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ORIGINAL ARTICLE

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Clinical and microbiological characteristics of community-acquired pneumonia among human immunodeficiency virus-infected patients in northern Thailand

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Abstract Human immunodeficiency virus (HIV) infections are prevalent in Thailand. However, the clinical and microbiological characteristics of community-acquired pneumonia (CAP) in such patients are not completely clear at present. In the present study, we analyzed the characteristics of CAP in 191 HIV-infected patients (192 episodes, 130 males and 61 females, mean age 32.9 years, range: 20–62) who had been admitted to Nakornping Hospital in northern Thailand between December 1996 and January 2002. The mean peripheral blood CD4 lymphocyte count was 68.5/mm^3 (range: 0–791). The most common organisms detected in the blood of the subjects were as follows: *Penicillium marneffei*, 13, *Salmonella* spp., 5, *Cryptococcus neoformans*, 4, *Staphylococcus aureus*, 3, and *Rhodococcus equi*, 3, and the most common organisms detected in sputum included *Haemophilus influenzae*, 38, *P. marneffei*, 10, *Streptococcus pneumoniae*, 10, *R. equi*, 9, and *S. aureus*, 9. Life-threatening meningitis in 5 (cryptococcal in 3 and tuberculous in 2), pneumothorax in 2, and tuberculous lymphadenitis in 1 were also noted, resulting in 21 fatalities (10.9%). The mean peripheral blood CD4 lymphocyte count for cases in which the subject died was 74.8/mm^3 (range: 0–340). Logistic regression analysis demonstrated that high age (odds ratio of over 40 years: 15.62) and *R. equi* infection (odds ratio: 8.14) are related to death of HIV-infected patients with CAP. The above findings indicate that various types of organisms, including mixed organisms, cause CAP in HIV-infected patients in northern Thailand, and high age and *R. equi* infection seem to be risk factors for death.

Key words Community-acquired pneumonia · AIDS · Thailand

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Introduction

Pulmonary infections are common complications and a major cause of mortality in human immunodeficiency virus (HIV)-infected individuals.[1-3] They significantly reduce the quality of life and longevity, and exert a great influence on the cost of medical care. In countries where the majority of the population have access to highly active antiretroviral therapy (HAART), a dramatic decrease in morbidity and mortality in HIV-infected persons has already been reported.[4,5] However, Thailand has not yet achieved that status during the period of this study, although HAART procedures have recently been initiated.[6] The proportion of HIV-infected patients continues to be high at present,[7] and it has been reported that Thailand has an estimated number of people living with HIV/AIDS of approximately 600 000.[6] Prophylaxis and the treatment of pulmonary infections complicating HIV-infected patients are very important in their overall management, but the clinical and microbiological characteristics of pulmonary infections among such patients have not been carefully evaluated in Thailand. The aim of the present clinical study was to investigate the state of community-acquired pneumonia (CAP) complicating HIV infections in Thailand.

Methods

All studies described herein were approved by the Human Ethics Review Boards of our institutions, and informed consent was obtained from each subject.

Patients

This study was performed in HIV-infected patients who had been admitted to Nakornping Hospital, which is a major community hospital and the center of HIV-infected patients in northern Thailand, between December 1996 and January 2002. Community-acquired pneumonia was diagnosed by new abnormal shadow likely infiltration on a chest roentgenogram with at least two of the following clinical and laboratory findings: fever (temperature >37.8°C), cough, the production of purulent sputum, dyspnea, and leukocytosis (WBC count >10000/μl). Although a few patients in this study had such chronic lower respiratory tract diseases as chronic bronchitis or bronchiectasis, most cases did not have such underlying diseases. Also, no cases in this study receive HAART. Patients were excluded from the study when the abnormal shadow on a chest roentgenogram was due to other causes such as congestive heart failure, pulmonary infarction or lung cancer.

Microbiological studies

On admission, two sets of blood cultures using BACTEC Plus Aerobic and BACTEC Myco/F lytic medium (Becton Dickinson Microbiology Systems, Sparks, MD, USA) were obtained, and when good-quality sputum, based on the criteria of Bartlett[8] was available, acid-fast staining, Gram staining, and a sputum culture using blood agar and chocolate agar for bacteria, Sabouraud dextrose agar for fungi, and a Lowenstein–Jensen medium for mycobacterium were performed using standard methods.

Clinical study

Serum samples were collected on admission and/or subsequently, to determine the CD4 lymphocyte count[9] and other laboratory tests. Cases with CAP were analyzed for differences in age, sex, microbiological results, treatment, clinical outcome, and complications.

Statistical analysis

Logistic regression analysis was performed by SYSTAT 10.2 (Hulinks, Tokyo, Japan).

Results

Patient characteristics

A total of 191 patients, including 130 males and 61 females, with a mean age of 32.9 years (range: 20–62), and a total of 192 episodes of CAP were enrolled in the present study. The mean peripheral blood CD4 lymphocyte count was 68.5/mm^3 (range: 0–791).

Microbiological results

Although approximately 10% of patients already received antibiotics such as penicillin before admission, blood, and sputum culture were collected in most cases. The most common organisms detected in the blood of the subjects were as follows: *Penicillium marneffei*, 13, *Salmonella* spp., 5, *Cryptococcus neoformans*, 4, *Staphylococcus aureus*, 3 and *Rhodococcus equi*, 3, and the most common organisms detected in the sputum were as follows: *Haemophilus influenzae*, 38, *P. marneffei*, 10, *Streptococcus pneumoniae*, 10, *R. equi*, 9, and *S. aureus*, 9 (Table 1). No significant organism could be detected in the 161 episodes (83.9%) from blood cultures and the 115 episodes (59.9%) from sputum cultures.

Complications and clinical outcome

Life-threatening meningitis in 5 (cryptococcal in 3 and tuberculous in 2), pneumothorax in 2, and tuberculous lymphadenitis in 1 were noted. One hundred and fifty patients (78.1%) improved and could be discharged, 21 patients (10.9%) failed to improve (i.e., transfer, discharge against advice, escape), and 21 patients (10.9%) died. The mean peripheral blood CD4 lymphocyte count for cases of patients who died was 74.8/mm^3 (range: 0–340) and the admission period was 1–52 (mean 12.0) days. The organisms isolated from cases of death were *H. influenzae* from the sputum, 5, *R. equi* from sputum, 3, *P. marneffei* from sputum, 3, *S. aureus* from sputum, 1, and sputum and blood, 1, *Klebsiella pneumoniae* from sputum, 2, *S. pneumoniae* from sputum, 1, and blood, 1, *Moraxella catarrhalis* from sputum, 1, and *Nocardia* spp. from sputum, 1.

Characteristics of CAP caused by various organisms

Community-acquired pneumonia caused by *H. influenzae* in 38 *P. marneffei* in 18, *S. pneumoniae* in 11, *R. equi* in 10, and *S. aureus* in 10 patients were compared (Table 2). The mean ages were almost similar. The ratio male to female in CAP caused by *S. pneumoniae* seemed to be lower, and mixed infections could occasionally be seen in each group (data not shown). The mean peripheral blood CD4 lymphocyte count for CAP caused by *R. equi* (7.9) was lower than those by other kinds of organisms, and the mortality rate of CAP caused by *R. equi* (30.0%) tended to be higher compared to other types of infections.

