発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hara M, Sakamoto T, Tanaka K	Influenza vaccine effectiveness among elderly persons living in the community during the 2003-2004 season.	Vaccine	26	6477-80	2008
鈴木幹三、林　嘉光	高齢者へのワクチン接種．感染制御	JICP	4(4)	345-50,	2008
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高山直子、鷲尾昌一、今村桃子、野口房子、小笹晃太郎、井手三郎	地域在住高齢者のインフルエンザ予防ワクチン接種状況と接種行動に影響を与える要因	臨牀と研究	85	281-4	2008
豊島泰子、鷲尾昌一、春口好介、今村桃子、井手三郎	高齢者入所施設におけるインフルエンザワクチンと肺炎球菌ワクチンの接種状況と感染予防対策 －特別養護老人ホーム、養護老人ホーム、老人保健施設、軽費老人ホームの比較－	臨牀と研究	85	1751-4	2008
鷲尾昌一、今村桃子、井手三郎、大浦麻絵、森　満	肺炎球菌ワクチンの公費補助の目的と背景 －全国の自治体の聞き取り調査より－	臨牀と研究	85	863-6	2008
鷲尾昌一、大浦麻絵、森　満	肺炎球菌ワクチンの公費補助と施設入所高齢者の肺炎 －北海道におけるインフルエンザシーズンの調査より－	臨牀と研究	85	1309-12	2008
鷲尾昌一、今村桃子、豊島泰子、中柳美恵子、荒井由美子	高齢者入所施設における入所者と看護・介護職員に対するインフルエンザワクチンと入所者に対する肺炎球菌ワクチンの接種状況 －福岡県での調査より－	臨牀と研究	85	1467-71	2008
春口好介、鷲尾昌一、豊島泰子、今村桃子、井手三郎	高齢者入所施設における看護・介護職員の業務に関する現状と課題 －福岡県における施設職員の業務と医療行為に関する実態調査－	臨牀と研究	85	1611-45	2008

III. 研究成果の刊行物・別刷り

A Practical Guide for Designing and Conducting Influenza Disease Burden Studies

World Health Organization
Western Pacific Region
November 2008

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A practical guide for designing and conducting influenza disease burden studies.

1. Influenza, Human 2. Cost of illness 3. Guideline

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1.1.7.2 Influenza managed in the outpatient setting

Disease burden:

Essential data:
- Total number of outpatient visits per week, month, year
- Proportion of outpatients with ILI per week, month, year
- Proportion of ILI patients with laboratory-confirmed influenza infection
- Age distribution of ILI patients and laboratory-confirmed influenza infections
- Seasonality: proportion of ILI cases with laboratory-confirmed influenza infection reported by week and month

Desirable data:
- If catchment population is known or can be estimated, incidence per 100 000 persons per year
- Distribution by age groups (0-2 years, 3-4 years, 5-17 years, 18-49 years, 50- 64 years, ≥ 65 years)
- Clinical data, including history, symptoms at presentation, medical intervention

Socioeconomic burden data:
- Direct treatment costs
- Lost work/school days due to laboratory-confirmed influenza infection
- Out-of-pocket costs to patient and families

1.2. ABSENTEEISM AS A MEASURE OF DISEASE BURDEN

Absenteeism at schools and workplaces can provide important information on the social and economic costs of influenza. Research has shown that trends in absenteeism often correspond to seasonal variations in influenza activity (16).

1.2.1 Absenteeism in Schools

As school-aged children are at high risk for influenza infection, data on absenteeism due to influenza-like illness (ILI) from schools may provide useful information on the seasonality, burden and social costs of influenza. Individual schools may be selected to act as sentinels and followed for one or more school years to determine the frequency of ILI-associated absenteeism. If laboratory diagnosis is available, an improved understanding of the burden and seasonality of laboratoryconfirmed influenza infection in the general community may also be gained by the study of school-age populations.

1.2.1.1 Methods and Materials

Study design:
Prospective, longitudinal, observational study

Study population:
All children attending selected schools in the survey area.
Selection of schools:
Schools should be randomly selected from among all schools in a specified administrative area.

