Method

Settings

Our study was performed at Aino Memorial Hospital (hospital A), which is affiliated with Nagasaki University, Japan. Hospital A includes 190 beds for geriatric wards. The average length of hospital stay was 110 days. In the study, informed consent was obtained from either the patients or their families.

Objectives

We had two main study aims. The first aim was to investigate the efficacy of infection control measures against nosocomial pneumonia in geriatric wards, including disinfection of the upper airway using povidone-iodine. The second aim was to clarify the patient-to-patient transmission of MRSA and the environmental contamination from colonizing MRSA; we then investigated the differences of MRSA types found in the patients and the environment using pulsed-field gel electrophoresis (PFGE) with the *SmaI* restriction enzyme.

Infection Control

Concerning the measures for infection control in hospital A, to reduce nosocomial pneumonia, we have added 3 active infection control measures in addition to the hand cleaning since October 1991. Hand cleaning is the gold standard of infection control worldwide. The first additional measure was isolating the patients with either colonization or infections with MRSA. For example, if a patient with MRSA colonization was found, he/she was immediately transferred to the 8-bed geriatric ward for MRSA-colonized patients. To prevent the formation of decubitus ulcers, the body position of bedridden patients was frequently changed. And if a patient had a moderate-to-severe decubitus ulcer, disinfection of the ulcer region using 3% povidone-iodine with 70% sugar was performed. Povidone-iodine treatment was applied to the patient once a day. To prevent nosocomial lower respiratory tract infection and pneumonia, disinfection of the upper respiratory tract by using 0.03–0.07% povidone-iodine in the oral cavity was applied to the bedridden patients and the MRSA-colonized patients. Povidone-iodine solution was sprayed into the patient's mouth twice a day using a Jackson-type hand nebulizer, and 5% povidone-iodine gel in the nasal cavity was applied to the bedridden patients twice a day through the nose.

We introduced disinfection of the upper respiratory tract because we found that that of bedridden aged patients was significantly colonized with MRSA and gram-negative rods.

Case Analysis

Cases of nosocomial pneumonia were retrospectively analyzed between January 1991 and March 1995. The study period was divided into four annual periods (periods 1, 2, 3 and 4). Period 1, January to December 1991, was used as the control.

During the study, the diagnosis of nosocomial pneumonia was based on the appearance of clinical symptoms 48 h after admission.

PFGE Study

For the first PFGE study, we performed a 12-week prospective culture survey to investigate the possible relationship between *S. aureus* types colonizing the respiratory tract and rectum of bedridden patients, and those isolated from the hospital environment. Regarding the sampling for methicillin-susceptible *S. aureus* (MSSA) and MRSA, the strains of *S. aureus* were isolated from the bedridden patients and the hospital environment in the geriatric ward. Culture specimens were prepared from simultaneously obtained swabs from the nasal cavity, pharynx and rectum. Sputum samples were collected using sterilized suction tubes. Culture samples from the environment of the investigated room were obtained using swabs from the floor surface (area, 100 cm^2) before ward cleaning and by agar plates placed for 3 h in 6 places throughout the room (one in each corner, one in the center and one at the entrance). Sampling was performed at 2-week intervals at a specific time (8–11 a.m.) on the surveillance days (days 1–7). A total of 40 strains of *S. aureus* were isolated. Of the 40, 23 strains (57.5%) of MRSA were isolated from both the bedridden patients and the environment (*S. aureus*: 21 from patients, 19 from the hospital environment). The relationship between oxacillin resistance and PFGE type was investigated in the 40 strains of *S. aureus* isolated from bedridden patients and the hospital environment [4].

For another PFGE study, a 4-week prospective culture survey for MRSA was performed for 12 patients as well as for the environment of the room of MRSA carriers in quarantine in the geriatric long-term care ward of hospital A. A total of 97 *S. aureus* strains (80 MRSA and 17 MSSA) were isolated during the periods of September 8–10, 23–25 and October 5–7, 1998. Twenty-five strains were from the respiratory tract, 4 strains from feces and 11 strains from decubitus ulcers. Fifty-seven strains were from the patients' environment. Molecular typing by PFGE with the *SmaI* restriction enzyme demonstrated that the predominant type of MRSA isolated from the environment changed by the minute [5].

