] Although genetic analysis of H5N1 virus isolated from humans in 1997, 2004, and 2005 revealed that all genes were of avian origin, limited person-to-person transmission was identified during the 1997 outbreak in Hong Kong, during 2004 in Thailand,[6] during 2005 in northern Vietnam, and during 2006 in Indonesia. Taken together, the risk of a pandemic caused by a reassortant virus with efficient and sustained human-to-human transmissibility has increased.

Although an anti-influenza drug—oseltamivir—has been effective against H5N1 in animals,[7] methylprednisolone and oseltamivir did not show obvious clinical benefits in some H5N1 patients.[8,9] Notably, H5N1 is resistant to M2 inhibitors and may rapidly develop resistance to neuraminidase inhibitors.[10] Considering the high mortality rate (>50%) associated with H5N1 avian influenza, there is an urgent need to develop new drugs to combat this disease.

In collaboration with Dr Ulrike Holzgrabe from University of Wurzburg, Germany, we synthesised and tested two chemical compounds—DBSC and BFDBSC—for their anti-H5N1 effects in cell culture system. DBSC did not show antiviral activity, whereas BFDBSC showed anti-H5N1 effect at a 50% inhibitory concentration (IC_{50}) of 80 μM, with a 50% cytotoxic concentration (CC_{50}) of about 7500 μM in Madin-Darby canine kidney (MDCK) cells and no toxicity in Balb/c mice with dose up to 5 mg/kg. As the non-halogenated mother compound, DBSC contained selenium and did not show anti-H5N1 activity, selenium in BFDBSC was also unlikely to show antiviral activity. Instead, the antiviral activity might be attributable to the compound's halogenated benzoyl residues. We therefore synthesised lipophilic bis-(p-fluorophenacyl) ester of BFDBSC (FP-BFDBSC) and three 4-bromo-2-fluorobenzoyl esters (ie BFB-borneol, BFB-menthol, and BFB-gallate) and tested their antiviral activity and toxicity in cell culture system.

Methods

FP-BFDBSC, BFB-borneol (from (1S)-(-)-borneol), BFB-menthol (from natural (-)-menthol) and BFB-gallate (from gallic acid) were chemically synthesised as described previously.[11]

The MDCK cells were used for culture and titration of H5N1 virus (A/Vietnam/1194/04) as described previously.[1,2,12,13] Briefly, about 90% confluent MDCK cells were infected with the virus at 37ºC for 1 hour, and then the virus solution was replaced by MEM with 1% FBS. After 48 to 72 hours at 37ºC,

the supernatants were collected, pooled, and stored at -80ºC for further study. The titres of virus in the supernatants were determined by $TCID_{50}$ assay based on observation of cytopathic effects after 72 hours of culture and/or a haemagglutination assay using turkey red blood cells.[1,2,12,13]

The compounds were serially two-fold diluted to seven dilutions and individually mixed with 100 $TCID_{50}$ of H5N1 virus at 1:1 ratio. The mixtures were inoculated to 96-well plates of MDCK cell cultures in triplicates. Compounds BFDBSC and DBSC were included as positive and negative controls, respectively. Serials of two-fold dilution of the compounds were also inoculated into the cell cultures as cytotoxicity controls of the compounds. Cytopathic effects in the cultures were monitored daily, and the supernatants were collected 48 hours post infection. The released virus in the supernatants was titrated using $TCID_{50}$,[1,2,12,13] whereas copies of viral RNA in the supernatants were measured by real-time RT-PCR as described previously,[1,2,12-17] Briefly, total RNA in the supernatants was extracted using RNeasy Mini kit (Qiagen, Germany) and reverse transcribed to cDNA using applied SuperScript II Reverse Transcriptase (Invitrogen, USA) and a primer 'Uni12' 5'-AGC AAA AGC AGG-3'. H5N1 viral NP gene was measured by SYBR green Mx3000 Real-Time PCR System (Stratagene, USA), using primers NP-Forward: 5'-GAC CAG GAG TGG AGG AAA CA-3', NP-Reverse: 5'-CGG CCA TAA TGG TCA CTC TT-3'.

The compounds diluted from the highest concentrations were inoculated to MDCK and Vero cells. Their potential toxicity was monitored by methylthiazolyldiphenyltetrazolium bromide and their 50% cytotoxic concentration (CC_{50}) was determined as described previously.[18] Amanitin at 30 μg/ml was used as a toxic control.

Results

Anti-H5N1 virus effects of the synthesised compounds (FP-BFDBSC, BFB-borneol, BFB-menthol, and BFB-gallate) were determined in MDCK cell cultures. BFDBSC and BDSC were included as positive and negative controls, respectively. One hour pre-incubation with different concentrations of the compounds potently inhibited the replication of H5N1 virus in MDCK cell cultures as measured by real-time RT-PCR (Fig a) and back titration using $TCID_{50}$ (Fig b). Two of the synthesised compounds (FP-BFDBSC and BFB-gallate) showed stronger antiviral effects than BFDBSC (positive control), whereas the other two compounds (BFB-borneol and BFB-menthol) showed less antiviral effects (Table 1). Notably, all these compounds were not soluble in water and had to be dissolved in DMSO. When the compounds in DMSO were further diluted in culture medium or PBS, BFB-borneol and BFB-menthol showed lower solubility than FP-BFDBSC and BFB-gallate (Table 2).