Data to be collected:
Number of children under surveillance

Number of absence events/episodes
Number of missed school days
Number of absence events associated with an ILI
Number of missed school days associated with an ILI
Number of absence events associated with laboratory-confirmed influenza
Number of lost school days due to laboratory-confirmed influenza infection

Survey procedure:

1. Selected schools keep daily attendance books throughout the survey time period.
2. All school absences are reported to the study coordinator on a daily basis (Annex 4).
3. The school nurse or designated study staff then contacts the child's family to inquire if the absence is related to illness. If yes, the student's parents will complete a reporting form (Annex 5). The reporting form details symptoms of influenza-like illness (ILI), such as fever, cough or sore throat as well as any visits to a medical provider. ILI-associated absences are recorded and tallied.
4. If the study has sufficient financial resources and access to a qualified diagnostic laboratory, children with an ILI-associated absence may be tested for the presence of an influenza virus infection. In this case, a health care worker from the study will visit the ill child in their home to collect clinical swab specimens for laboratory diagnosis. If available, a rapid influenza test may be used to diagnose influenza infection during the home visit.

1.2.1.2 Data Storage and Analysis

An efficient system for storage and management of data should be established prior to the commencement of the survey. Weekly tallies of the number of ILI-associated absences can be used as an indicator of increased influenza activity.

There are three levels of potential data:

1. Total (All-cause) Absence Episodes
2. Total ILI-Associated Absence Episodes
3. Total Laboratory-confirmed Influenza Infection Absence Episodes

Data analysis:

1. Calculate the proportion of absentees by:

$$\frac{\text{Total number of students absent at the school during the week}}{\text{Total number of students attending the school on the first day of the week}}$$

   If a student is absent across more than one week, he/she contributes to the numerator for both weeks

2. Attempt to identify the commencement of the influenza season by increases in absenteeism.

3. Estimate influenza burden by comparing the proportion of absentees during the influenza season with those during the non-influenza season (excess influenza absenteeism).

4. Estimate the relative influenza burden by calculating the proportion of absenteeism due to influenza, based on results of influenza diagnostic laboratory tests.

1.2.1.3 Monitoring

An ongoing comparison of school ILI-associated absenteeism with the reported incidence of influenza from the national surveillance system will allow an assessment of the reliability of the survey results.

1.2.1.4 Ethical Considerations

This type of study will, in most cases, require a formal protocol and clearance from the relevant Institutional Review Board(s).The coordinators of the survey must protect the privacy of individuals when reporting and publishing data. Before commencing such a survey, the coordinators must thoroughly explain the purpose, protocols, and material of the survey to the school administration and to parents. Written consent should be obtained, and signed and dated by both the school caretaker and the study coordinator. The titles, names, and contact information of the signatories should be clearly provided. The original copies of the document are filed at the school and at the local health care centre. Informed consent must also be obtained from a parent or legal guardian of the absentee.

1.2.2 Absenteeism in Workplaces

Selected workplaces are identified to act as sentinels and monitored prospectively for absences due to ILI. People of working age are considered to be at lower risk for influenza morbidity and its complications than other age groups. Workers may continue to work while ill or return to work before they are fully recovered. Consequently, workplace absenteeism survey data may underestimate the true burden.

1.2.2.1 Methods and Materials

Study design:
Prospective, longitudinal, observational study

Study population:
All employees working in selected workplaces

Selection of workplaces:
When possible, workplaces should be randomly selected from all workplaces within a given region.

Data to be collected:
Number of all-cause absentee episodes
Number of ILI-associated absentee episodes
Number of absentees episodes with laboratory-confirmed influenza
Costs incurred due to medical assessment/intervention

Survey procedure:
1. Selected workplaces keep daily attendance books throughout the survey year. The number of absentees is recorded and reported to the study coordinating centre every day (Annex 6).