Criteria for Bacterial Strain Typing

The band types were compared according to the criteria for bacterial strain typing described by Tenover et al. [6]. We designated strains as indistinguishable if the number of fragment differences was 0, as closely related if the fragment differences were 1–3, as possibly related if the fragment differences were 4–6 and as different if more than 7 fragments were different. The most frequently detected pattern in the outbreak was designated pattern A. Patterns that were closely or possibly related to the outbreak pattern were considered subtypes of A and were designated subtype A1, subtype A2 and so on. Patterns that were classified as different were designated pattern B, pattern C and so on [6].

Results

Underlying Diseases

The underlying diseases were diagnosed in the study population in each period. The average age was about 78 years. In geriatric wards, the major underlying diseases were cerebrovascular disease, decubitus ulcer and respiratory diseases. Most of the patients were bedridden and could not eat anything orally in severe cases. Therefore, they accepted either tube feeding or intravenous hyperalimentation.

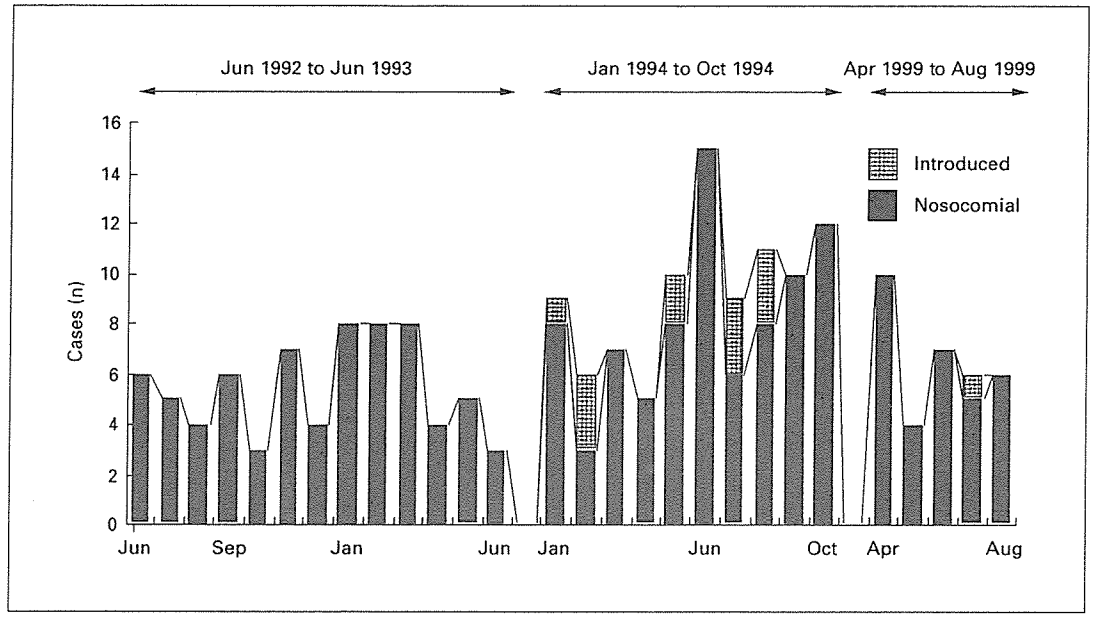

Fig. 1. The monthly new cases with MRSA colonization in geriatric wards. Several new cases were observed in hospital A each month.

Incidence of Nosocomial Pneumonia

Nosocomial pneumonia significantly decreased (period 1 vs. periods 2, 3 and 4, $p < 0.05$, $p < 0.05$, $p < 0.05$, respectively). Major causative organisms of nosocomial pneumonia were MRSA and *Pseudomonas aeruginosa*. A significant reduction of nosocomial pneumonia was observed in the cases with MRSA and those with *P. aeruginosa* (MRSA: period 1 vs. periods 2, 3 and 4, $p < 0.05$, $p < 0.05$, $p < 0.01$, respectively; *P. aeruginosa*: period 1 vs. period 3, $p < 0.01$, period 2 vs. periods 3 and 4, $p < 0.01$, $p < 0.05$, respectively). Interestingly, an improvement in the incidence of decubitus ulcers was associated with a significant reduction in nosocomial pneumonia (period 1 vs. periods 2 and 3, $p < 0.05$ and $p < 0.05$, respectively). Regarding the outcomes of MRSA-induced pneumonia, the number of cases with MRSA-induced pneumonia decreased in the periods 2, 3 and 4. On the other hand, the mortality rate increased.