Statistical analysis concerning death

Some kinds of factors were compared between survivor and dead cases with CAP (Table 3). The mean CD4 and prevalence of septicemia seems to be similar between these two groups, but ratio male to female, high age, and *R. equi* infection appear to be higher in dead cases. Logistic regression analysis demonstrated that high age (odds ratio of over 40 years: 15.62) and *R. equi* infection (odds ratio: 8.14) are

Table 1. Pathogens isolated from blood and sputum from HIV-infected subjects with community-acquired pneumonia

Blood culture		Sputum culture	
Penicillium marneffei	11	*Haemophilus influenzae*	27
P. marneffei+Rodococcus equi	1	*H. influenzae+Moraxella catarrhalis*	1
P. marneffei+Cryptococcus neoformans	1	*H. influenzae+Klebsiella pneumoniae*	1
Salmonella spp.	5	*H. influenzae+Staphylococcus aureus*	1
Cryptococcus neoformans	3	*Rhodococcus equi*	6
Staphylococcus aureus	3	*R. equi+Salmonella enteritidis*	1
Rodococcus equi	2	*R. equi+Escherichia coli*	1
Pseudomonas aeruginosa	2	*R. equi+Penicillium marneffei*	1
Streptococcus pneumoniae	1	*Penicillium marneffei*	4
Enterobacter cloacae	1	*P. marneffei+H. influenzae*	2
Mycobacterium tuberculosis	1	*P. marneffei+K. pneumoniae*	1
Negative	161 (83.9%)	*P. marneffei+Cryptococcus neoformans*	1
		P. marneffei+H. influenzae+S. pneumoniae+K. pneumoniae	1
		Streptococcus pneumoniae	4
		S. pneumoniae+H. influenzae	1
		S. pneumoniae+Pseudomonas aeruginosa	1
		S. pneumoniae+H. influenzae+S. aureus	1
		S. pneumoniae+H. influenzae+S. aureus+K. pneumoniae	1
		Staphylococcus. aureus	6
		Escherichia coli	3
		Mycobacterium tuberculosis	2
		M. tuberculosis+S. pneumoniae	1
		M. tuberculosis+H. influenzae	1
		Pseudomonas aeruginosa	2
		P. aeruginosa+M. catarrhalis	1
		Nocardia spp.	2
		Nocardia spp.*+H. influenzae*	1
		Salmonella enteritidis	1
		Klebsiella pneumoniae	1
		Negative	115 (59.9%)

Table 2. Comparison of characteristics among community-acquired pneumonia caused by various organisms

	Haemophilus influenzae (n = 38)	*Penicillium marneffei* (n = 18)	*Streptococcus pneumoniae* (n = 11)	*Rhodococcus equi* (n = 10)	*Staphylococcus aureus* (n = 10)
Age distribution, years (mean)	20–48 (30.2)	24–44 (33.5)	23–38 (31.4)	21–42 (31.6)	25–35 (29.6)
Male/Female	24/14	15/3	5/6	9/1	7/3
Detection site					
sputum and blood	0	5	0	2	2
sputum	38	4	10	7	7
blood	0	4	1	1	1
others	0	5[a]	0	0	0
CD4 distribution (mean)	0–708 (84.2)	0–114 (37.9)	0–114 (30.9)	0–21 (7.9)	6–431 (84.5)
Dead cases (%)	5 (13.2%)	3 (16.7%)	2 (18.2%)	3 (30.0%)	2 (20.0%)

[a]Three from blood and skin, 1 from skin, and 1 from sputum, blood, and skin

Table 3. Comparison of characteristics between survivor and dead cases of HIV-infected subjects with community-acquired pneumonia

	Survivors (n = 171)	Dead cases (n = 21)
Mean CD4	67.9	74.8
Ratio male to female	1.9	6.0
Age, %		
20–29	38.2	19.0
30–39	45.3	47.6
over 40	16.5	33.3
Septicemia	29	2
Rhodococcus equi infection	7	3

related to death, but septicemia (odds ratio: 0.83) and CD4 (odds ratio of below 50: 0.14) are not concerned with death in our study (Table 4).

Discussion

The incidence of specific opportunistic infections in HIV-infected individuals varies in different countries, since the prevalence of microorganisms in a given environment determines the patterns of the invading pathogens. In the

Table 4. Logistic regression analysis to predict death in HIV-infected subjects with community-acquired pneumonia

	Odds ratio	95% confidence interval
Age group (years)		
20–29	1.0	–
30–39	5.48	0.55–55.04
over 40	15.63	1.31–186.1
Rhodococcus equi infection	8.14	1.02–65.06
Male	1.76	0.32–9.71
Septicemia	0.83	0.14–4.92
CD4		
below 50	0.14	0.01–1.89
50–199	0.59	0.05–7.49
over 200	1.0	–

United States, disseminated *Mycobacterium avium* complex disease was reported to be the most common opportunistic infection in homosexual patients with acquired immunodeficiency syndrome (AIDS).[10] On the other hand, in South Korea tuberculosis was the most frequent opportunistic infection in HIV-infected subjects.[11] Thailand has one of the most explosive HIV/AIDS epidemics in the world. Penicilliosis due to *P. marneffei* was the third most frequent AIDS-defining infection after tuberculosis and cryptococcosis in northern Thailand prior to the introduction of HAART, and is endemic in Southeast Asia.[12] In our study, CAP caused by *P. marneffei* in HIV-infected patients was frequent, different from other countries. Community-acquired pneumonia caused by *R. equi* was also frequently seen in our study, which tended to appear in the later stages of HIV infections and was fatal compared to other types of infections. In fact, the CD4 count of patients with CAP caused by *R. equi* was lower than those of other types of infection and the mortality rate was significantly high compared to other kinds of infection in our study. A marked increase in the incidence of infections caused by *R. equi* has been reported since the start of the HIV epidemic in 1981,[13,14] and the outcome has been reported to be fatal in 60% of HIV-infected patients and in 28% of HIV-negative individuals.[15] Recent studies indicate that pulmonary infections caused by *R. equi* are not uncommon in HIV-infected patients in northern Thailand.[16] Since Chiang Mai is surrounded by much farmland in northern Thailand, many farmers were involved in our study. This might be one reason that the rate of *R. equi* infection as zoonosis was relatively high. In pulmonary nocardiosis and CAP caused by *R. equi*, cavitary pulmonary lesions, similar to pulmonary tuberculosis, were noted.[17] Therefore, a misdiagnosis of pulmonary tuberculosis should be avoided by a careful examination, laboratory tests, and a radiological workup. Other common pathogens causing CAP in our study were *H. influenzae*, *S. pneumoniae*, and *S. aureus*, and these organisms are similar to those reported in previous reports on non HIV-infected[18,19] and HIV-infected patients.[1,2] In addition to differences in pathogenic microorganisms and the proportion of mixed infection between deceased cases in the present study and survivors, we should also recognize differences in the general condition of a patient and accompanying socioeconomic problems. Although high age was significantly related to death among HIV-infected patients with CAP, CD4 was not concerned with mortality. Since the CD4 was already low in most patients of our study thus meaning that most cases were already in the later stages of HIV infections, it might have influenced the result. Although HAART has recently been started,[7] its effect has not been fully evaluated in Thailand. In such situations, immunization and prophylaxis defined in a number of studies should be considered.[20–22] In conclusion, various types of organisms, including mixed organisms, cause CAP in HIV-positive patients in northern Thailand.

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Original Article

Drug-resistant pneumococci in children with acute lower respiratory infections in Vietnam

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Abstract ***Background***: Acute lower respiratory infections (ALRI), primarily pneumonia, are the leading cause of death in children under 5 years of age. Most of these deaths occur in Africa and southeast Asia. Increasing rates of drug resistance in pneumococcal strains emphasize the necessity of prevention of pneumococcal vaccines. The aim of the present study was to determine the frequency of drug resistance and the distribution of serotype of pneumococcal strains isolated from pediatric patients with ALRI in Vietnam.

Methods: Two hundred and twenty pediatric patients with ALRI under 5 years of age were enrolled in Hanoi, Vietnam between 2001 and 2002. Bacterial pathogens with a heavy growth (10^6 c.f.u./mL) were isolated from nasopharyngeal secretions on quantitative culture. Fifty-three pneumococcal strains isolated from the nasopharynx of pediatric patients were examined for antibiotic susceptibility including drug-resistant genes and serotyping.

