IgA in BAL fluids in most types of the three serial IT immunizations in the present study indicates the generation of cross-reactive IgA in the airways.

The three serial IT immunizations of a single strain significantly enhanced the bacterial clearance of the homologous strain from the lungs of mice (Fig. 4). No cross-protection was found in mice that had received three serial IT challenge of strain H04-06. These data suggest that the enhancement in bacterial clearance is primarily strain-specific. We also found that the three serial IT immunizations with strain H05-19 or H99-115 induced a cross-protective effect in the lungs of mice (Table 4). This cross-protective effect between strain H05-19 and H99-115 was associated with strain-specific IgA as well as IgG in BAL fluids (Table 3). Since there is a distinction in the amino acid sequence of loops 5 and 6 in the P2 molecule between these two strains, a cross-protective effect may be conferred by surface antigens other than P2. Possible surface antigens responsible for this cross-protective effect may include P5 adhesin and lipooligosaccharide (Sethi and Murphy 2001; Hirano et al. 2003; Novotny and Balaletz 2003). Although bacterial clearance in the lung also tended to be enhanced after an IT challenge of live H05-19 or H99-115 strain in mice that previously received the three serial IT immunizations of three different strains, the levels of enhancement were not significant in the lung of these mice (Fig. 4). Collectively, these data demonstrate that three serial IT immunizations of a single strain could lead to the production of strain-specific IgA as well as IgG, subsequently leading to an enhanced bacterial clearance of the homologous strain in the lung. The association of the enhanced bacterial clearance of strain H04-06 in the lungs of mice after three serial repeated IT challenge of the homologous strain with the presence of strain-specific IgA, but not IgG, may underscore the importance of strain-specific IgA in BAL fluids for inhibiting bacterial adherence in the airway (Taylor et al. 1990; Kurono et al. 1991).

The presence of strain-specific IgA and IgG in BAL fluid was not always associated with an enhanced bacterial clearance in lungs, because the serial IT challenges of three different strains could lead to the production of strain-specific IgA and IgG against all three strains, but none of them developed protective immunity against NTHi. These findings provide support for the view that three serial IT challenges of a single strain is sufficient to induce the production of strain-specific IgA which is capable of inhibiting the adherence of the homologous strain to the airway epithelium, while repeated IT challenges by three different strains generate strain-specific IgA which lacks such activity (Taylor et al. 1990; Kurono et al. 1991). We, therefore, examined the issue of whether strain-specific IgA and its avidity were associated with an enhanced bacterial clearance in the lungs of mice (Kauppi-Korkeila et al. 1996; Breukels et al. 2002). As we expected, the increased IgA and its avidity specific to OMP of NTHi were associated with an enhanced bacterial clearance in the lungs of mice that had received serial three IT challenges of a single strain (Table 4). No significant bacterial clearance, however, was found in the lungs of mice that had received serial three IT immunizations with different strains despite the increased IgA and its avidity in BAL fluid. Further examinations are required to elucidate this discrepancy in mice that received serial three IT immunizations by different strains.

In summary, three serial airway immunizations with a single or three different strains of NTHi stimulated the production of cross-reactive IgG and IgA in BAL fluids, but only three serial IT challenges of a single strain could induce the enhancement in bacterial clearance of the homologous strain in the lung. This enhancing effect on bacterial clearance in the lungs is, therefore, primarily induced in a strain-specific manner. In addition, enhanced bacterial clearance of a hetrologous strain was also found after three serial IT immunizations of a single strain among two of a the three strains employed for bacterial challenge. Increased strain-specific IgA and its avidity in BAL fluids was associated with an enhanced bacterial clearance in mice that had received serial IT immunizations with a homologous strain, but not in mice that had received serial IT immunizations

with heterologous strains. The data herein suggest that P2 molecules and surface antigens other than P2 are involved in the development of pulmonary defense against NTHi. Our data also suggest that a host previously infected by a NTHi continues to be susceptible to infections by other strains of NTHi, and may explain the mechanism of recurrent bacterial exacerbations of COPD. Since only three strains of NTHi with different P2 epitopes were employed in this study, the conclusions drawn are limited. Further studies will be required for a complete understanding of the strain-specific pulmonary defense against NTHi.