All the synthesised compounds at their maximum soluble concentration did not show toxicity in MDCK cell cultures (Table 2). However, the toxicity test was limited by low solubility of the compounds.

Discussion

All four synthesised compounds exhibited antiviral effect against H5N1 infection in MDCK cell cultures. The antiviral activity may be attributable to the halogenated benzoyl residues. Although antiviral effects of FP-BFDBSC and BFB-gallate were about 1-fold higher than that of the positive control (BFDBSC), further studies and applications were limited by their poor solubility, which was about 7- to 10-fold lower than that of the positive control. Thus, new

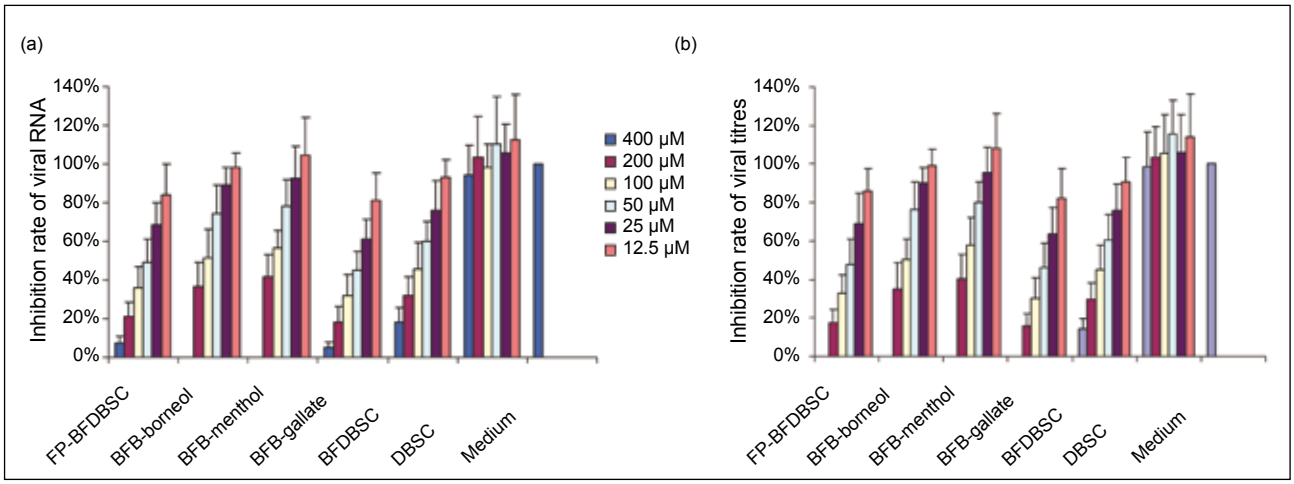

Fig. Inhibition of H5N1 virus infection in Madin-Darby canine kidney (MDCK) cell cultures
Six compounds are mixed with 100 $TCID_{50}$ of H5N1 virus and then inoculated to MDCK cells in triplicates. Antiviral effects of the compounds were determined by measuring viral RNA copies (relative viral RNA copies yielded as compared to untreated controls) using (a) real-time RT-PCR and (b) viral titres (relative infective virus yielded as compared to untreated controls) by titration using $TCID_{50}$ in culture supernatant 48 hours post infection. Three triplicates are tested.

Table 1. 50% inhibitory concentration (IC_{50}) of the compounds against H5N1 viral infection

Compounds	Mean±SD IC_{50} (μM)	
	Real-time RT-PCR	$TCID_{50}$
BFB-borneol	49.2±5.3	48.1±4.8
BFB-menthol	106.8±17.6	102.4±19.3
FP-BFDBSC	172.5±18.8	177.4±17.5
BFB-gallate	45.4±7.1	46.8±5.5
BFDBSC	82.6±8.7	80.8±7.2

Table 2. Solubility and toxicity of the compounds

Compounds	Solubility in 10% DMSO-PBS (μM)	50% cytotoxic concentration (μM)
BFB-borneol	2000	>2000
BFB-menthol	200	>200
FP-BFDBSC	200	>200
BFB-gallate	3000	>3000
BFDBSC	20 000	7478±127

compounds containing the halogenated benzoyl residues with higher solubility should be designed. Furthermore, as over 80% of human influenza infections in Hong Kong appear resistant to the current anti-influenza drug Tamiflu, it is also worth evaluating any potential emergence of drug resistance for the newly designed compounds. This preliminary study has provided the theoretical and practical basis for development of new drugs to combat H5N1 avian influenza.

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Effects of built environment on walking among Hong Kong older adultsI

Key Messages

1. Reliable and valid interviewer-administered questionnaires were developed to investigate associations of perceived neighbourhood attributes of Hong Kong older adults with their walking for transportation and recreation.
2. Access to and availability of different types of services and destinations, provision of facilities for resting/sitting in the neighbourhood, and easy access to/from residential buildings may help maintain an active lifestyle by facilitating walking for transport in the neighbourhood.
3. Access to services, indoor places for walking, environmental aesthetics, low traffic, and absence of physical barriers may promote recreational walking.