Results: A total of 73.6% of pneumococcal strains were genotypic penicillin-resistant *Streptococcus pnemoniae* (gPRSP), possessing altered penicillin-binding protein genes *pbp 1a + 2x + 2b*; 67.9% of these strains were gPRSP and simultaneously had the *ermB* gene, which is responsible for high resistance to erythromycin. The majority of gPRSP strains were serotype 19F or 23F.

Conclusion: gPRSP strains with serotype 19F or 23F are highly prevalent among pediatric patients with ALRI under 5 years of age in Hanoi, Vietnam.

Key words acute lower respiratory infection, children, drug resistance, serotyping, *Streptococcus pneumoniae*.

Acute lower respiratory infections (ALRI), primarily pneumonia, are the leading cause of death in children under 5 years of age, but diagnosis is difficult both in industrialized and developing countries. A recent report indicated that 1.9 million children died from ALRI in 2000, worldwide, and that 70% of these deaths occurred in Africa and southeast Asia.[1] The two leading bacterial pathogens of pneumonia are *Streptococcus pneumoniae* and *Haemophilus influenzae*.[2,3]

An increasing prevalence of pneumococcal strains resistant to β-lactams has been observed in both developing and developed countries during the last decade.[4] Recent studies have also found high frequencies of penicillin- and macrolide-resistant pneumococci as a respiratory pathogen in several Asian countries, including Japan.[5,6] While the resistance of *S. pneumoniae* to β-lactams has been shown to be associated with mosaic mutations in the penicillin-binding protein genes *pbp 1a*, *pbp 2b* and *pbp 2x*,[7,8] macrolide resistance is mediated by methylation of the 23S rRNA methylase encoded by the *ermB* gene and macrolide efflux via the *mefA* gene.[9,10]

In children, bacterial pneumonia is generally preceded by asymptomatic bacterial colonization. It is well understood that bacterial colonization plays a central role in bacterial pneumonia.[11] Previous studies used nasopharyngeal swab samples to examine antibiotic resistance and serotyping for treatment and vaccine formulation among children.[12–15] Increasing rates of drug resistance in pneumococcal strains emphasize the necessity of prevention of pneumococcal vaccines. Although two previous studies have recently reported that the pneumococcal conjugate vaccine is effective in reducing the incidence of pneumonia as well as invasive pneumococcal pneumonia among African infants,[16,17] few data on the drug resistance or serotype distribution of penumococcal strains isolated from pediatric patients with ALRI are currently available in developing countries in Southeast Asia. The aim of the present study was therefore to examine antibiotic susceptibility, including drug-resistant genes and serotyping of pneumococcal strains isolated from the nasopharynx of patients with ALRI, among children in Hanoi, Vietnam.

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Methods

Patient population and study design

Two hundred and twenty pediatric patients with ALRI were enrolled at the National Pediatric Hospital (NPH), a 600-bed hospital, or the Bach Mai Hospital (BMH), a 1400-bed hospital, the two tertiary hospitals in Hanoi, Vietnam, between January 2001 and December 2002. The distance between the two hospitals is 6 km, and each hospital covers Hanoi and its suburban area. The criteria for inclusion were: (i) age under 5 years and a diagnosis of ALRI made within 24 h of admission; (ii) clinical symptoms of a productive cough, fast breathing and fever >37.5°C; and (iii) crackle in the lung fields on auscultation. Exclusion criteria were: (i) age above 5 years; (ii) illness of non-infectious etiology; (iii) failure to provide consent. The present study was approved by the Institutional Review Boards of National Institute of Hygiene and Epidemiology, and a signed consent form was obtained from each subject.

Bacteriological examinations

A previous study reported the isolation of bacterial pathogens on quantitative culture using oropharyngeal swab samples from pediatric patients.[18] Because nasopharyngeal samples are regarded to be superior to oropharyngeal samples for the detection of the two leading pathogens (*S. pneumoniae* and *H. influenzae*),[19] we examined nasopharyngeal secretions from 220 patients with ALRI using two flexible swabs (Medical Wire & Equipment, Wiltshire, England, UK) for quantitative bacterial culture. After weighing the nasopharyngeal swab samples on a microbalance, the volume of the sample was determined to be approximately 0.01 mL. This sample was diluted in brain–heart infusion broth (BBL, Becton Dickinson, Cockeysville, MD, USA), and a 10-fold dilution was then prepared in saline as described previously.[18,20] A quantitative bacterial culture was carried out on trypticase soy agar (BBL, Becton Dickinson) containing 7% defibrinated rabbit blood and incubated in a 5% CO_2 incubator at 37°C overnight. Possible bacterial pathogen with a heavy growth (10^6 c.f.u./mL) was isolated.[21]

Fifty-three strains of *S. pneumoniae* isolated from 220 pediatric patients with ALRI were investigated for antibiotic susceptibility including analysis of drug-resistance genes and serotyping. Forty isolates were from NPH and 13 isolates were from BMH, respectively. The minimum inhibitory concentrations (MIC) were determined using agar dilution (National Committee for Clinical Laboratory Standards 2001),[21] and serotyping was done on the basis of the quellung reaction with capsular antisera (Statens Serum Institut, Copenhagen, Denmark). Three penicillin-binding protein genes (*pbp1a, pbp2x, pbp2b*) only in susceptible strains and macrolide-resistant genes (*mefA* and *ermB*) were amplified on polymerase chain reaction (PCR; Wakunaga Pharmaceutical, Hiroshima, Japan) according to the manufacturer's instructions.[7,22]

Results

The 50% MIC (mic_{50}) and 90% MIC (mic_{90}) (μg/mL) against the 53 strains of *S. pneumoniae* are listed in Table 1. The MIC for penicillin G and ceftriaxone against 53 pneumococcal isolates (one strain per patient) based on PCR results are shown in Figure 1. The *in vitro* activity of penicillin G against these strains indicated that 34 patients were associated with penicillin-resistant strains (MIC, 2 μg/mL in 20 strains; 4 μg/mL in 14 strains, penicillin G; Fig. 1a). The grade of resistance was much milder for a third-generation cephem, ceftriaxone, than for penicillin G (Fig. 1b). According to a new breakpoint for ceftriaxone,[23] 50 (94.3%) of the 53 strains were regarded as susceptible. Interestingly, of the 53 strains, 39 (73.6%) were genotypic penicillin-resistant *S. pnemoniae* (gPRSP), possessing altered *pbp 1a + 2x + 2b* genes (Table 2).[24] Eight strains possessed altered *pbp 2x + 2b* genes. Lack of mutation was found in only six strains. These pneumococcal strains were also frequently associated with macrolide-resistant genes, such as *mefA and ermB*. Twenty-one strains were found for both *mefA* and *ermB*, 21 strains for *ermB* alone, and four strains for *mefA* alone, and seven strains for no mutation. Thirty-six pneumococcal strains (67.9%), therefore, were gPRSP and simultaneously had the *ermB* gene, which is responsible for high resistance to erythromycin. Relationship between the genotype patterns of the *pbp* gene (Table 3) or the macrolide-resistant gene and serotype distribution among 53 isolates are shown in Table 4). The most frequently observed serotypes were 19F (47.2%), 23F (32.1%) and 6B (5.7%). The rate of coverage of 7-valent conjugate vaccine was 88.7%. Non-covered serotypes in these strains were 6B (three strains), 9L (two strains) and others. More importantly, 92.3% (36/39) of gPRSP and 94.4% (34/36) of gPRSP associated with *ermB* gene were serotype 19F or 23F. We also compared the frequency of drug-resistant genes and the serotype distribution between 40 isolates from NPH and 13 isolates from BMH. Although six strains with no *pbp* gene mutation were found only in isolates from NPH, no difference was found in the frequency of *pbp* genes including gPRSP or macrolide-resistant genes and in the frequency of major serotypes, such as 19F and 23F, between pneumococcal isolates from the two hospitals (data not shown).