Acknowledgments

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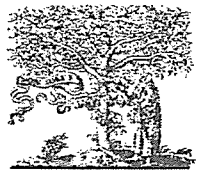

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Epidemiology of influenza in Hanoi, Vietnam, from 2001 to 2003

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Summary *Objective:* The aim of this study was to clarify the epidemiology of laboratory-confirmed influenza in Hanoi, Vietnam.
Methods: Influenza was detected by virus isolation from nasopharyngeal swabs of influenza-like-illness (ILI) patients who reported to outpatient clinics in Hanoi, Vietnam between 2001 and 2003, before the start of avian influenza A/H5N1 outbreaks. Influenza isolates were characterized by hemagglutinin inhibition test.
Results: A total of 4708 nasopharyngeal swabs were collected from patients with ILI. Influenza was positive in 119 (2.5%) samples by virus isolation. Influenza circulated throughout the year, with possible two peaks in summer and winter. Influenza B viruses and A/H3N2 predominated in 2001 and 2002, respectively, and mixed circulation of A/H1N1, A/H3N2 and B were observed in 2003. The seasonality of influenza roughly matched with clinical case reports in the North Region by National Communicable Disease Surveillance in Vietnam.
Conclusions: The findings of year-round and biannual peak circulation of influenza in a subtropical area were in accordance with the results of previous studies in tropical and subtropical regions. Our observations indicated that establishment of laboratory-based surveillance in tropical and sub-tropical countries is important for taking actions for pandemic strategies, and links to the WHO global influenza network.
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Introduction

Influenza epidemiology in tropical and sub-tropical countries is not well documented,[1] and the World Health Organization (WHO) is urging intensification of influenza surveillance as a part of contingency planning for responses to influenza pandemics.[2] In the plan, establishing influenza surveillance with epidemiological and virological links is stressed, with the aims of determining the burden of diseases in nations, increasing the ability to identify influenza virus strains with epidemic and pandemic potential, and contributing to collection of influenza isolates to support global influenza vaccine strain selection.

The National Communicable Disease Surveillance of influenza-like-illness (ILI) has been conducted in Vietnam, but solely based on clinical case reports. The importance of the area is stressed because Vietnam and neighboring countries have been experiencing avian influenza A/H5N1 outbreaks since the end of 2003. Also the area is in close proximity to China, historically an epicenter for new strains, such as A/H2N2 in the 1950s, and A/H3N2 in the 1960s, while A/H5N1 occurred in Hong Kong in 1997. Thus, we conducted a laboratory based influenza study to monitor the circulation patterns of the virus in Hanoi, Vietnam from 2001 to 2003.

Materials and methods

Geographic and climatic background

Vietnam consists of 64 provinces, and are commonly categorized into four regions; the North, the Central, the West Highland, and the South. Geographic, demographic and climatic features differ much among the areas. Hanoi is the capital of the country, situated in the North Region with a population of 4 million, in a sub-tropical climate with four seasons. Summer is hot and rainy lasting from May to October, and winter lasts from December to March. Southern parts of the country have more tropical climates with only two seasons, rainy and dry.

Study population

We organized a laboratory-based influenza surveillance in Hanoi from 2001-2003. Ten outpatient private clinics and two hospitals (Bach Mai and National Institute of Pediatrics), mainly pediatrics, participated in the study. ILI was defined on the basis of sudden onset of fever (>37.8 °C) and any signs or symptoms of acute respiratory infection such as coughing, a sore throat, a runny, or stuffy nose. Participants were asked for oral informed consent at the time of enrolment. Patient demographic details such as name, sex, age, address, and clinical symptoms, were recorded at hospitals and clinics upon collection, then the samples and data were sent to the Respiratory Virus Section, Virology Department in National Institute of Hygiene and Epidemiology (NIHE), which is a governmental institution in Hanoi affiliated to the Ministry of Health in Vietnam.