Employees absent from work provide a completed reporting form (Annex 7) at the time of return. The reporting form details symptoms of influenza-like illness (ILI), such as fever, cough or sore throat as well as any visits to a medical provider. ILI-associated absences are recorded and tallied.

2. If the study has sufficient financial resources and access to a qualified diagnostic laboratory, workers with an ILI-associated absence may be tested for the presence of an influenza virus infection. In this case, a health care worker from the study will visit the worker in their home to collect clinical swab specimens for laboratory diagnosis. If available, a rapid influenza test may be used to diagnose influenza infection.

3. Workplaces should calculate employees' medical costs associated with these illnesses.

1.2.2.2 Data Storage and Analysis

An efficient system for storage and management of data should be established prior to the commencement of the survey. Weekly tallies of the number of ILI-associated absences can be used as an indicator of increased influenza activity.

Data analysis:

1. Calculate the proportion of absentees by:

$$\frac{\text{Total number of employees absent at the workplace during the week}}{\text{Total number of employees attending the workplace on the first day of the week}}$$

If an employee is absent across more than one week, he/she contributes to the numerator for both weeks.

2. Attempt to identify the commencement of the influenza season by increases in absenteeism.

3. Estimate influenza burden by comparing the proportion of absentees during the influenza season with those during the non-influenza season (excess influenza absenteeism).

4. Estimate the relative influenza burden by calculating the proportion of absenteeism due to influenza, based on results of influenza diagnostic tests.

5. Calculate excess medical expenses due to influenza by comparing absentees' medical expenses with non-absentees' medical expenses during influenza season. It is important to recognize that the proportion of people with underlying disease may be larger among absentees than among non-absentees. Thus, excess medical expenses due to influenza can also be estimated by comparing medical costs of absentees during the influenza season with that during the non-influenza season.

1.2.2.3 Monitoring

An ongoing comparison of ILI-associated workplace absenteeism with the reported incidence of influenza from the national surveillance system will allow an assessment of the reliability of the survey results.

1.2.2.4 Ethical Considerations

This type of study will require a formal protocol and clearance from the relevant Institutional Review Board(s).The coordinators of the survey must protect the privacy of individuals when reporting and publishing data. Before commencing such a survey, the coordinators must thoroughly explain the purpose, protocols, and material of the survey to the business administration and to the workers. Written consent should be obtained, and signed and dated by both all workers. The titles, names, and contact information of the signatories should be clearly provided. The original copies of the documents are filed at the study coordinating centre.

Annex 4:

Health Care Center Recording Form (School Absenteeism)

Date of Information : __/__/_____ (dd/mm/yy)

Name of School:

School Close : Yes _____ No _____

	Grade 1		Grade 2		Grade 3		Grade 4		Grade 5		Grade 6	
Number of absentees	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Number of absentees from febrile disease												
Number of laboratory confirmed influenza												
Number of AH1 influenza												
Number of AH3 influenza												
Number of B influenza												

Annex 5:

Student Absentee Reporting Form

Name of school :

Grade :

Name :

Periods of absenteeism : from __/__/__ (dd/mm/yy) to __/__/__ (dd/mm/yy)

Consultation in a clinic (yes • no)

Symptoms during the period of absenteeism.

1. Max temperature : degrees
2. Nasal discharge ; (presence • absence)
3. Cough : (presence • absence)
4. Sputum : (presence • absence)
5. Sore throat : (presence • absence)
6. Joint pain : (presence • absence)

Diagnosis	:	Influenza : others ( )
Rapid diagnostic test	:	done (A • B • negative) • not done
Pneumonia	:	presence • absence
Encephalopathy	:	presence • absence
Otitis Media	:	presence • absence

Annex 6:

Health Care Center Recording Form (Workplace Absenteeism)

Date of Information : ___/___/_____ (dd/mm/yy)

Name of work place :

Age	< 20 years old		20 - 29		30 - 39		40 - 49		50 - 64		65+	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Number of absentees												

Annex 7:

Employee Absentee Reporting Form

Name of workplace :

Unit / Department :

Name :

Periods of absenteeism : from __/__/__ (dd/mm/yy) to __/__/__ (dd/mm/yy)

Consultation in a clinic (yes • no)

Symptoms during the period of absenteeism.