Monthly New Cases with MRSA Colonization in Geriatric Wards

Figure 1 shows the monthly new cases with MRSA colonization in geriatric wards. Several new cases were observed in hospital A each month. Before and after the preventive measures we investigated the number of MRSA-colonized patients. We found that MRSA-colonized cases mainly originated from the geriatric ward. The cases with MRSA introduced from the other hospital departments were screened and picked up on admission, but they were not the main cause of MRSA colonization.

It is important to note that cleaning the upper respiratory tract using povidone-iodine contributes to the reduction of the number of cases with MRSA colonization.

In hospital A, MRSA colonization was found in less than 20 cases, although several new cases were observed each month.

PFGE Study

In the first PFGE study, 6 PFGE types were distinguished (A–F). Of the 6 types, 2 (B and C) were mainly found in both the patients and the hospital environment. Type B strains were mainly MRSA. All of the type C strains were susceptible to oxacillin. The rates of MRSA isolated were about 70% in patients and about 30% in the environment. From day 1 to 3, six PFGE types (A–F) were observed. Two types (B and C) were found in both the bedridden patients and the hospital environment. Of the subtypes, subtype C1 strains were simultaneously found in both the patients and the environment. All the

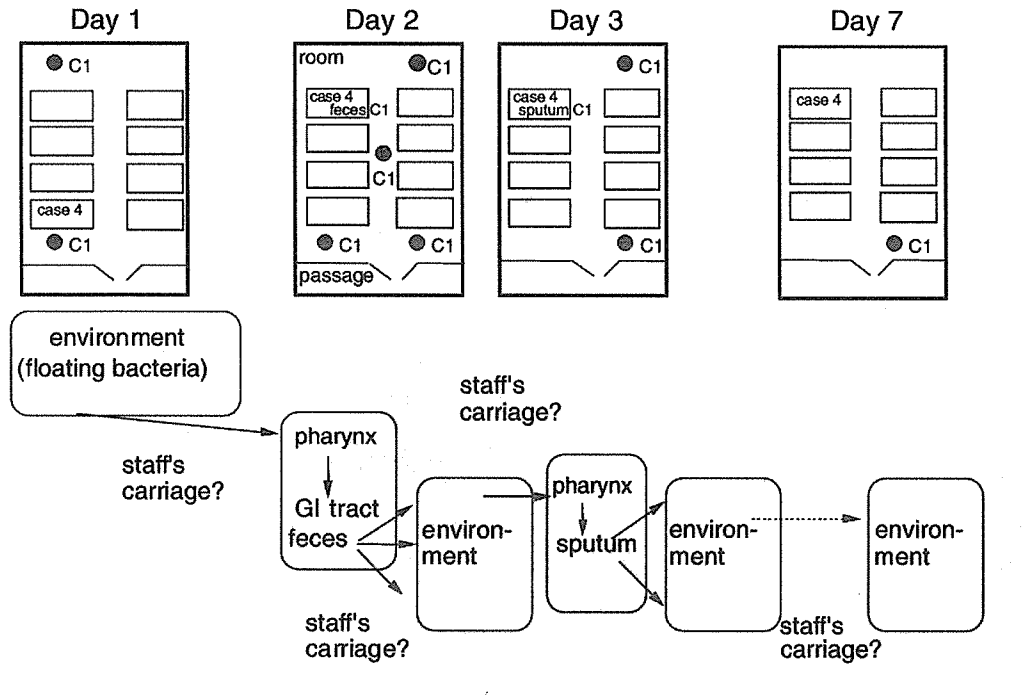

Fig. 2. The transmission of subtype C1 strain in a geriatric ward.

type C strains were susceptible to oxacillin. Subtype B2 strains were found in the patients, and were MRSA.

From day 4 to 7, subtype B2 strains were simultaneously found in both the patients and the environment. Subtype C1 strain was found in the environment.

On day 2, subtype C1 strains were isolated from feces and the environment. On day 3, subtype C1 strains were isolated from the sputum and the environment. On day 4, subtype B2 strains were isolated from the pharynx and the environment.