Table 1 MIC of 53 strains of *Streptococcus pneumoniae*

Antibiotic	MIC (μg/mL)		
	Range	50%	90%
Penicillin G	0.01–4.00	2	4
Ampicillin	0.01–16.00	4	8
Cefuroxime	0.02–16.00	4	8
Ceftriaxone	0.01–4.00	1	1
Erythromycin	0.01–128	64	128
Minocycline	0.032–16.00	8	8
Gentamicin	2.00–16.00	8	8
Chloramphenicol	1.00–16.00	4	8
Ofloxacin	0.50–4.00	2	4
Co-trimoxazole	2.00–128	128	128

MIC, minimum inhibitory concentration.

Discussion

The present findings indicate that *S. pneumoniae* isolated from pediatric patients with ALRI were highly resistant to penicillin, erythromycin and co-trimoxazole. A similar finding of antibiotic resistance to these drugs was found for *H. influenzae* strains isolated from these patients.[25] An unique finding in the present

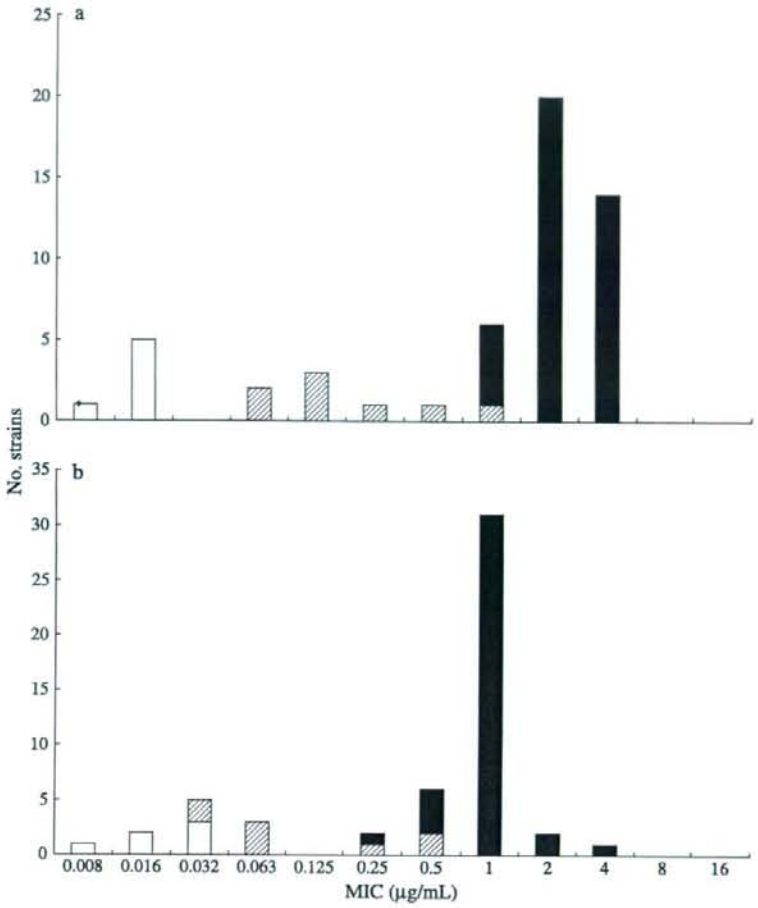

Fig. 1 Relationship between minimum inhibitory concentration (MIC) for two β-lactam antibiotics, (a) penicillin G and (b) ceftriaxone, and genotype of the penicillin-binding protein (*pbp*) genes in 53 clinical isolates of *Streptococcus pnemoniae* from pediatric patients with acute lower respiratory infections. (□) No mutation; (▨) *pbp 2x + 2b* ; (■) *pbp 1a + 2x + 2b*.

study is that most of the pneumococcal isolates from pediatric patients with ALRI were gPRSP with macrolide-resistant genes, which are associated with serotype 19F or 23F. A previous study reported that two international multidrug-resistant clones Spain 23F-1 and Taiwan 19F-14 were spreading among children with upper respiratory tract infections in Hanoi, Vietnam.[26] Another study on surveillance of drug-resistant pneumococcal strains also found the spread of Spain 23F-1 clone among patients with invasive pneumococcal infections in Ho Chi Minh City, Vietnam.[27] The frequency of serotype 19F or 23F (79.3%) in pediatric patients with ALRI in the present study was much higher than that of pediatric patients with upper respiratory tract infections (53%)[26] or invasive pneumococcal infections (43.3%) in Vietnam.[27] The most frequently observed serotype was 23F, but not 19F, in those studies. The present data, therefore, suggest a recent increase of serotype 19F multidrug-resistant pneumococcal strains in pediatric patients with ALRI in Hanoi, Vietnam.

Table 2 Genotype of drug-resistant genes and MIC in 53 strains of *Streptococcus pneumoniae*

Genotype	*n* (%)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
			Penicillin	
No mutation	6 (11.3)	0.01–0.02	0.02	0.02
pbp 2x + 2b	8 (15.1)	0.01–1.0	0.13	1
pbp 1a + 2x + 2b	39 (73.6)	1.0–4.0	2	4
			Erythromycin	
No mutation	7 (13.2)	0.01–0.06	0.03	0.06
MefA	4 (7.5)	0.5–4.0	1	4
ErmB	21 (39.6)	1.0–128	32	128
mefA + ermB	21 (39.6)	4.0–128	128	128

MIC, minimum inhibitory concentration.

The high resistance of the two major pathogens (*S. pneumoniae* and *H. influenzae*) to co-trimoxazole in the present study and in

Table 3 *pbp* genotype and serotype among 53 strains of *Streptococcus pneumoniae*

Genotyping	No. isolates with serotype									
	19F	23F	6B	9L	14	19B	9A	17F	Non-typeable	Total
No mutation	3			2				1		6
pbp 2x + 2b	1	2	3		2					8
pbp1a + 2x + 2b	21	15				1	1		1	39
Total	25	17	3	2	2	1	1	1	1	53

pbp, penicillin-binding proteins.

Table 4 Genotype of macrolide-resistant genes and serotype among 53 strains of *Streptococcus pneumoniae*

Genotyping	No. isolates with serotype									
	19F	23F	6B	9L	14	19B	9A	17F	Non-typeable	Total
No mutation	2	2		2				1		7
mef	1	1			1				1	4
ermB	5	12	3		1					21
mefE + ermB	17	2				1	1			21
Total	25	17	3	2	2	1	1	1	1	53

our previous report may facilitate attempts to use penicillin as an antibiotic for treating pneumonia among children.[25] Although treatment with oral amoxicillin (45 mg/kg) has been shown to be equally effective to that of injectable penicillin for severe pneumonia among children in developing countries,[28] it might be recommended to give a high dose of oral amoxicillin or injectable penicillin or third-generation cephem for children with pneumonia because of the high prevalence of gPRSP or TEM-1 type β-lactamase-producing *H. influenzae* among children in Hanoi, Vietnam.[25] A recent study also reported that drug resistance of nasal carriage isolates of *S. pneumoniae* was increasing in rural areas as well as in urban areas in Vietnam.[29] To prevent the progress of antibiotic-resistant respiratory pathogens, the use of antibiotics without a prescription should be limited in Vietnam.[13]

More importantly, the early introduction of pneumococcal conjugate vaccine is strongly recommended in Vietnam, because the coverage of serotypes by a 7-valent conjugate vaccine was high (88.7%) in pediatric patients with ALRI in the present study. Furthermore, although 7-valent or 9-valent pneumococcal conjugate vaccine are currently available, a new formulation of pneumococcal conjugate vaccine with two serotypes, such as 19F and 23F, may be a possible strategy for reducing the cost of this vaccine in Vietnam.