Laboratory examinations

Specimen collection and transportation
All respiratory swabs were placed in viral transport media and transported to NIHE on the day of collection. Specimens were aliquoted in cryotubes and kept at −80 °C until the time of laboratory examination.

Virus isolation and identification
From each sample, 100 μl of supernatant was inoculated into Madin–Darby canine kidney (MDCK), and Hep-2 cells in 48 multi-well plates for influenza and other respiratory virus isolation.[3] The plates were kept at 34 °C under a 5% CO_2 atmosphere for up to 10 days to observe cytopathic effect (CPE). Fifty-microliter aliquots of supernatant in CPE-positive samples were passaged twice to obtain enough virus titers to perform virus identification, and to stock strains. All influenza isolates were typed and subtyped by hemagglutination inhibition (HAI) assay with WHO influenza reagent kits, provided by the Centers for Disease Control and Prevention, Atlanta, GA, USA.[4] HAI testing was achieved with chicken red blood cells. Identification of other respiratory viruses such as adenovirus, enterovirus, respiratory syncytial virus (RSV) was conducted simultaneously by microneutralization test or PCR (data not shown). Selected influenza isolates were sent to National Institute of Infectious Diseases, the WHO Collaborating Influenza Reference Center in Tokyo, Japan for confirmation.

National Communicable Disease Surveillance

In Vietnam, the Ministry of Health employs the National Communicable Disease Surveillance. Twenty-four communicable diseases, including ILI are routinely reported each month by public health care facilities. ILI is based upon clinical diagnosis without laboratory confirmation that includes fever and other signs such as chills cough, stuffy nose, or headaches. The number of cases and death was sent from the public health care facilities to commune, district, provincial, and eventually regional level public health institutions. Epidemiology Department in NIHE integrates all information, and the number of cases and deaths was tabulated by disease, province and month.

We calculated the number of ILI adjusted by 100,000 population to clarify the regional seasonality and disease burden of ILI by the National Communicable Disease Surveillance in Vietnam to compare with our virological data.

Statistical test

The Scheffe test was used as statistical test to compare the means between pairs of groups more than three. Tukey's test was employed to compare proportions among multiple groups. All p values were two tailed, and $p < 0.05$ was considered statistically significant. All tests were performed using statistical software developed in the Department of Public Health, Niigata University, Japan, according to the methodologies for each test.[5]

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Results

A total of 4708 nasopharyngeal swabs were collected from 2001 to 2003, with yearly figures of 2182, 1462, and 1064, respectively (Table 1). The mean age of participants was 6.5 ± 10.0 years old in three years, and 64% were children under 5 years old. The number of males was almost equal to females.

One hundred and eleven (2.5%) of the 4708 samples were confirmed to be influenza by virus isolation. Influenza viruses were detected in 74, 13, and 32 samples in 2001, 2002, and 2003, respectively. The positivity was significantly lower for 2002 compared to those for 2001 and 2003 ($p < 0.01$) (Table 1).

Influenza virus was detected almost throughout the year, with a possible peak in June–August during the hot rainy season, and another in December-January during winter in this study in Hanoi (Fig. 1). The rainy season peak was unclear in 2003 due to the low activity of virus isolation after temporal closure of the laboratory during the late March to May by SARS. The predominant influenza virus was influenza B in 2001, and A/H3N2 in 2002, and then A/H1N1, A/H3N2 and B co-circulated in 2003. The first detected human case of A/H5N1 in Vietnam involved a young child in the North Region in December 2003 (data not shown).[6] No A/H5N1 was detected in our samples.