1. Max temperature : degrees
2. Nasal discharge : (presence • absence)
3. Cough : (presence • absence)
4. Sputum : (presence • absence)
5. Sore throat : (presence • absence)
6. Joint pain : (presence • absence)

Diagnosis	:	Influenza : others ( )
Rapid diagnostic test	:	done (A • B • negative) • not done
Pneumonia	:	presence • absence

Immunogenicity of Trivalent Inactivated Influenza Vaccine Among Children Less Than 4 Years of Age

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This study investigated the immunogenicity of trivalent inactivated influenza vaccine. Subjects comprised 259 children under 4 years of age who visited 6 pediatric clinics to receive influenza vaccine. Age distributions were: 64 in <1.0 year, 65 in 1.0-1.9 year, 64 in 2.0-2.9 year and 66 in 3.0-3.9 year age group. Two doses of vaccine were given subcutaneously at 4 weeks apart. Dosage was 0.1 ml for children <1-year-old, while dosage was 0.2 ml for children ≥1-year-old, in accordance with standard Japanese recommendations. To measure hemagglutination inhibition (HAI) antibody titer, triplet sera were obtained before vaccination (S0), 4 weeks after first vaccination (S1) and 4 weeks after second vaccination (S2). The geometric mean of HAI antibody titer and seroprotection proportion (postvaccination titer ≥1:40) were calculated by age group. Analysis of variance was also employed to estimate the independent effects of age and prevaccination titer on the fold-rise in antibody. Geometric means of HAI titer were lower among the two younger age groups than among the two older age groups, regardless of vaccine strain or when blood samples were collected. Seroprotection proportion after 2 doses of vaccine in <1.0 year, 1.0-1.9 years, 2.0-2.9 years and 3.0-3.9 years were: 38%, 58%, 89% and 85% against A(H1); 52%, 54%, 81% and 73% against A(H3); and 23%, 49%, 67% and 71% against B, respectively. Regarding analysis of variance, prevaccination titer consistently indicated strong effects on antibody fold-rise, regardless of vaccine strain or combinations of paired sera. After 2 doses of vaccine (S2/S0), significant effects of age on antibody induction were shown against A(H1) and B (P=0.000 and P=0.002, respectively). Thus, the immunogenicity of trivalent inactivated influenza vaccine was strongly affected by prevaccination titer and age. Even after 2 doses of vaccination, a protective level of antibody could not be achieved in about 50-80% of subjects among infants aged <1-year-old, and 40-50% among children at 1.0- to 1.9-years-old.

Introduction

Recently, several studies have documented that the risk of hospitalization from influenza are higher among infants[1-3]. In addition, about half of influenza-related pediatric deaths from the United States during the 2003/2004 season, had no underling medical condition previously associated with an increased risk for influenza-related complications[4]. These results lead to recognition that preventing influenza is important also in healthy children without underlying medical condition. As a result, the US Advisory Committee on Immunization Practices (US-ACIP) began recommending that infants aged 6-23-months should be vaccinated from the 2004/2005 season[5]. Furthermore, US-ACIP decided that recommendation for vaccination of children was extended from infants aged 6-23-months to all children aged 6-59-months in the 2006/2007 season[6]. However, the immunogenicity and efficacy of influenza vaccines in infants have not necessarily been established[7-9]. The reason is that infants differ from adults in terms of maturity of the immune system, history of influenza virus infections and history of vaccinations. Particularly in Japan, vaccine dosage by standard recommendation is lower than that in other countries [6]. It has been controversial whether current vaccine dosage in Japan elicits sufficient immunological responses for preventing influenza infection. However, due to the logistic difficulties, few investigations have examined immunogenicity in infants and children.