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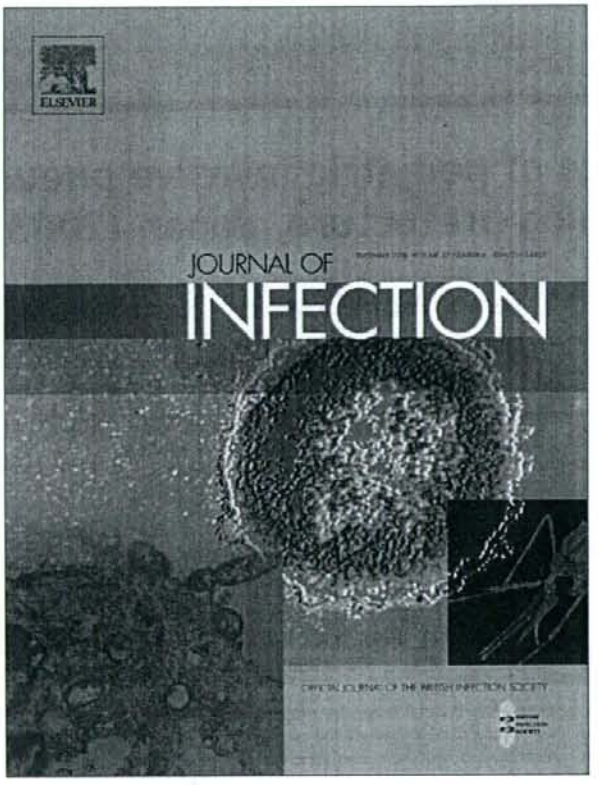

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The incidence of pediatric invasive pneumococcal disease in Chiba prefecture, Japan (2003–2005)

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Summary *Objectives:* The purpose of the study is to evaluate the incidence, spectrum of clinical manifestations and outcome of invasive pneumococcal disease (IPD) in children in Chiba prefecture, Japan.
Methods: To determine the precise incidence of IPD in Chiba prefecture, we implemented a retrospective survey of the period from 2003 to 2005. A written questionnaire was sent to 45 hospitals that have pediatric wards, and information was obtained from all hospitals. The questionnaire included the clinical diagnosis, patient's age, underlying disease, prognosis and antimicrobial susceptibility of the isolated strains.
Results: During the 3 study years, 130 patients were diagnosed with IPD. The mean annual incidence rates of IPD among children <2 and <5 years were 19.5–23.8 and 12.6–13.8 per 100,000, respectively. Among 130 patients with systemic infection, 66 patients had bacteremia, 39 had pneumonia and 16 had meningitis. Five patients had neurological sequelae and 2 patients died. Seventy-four out of 115 isolates (64.3%) exhibited resistance to penicillin G.
Conclusions: The annual incidence of pediatric IPD has remained constant during the study period. Two-third of isolated strains were at least partially resistant to penicillin G. Establishment of appropriate antibiotic therapy against IPD due to penicillin-resistant strains and the introduction of pneumococcal conjugate vaccines are emergent issues in Japan.

Introduction

Streptococcus pneumoniae is one of the leading causes of serious invasive infection, with high mortality and morbidity in children, due to meningitis, septicemia and pneumonia. Over the past decades, the incidence of serious infections due to strains of *S. pneumoniae* with decreased susceptibility to

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penicillin G and broad-spectrum cephalosporin has been steadily increasing worldwide.[1] The number of cases of antibiotic-resistant invasive pneumococcal disease (IPD) has also increased, along with an increasing number of antimicrobial-resistant pneumococcal strains.[2] The emergence of these strains has made the selection of antibiotics for the treatment of IPD more difficult. Recently, the introduction of pneumococcal conjugate vaccines has dramatically decreased the incidence of IPD including antibiotic-nonsusceptible IPD in the United States.[3,4] Accurate up-to-date information on the incidence of IPD is critical to assist decision making for the introduction of pneumococcal vaccines that are appropriate to the country. However, there is little information regarding the incidence of IPD in Japan and the pneumococcal conjugate vaccine has not yet been introduced in Japan. The purpose of this study was to clarify the incidence of IPD in children in the prefecture of Chiba, Japan, using population-based surveillance.

Subjects and methods

To determine the precise incidence of IPD in Chiba prefecture in Japan, we implemented a retrospective survey for the period from 2003 to 2005. Chiba prefecture is one of the 47 prefectures in Japan and is located in the middle of Japan. The population in Chiba prefecture is about 6 million, which represents about 5% of the population of Japan. The population of children less than 2 years of age in Chiba prefecture in 2003, 2004 and 2005 was 109,448, 107,806 and 106,017, respectively. The population of children less than 5 years of age in 2003, 2004 and 2005 was 278,280, 276,108 and 273,468, respectively.[5–7]

A written questionnaire was sent to 45 hospitals in Chiba prefecture that have pediatric wards, and information was obtained from all 45 hospitals either from the clinical records or laboratory records.

IPD was defined as isolation of *S. pneumoniae* from blood cultures in patients with or without focus of infection. The positive rate of blood culture in each hospital was not clarified in this study. The questionnaire included the clinical diagnosis, patient's age, underlying disease, prognosis and antimicrobial susceptibility of the isolated *S. pneumoniae* strains.

Results

During the 3 study years, 130 patients were diagnosed with IPD. The number of cases of IPD in 2003, 2004 and 2005 were 36, 47 and 47, respectively. Among 130 patients with IPD, 66 (50.8%) patients had bacteremia without a focus, 39 had pneumonia, 16 had meningitis and 5 had cellulitis. The mean annual incidence rates of IPD among children less than 2 years of age and less than 5 years of age were 19.5–23.8 and 12.6–13.8 per 100,000, respectively (Table 1). The ages at the onset of IPD were available in 124 patients; 71 (57.3%) in the 0–1-year-old subgroup, 41 (33.1%) in the 2–4-year-old subgroup, 12 (9.7%) in the 5 years or older subgroup. Of the 16 patients with meningitis, 10 (62.5%) were in the 0–1-year-old subgroup, 2 (12.5%) were in the 2–4-year-old subgroup and remaining 4 (25.0%) were in the 5 years or older subgroup (Fig. 1). Fifty-eight percent of the subjects (72/125) were male. At least one underlying condition was documented in 27 (21.8%) of 125 patients with IPD. These included congenital anomaly/syndrome ($n = 6$), bronchial asthma ($n = 4$), malignancy ($n = 4$), congenital heart disease ($n = 3$), neurological disorder ($n = 3$) and others ($n = 7$) in children with IPD. Of the 125 study patients, 5 patients (4%) developed permanent neurological complications. All the patients with neurological sequelae had meningitis. Two study patients (1.6%) died, of which one was aged 3 years with congenital heart disease and the other was aged 17 years with a brain tumor. These fatal cases were diagnosed with fulminant sepsis and pneumonia, respectively. The precise antimicrobial susceptibility of the isolated *S. pneumoniae* strains was known for 115 strains (115/130; 88.5%). Minimal inhibitory concentrations of penicillin G against these 115 *S. pneumoniae* strains were determined by micro-broth dilution method. Of 115 isolates, 49 isolates (42.6%) exhibited intermediate penicillin G resistance and 25 (21.7%) were penicillin G resistant (Fig. 2).