Figure 2 shows the transmission of subtype C1 strain in a geriatric ward. We speculate that subtype C1 strains were in the environment. Between days 1 and 2, subtype C1 strain might have been transmitted to the pharynx. The subtype C1 strain that colonized the pharynx might then have been swallowed and colonized the gastrointestinal tract. Then, the subtype C1 strain might have been dispersed from feces to the environment. As another transmission mode, the subtype C1 strain that colonized the respiratory tract might have been dispersed to the environment when the patients accepted oral care such as suctioning of secretion.

In these processes there might have been either direct or indirect carrier effects of *S. aureus* by the hospital staff.

In another PFGE study, the samples of the hospital environment were more vigorously collected in a geriatric room by using a settled agar plate for 48 h. Identical and closely related MRSA strains were found in both the patients and the environment. The patterns of 42 MRSA strains isolated from the environment were identical in 26 (61.9%), closely related in 15 (35.7%) and possibly related in 1 (2.4%) of the cases of those simultaneously isolated from patients. The 97.6% of MRSA strains collected indicated an identical or closely related pattern. There were no strains of MRSA classified as different. There was no correlation between patients and the environment with the 17 MSSA isolates.

This PFGE analysis revealed that, if the samples from the patients and the environment were vigorously collected at the same time in a geriatric room, the PFGE types of all MRSA strains isolated from the environment were identical or had close or possible relationships with those from the bedridden patients (or the PFGE types of

all MRSA strains isolated from the environment were closely associated with those from the bedridden patients).

Conclusions

Our study shows that stringent infection control measures, including disinfection of the upper airways by povidone-iodine, are necessary in geriatric wards to reduce the incidence of nosocomial pneumonia, which was mainly caused by MRSA and *P. aeruginosa.*

On the other hand, our PFGE analyses demonstrated that MRSA in the airways of patients can contaminate the environment and that MRSA from the environment may be transmitted to patients. These results support the importance of disinfection of the upper airway in the prevention of nosocomial pneumonia and in the prevention of transmission of MRSA in geriatric wards.

Acknowledgments

We thank Chieko Shimauchi (Miyazaki Prefectural College), Matsuhisa Inoue (Kitasato University School of Medicine) and Akihiro Wada (Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University) for their help in the completion of the PFGE studies.

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Case Report

A mixed bacterial infection of a bronchogenic lung cyst diagnosed by PCR

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An unusual paediatric case of a bronchogenic cyst infected with both *Haemophilus influenzae* type b and *Streptococcus pneumoniae* is described, which was detected not by culture of the purulent cyst fluid, but by real-time PCR amplification for several potential pathogens of DNA extracted from the fluid.

Case report

A 6-year-old girl was referred to Chiba University Hospital because of fever and left epigastric pain. She had not been vaccinated against *Haemophilus influenzae* type b (HIB) or *Streptococcus pneumoniae*. She had been hospitalized at 3 years of age for treatment of a left lung abscess with parenteral antibiotic therapy and percutaneous drainage. Culture of the abscess fluid, collected prior to the administration of antibiotics, revealed the presence of *S. pneumoniae*. Although her symptoms resolved with treatment, a chest radiograph and computed tomography (CT) scan obtained just before discharge from the hospital revealed a residual cystic mass in the superior segment of the inferior lobe (S6) of the left lung. Although the cystic lesion persisted for 3 years, she remained asymptomatic until her second hospital admission.

On admission, the patient's axillary temperature was 38·9 °C, breath sounds were diminished over the left lower lung field, and coarse crackles were heard upon auscultation. Her white blood cell count was elevated to 25 100 mm^{-3} with 90 % neutrophils, and her C-reactive protein (CRP) was 8·7 mg dl^{-1} (upper limit of normal, 0·3 mg dl^{-1}). A chest radiograph and CT scan showed an air-fluid level within the cyst, which was 6 cm in diameter and circumscribed with a thick wall, in the S6 segment of the left lung (Fig. 1a).

Transthoracic aspiration of the cyst using ultrasonographic guidance failed to yield fluid for culture or other tests. Empiric antimicrobial therapy was started with panipenem-betamipron (60 mg kg^{-1} per day), which is a parenteral carbapenem available commercially only in Japan, and the patient's symptoms and laboratory values improved within 2 days. Blood, sputum and nasopharyngeal culture performed on the day of admission was negative. Over the 10 days of antibiotic therapy, the patient was afebrile, and her serum CRP dropped to 0·5 mg dl^{-1}, but the fluid volume in the cyst increased gradually (Fig. 1b).