During the study period, all A/H1N1 isolates were antigenically characterized as A/New Caledonia/20/99 (H1N1)-like strains, and A/H3N2 were A/Panama/2007/99-like (Table 2). One A/H3N2 strain collected in October 2003 reacted with both A/Panama/2007/99 and A/Korea/770/2002 (A/Fujian/411/2002-like) in HAI. Influenza B strains in 2001 were categorized as B/Johannesburg/5/99-like or B/Yamanashi/166/98-like in the Yamagata lineage, and in 2003 B/Hong Kong/330/2001-like strains in the Victoria lineage were found.

According to the National Communicable Disease Surveillance conducted by the Ministry of Health in Vietnam, the average number of cases nationwide was 2045.2 per population per year during 2001–2003, and the category was ranked as the highest among the 24 communicable diseases. Each region had a different seasonality and numbers of ILI (Fig. 2).The North Region had 2725.8 cases per 100,000 population per year, and showed seasonality with a high peak from May to September. Average number of ILI cases was the highest in the West Highland region, 3670.6 cases per 100,000 population year, with a possible peak in August–September. The Central and the South did not show clear seasonality, and the ILI cases were lower than the others.

Discussion

Our results indicate that influenza viruses circulate throughout the year in Hanoi, Vietnam, with possible two peaks, one in the rainy seasons and another in the cold winter. This finding was consistent with previous reports in tropical and subtropical climate countries in Asia, such as Thailand, Indonesia, Myanmar, Singapore, and the southern part of China including Taiwan.[1,7–15] However, information from these areas is still limited, probably due to lack of organized influenza national surveillance networks, equipment and skilled staff in laboratories, and financial resources.[13] For example, in our study, the number of virus isolates was significantly low in 2002, due to a serious shortage in laboratory staff and equipment. Then in 2003, the virus isolation was stopped from late March to May due to SARS outbreaks in Hanoi, and still low in June–August. One A/H1N1 was isolated in March before the temporal closure of the laboratory.

According to the influenza activity reports by WHO and Collaborating Centers, the predominant types/subtypes in South-East Asia and Oceania were mixtures of A/H1N1, H3N2 and B during 2001 and 2002, this changing to A/H3N2 predominance with a small proportion of A/H1N1 and B in 2003.[16–18] In our study, the predominant types/subtypes of influenza viruses circulated in Hanoi over the three-year period were slightly different from those in surrounding areas in Asia, but the antigenic characteristics were similar.[16–20] A change of predominant strains in A/H3N2 from A/Panama/2007/99 to A/Fujian/411/2002 caused a big epidemic world-wide in 2003–2004.[18,19,21,22] Even one A/H3N2 strain in our study collected in October 2003 indicated the invasion of A/Fujian/411/2002-like strain to Hanoi as in other areas in Asia.[23,24] Influenza B strains collected during February to October in 2001 were a mixture of two strains, B/Yamanashi/166/98, which was 2000–2001 vaccine component, and B/Johannesburg/5/99, a component in the following 2001–2002 season. Thus, our results suggested that strains in Hanoi fairly matched with global vaccine strains, and can provide a strong support for the global virologic surveillance by WHO Influenza Surveillance Network.[25]

Table 1 Demographic details of influenza-like-illness patients and number of confirmed influenza cases

	Year			
	2001 ($N = 2182$)	2002 ($N = 1462$)	2003 ($N = 1064$)	Total ($N = 4708$)
Age (mean years ± SD)[a]	7.4 ± 11.2[d]	5.9 ± 9.2	5.3 ± 8.2	6.5 ± 10.0
Sex, Male (%)[b]	1152 (53.2)[e]	794 (54.7)	401 (56.5)	2347 (54.2)
Influenza positive (%)[c]	74 (3.4)	13 (1.0)[f]	32 (3.0)	119

[a] 37 participants of unknown age were excluded from the analysis.
[b] 380 participants of unknown sex were excluded from the analysis.
[c] Influenza was confirmed by virus isolation.
[d] Mean age of participants for 2001 was significantly higher than those for 2002 and 2003, respectively ($p < 0.01$).
[e] Proportion of male did not have significant differences between the years.
[f] Proportion of influenza positives for 2002 was significantly lower than those for 2001 and 2003, respectively ($p < 0.01$).