Discussion

Using hospitalized population-based surveillance, this study determined that the annual incidence of IPD among Japanese children younger than 2 and younger than 5 years

Table 1 Annual incidence of invasive pneumococcal infections by clinical diagnosis in Chiba prefecture, Japan

	Number of cases/ 3 years	2003		2004		2005	
		Annual incidence[a]	Annual incidence[b]	Annual incidence[a]	Annual incidence[b]	Annual incidence[a]	Annual incidence[b]
Total IPD	130	23.8	12.6	19.5	13.8	22.6	13.5
Bacteremia	66	13.7	7.2	8.3	7.6	10.4	7.3
Pneumonia	39	3.7	2.2	8.3	4.7	9.4	5.5
Meningitis	16	4.6	2.2	1.9	1.1	2.8	1.1
Cellulitis	5	1.8	0.7	1.9	0.7	0	0.4
Others	4	0	0.4	0	0	0	0

[a] Cases/100,000 children younger than 2 years old.
[b] Cases/100,000 children younger than 5 years old.

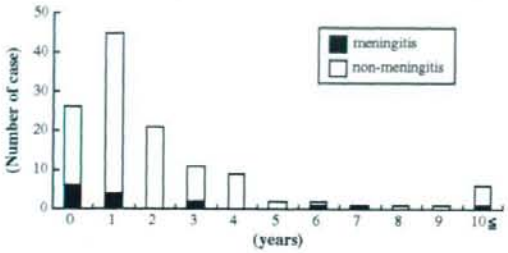

Figure 1 Age distribution of invasive pneumococcal diseases in children.

of age (19.5–23.8 and 12.6–13.8 per 100,000, respectively) was similar to those rates reported in a study conducted in Germany,[8] but was much lower than the incidence reported in the pre-pneumococcal conjugate vaccine era in the United States (166 cases/10^5 children younger than 2 years of age).[9] Several hospital-based European studies have also reported higher rates in children less than 2 years of age, including: the United Kingdom,[10] 42.1; Denmark,[11] 32.5; Finland,[12] 45.3; and Spain,[13] 59.6 cases/10^5/year. In most cases, IPD is detected by blood culture. Thus, validation of the local blood culture practices could affect the incidence rates of IPD.[14] The incidence of IPD reported in the present study was obtained from only the hospitals that have pediatric wards. If our study were to include clinics and hospitals that only have outpatient clinic, the incidence of IPD would be increased. In Japan, primary care physicians tend to easily prescribe antibiotics for febrile pediatric patients. This factor also may influence the incidence of IPD in our study.

This study is the first in the English literature to report the population-based incidence of IPD among Japanese children. One limitation of this study is that surveillance was restricted to children who lived in only 1 prefecture in Japan. Sakata previously evaluated the incidence of IPD in 8 hospitals located in 2 sub-prefectures in Hokkaido, northern part of Japan, from 1999 to 2004. He estimated that the incidence of IPD was 35.5 cases/10^5 children younger than 5 years of age.[15] It was higher than the rate reported in our study. To clarify the incidence of IPD in Japan, officially implemented active surveillance system should be established to monitor the occurrence of IPD throughout overall pediatric population in Japan.

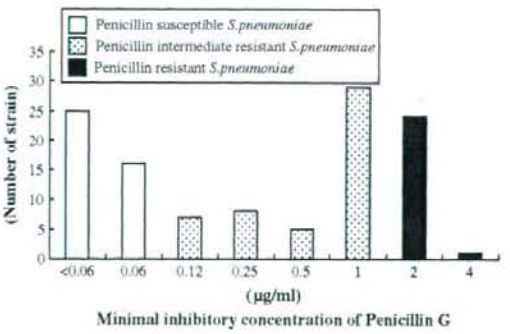

Figure 2 Antimicrobial susceptibility of isolated strains from invasive pneumococcal disease in children.

In this study, 57% of all patients of IPD cases and 63% of the pneumococcal meningitis cases involved children aged less than 2 years of age. Similar to the previous investigations, we found that almost half of the children with IPD were less than 2 years of age and that meningitis occurred more frequently in younger children than in older children.[10–12,16,17]

Our data also indicated that 21.8% of the patients with IPD have various underlying diseases and the remaining 78.2% of the patients had no underlying conditions. The most common underlying disorders in our study were congenital anomaly/syndrome, bronchial asthma and malignancy. We don't have any data on the incidence of these underlying disorders in the community. The underlying disorders in our study were different from those in other studies.[18,19] For instance, the most common underlying disorders in Bennett et al.'s and Levine et al.'s studies were sickle cell anemia and chronic pulmonary disorder, respectively. This finding supports that pneumococcal conjugate vaccine should be recommended not only for children with identifiable risk factors but children who do not exhibit any underlying disorder.

In our study, 42.6% of isolates exhibited intermediate penicillin G resistance and 21.7% of the isolates were penicillin G resistant. Although pneumococcal isolates that are resistant to antimicrobial drugs have been detected on all continents, the rate of beta-lactam resistance in Japan is one of the highest in the world.[20,21] Several reasons for the high beta-lactam resistant rate observed in Japan are considered. One reason is that oral cephalosporin antibiotics are overprescribed for outpatients as a first-choice antibiotic. The prescription of antibiotics for pediatric outpatients in Japan differs from patterns in other countries.[22,23] The problem is reflected by the observation that many *S. pneumoniae* isolates in Japan decrease their susceptibility to cephalosporin antibiotics.[24] The relatively high population density in Japan is a secondary factor, which results in easy transmission of resistant strain among children. Studies conducted in areas with a higher prevalence of penicillin-resistant *S. pneumoniae* have shown this to be associated with increased mortality.[25] In this study, pneumococcal meningitis was the most important cause of long-term morbidity, which contributed to 100% of the sequelae. The mortality rate for our study (1.6%; 2/125) was low, similar to those of other studies, i.e., 1.3%,[12] 2.0%,[26] 2.2%,[27] but not zero. There were 2 fatal cases in our study. One was fatal sepsis and the other was pneumonia. Both cases had severe underlying disease. As such, vaccination is one of the possible ways to prevent this type of infection.

At present, 7-valent conjugate pneumococcal vaccines are not yet being used in Japan. Serotyping was not performed on all of the isolated strains in each hospital in this study period and all strains were not stocked. Therefore, regrettably, serotyping of the isolated strains was not done in this study. However, another study in Japan revealed that 76.2% of the isolated strains from pneumococcal meningitis were covered by the 7-valent pneumococcal conjugate vaccine. The most frequent serotypes were 6B, 19F, 23F, 6A

and 14 in children and the same serotypes predominated among penicillin-resistant strains.[21] Serotyping of *S. pneumoniae* has to be included in the IPD surveillance system in Chiba prefecture that started in 2007.

In conclusion, the annual incidence of pediatric IPD has remained constant during the study period. Two-third of isolated strains were at least partially resistant to penicillin G. Establishment of appropriate antibiotic therapy against IPD due to penicillin-resistant strains and the introduction of pneumococcal conjugate vaccine for the prevention of IPD are the emergent issues in Japan. However, before deciding to introduce such a routine immunization program in Japanese children, nationwide surveillance for incidence of IPD, antibiotic susceptibilities and serotypes of *S. pneumoniae* isolated from IPD patients should be done.

Competing interests

The authors have no competing interests. No funding has supported this work.

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In Vivo and In Vitro Alterations in Influenza A/H3N2 Virus M2 and Hemagglutinin Genes: Effect of Passage in MDCK-SIAT1 Cells and Conventional MDCK Cells▽

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No mutations were detected in the hemagglutinin gene of influenza A/H3N2 virus isolates from patients undergoing short-term amantadine treatment. However, genetic changes occurred after serial passage in either MDCK or MDCK-SIAT1 cells. Our results showed that only a few mutations were observed in MDCK-SIAT1-passaged isolates in the presence of amantadine.