Materials and Methods

Subjects. Subjects comprised children <4-years-old who visited one of 6 pediatric clinics for vaccination from October to November 2005. Children with an acute febrile illness or signs of severe acute illness at the time of vaccination, past history of anaphylaxis due to vaccine components, or other inappropriate condition to receive vaccination were excluded. After explaining about the study, written informed consent was obtained from the legal guardian (mainly parents) of each subject. Eventually, a total of 259 children were enrolled. Age distributions were: 64 in <1.0 year, 65 in 1.0-1.9 year, 64 in 2.0-2.9 year and 66 in 3.0-3.9 year age group. All subjects completed the study protocol including collection of blood samples at three times.

Vaccination. The vaccines administered were the commercially available inactivated trivalent influenza vaccine for the 2005/2006 season (Biken HE01A). The vaccine contained A/New Caledonia/20/99 (H1N1), A/New York/55/2004 (H3N2) and Shanghai/361/2002 (B), and the antigen level for each strain was 30 μg/ml. Vaccine dosage were given according to the standard Japanese recommendation (i.e., 0.1 ml for <1-year-old and 0.2 ml for 1.0- to 5.9-years-old). Two doses of vaccine were administered subcutaneously 4 weeks apart. The second dose was completed before the end of November.

Serum collection and antibody titer measurement. Blood samples were collected for the following 3 points: before vaccination (S0), 4 weeks after first vaccination (S1), and 4 weeks after second vaccination (S2). Serum samples were stored at between -70 and -80°C, and hemagglutination inhibition (HAI) antibody titers at above triplet point were measured according to the conventional method.

Analysis. The geometric mean of HAI titer and seroprotection

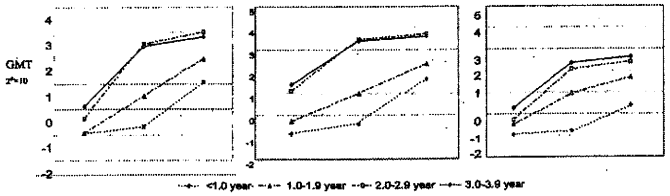
proportion (proportion of subjects with ≥1:40 HAI titer)[10,11] were calculated by age group. In these calculation, HAI titers of <1:10 were regarded as 1:5. The Mantel-extension method was used to assess the association between age and seroprotection proportion. Furthermore, to ascertain the effects of prevaccination HAI titer and age on immunological responses, analysis of variance was conducted using the fold-rise in HAI titer as a dependent variable, and age (4 levels) and prevaccination HAI titer (3 levels) as independent variables. In this analysis, HAI titers were subjected to logarithmic conversion. All tests were two-sided, and level of significance was set at 5%. All statistical analyses were performed using SAS version 9.1.3 (SAS Institute Inc.).

Ethical considerations. The study protocols were approved by the Clinical Study Review Board of Medical Co. LTA Kyushu Clinical Pharmacology Research Clinic.

Results

Geometric mean of HAI titer (Figure 1). Geometric means of HAI titer were lower among the two younger age groups than among the two older age groups, regardless of vaccine strain or when blood samples were collected. For children aged 2 and 3-years, geometric mean markedly increased after the first dose, confirming a favorable HAI titer rise, but degree of HAI titer rise following the second dose was not marked. Conversely, for children aged 0 and 1-year, degree of HAI titer rise following the first dose was small, while degree of HAI titer rise following the second dose was large. After 2 doses of vaccine, antibody titer for children aged 0 and 1-year was lower than that for children aged 2 and 3-years.

Figure 1. Geometric mean of HAI antibody titer against inactivated influenza vaccine by age group.



Blood samples were collected before first vaccination (S0), 4 weeks after first vaccination (S1) and 4 weeks after second vaccination (S2).

Seroprotection proportion (Table 1). The range of seroprotection proportion following 2 doses was 23-52% for infants aged <1.0 year, 49-58% for children aged 1-year, 67-89% for those aged 2-years and 71-85% for those aged 3-years. Thus, seroprotection proportion (S2) for the two younger age groups was lower than that for the two older age groups.