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Introduction

The number of senior residents (aged 65+ years) in Hong Kong is projected to reach 2.3 million in 2013, corresponding to 27% of the total population.[1] Regular engagement in physical activity contributes to healthy ageing. Walking is recommended for seniors because it is versatile, affordable and relatively safe. The accrual of 30 minutes of walking on most days of the week is deemed to have significant health benefits.[2]

The neighbourhood environment plays a decisive role in facilitating residents' walking.[3,4] Knowledge of built environmental characteristics conducive to an active lifestyle can inform policies on public health, land use, and transportation. Neighbourhood safety, aesthetics, access to facilities, and street connectivity (number of intersections) are positively related to walking in seniors.[4] In western countries, the impact of the neighbourhood environment on the physical activity levels of seniors has been studied. It is unknown whether the associations can be generalised to an Asian urban context, which is markedly different in culture and built form. Hence, this study aimed to develop and validate instruments for investigating the associations between the built environment and walking in senior residents of Hong Kong and other similar Chinese cities, and to provide effects of perceived attributes of the neighbourhood on walking for different purposes.

Methods

This study was conducted from October 2006 to July 2008. It consisted of two stages: (1) translation/development and validation of self-report instruments of the perceived neighbourhood environment, and (2) assessment of walking within and outside the neighbourhood. The original measures for adaptation to a Chinese-speaking senior population were the abbreviated Neighbourhood Environment Walkability Scale (NEWS-A)[3] and the walking section of the Neighbourhood Physical Activity Questionnaire (NWQ).[5]

A multi-disciplinary panel of experts adapted the original NEWS-A to reflect the built environment of Hong Kong and needs of seniors. The adapted NEWS-A and NWQ were translated into Chinese, back-translated into English, and pilot tested on a sample of Hong Kong seniors aged 65+ years who were able to walk without assistance, had no cognitive impairment, and resided in a priori selected locations. They were recruited from Elderly Health Centres (EHC) in Wan Chai, Nam Shan, Tseung Kwan O, and Yuen Long.

These EHCs were selected based on walkability and socio-economic status (SES) of their catchment areas (districts). Based on information from the Census and Statistics Department and the Centamap (www.centamap.com), median household income and percentage of owner-occupiers were used as measures of SES, whereas household density, intersection density, and commercial and service destinations density were used as measures of walkability. Eight street blocks were randomly selected in each area (total 32 street blocks). The median household income ranged from HK$12 200 to 25 000 and the percentage of owner-occupiers ranged from 42% to 62%. Approximately 15 EHC members

were recruited from each street block.

The Chinese-versions of the NEWS-A and NWQ (hereafter named NEWS-ACS, and NWQ-CS, respectively), and the long version of the International Physical Activity Questionnaire (IPAQ)[3,5] were interviewer-administered to consenting participants (recruitment rate, 78%). Socio-demographic information was also collected. Participants were asked to wear an accelerometer (motion sensor) for a week, keep a diary of walks, and be reassessed 2 weeks after the first assessment. Of 96 consenting participants recruited (three per street block), 94 provided valid accelerometry/diary data. The built environment (400 m radius circles around 32 street blocks corresponding to ~13-minute walking distance for Hong Kong older adults) was collected via environmental audits.

The original NEWS-A was a 54-item questionnaire assessing perceived environmental characteristics related to walking.[3] The NEWS-ACS included 22 additional items describing features of the environment relevant to the study setting and target population. The NWQ-CS consisted of the same items of the original NWQ, which assessed usual walking within and outside the neighbourhood (defined as a 10-15 minute walk from home).[5] Participants reported the frequency, duration, destinations of walking for recreation and transport within and outside the neighbourhood. Physical activity was assessed using the long version of the IPAQ,[3,5] which consisted of five domains: work, transportation, housework and house maintenance and caring for family, leisure-time, and sitting. Respondents reported the number of days per week and the time per day they usually spent doing these activities.

A weekly diary of walks was used to record within and outside neighbourhood walking. Accelerometers (motion sensors), worn for a week during waking hours, were used to measure ambulatory activities. The numbers of minutes of non-sedentary activity, within the timeframe of each walking trip/period recorded in the diary, were computed for each participant and summed for each of the four categories of walking for transport and recreation within and outside the neighbourhood. A validated audit tool (modified for Hong Kong so as to provide data comparable to those collected via the NEWS-ACS) was used to collect multiple-rater objective environmental data on the participants' neighbourhood environment (every street within a selected 400 m buffer zone).

Results

There were 50 subjects in the pilot study and 484 (283 women and 201 men; 67% aged 65-74, 31% aged 75-84, and 3% aged 85+) in the validation study.

Factor analysis indicated that the NEWS-ACS consisted of 13 multi- and 4 single-item inter-correlated constructs: residential density, heterogeneity of land use, access to services, physical barriers to walking, street connectivity, human and motorised traffic, infrastructure for walking, indoor places for walking, aesthetics, social disorder/litter, traffic speed, presence of people, crime, fence separating traffic from sidewalks, bridge/overpass connecting to services, easy access of residential entrance, and sitting facilities. Most items showed moderate-to-excellent levels of test-retest reliability (intraclass correlation [ICC], >0.40).