The patient was referred for surgical treatment, and on hospital day 26, a left lower lobectomy was performed under general anaesthesia through a lateral thoracotomy (Fig. 2). The patient's recovery was uneventful and she was discharged on postoperative day 10.

Histology of the specimen showed ciliated epithelium on the lumen of the cyst, and the pathologic diagnosis was bronchogenic cyst.

A white–brown purulent fluid was aspirated from inside the extracted cyst (Fig. 2). No micro-organism was observed by Gram or Ziehl–Neelsen staining. Cultures of the fluid on blood and chocolate agar, and in BacT/Alert (bioMérieux) blood-culture bottles incubated under aerobic and anaerobic conditions were negative. Culture for mycobacteria was negative.

An aliquot of the purulent fluid (stored at −20 °C) was sent to the Gifu University Department of Microbiology for molecular diagnosis by PCR. PCR assays for several pathogens associated with respiratory tract infections have been developed. The nucleotide sequences of the primers used in this study are listed in Table 1. DNA was extracted

Abbreviations: CRP, C-reactive protein; CT, computed tomography; HIB, *Haemophilus influenzae* type b.

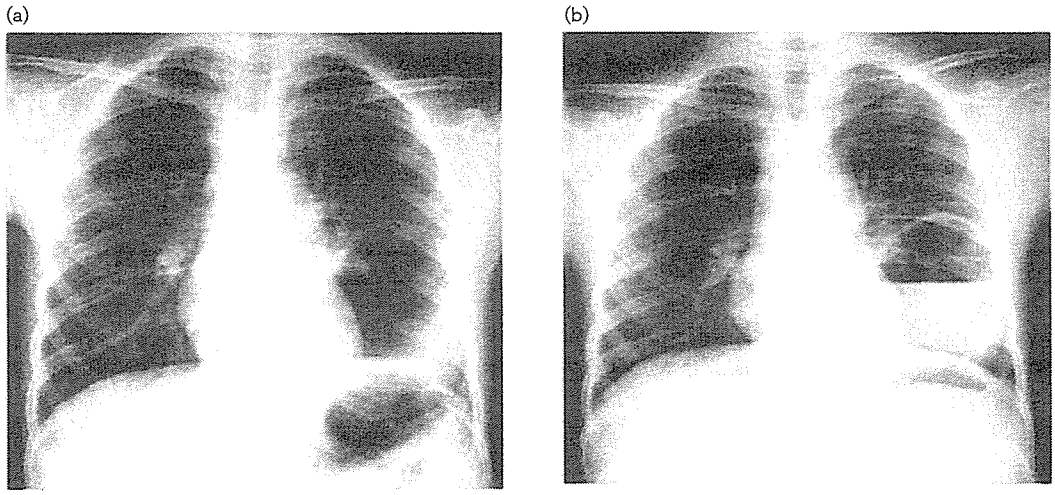

Fig. 1. Chest radiograph performed on the day of admission, showing a cystic lesion with air-fluid level in the left lower lobe (a), and chest radiograph following the 17 days of antibiotic therapy, showing increased fluid level within the cyst (b).

with the MORA-EXTRACT kit (Kyokuto) according to the manufacturer's instructions. Conventional PCR performed on the extracted DNA was positive for *H. influenzae* (16S rRNA gene) and *S. pneumoniae* (*lytA* gene), but negative for *Bordetella pertussis*, *Chlamydophila* sp., *Coxiella burnetii*, *Legionella* sp., *Moraxella catarrhalis*, *Mycoplasma pneumoniae* and *Staphylococcus* sp. In addition, HIB *cap* genes were identified with primers that amplified HIB-specific DNA. The *S. pneumoniae* gene encoding pneumolysin (*ply*) was also detected by conventional PCR.