Analysis of variance. Irrespective of vaccine strain or combinations of paired sera, effects of prevaccination HAI titer on fold-rise in antibody were always significant (P=0.000-0.013). Age and prevaccination HAI titer represented independent significant factors for titer rise after the first dose (S1/S0). After 2 doses of vaccine (S2/S0), significant effects of age on antibody induction were shown against A(H1) and B (P=0.000 and P=0.002, respectively), but not against A(H3) (P=0.766). Thus, the immunogenicity of trivalent inactivated influenza vaccine was strongly affected by prevaccination titer and age.

Table 1. Seroprotection proportion as frequency of subjects with postvacccination HAI titers 1:40.

		A/New Caledonia/20/99(H1N1)			A/New York/55/2004(H3N2)			B/Shanghai/361/2002		
Age (years)	N	S0	S1	S2	S0	S1	S2	S0	S1	S2
<1.0	64	1 (2)	2 (3)	24 (38)	2 (3)	2 (3)	33 (52)	0 (0)	3 (5)	15 (23)
1.0–1.9	65	0 (0)	18 (28)	38 (58)	9 (14)	18 (28)	35 (54)	8 (12)	22 (34)	32 (49)
2.0–2.9	64	3 (5)	50 (78)	57 (89)	23 (36)	47 (73)	52 (81)	8 (13)	39 (61)	43 (67)
3.0–3.9	66	11 (17)	51 (77)	56 (85)	29 (44)	45 (68)	48 (73)	15 (23)	43 (65)	47 (71)
Total	259	15 (6)	121 (47)	175 (68)	63 (24)	112 (43)	168 (65)	31 (12)	107 (41)	137 (53)
Trend P*		0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000

Discussion

This study shows that younger age was associated with more difficult acquisition of protective-level HAI titers. This results is consistent with that in past studies[12,13]. Even after second vaccination, about 50-80% of 0-year-olds and 40-50% of 1-year-olds had not achieved protective-level of HAI titers. In terms of the geometric mean of HAI titer, degree of HAI titer rise following the first dose was more favorable for the two older age groups than for the two younger age groups. The reason for this was that older children already had antibodies before vaccination. In addition, degree of HAI titer rise following the second dose was lower for older children than for younger children. This represents the phenomenon called the law of initial value or negative feedback[10]. In other words, small immunological response following the second dose might be results from highly achieved HAI titers after the first dose among older children. 24% of total subjects possessed protective-level HAI titers against A(H3) before vaccination. The proportion of subjects with protective-level HAI titers before vaccination was higher for A(H3) than for A(H1) or B. The reason is that A(H3) represented the main epidemic strain in 2 of the past 3 seasons. If subgroup analysis is performed by limiting subjects without antibody, the effects of age alone could have been ascertained. To do such analysis, however, more sufficient numbers of children would be needed to enroll. Therefore, in this study, effects of age and prevaccination HAI titer were simultaneously considered to investigate the independent effects of these factors by using the analysis of variance. The analyses showed that the effects of prevaccination HAI titer on fold-rise in titer were always significant, regardless of vaccine strain or combinations of paired sera, and marked effects were independent of age. The effects of age on titer rise following the two doses (S2/S0) were significant for A(H1) and B, but not for A(H3). However, the effects of age for A(H3) were significant following the first dose (S1/S0) and second dose (S2/S1). This could be explained as follows: the HAI titer rise following first dose (S1/S0) was slight for younger children and favorable for older children, but degree of HAI titer rise following second dose (S2/S1) was favorable for younger children and slight for

older children. As a result, the effects of age were significant when separately analyzing first and second doses, but were not significant when combining both doses. This tendency was marked for A(H3), as prevaccination HAI titers for A(H3) were high, particularly among children aged 2 and 3-years. These findings suggest that both existing antibody and age are closely related to immunological response to vaccines.

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Does Influenza Viral Population Change in a Patient Infected With Influenza?