Regression models were used to examine the associations of perceived neighbourhood attributes (measured by the NEWS-ACS) with matching attributes from objective environmental audits. Most correlations were significantly positive and ranged from small ($0.11>r>0.24$) to large ($r>0.37$). This suggested that perceived neighbourhood attributes could be proxies of objective environmental characteristics.

Regression models were used to examine the extent to which perceived neighbourhood attributes (measured by the NEWS-ACS) were related to walking for different purposes (measured by the NWQ-CS), adjusting for demographic characteristics and total weekly minutes of physical activity other than walking (measured by the IPAQ) [Table]. Absolute values of >0.37 represent strong associations, whereas values of 0.10 to 0.24 represent moderate associations. Weekly minutes of walking for transport was positively associated with perceived heterogeneity of land use, access to services, human and motorised traffic, crime, easy access to residential entrance, and sitting facilities in the neighbourhood. Walking for recreation was positively associated with access to services, street connectivity, indoor places for walking, and bridge/overpass connecting to services. Physical barriers to walking was negatively associated with human and motorised traffic. These

Table. Associations of perceived neighbourhood attributes with self-reported weekly minutes of walking within the neighbourhood

Attribute	Walking for transport (*r*)	Walking for recreation (*r*)
Residential density	0.07	-0.06
Heterogeneity of land use	0.15‡	0.01
Access to services	0.11*	0.10*
Physical barriers to walking	0.06	-0.09*
Street connectivity	0.06	0.09*
Human and motorised traffic	0.12†	-0.09*
Infrastructure for walking	0.06	0.01
Indoor places for walking	0.07	0.12†
Aesthetics	0.01	0.10*
Social disorder/litter	0.06	0.01
Traffic speed	-0.05	-0.07
Presence of people	0.03	-0.06
Crime	0.11*	0.01
Fence separating traffic from footpath	0.01	0.03
Bridge/overpass connecting to services	-0.03	0.09*
Easy access of residential entrance	0.11*	0.08
Sitting facilities	0.20‡	0.03

* P<0.05
† P<0.01
‡ P<0.001

findings indicated that some environmental characteristics influenced the walking behaviour of Hong Kong seniors.

Moderate-to-excellent test-retest reliability was noted for all NWQ-CS items, except for weekly minutes of walking for transport outside the neighbourhood. The same variable had the weakest association with walking measured via an accelerometer/diaries (ICC=0.35, P<0.001) owing to the participants' tendency to underreport this type of walking in the NWQ-CS. No other significant differences were noted between mean weekly minutes of walking collected using the NWQ-CS and accelerometry/diary data. The associations between NWQ-CS and accelerometry/diary measures of other types of walking ranged from 0.52 to 0.77. Overall, the associations were higher than those observed for other self-reported measures of physical activity, and this supported the validity of the NWQ-CS.

Discussion

Accelerometry/diary data recorded walking in the last 7 days, whereas the NWQ-CS measured habitual walking. This might have yielded lower levels of concurrent validity than when the NWQ-CS had captured walking in the last 7 days. Studies using the NWQ-CS as a measure of context-specific walking for different purposes need to interpret estimates of transport-related walking outside the neighbourhood with caution.

Validity of the NEWS-ACS was based on associations between perceived environmental characteristics and walking behaviour. Access to and availability of services,[3,4] access to residential entrance, and the presence of sitting facilities in the neighbourhood were potential determinants of walking for transport in Hong Kong seniors. Several other perceived environmental attributes tended to be related to walking for transport in the expected direction. Perceived crime, and human and motorised traffic were positively associated with walking for transport. This may be due to the fact that residents may be more aware of crime in their local areas, and destination-dense, busy areas tend to attract more crime and have higher levels of traffic.[3] Environmental aesthetics (presence of greenery and attractive sights), access to services, street connectivity, indoor places for walking, and bridge/overpass connecting to services were potential determinants of recreational walking.[3,4] In contrast, human/motorised traffic and physical barriers to walking were possible deterrents of recreational walking.[3] Although most correlates of walking in older adults were similar to those observed elsewhere and in younger cohorts,[3,4] neighbourhood attributes that were peculiar to Hong Kong (access to residential entrance—lifts in high-rise buildings) and seniors (facilities for sitting and resting) were identified. Thus, an environment supportive of walking may help Hong Kong older adults maintain an active lifestyle.

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Physical activity for children in special school environmentl

Key Messages

1. We assessed children's physical activity (PA) in structured (physical education) and unstructured (recess, lunch, before and after school) periods in special schools and examined its association with modifiable area contextual characteristics.
2. Children with disabilities were not highly active, but were more active during recess and lunch periods than at other times including physical education classes.
3. Areas were often not accessible during unstructured settings. Children were more active in areas when supervision and organised activities were provided.
4. Providing an interactive game during free play did not significantly increase group's PA.
5. Children's PA accrual is influenced by contextual characteristics of the school environment. There is a need to make areas more accessible and to use social marketing and programming to attract more users. School and health professionals should modify contextual characteristics by providing more direct supervision and organised activities during free play.