For detection and quantification of HIB- and *S. pneumoniae* (*lytA*)-specific DNA, real-time PCR assays were performed in an iCycler iQ system (Bio-Rad Laboratories) using SYBR green. The amplification protocol consisted of an initial incubation of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, with Ex *Taq* DNA Hot Start polymerase (TaKaRa Bio). The specificity of the assays was assessed by testing with nucleic acid extracts from related organisms and a variety of micro-organisms commonly isolated from respiratory specimens. No signal in the HIB or *S. pneumoniae* (*lytA*) real-time PCR was observed with any of the control organisms tested. For the preparation of each external standard DNA, *H. influenzae* ATCC 10211 (HIB) or *S. pneumoniae* ATCC 49619 was used. The bacteria were suspended in Tris/EDTA buffer to a density of 0·5 McFarland units. DNA was extracted as described above by means of the MORA-EXTRACT kit. Six dilutions from 10^8 to 10^3 organisms ml^{-1} were prepared and run in duplicate as external standards in parallel

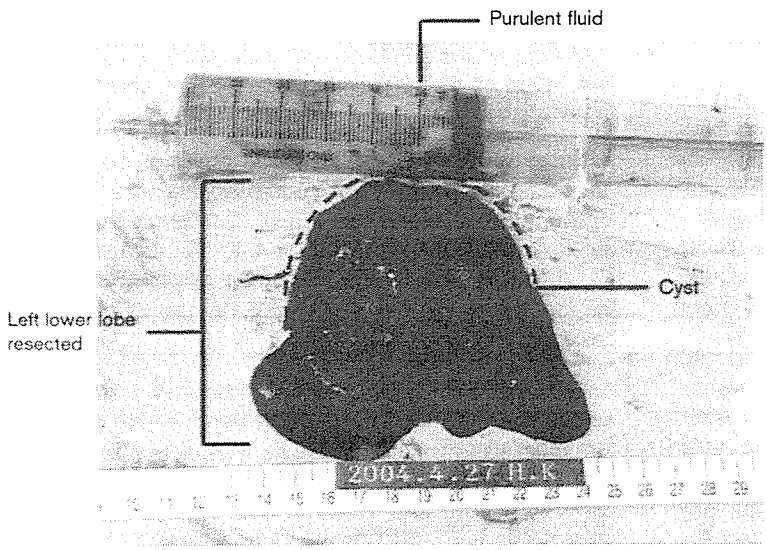

Fig. 2. Resected left lower lobe including the cyst. The cyst was 8 × 8 × 6·5 cm in size, situated in the S6 segment. Abundant white–brown purulent fluid was obtained by aspiration from the inside of the extracted cyst.

Table 1. Oligonucleotide PCR primers used in this study

Organism	Gene	Primer name	Sequence (5′–3′)	Product size (bp)	Reference
Haemophilus influenzae	16S rRNA	HI 16S-F	GGAATCTGGCTTATGGAG	336	This study
		HI 16S-R	CACATCAACCTTCCTCAA		
H. influenzae type b	*cap*	Hib cap-F	GATACCTTAATTCGCCGCTCGTCAT	152	This study
		Hib cap-R	ATATCTCGCACATCGTGTTCAGCAC		
Streptococcus pneumoniae	*lytA*	Sp lytA-F	AACCATATAGGCAAGTACAC	429	This study
		Sp lytA-R	ATCATGCTTAAACTGCTCAC		
S. pneumoniae	*ply*	Sp ply-F	TGCAGAGCGTCCTTTGGTCTAT	81	Corless *et al.* (2001)
		Sp ply-R	CTCTTACTCGTGGTTTCCAACTTGA		
Bordetella pertussis	*prn*	Bpt-F	CATCCGACTGGAACAAC	427	This study
		Bpt-R	CGTTGGACCGTGACATTG		
Chlamydophila sp.	16S rRNA	Chp-F	CGTGGATGAGGCATGCAA	290	This study
		Chp-R	CAGTCCCAGTGTTGGCGG		
Coxiella burnetii	*groEL*	Cob-F	CTGAAGAAGTCGGATTGT	574	This study
		Cob-R	CCCATCTCAATCATGTCA		
Legionella sp.	16S rRNA	Leg-F	GAGTAACGCGTAGGAATA	337	This study
		Leg-R	TCCTCCCCACTGAAAGTG		
Moraxella catarrhalis	16S rRNA	Mcat-F	GAACGAAGTTAGGAAGCT	473	This study
		Mcat-R	TTGCACCCTCTGTATTAC		
Mycoplasma pneumoniae	16S rRNA	Mpn-F	CGTATCCAATCTACCTTATA	360	This study
		Mpn-R	CATTACCTGCTAAAGTCATT		
Staphylococcus sp.	16S rRNA	Sta-F	TAATACATGCAAGTCGAG	294	This study
		Sta-R	GGACCGTGTCTCAGTTCCA		