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Introduction

New epidemic strains of influenza are always generated and seasonal influenza epidemics occur every year in the world because influenza viruses continually change genetically to evade host immunological attacks [1]. However, little is known about when and where influenza viruses change in the world. There are two ideas; a new strain comes into the human population in one place on the earth and spreads around the world from there or some new strains appear somewhere in the world at about the same time and spread from local areas all over the world. In both cases, the viral genetic variation must occur in a reservoir host infected with influenza virus. It is thought that an influenza virus isolated from an individual is genetically heterogeneous. The composition of the influenza viral population (quasispecies) at an early infection phase can differ from that at a later infection phase. Our objective for this study was to observe the changes of the influenza virus dominant population during a natural clinical course from a patient infected with influenza. Thus, we cloned and sequenced the RT-PCR products of the HA1 region of the HA gene in influenza virus isolates and compared the composition of the viral population between the early and late phase of infection in patients.

Materials and Methods

We collected nasal washes from patients with influenza-like illness at the early and late phase (from one to five days after onset of illness) of the clinical course at a pediatric clinic in Kadoma, Osaka, during the 2001-2004 influenza seasons. We inoculated specimens onto MDCK cells and harvested supernatants when CPE was observed. We identified isolates using the PAP staining technique with type and sub-type specific monoclonal antibodies to influenza viruses [2] or using the hemagglutination inhibition test with type specific polyclonal chicken sera (Denka-Seiken, Tokyo, Japan) [3]. Viral RNA from isolates was extracted with QIAamp Viral RNA Mini Kit (Qiagen Japan, Tokyo, Japan). We performed RT-PCR on the HA1 region of HA gene in H3N2 influenza virus by using Ready-to-Go RT-PCR Beads (GE Healthcare, UK) with specific primers (5'-CTATCATTGCTTTGAGCTAC-3' and 5'-GTTTCTCTGGTACATTCCGC-3'). The PCR products of 1,023 base pairs were cloned with TOPO TA-Cloning Kit for Sequencing (Invitrogen, California USA) or sent to a commercial laboratory (Takara-bio, Otsu, Japan) where each RT-PCR product was cloned and sequenced. We analyzed a 981 base pair nucleotide sequence of the HA1 gene with Genetyx software (Genetyx, Tokyo, Japan).

Results

Preliminary experiment. There is a chance to change the viral population by passage in MDCK cells. To confirm this possibility, compositions of viral populations among nasal washes, primary viral cultures in MDCK cells and secondary passage viral cultures in MDCK cells were compared. We undertook an investigation of two cases during the 2005-06 season. More than 45 clones of each PCR product of 6 specimens (two nasal washes, two primary viral cultures and two secondary viral cultures) were sequenced and analyzed. No changes of the dominant population among the three isolation procedures were observed in the two experiments (Table 1). Therefore, we used primary viral cultures as the first choice or secondary viral cultures if primary viral cultures were not available, to amplify the isolates instead of nasal washes to achieve our objective.

Table 1. Changes of the dominant population between nasal washes and viral cultures.

	Dominant population clone / total clones sequenced (%)		
		Viral culture with MDCK cells	
	Nasal wash	Primary	2nd passage
Examination 1	36/45 (80.0)	44/54 (81.5)	42/48 (87.5)
Examination 2	48/52 (92.3)	38/51 (74.5)	44/47 (93.6)

Background of cases. Ten infant/adolescent cases were investigated. Backgrounds of all cases are described below: Case 1; a 3-month-old boy, sampling dates on 2 and 5 Feb. 2002, Case 2; a 3-year-old girl, sampling dates on 2 and 5 Feb. 2002, Case 3; a 5-month-old boy, sampling dates on 13 and 16 Feb. 2002, Case 4; a 1-year-old girl, sampling dates on 12 and 16 Feb. 2002, Case 5; a 1-month-old boy, sampling dates on 29 Mar. and 1 Apr. 2002, Case 6; a 6-year-old girl, sampling dates on 2 and 5 Feb. 2003, Case 7; a 4-year-old boy, sampling dates on 20 and 23 Jan. 2004, Case 8; a 2-year-old girl, sampling dates on 19 and 23 Feb. 2004, Case 9; a 4-year-old girl, sampling dates on 20 and 23 Feb. 2004, Case 10; a 4-year-old girl, sampling dates on 9 and 12 Feb. 2004. Case 1 and 2 were siblings. Additionally, a specimen from the father of Case 5, sampling dates on 1 Apr. 2002, was investigated, because he appeared to catch influenza from his baby. H3N2 influenza viruses were isolated from all of the specimens above. All cases completely recovered from the disease and there were no complications.