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Introduction

Physical activity (PA) is an important part of healthful lifestyle for children. Most children with disabilities are insufficiently active.[1] To minimise health risks associated with sedentary living among children with disabilities, health professionals should know about the settings in which children with disabilities accrue PA.[2]

Schools are important settings for promoting PA during structured (physical education) and unstructured (recess, lunch, before and after school) periods. School environments such as space size, equipment, and supervision are closely linked with PA accrual by children.[3] Providing children with prompts for PA by supervisors and adding game equipment may be effective in increasing their PA levels at school.[4]

This study aimed to examine the special school environments in promoting PA among children with disabilities. Children's PA was measured throughout the school day, and its association with contextual variables determined. In addition, the influence of innovative electronic game equipment on children's activity levels was assessed in a small-scale intervention study.

Methods

This study was conducted from December 2007 to December 2009. It consisted of an observational study and an interventional study.

The observational study was conducted on 5 normal school days in 10 special schools. Observations were delimited to schools for children with sensory impairment, physical disabilities, mild or moderate-to-severe intellectual disabilities, and impaired social development. Systematic observation was carried out throughout the day (ie during physical education, recess, lunch, before school, and after school). Each target area that permitted PA was observed periodically using a scanning technique. The activity level of each boy and girl was coded as sedentary (lying down/sitting/standing), walking, or vigorous, thereby energy expenditure could be estimated. The environmental characteristics of each target area were recorded in terms of accessibility (eg not locked), usability (eg not excessively wet or roped off for repair), and presence of equipment (eg balls and jump ropes provided by the school), supervision (ie closely monitored by school staff), and organised activities (eg a scheduled event or exercise class led by school staff). A total of 135 physical education lessons, 115 recess periods, 50 lunch periods, and 50 before- and 50 after-school periods (400 area observations and 7074 child observations) were made in 67 different activity areas on 5 normal school days over 3 months. Data were collected between mid February and April 2008.

For the intervention study, three special schools for children with mild intellectual disabilities were randomised to the intervention (n=2) and control (n=1) groups. Each intervention school received the J-mat running game (an interactive electronic game) for children to play in a predetermined target area. Children were allowed to use the game during free time. All schools had no extra game equipment at baseline. The control school received no game and continued with their usual programmes. A total of 150 area observations and 5335 child

observations were made during 5 normal school days over a month at baseline and 3-month post-intervention.

The System for Observing Play and Recreation in Communities[5] (SOPARC) was modified to document children's PA in structured and unstructured settings. It is a reliable measure for assessing children's PA in diverse and open activity environments (target areas) in both indoor and outdoor settings. Six observers were trained to use the SOPARC guidelines. Prior to data collection, reliability tests were conducted on 1 normal school day in each participating school by multiple independent observers. Inter-observer agreement for the number of area users was 97% for both girls and boys, for characteristics of the target areas were 98% for girls and 97% for boys, and for activity levels were 97% for girls and 96% for boys.

Results

A total of 1230 and 5844 child observations were made on 5 observation days during 135 physical education lessons and during free play periods, respectively. Overall, children were sedentary about half the time during physical education and free play (Fig 1). Children were less sedentary (P<0.0001) and were more likely to engage in walking (P<0.0001) during free play than physical education.

Some activity areas at lunch, as well as before and after school were vacant (data not shown). When summing the walking and vigorous categories, boys engaged in moderate-to-vigorous PA (MVPA) more often than girls before school (P<0.0001) and during recess (P<0.0001), but less often after school (P<0.05). Children with sensory impairments and mild intellectual disabilities tended to engage in more MVPA during physical education than those with moderate-to-severe disabilities (P<0.01) [data not shown].

A total of 67 target areas in the 10 special schools were identified, and the contextual characteristics (ie accessibility, usability, and presence of supervision, activity organised, and equipment) of each area were assessed during each visit (ie 265 observations x 67 areas = 17755 area visits). Overall the target areas were accessible to students during 47.1% (standard deviation [SD], 23.9) of the area visits. Meanwhile, target areas were usable 92.3% (SD, 11.0) of the observations, whereas 18.1% (SD, 13.7) were supervised, 2.8% (SD, 4.8) provided organised activities, and 32.5% (SD, 30.7) were equipped. Figure 2 illustrates these characteristics for the four free play periods. Areas were more likely to be accessible and supervised during recess periods than others (P<0.01).

A summary score for activity intensity—energy expenditure rate (EER)—was estimated for each unstructured setting using a standard calculation.[5] Overall, children were more physically active (ie had a higher mean EER) when the activity areas were supervised (and before and after school) and being organised (and at lunchtime and after school) [Table].

The mean EER during overall free play periods was significantly higher in children in intervention schools than in the control school (P<0.01). Overall, children had a significant reduction in mean EER during free play periods (P<0.01), before school (P<0.05), and during recess (P=0.001), indicating the minimal effects of the interactive electronic game on children's activity accrual.