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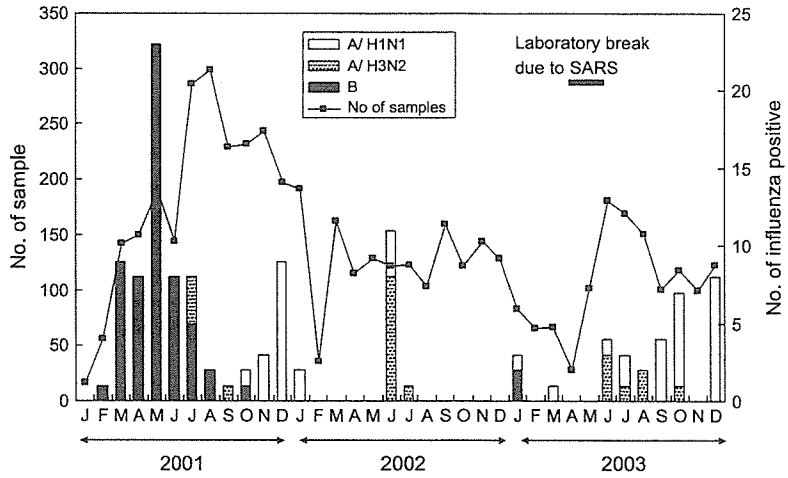

Figure 1 Monthly number of samples collected and influenza positives by virus isolation from 2001 to 2003, in Hanoi, Vietnam.

The WHO emphasizes the use of existing mechanisms and infrastructures; strengthening and adopting to newly emerging diseases is regarded as the most expedient way to improve capacity for the emergent situations.[26] After reconstruction of virus isolation systems and introduction of reverse transcription (RT)—PCR techniques and equipment in our collaboration between NIHE and Niigata University, we were able to isolate influenza viruses and additionally detect influenza genomes by RT—PCR. As a series of studies, 679 samples were selected from virus isolation negative samples in this study, and 72 (10.6%) were positive for either influenza A/H1, H3 or B by RT—PCR. The year-round distribution of influenza virus, and a large peak in rainy season was clearer in combination with the RT—PCR results. However, we did not incorporate these results in this study due to the limited number of samples tested. Currently, seasonal influenza and highly pathogenic avian influenza A/H5N1 co-circulate in some tropical countries, thus higher biosafety levels (BSL3) are required for laboratory safety for virus isolation. Although virus isolation is a gold standard for influenza detection for surveillance, it is labor-intensive and requires specialized skills. RT—PCR is more sensitive, safer, and easy to introduce in laboratories in developing countries, where conditions are not always optimal.[13]

The laboratory contributed tremendously during SARS and A/H5N1 outbreaks in Hanoi. Our experience showed that successful capacity building in laboratories needs time, so the government and international agencies should look for both emergency solutions to the present crisis and longer-term ways to strengthen fundamental capacity. WHO recommends indirect fluorescent assays (IFA) as a screening method in developing countries, where there is a skill shortage in laboratory personnel and a lack of laboratory equipment.[27] As an alternative method, recent studies in Myanmar and Thailand showed successful utilization of rapid diagnostic tests for initial screening in medical facilities.[13,14]

The seasonality of ILI roughly matched with our laboratory data in the North Region by National Communicable Disease Surveillance. The figure was higher than that in Thailand (1420/100,000),[12] suggesting a high burden of ILI in our country. However, it may be confounded by many factors. Firstly, testing for influenza infection is not routinely available, and very few reported cases are ever confirmed in the laboratory.[8] As with many passive surveillance systems, underreporting is frequent. Alternatively, because the majority of ILI cases are not caused by influenza, the lack of laboratory confirmation could also result in overestimates of influenza infections. Thus