Viral population and nucleotide sequences of dominant population. Nucleotide sequenced clones from each isolate were classified into dominant population (a large number of identical clones), minor population (a small number of identical clones) and no cluster (a group of unique clones). The percentage of dominant populations in each isolate ranged from 54.5 to 90.0 percent (Table 2). At the early infection phase, all isolates consisted of one dominant population and some unique clones. At the late infection phase, all isolates had one dominant population, a few or no minor populations and no cluster clones. As far as we tested, the dominant population in isolates at early and late infection phases from individual

patients demonstrated the same nucleotide sequences. However, the sequences of the dominant populations from all cases differed from one another except for case 1 and 2 (siblings) and case 5 and his father. As for the deduced amino acid sequences of the dominant populations, comparable results were obtained.

Table 2. The Percentage of the dominant population in each isolate from patients infected with influenza.

	Early phase		Late phase	
Case	Total number of sequenced clones	Dominant population clones (%)	Total number of sequenced clones	Dominant population clones (%)
1	58	45 (77.6)	46	29 (63.0)
2	51	40 (78.4)	60	44 (73.3)
3	57	39 (68.4)	52	35 (67.3)
4	45	35 (77.8)	50	34 (68.0)
5	45	30 (67.8)	55	30 (54.5)
6	20	17 (85.0)	20	14 (70.0)
7	20	14 (70.0)	20	14 (70.0)
8	20	18 (90.0)	20	16 (80.0)
9	20	13 (65.0)	20	13 (65.0)
10	20	17 (85.0)	20	15 (75.0)

Chronological analysis. The changes to the nucleotide and deduced amino acid sequences of the HA1 region of the HA gene of the dominant viral population occurred chronologically. Influenza isolates have been proceeding genetically like as on the natural course. Of the antigenic epitopes in HA of H3N2, the variations on site A and B were regularly occurred and accumulated (Table 3).

Table 3. Chronologically change of deduced amino acid in antigenic site of HA1 region of the dominant viral population.

	Sampling	Site A		Site B				
	date	Position number		Position number				
Case	M-Y	144	145	155	156	159	173	189
1	02-02	N	K	H	Q	Y	E	S
2	02-02	N	K	H	Q	Y	E	S
3	02-02	D	K	H	Q	Y	K	S
4	03-02	D	K	H	Q	Y	K	S
5	03-02	D	K	H	Q	Y	K	S
6	02-03	N	K	T	H	Y	K	S
7	01-04	N	K	T	H	Y	K	S
8	02-04	D	K	T	H	F	K	N
9	02-04	N	N	T	H	F	K	N
10	02-04	N	K	T	H	F	K	N

Discussion

The dominant population of influenza virus from an infected individual does not change significantly during the natural clinical course or during transmission, as was suggested from those cases that were relatives, whereas isolated influenza viruses from each patient are not identical. Influenza viruses chronologically lined have been proceeding genetically. Our data show that influenza virus does not change as rapidly as one may predict. However, because influenza virus is believed to mutate in a human host, the data suggest that in this study we are likely to have missed the time and place of such change. Therefore, this study suggests that in order to significantly diverge genetically and to accumulate measurable variation, influenza viruses must go through numerous cycles of human-to-human transmission. Paradoxically speaking, influenza virus would, therefore, not evolve according to this paradigm if we were able to interrupt human-to-human transmission. In order to slow evolution of influenza, it is of utmost importance to prevent influenza infection and transmission, which from a public health standpoint indicates that prophylaxis may be more suitable than therapy for influenza control.

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