Discussion

In unstructured settings, children's PA behaviour is voluntary, spontaneous, and intermittent in nature. Their participation in PA is therefore highly related to the

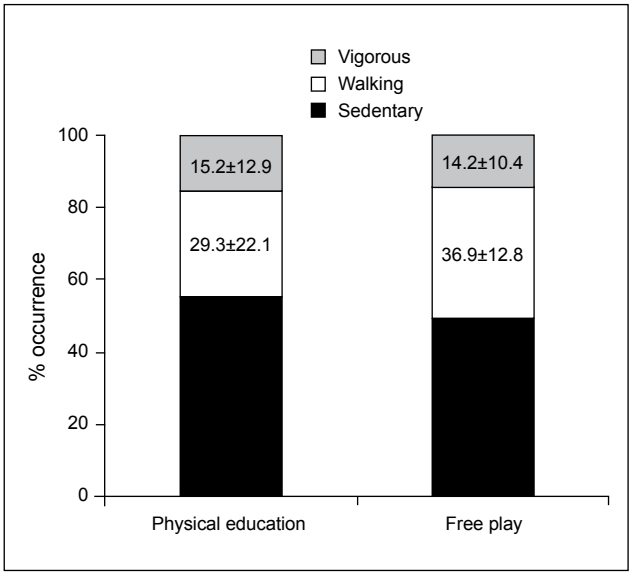

Fig 1. Proportion of children in three activity levels during physical education and free play settings

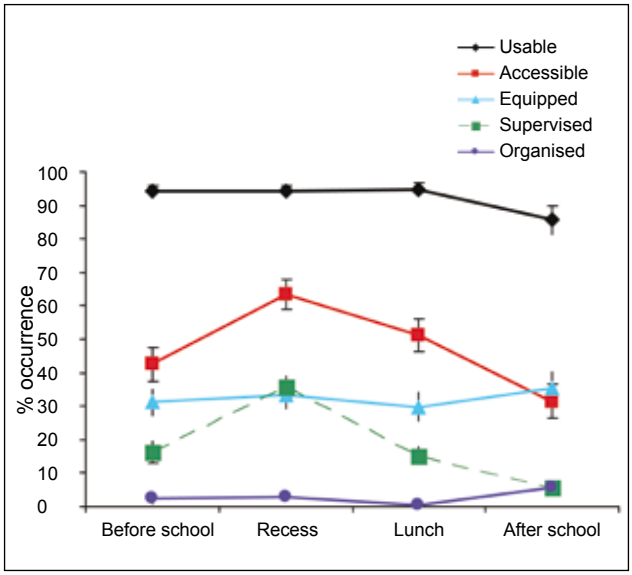

Fig 2. Contextual characteristics of 67 activity areas during free play times

Table. Associations (Spearman's rho) of contextual characteristics of activity areas with activity levels (mean estimated energy expenditure rate [kcal·kg^{-1}·min^{-1}]) in unstructured settings

Contextual characteristics	Overall	Before school	Recess	Lunch	After school
School size (m^2)	0.01	-0.31*	-0.33*	0.08	0.35*
% Accessibility	-0.03	0.26	0.15	0.01	-0.24
% Usability	-0.12	-0.06	0.46†	-0.20	-0.26
% Supervision	0.72‡	0.64‡	-0.01	0.47†	0.32*
% Activity organised	0.49‡	0.02	-0.15	0.36†	0.58‡
% Equipment	0.12	0.08	0.03	-0.12	-0.13

* P<0.05
† P<0.01
‡ P<0.0001

surrounding environments, especially when opportunities and prompts for PA are immediately available.[4] Potentially modifiable contextual characteristics varied greatly during unstructured settings. Despite usable about 90% of the time, the activity areas were far less often accessible, equipped, supervised, and provided organised activities. Children with disabilities were more active in the areas when supervision and organised activities were provided. Teachers or playground supervisors should promote children's active behaviour by providing more choices of organised activities to facilitate their MVPA while ensuring safety. However, provision of an innovative game session was not effective in facilitating active behaviour during free play periods. The J-mat running game only involved one child completing a game in a 5-minute interval. Only a limited number of children could play in such a short period, while others were mere observers. A more sophisticated intervention design and providing more game equipment in several activity areas might result in a greater impact on children's activity levels. Other forms of PA interventions at school, in particular those involving the family, need to be further explored.

Children's activity accrual is influenced by school contextual characteristics. In special schools, areas are often not accessible, for which policy changes should be implemented to enhance accessibility. In addition, areas are frequently vacant and it is important for school policy makers to attract more users through social marketing and programming. There is room for modifying programming to provide more active games and for teaching playground supervisors to promote PA. Future research could consider including more special schools and extending longer observation periods. Studies to examine the PA level of children with special needs attending inclusive/mainstream schools are also needed.

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Risk factors and outcomes of childhood obesity in Hong Kong: a retrospective cohort studyl

Key Messages

1. Onset of obesity is related to age, gender, pubertal stage, dietary habits, and parental occupation. Targeting the high-risk groups may help curb obesity in children.
2. Obesity may lead to poor self-esteem and potential psychosocial risk. The psychosocial impact of obesity could be more pronounced in girls than boys.
3. The association between obesity and psychosocial health could be bi-directional. Improving psychosocial health could be beneficial in weight management for normal-weight and obese children.
4. Obesity is associated with higher blood pressures.