with the test DNA. To generate a standard curve, the threshold cycle (Ct) of the standard dilutions was plotted against the organism cell numbers. The linear correlation (r^2) coefficients between the Ct values and organism cell numbers were 0·998 and 0·996 for the HIB and *S. pneumoniae* PCR targets, respectively. The slopes were −3·506 and −3·983 for HIB and *S. pneumoniae* PCR targets, respectively. The real-time quantitative measurements indicated that the cyst fluid contained amounts of DNA equivalent to $1{\cdot}1 \times 10^7$ and $1{\cdot}2 \times 10^6$ organisms ml^{-1} of specimen for HIB and *S. pneumoniae*, respectively. The sizes and the melting curves of the PCR products were as expected, based on the primer designs. In addition, the PCR products were sequenced using the Big Dye-Terminator Cycle Sequencing kit (Applied Biosystems). Samples were analysed on an ABI Prism 3100 DNA genetic analyser (Applied Biosystems). In each instance, the sequence of the PCR product was identical to the sequence of the reference strain and confirmed the identity of the pathogens present in the cyst fluid.

Discussion

Bronchogenic cysts account for 14–22 % of congenital cystic diseases of the lung, and often present with infectious complications in older children and adults (Lierl, 1993; Laberge *et al.*, 2004). However, pathogens of the infected cysts frequently remained unidentified. Ribet *et al.* (1996) reported that culture of the purulent contents of bronchogenic cysts revealed *Streptococcus* sp., pneumococcus and *Escherichia coli* in each of three cases, and there are two reports of *H. influenzae* infection of an existing cyst (Klapper & Sherman, 1986; Wewers *et al.*, 1982). Recently, *Mycobacterium* species and fungus have been identified in infected cysts (Minami *et al.*, 2004). Nevertheless, the pathogenic organisms are often unidentified in the cases of infected cysts, and the frequency of infection by specific pathogens is not clear.

S. pneumoniae and capsulated *H. influenzae* are both important causes of bacteraemia and meningitis. In Japan, HIB is still responsible for serious disease because HIB vaccines are not currently used. These organisms are usually found colonizing the upper airway, and occasionally result in disseminated bloodstream infection. In the case reported here, culture of blood, sputum and nasopharyngeal specimens was negative. On the basis of quantitative real-time PCR analysis, however, we concluded that the inflammation was due to simultaneous infection with HIB and *S. pneumoniae*. We were careful to avoid cross-contamination and sample carryover by taking standard precautions, including performing pre- and post-PCR procedures in separate rooms. DNA amplification was performed with primers targeting two different genes, 16S rRNA and the *cap* region for HIB, and *lytA* and *ply* for *S. pneumoniae*. In addition, the specificity of the PCR products was confirmed by sequence analysis.

In cases of purulent lung cyst, the source of bacterial infection is often difficult to identify by culture because

of previous or concurrent administration of antibiotics. Therefore, PCR analysis for several likely pathogens should be considered for culture-negative specimens.

To our knowledge, this is the first reported case of a bronchogenic cyst coinfected with HIB and *S. pneumoniae* that has been diagnosed by quantitative real-time PCR.

Acknowledgements

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An Off-Seasonal Amantadine-Resistant H3N2 Influenza Outbreak in Japan

Reiko Saito, Danjuan Li, Chieko Shimomura,[1] Hironori Masaki,[2] Mai Q. Le,[3] Hang L.K. Nguyen,[3] Hien T. Nguyen,[3] Tu V. Phan,[4] Tien T.K. Nguyen,[4] Maki Sato, Yasushi Suzuki and Hiroshi Suzuki

Department of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan, [1] Shimomura Clinic, Nagasaki, Japan, [2] Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, [3] National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, and [4] Pasteur Institute, Ho Chi Minh City, Vietnam