Table 2 Characteristics of influenza virus isolates

Year	Influenza virus A H1N1		Influenza virus A H3N2		Influenza virus B		Total
	No. of positives	Strain[a] (no.)	No. of positives	Strain[a] (no.)	No. of Positives	Strain[a] (no.)	
2001	13	A/New Caledonia/20/99 (13)	4	A/Panama/2007/99 (4)	57	B/Yamanashi/166/98 (11) B/Johannesburg/5/99 (46)	74
2002	5	A/New Caledonia/20/99 (5)	8	A/Panama/2007/99 (8)	0		13
2003	23	A/New Caledonia/20/99 (23)	7	A/Panama/2007/99 (6), A/Panama/2007/99 and A/Korea/770/02 (1)[b]	2	B/Hong Kong/330/2001 (2)	32
Total	41		19		59		119

[a] The strain type was characterized with the hemagglutinin inhibition test.
[b] A/Korea/770/02 is antigenically similar to A/Fujian/411/02.

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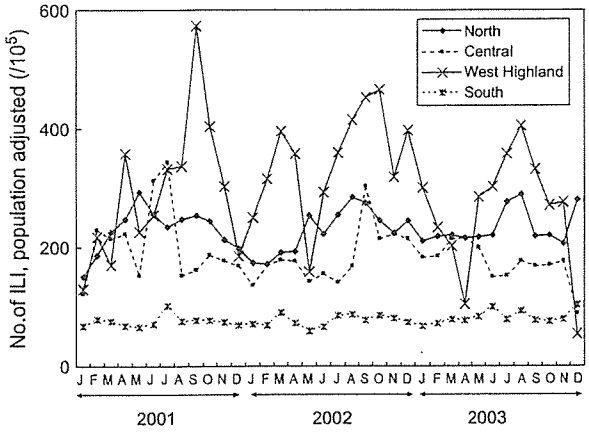

Figure 2 Population adjusted (per 100,000) number of reported influenza-like-illness in the National Communicable Disease Surveillance by region in Vietnam from 2001 to 2003.

we need rapid establishment of more influenza-specific national surveillance with a link between laboratory and clinical data to evaluate the actual burden of disease in the area.[12]

Currently, a national policy for influenza vaccination in Vietnam has yet to be decided due to lack of information on the burden of disease and the seasonality of influenza. Morbidity and mortality rates of influenza infections by age group are essential for selecting target populations for immunization. Increasing evidence suggests that disease burden in tropical and subtropical regions may be much higher than those in the USA.[1,12,28] In studies in Taiwan, successful reduction of hospitalization and mortality in the elderly suggested significant cost savings expected through vaccination programs in tropical and subtropical regions.[29,30] Also, optimal timing for vaccination and strain selection are important factors for immunization programs. It is difficult to decide whether Northern or Southern Hemisphere vaccines should be used in Hanoi due to the two influenza peaks. In Singapore, an annual vaccination can be taken a few weeks before either one of the two influenza infection peaks seen in May–July and November–January,[15] while Hong Kong and Taiwan have a policy of vaccination before the start of winter influenza season despite biannual peaks in these areas.[28,29] Further information on circulating patterns in equatorial tropical climate areas in Asia as well as those of various parts of Vietnam is needed for proper timing of vaccination.

In summary, our laboratory-based influenza surveillance study conducted in Hanoi, Vietnam, over a 3-year period has provided valuable data on characteristics of influenza seasonality and circulating viruses in the area. The importance of strong laboratory diagnosis capacity as a support to outbreak detection has been repeatedly emphasized.[26] Our activity in NIHE is a seed from which to establish sentinel surveillance systems, including laboratory development at the provincial levels. Enhancing the ability to detect influenza viruses should contribute to prevention and control of outbreaks, and to provide a driving force to fight against A/H5N1 or any new types of influenza that may start a pandemic. Finally, it should support global influenza vaccine strain selection, for both seasonal and avian influenza, as a part of the WHO influenza program.

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