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Introduction

Childhood obesity has become a major public health concern in Hong Kong. The prevalence of obesity among secondary and primary school students has been increasing, reaching 17.9% in 2003/04. The problem is more severe in primary schools and in boys than girls.[1]

The obesity pandemic is largely due to over-eating and under-activity. Obesity is also socially patterned; socioeconomic status may contribute to the temporal rise.[2] Obese children are more likely to experience psychological and physiological problems.[3] Persistent obesity from childhood predicts higher risks of morbidity and premature mortality in adulthood, which increases public health expenses in the long run.[4]

The Student Health Service (SHS) of the Department of Health has offered health assessments for local primary and secondary school students since 1995/96. Its readily accessible data facilitate retrospective cohort studies. The SHS has a consistently high overall participation rate (>80%). Hence, this database is representative of the primary school children in Hong Kong. The present study used the SHS database to investigate the prospective relationship between potential risk factors and childhood obesity. Possible health consequences of obesity later in childhood and early adolescence were also identified.

Methods

This retrospective cohort study was conducted from August 2007 to October 2008. All primary (P) 4 students (n=114 947) aged 8 to 12 years who participated in the SHS in the school years of 1998/1999 and 1999/2000 were included. The records of these students in subsequent academic years were traced until secondary (S) 2, using unique identifiers.

Height, weight, and blood pressure were measured by well-trained health care workers or nurses following standard protocols. Pubertal development was assessed by trained doctors using the progressive rating method based on Tanner stages of pubic hair development. Both blood pressure and puberty were assessed at odd-numbered grades only (ie P5, S1, S3, and S5).

Weight groups were determined by both the International Obesity Task Force (IOTF) standard and the local weight-for-height (WFH) reference. Under the IOTF standard, BMI cutoffs for childhood overweight and obesity are ≥25 and ≥30 kg/m^2, respectively, which are equivalent to those for western adults at age 18 years. For the local WFH reference, obesity, irrespective of age, was defined as above 120% of the median figure derived from Hong Kong reference data.

Starting from P4, every participant of even-numbered school grade (P4, P6, S2, S4, and S6) completed a self-administered standardised health assessment questionnaire. Closed answers were used to assess dietary and physical activity behaviours. Variables for analysis included four questions on dietary habits (milk consumption, breakfast habit, junk food intake, and fruit/vegetable intake), one on physical activity (frequency of aerobic exercise), and another on inactivity

(TV time). The last 60 questions in the primary school questionnaire addressed psychosocial health using the Chinese version of the form A of the culture-free Self-Esteem Inventories (SEI) for Children, which included four subscales: general self-esteem (SE), social SE, academic SE, and parent-related SE; the sum of these was total SE. According to SHS guidelines, students with a total score of ≤19 or having 'very low' scores in any subscale were classified as being in the 'high psychosocial risk' group. The last 112 questions in the secondary questionnaire consisted of the Chinese version of the Achenbach's Youth Self-Report (YSR), which measures psychological and behavioural problems in adolescents. There were eight subscales: withdrawal, somatic complaints, anxious/depressed, social problems, thought problems, attention problems, delinquent problems, and aggressive problems. Students with total score or any subscale score reaching the local screening cutoffs were classified as having potential psychosocial risk.

After excluding ineligible subjects, the 2-year cohort included 69 045 P4 students (60.9% of the eligible subjects) who were successfully followed into P6. The traced and untraced subjects were similar in terms of socio-demographics, physiological and lifestyle characteristics (Cohen effect sizes ranged from 0.07-0.14) except for age. The subjects were also similar to the corresponding population group in Hong Kong in terms of gender, residential district, and housing type (all Cohen effect sizes <0.1). The 4-year cohort included all P4 students who were followed into F2. The final sample consisted of 36 599 subjects (32.3% of the original sample). Similarly, the characteristics of the traced and untraced subjects were very alike; the traced subjects were also comparable to the corresponding Hong Kong population.

Results

According to the IOTF standard, there was a higher prevalence of overweight (19.3% vs 13.8%) and obesity (6.4% vs 3.8%) in boys than girls in P4. Similar results were obtained based on the WFH reference (26.8% in boys vs 17.0% in girls). As the WFH system does not differentiate overweight from obesity, only the IOTF standard was used in subsequent analyses. In P6, the prevalence of overweight and obesity remained similar. In S2, the respective prevalences dropped noticeably to 15.8% and 3.6% in boys and 9.4% and 1.4% in girls.

Socio-demographic predictors of childhood obesity

In the P4-P6 cohort, older and female normal weight children were less likely to develop overweight/obesity (hereafter referred as obesity) 2 years later. Using primary parental education as a reference, children with parents having secondary level schooling were 12% less likely to develop obesity. Parental occupation was not a significant predictor of obesity onset. Overall, children who already started puberty at P5 had 32% higher risk of developing obesity at P6 than their prepubertal counterparts. However, the results were influenced by gender (P<0.001); pubertal boys were 47% less likely to develop obesity, but pubertal girls were 63% more likely to develop obesity.

In the P4-S2 cohort, pubertal stage at S1 was a negative predictor of obesity onset. Children with onset of puberty were 36% less likely to develop obesity than their prepubertal counterparts. Again, this was influenced by gender (P<0.001) such that the association was significant in boys but not in girls. The risks of obesity for children were 1.71 and 1.67 times higher, respectively, when their parental occupation was classified as 'service/clerical' and 'professional/managerial', compared with 'unemployed' parents.