Viral resistance to amantadine emerged quickly in vivo when patients received amantadine for the treatment of influenza A virus infection (3, 5). The genetic basis of resistance to amantadine is associated with amino acid substitutions in the transmembrane region of the M2 gene (2). We documented a high frequency of amantadine-resistant influenza A/H3N2 virus isolates with a Ser-31-Asn mutation in the M2 gene and dual changes in the hemagglutinin (HA) gene at residues 193 and 225 (clade N lineage) (10). However, little is known about whether the HA changes (i) were synergistic with those occurring in the M2 gene in response to drug selection pressure or (ii) occurred separately and were randomly associated with fitness-improving mutations. To address this question, we analyzed changes in the M2 and HA genes of influenza A viruses from clinical samples and from the same isolates that were serially passaged in Madin-Darby canine kidney (MDCK) cells or SIAT1-transfected MDCK cells in the presence or absence of amantadine. The MDCK cell line is commonly used for influenza virus isolation, while the MDCK-SIAT1 cell line is a recently introduced MDCK variant characterized by an overexpression of sialyl-α2,6-galactose that improves the binding of human influenza A virus to the cell receptor (6).

Nasopharyngeal swabs were collected from patients with an influenza-like illness who visited a pediatric outpatient clinic in Niigata City, Japan, from 2000 to 2002. Samples were collected at the first visit and at the second visit after 3 to 5 days of amantadine treatment. One hundred microliters of each sample was inoculated into MDCK cells for virus isolation. Antigenic characterization was performed by hemagglutination inhibition test (12). Influenza A viruses were screened for amantadine susceptibility by the 50% tissue culture infective dose/0.2-ml method (5) and verified by M2 gene sequencing of the transmembrane region to a detect mutation at position 26, 27, 30, 31, or 34 that confers resistance (12). After screening, influenza A/H3N2 viruses that were originally amantadine sensitive and became amantadine resistant after drug treatment were selected and analyzed for this study (in vivo).

MDCK-SIAT1cells (kindly donated by Mikhail Matrosovich, Institute of Virology, Philipps University, Marburg, Germany) were passaged as described elsewhere (6). The selected parental amantadine-sensitive strains were inoculated into MDCK cells or MDCK-SIAT1 cells and sequentially passaged 10 times in the presence or absence of amantadine at a final concentration of 2.0 μg/ml. The viruses were analyzed after the 3rd and 10th passages in the absence or presence of amantadine in MDCK or MDCK-SIAT1 cells (in vitro). Viral RNA extraction and cDNA synthesis were performed as described elsewhere (1). After amplification of the M2 and HA genes, direct sequencing of PCR products was performed with an ABI 3100 DNA sequencer (11). The transmembrane region of the M2 channel protein and the coding regions of the HA1 domain (amino acid residues 1 to 329) and the HA2 domain (amino acid residues 1 to 208) were analyzed. No virus plaque purification and cloning of PCR products was performed prior to sequencing.

Our in vivo study showed that all of the virus isolates ($n = 7$) obtained from patients treated with amantadine possessed M2 gene mutations, whereas HA changes were not observed after amantadine treatment (Table 1).

Our in vitro study showed that all of the viruses developed M2 gene mutations after 10 passages in both MDCK and MDCK-SIAT1 cells in the presence of amantadine, but the M2 mutation sites differed between the two cell lines for five of the isolates (Table 1). On the other hand, no mutations were observed in amantadine-free cultures after 10 passages, except for one isolate that showed an A30T substitution in M2 (conferring amantadine resistance) when grown in MDCK cells.

Analysis of the HA gene showed that six out of seven isolates developed mutations after three passages in MDCK cells, and eventually all of the isolates showed mutations in the HA1 and HA2 domains after 10 passages in the presence or absence of amantadine (Table 1). Most of the HA mutation sites and the type of amino acid substitutions were similar between the iso-

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lates passaged with or without amantadine and between 3rd- and 10th-passage isolates. However, the number of mutation sites increased after the 10th passage when cells were cultivated in the presence of amantadine.

HA changes were not observed in viruses after the 3rd passage in MDCK-SIAT1 cells and occurred in only one or two viruses after the 10th passage without or with amantadine, respectively (Table 1). The HA mutation sites and types of amino acid substitutions found were similar to those found in MDCK-passaged viruses but were far less numerous.

Our clinical surveillance results suggested that the short-term amantadine treatment did not drive HA mutations, and thus, the appearance of clade N was due to the combined events of reassortment (7, 13), amantadine-driven point mutations in the M2 gene, and fitness-improving mutations in the HA gene (11).

The HA gene was more variable in MDCK-passaged viruses than that in their MDCK-SIAT1-passaged counterparts. Mutations in amantadine-free culture accumulated mostly at the receptor-binding (RB) pocket in the HA1 subunit (residues 156, 220, and 229) (14, 15), whereas changes in the HA2 subunit did not have any functional significance. We assume that frequent mutations near RB sites can be attributed to adaptation of the viruses to MDCK cells, which express larger amounts of NeuAcα2,3Gal and smaller amounts of NeuAcα2,6Gal than do human airway epithelial cells (9). Egg-adapted influenza viruses also showed high specificities of binding to NeuAcα2,3Gal, presumably resulting from key amino acid changes at the RB site (at position 226) (4). On the other hand, MDCK-SIAT1 cells, engineered to overexpress NeuAcα2,6Gal, led to fewer HA mutations and could thus be more reliable for multipassage analysis of human influenza virus. Similar results for MDCK-SIAT1-passaged viruses were recently demonstrated by Oh et al., but different amino acid substitutions in the HA1 subunit were observed (8).

In our study, amantadine drove more HA1 mutations in both cell lines (more stable in MDCK-SIAT1 cells) than under amantadine-free conditions, and most of them were key amino acids at or near RB sites (positions 144, 156, 183, 186, 199, 220, 221, 226, 229, and 236) (13–15). Thus, amantadine may contribute to the appearance of a mutated HA gene with an RB site property altered by an unknown mechanism. While our clinical study demonstrated that amantadine did not affect the HA gene in the short term, the in vitro results suggested the possibility that longer exposure to the drug affects the HA gene in vivo, since MDCK-SIAT1 cell-passaged viruses also underwent changes in the HA gene in the presence of amantadine.

Given the small number of samples tested, our results elucidated the differences in the HA gene changes seen in vivo and in vitro in a comparison of two cell lines during the development of amantadine resistance. These results suggested that careful interpretation is needed after consecutive passages in MDCK cells, but MDCK-SIAT1 cells are more favorable for analysis of the RB domain and the molecular epidemiologic phylogeny of the HA gene.

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We declare that none of us have any conflict of interest.

TABLE 1. Sequence analysis of H3N2 variants selected in vivo and in vitro

Patient	In vivo				In vitro[a]											
	Virus from patient before treatment		Virus from patient after amantadine treatment		MDCK cells								SIAT1-MDCK cells			
					Without amantadine				With amantadine				Without amantadine			
					3rd-passage virus		10th-passage virus		3rd-passage virus		10th-passage virus		3rd-passage virus		10th-passage virus	
	M2	HA	M2	HA	M2	HA	M2	HA	M2	HA	M2	HA	M2	HA	M2	HA
1	ND[b]	ND	S31N	—[c]	ND	R229G	ND	R229G	ND	P221L	S31N	S137Y, P221L	ND	—	ND	S31N
2	ND	ND	S31N	—	ND	R229I	ND	R229I	ND	R229I	S31N	R229I	ND	—	ND	S31N
3	ND	ND	A30T	—	ND	R229G	ND	R229G	ND	R229K, I236V	S31N	R229K, I236V	ND	—	ND	I26F
4	ND	ND	V27A	—	ND	R229K	ND	R229K	ND	R229K, I236V	V27A	R50G, V106A, D144N, G186S, S199P, R229L	ND	—	ND	A30V
5	ND	ND	S31N	—	ND	V226I	ND	V226I	ND	V226I	S31N	S247C, P278L	ND	—	ND	V27G
6	ND	ND	S31N	—	ND	—	ND	R83K, H183L, A147T[d]	ND	—	V27A	R83K, H183L, A147T[d]	ND	—	ND	S31N
7	ND	ND	S31N	—	ND	H183L	A30T	H156N, T147A[e], G150E[e]	ND	H183L	S31N	H156N, T147A[e], G150E[e]	ND	—	ND	A30V

[a] Viruses used in the in vitro study were those obtained from patients before amantadine treatment.