Lifestyle predictors of childhood obesity

'Breakfast eating' and 'milk consumption' were significantly associated with obesity onset 2 and 4 years later. Eating breakfast at home predicted ≥26% reduction in risk. However, children having breakfast outside home (eg fast food stall, cafeteria, or elsewhere) did not differ from those who skipped their breakfast (P=0.60, data not shown). Daily milk consumption also predicted 14% and 21% lower chance of obesity onset at P6 and S2, respectively, compared with less frequent or no consumption.

Self-esteem and obesity

Having lower SEI scores at P4 predicted a higher risk of obesity onset at P6 for each of the four subscales and total SEI score (P for trend all <0.01). Using overall psychosocial risk as a predictor, High-risk children had 32% (P<0.01) higher chance of becoming obese at P6. Among the baseline overweight/obese children, having high/very high total, general, and social SE independently predicted 26 to 37% higher chance of returning to normal weight (P for trend all <0.01) 2 years later. However, parent-related SE seemed not to be predictive of weight status at follow-up among the already obese group.

Conversely, weight status at baseline was used to predict SE 2 years later. Compared with normal weight children, overweight and obese children had 12 to 33% higher risk of developing low/very low total, general, social, and academic SEI scores (P for trend all <0.001). However, weight status was not predictive of parent-related SE at follow-up. Regarding overall psychosocial risk, the overweight and obese children had 16% and 44% higher risk of having psychosocial problems at P6. Stratification analysis by gender suggested that obese girls might have double the excess risk as boys (78% vs 31%), although the difference was not significant (P=0.50).

Youth Self-Report outcomes of childhood obesity

Overall, body fatness at baseline was positively associated with the YSR total score at S2 but not significantly (P=0.07). The obese and overweight respectively had 16% and 34% higher chance of meeting the screening cutoffs,

compared to the normal weight, although such did not reach significance. Nor were the associations for each of the eight YSR subscale scores.

Regarding potential psychosocial problems, the overweight had 23% higher chance of meeting the overall screening criteria at S2. The interaction between weight status and gender was not significant (P=0.63). Nevertheless, stratification analysis showed that the association was more pronounced and consistent in girls (P for trend=0.01), whereas the association almost disappeared in boys. In particular, obese girls had 61% (P<0.05) higher chance of having psychosocial risk compared with their normal weight counterparts.

Blood pressure and obesity

Cross-sectionally, systolic and diastolic blood pressures increased progressively from normal weight to overweight and obese (all P<0.001). From P4 to P5, increasing blood pressures was associated with increasing body fatness. Compared with normal weight children, overweight and obese children respectively had higher systolic blood pressure by 5.3 (95% confidence interval [CI], 5.2-5.5) and 9.2 (95% CI, 8.9-9.6) mm Hg, and higher diastolic blood pressure by 2.0 (95% CI, 1.9-2.1) and 3.7 (95% CI, 3.5-3.9) mm Hg (all P<0.001).

Discussion

Our study identified several risk factors of childhood obesity, namely socio-demographic factors, lifestyle, and psychosocial health. Obesity could lead to undesirable health consequences both psychosocially and physiologically.

Determinants of obesity

It is important to prevent obesity early in life and target boys. In our study, sexual maturation was associated with onset of puberty, and sex differences existed. Pubertal girls were more likely to become overweight/obese than later-maturing girls. In contrast, pubertal boys had reduced risk of obesity. Clinical guidelines for screening child obesity should take into consideration maturational stages.

In contrast to usual findings in developed countries, our findings showed that higher parental occupation status was associated with higher risk of childhood obesity 4 years later. This could be due to confounding by parental fatness. Fatness is commonly perceived as fortune among older Chinese, and people from higher socio-economic status can afford more nutritious food leading to a higher risk of obesity. Further research on the micro-environment of Chinese families and the influences of parenting on child-eating behaviours may help elucidate such observations.

Two modifiable lifestyle determinants of childhood obesity were identified: breakfast eating and milk consumption. Children eating breakfast at home had lower risk of obesity, whereas children eating breakfast outside home did not fare better than those skipping breakfast. The finding may help elucidate some of the inconsistent results in overseas research. It is important to differentiate the location of breakfast eating during data collection. Daily milk consumption was protective of obesity. The observation was consistent with some epidemiologic and clinical evidence in western populations. In light of the increasing milk consumption among Chinese children, further research with more vigorous measurement of milk consumption is warranted.

Psychosocial health

The bi-directional relation between self-esteem and obesity was demonstrated. Poor esteem could be both a cause and consequence of obesity. Higher self-esteem may help children who are already obese to return to normal weight. Improving self-esteem could be beneficial in weight control and management for both normal and overweight children.

Increasing body fatness predicted higher YSR psychosocial risk in obese girls only. Similar to other population-level studies, the association between emotional health and obesity may not be strong. Whether the measurement is sensitive enough to identify subtle psychosocial problems is unclear. In general, the undesirable psychosocial consequences were more pronounced in girls than boys. This has important implications for the planning of future health education programmes.

Physiological outcome

Obese children had substantially higher blood pressures than their non-obese counterparts, both cross-sectionally and prospectively. However, as the period of follow-up was relatively short (1 year), further research on cardiovascular health and other clinical outcomes is needed.

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AUTHOR INDEX

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