[b] ND, mutation not detected.

[c] —, No mutation was detected compared to the virus collected from the patient at the first clinic visit.

[d] Substitutions which are located within the HA2 subunit.

[e] Substitutions which are located within the HA1 subunit.

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ORIGINAL ARTICLE

Molecular evolution of human influenza A viruses in a local area during eight influenza epidemics from 2000 to 2007

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Abstract A total of 1,041 human influenza A virus isolates were collected at a clinic in Niigata, Japan, during eight influenza seasons from 2000 to 2007. The H3N2 subtype accounted for 75.4% of the isolates, and the rest were H1N1. Extremely high rates of amantadine-resistant strains of H3N2 subtype were observed in 2005/2006 (100%) and 2006/2007 (79.4%), while amantadine-resistant strains of H1N1 subtype were only detected in 2006/2007 (48.2%). Sequence and phylogenetic analysis of the HA1 subunit of the hemagglutinin (HA) gene revealed a characteristic linear trunk in the case of H3N2 viruses and a multi-furcated tree in the case of H1N1 and showed a higher sequence diversity among H3N2 strains than H1N1 strains. Mutations in the HA1 from both subtypes were mainly found in the globular region, and only one-third of these were retained for two or more successive years. Higher diversity of H3N2 viruses was mainly attributable to a higher fixation rate of non-synonymous mutations and to a lesser extent to a higher nucleotide substitution rate than for H1N1. Our analysis showed evidence of four positively selected sites in the HA1 of H1 and five sites in that of H3, four of which were novel. Finally, acquisition or loss of *N*-glycosylation sites was shown to contribute to the evolution of influenza A virus, especially in the case of H3N2, which had a higher tendency to acquire new glycosylation sites.

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Introduction

Type A influenza viruses are major pathogens for humans. Annual influenza epidemics are estimated to affect around 3–5 million of the world's population. During the last century, four influenza pandemics alone claimed more than 50 million lives [35]. Vaccination remains the primary measure for mitigating the outcomes of annual influenza epidemics, but vaccine strains have to be updated every year due to the continuous evolution of the viral proteins [6]. Antiviral drugs like M2 channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) provide an alternative for controlling influenza infections, although high resistance rates to the former have widely limited its use [10, 17, 28].

Influenza A viruses are negative sense single-stranded RNA viruses belonging to the family *Orthomyxoviridae* and possess a genome of eight single-stranded segments. Influenza A virus is further classified based on the antigenic properties of its surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA). Influenza A/H3N2 and A/H1N1 subtypes are the major subtypes currently circulating in human populations [24, 37]. HA is of special interest due to its role in the viral entry mechanism and immune recognition. It consists of two subunits: HA1, which contains the receptor-binding and antigenic domains, and the HA2 subunit, which is responsible for the fusion of the virion with the endosomal membrane in the host cell [40, 41]. The HA1 subunit undergoes a process

termed positive Darwinian selection through continuous antigenic mutations that allow the virus to evade the host's humoral immune response [12].

Since the last decade, there has been an explosive increase in sequence data for influenza virus. The availability of high-throughput molecular biology and computational technologies and software provides powerful tools for phylogenetic and evolutionary analyses. Many studies have analyzed the evolution of the HA1 subunit of influenza A/H3N2 viruses and fewer have examined that of A/H1N1 [24]. However, in most of these studies, geographically dispersed isolates were analyzed using routine phylogeny, and issues like evolution rate and positive selection were explored in only a few studies [4, 5, 13, 25]. Moreover, the aim of surveillance programs usually focuses on identifying serologically novel strains and determining their genetic variation, which might bias the results of studies in which the evolution of influenza virus is analyzed [43]. In this study, we analyzed and compared the genetic diversity and mechanisms underlying the evolutionary dynamics of the HA1 of both influenza A/H3N2 and A/H1N1 viruses isolated in a single area in Niigata, Japan, from 2000 to 2007, using evolutionary models and analytical bioinformatics tools [26, 45, 46].

Materials and methods

Sample collection and isolation

During eight influenza seasons from January 2000 to April 2007, nasopharyngeal swabs were obtained from patients with influenza-like illness symptoms at a pediatric clinic in Niigata City, the capital of Niigata prefecture, Japan. The clinic is located in the central part of the city with ~2000 outpatient visits per month. All of the patients from whom swabs were obtained were local inhabitants, with the majority of them residing within a 10-km radius of the clinic. None of the patients in this study received anti-influenza (amantadine) treatment before the swab was obtained. Swabs were checked by using an influenza rapid diagnostic kit such as QuickVue Rapid SP influ (DS Pharma Biomedical Co., Ltd, Osaka), Espline Influenza A&B–N (Fujirebio Inc., Tokyo), or Quick S-Influ A/B "Seiken" (Denkaseiken Co., Ltd, Tokyo). Another swab was obtained from each influenza A-positive patient upon signing an informed consent statement. Swabs were suspended in viral transport medium and kept at 4°C until transportation to our laboratory within 1 week. For influenza virus isolation, 100-μl aliquots of supernatant of nasopharyngeal swabs were inoculated onto MDCK cells and then kept at 34°C under 5% CO_2 until a specific cytopathic effect was detected. The viruses were passaged three times to obtain sufficient virus titers for virus identification. Isolates were then antigenically subtyped by hemagglutination inhibition test [8]. Virus isolates were then stored at −80°C until further analysis.

RNA extraction, PCR, and nucleotide sequencing

One-hundred-microliter aliquots of the supernatant after the third culture passage were used for viral RNA extraction with an Extragen II kit (Kainos, Tokyo, Japan), according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA with the influenza A virus universal primer Uni12, as described elsewhere [18]. PCR was performed with M gene-specific primers to amplify the M2 fragment, a 231-bp product covering nucleotides 680–910 [23]. The PCR products were sequenced to examine amino acid mutations at positions 26, 27, 30, 31, and 34, which confer resistance to amantadine [2, 15]. Following the initial characterization, representative viruses from each subtype were selected at random, but distributed over the epidemic of each season (2–4 isolates each from the beginning, middle, and end of the epidemic in each season) to avoid capturing samples from one outbreak. In the case of the 2006–2007 season, when both amantadine-sensitive and amantadine-resistant H3N2 and H1N1 viruses co-circulated, the same selection criteria was used, but this time relevant numbers of samples from both sensitive and resistant lineages were selected. The HA1 fragments of the selected samples of H3N2 and H1N1 viruses were amplified with specific primers [3]. The PCR products were purified using an MSB spin PCRapace purification kit (Invitek, Berlin, Germany), labeled by the use of a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster, CA) according to the manufacturer's instructions, and then analyzed using an ABI 3100 automatic DNA sequencer.

Sequence alignment and phylogenetic analysis

The sequences were assembled, edited, and aligned using BioEdit 7.0.9 [14]. The genomic sequences of the reference vaccine strains used in this study were obtained from the WHO Influenza Sequences Database (http://www.flu.lanl.gov). Phylogenetic analysis of HA1 was performed using a region of 827 bp corresponding to amino acids 21–295 (covering 84% of the HA1 subunit) for H3N2 and 829 bp corresponding to amino acids 17–292 (covering 85% of the HA1 subunit) for H1N1 viruses. Phylogenetic trees were constructed by neighbor-joining method bootstrap analysis ($n = 1{,}000$) using the MEGA (version 4.0) program [21]. For clarity, H3 numbering (based on A/Aichi/2/68 [42]) of the amino acid positions of the HA1 subunit was used for both H3 and H1.