Saito, R., Li, D., Shimomura, C., Masaki, H., Le, M.Q., Nguyen, H.L.K., Nguyen, H.T., Phan, T.V., Nguyen, T.T.K., Sato, M., Suzuki, Y. and Suzuki, H. *An Off-Seasonal Amantadine-Resistant H3N2 Influenza Outbreak in Japan.* Tohoku J. Exp. Med., 2006, **210** (1), 21-27 —— An off-season community influenza outbreak with high prevalence of amantadine-resistant influenza A/H3N2 occurred during September-October 2005 in Nagasaki Prefecture, Japan, prior to standard influenza circulation. A total of 48 patients with influenza-like-illness (ILI) visited a clinic during the outbreak and 27 (69.2%) of 39 ILI patients were positive for influenza A with rapid antigen testing (Quick Vue Rapid SP Influ). Nine patients were not tested because their symptoms were compatible for influenza without examination. Nasopharyngeal swabs were obtained from 4 of 27 rapid test positive patients, and influenza H3N2 strain was isolated from one out of four. The 4 nasopharyngeal samples were positive for influenza A M2 gene in polymerase chain reaction, and sequencing results all showed identical mutation at position 31, serine to asparagine (S31N) in the gene, conferring amantadine resistance. The phylogenetic tree analysis demonstrated that the hemagglutinin (HA) gene sequences of the 4 samples formed a distinct cluster (named clade N) from recent circulating H3N2 strains, characterized by dual mutations at position 193, serine to phenylalanine (S193F), and at position 225, asparatic acid to asparagine (D225N). Our findings suggested that an off-season community influenza outbreak in Nagasaki was caused by a distinct clade in H3N2 (named clade N), which possessed characteristics of amantadine resistance. ——— influenza; amantadine; antiviral resistance; outbreak

Influenza in zones with a temperate climate is characterized by the occurrence of annual epidemic during the winter months and influenza season falls in November through April in Japan. However, several off-season community influenza outbreaks occurred in 2005 (Hirano et al. 2005;

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Inoue et al. 2005; Morikawa et al. 2005; Taira et al. 2005), and we had a chance to study one of those outbreaks.

M2 inhibitors, amantadine and rimantadine, are effective for prevention and treatment of influenza A infections. The medicines are economical and chemically stable, but emergence of resistance and adverse effect are matters of concern (Suzuki et al. 2003). The genetic basis for resistance to these drugs is associated with amino acid substitutions at positions 26, 27, 30, 31 or 34 in the transmembrane region of the M2 protein (Pinto et al. 1992; Holsinger et al. 1994). It was reported that roughly 1/3 of patients develops resistance after treatment (Hayden and Hay 1992; Saito et al. 2002), but resistance in pre-treatment samples, or community prevalence, remained low in the past, as 0-3% in Japan (Suzuki et al. 2001, 2003), and roughly 1% in the United States and the United Kingdom (Ziegler et al. 1999; Tooley 2002). However, Bight and colleagues (Bright et al. 2005, 2006) recently highlighted a dramatic increase in the prevalence of amantadine resistant H3N2 influenza strains in Asian countries and USA.

Upon our epidemiological study of an off-season community influenza outbreak occurred in Nagasaki Prefecture in 2005, we found high prevalence of amantadine resistant H3N2. Here, we report this outbreak, and tried to clarify the relationship between the genetic characteristics of amantadine-resistant H3N2 influenza strains and its high prevalence.

Materials and Methods

Epidemiological information and specimen collection

During September-October 2005, an influenza outbreak was reported in Nagasaki Prefecture, Japan (Hirano et al. 2005). This study was conducted with patients consulted to Shimomura Clinic during the outbreak in Tokitsu-cho town with a total population of approximately 30,000, Nagasaki Prefecture.

Influenza-like illness (ILI) cases were defined on the basis of a sudden fever, cough and sore throat. Their nasopharyngeal swabs were examined with rapid antigen test kits for diagnosis of influenza A or B (Quick Vue Rapid SP Influ, DS Pharma Biomedical Co., Ltd., Osaka) prior to antiviral drug treatment (oseltamivir) at the initial office visits. Upon provision of oral informed consent, precise patient information and nasopharyngeal swabs were obtained from selected influenza A positive patients for further virological testing. They were stored at 4°C in viral transport media until transferred to the Department of Public Health, Niigata University, Graduate School of Medical and Dental Sciences, Niigata City, Japan.

Virus isolation

One hundred μl of supernatant of nasopharyngeal swabs was inoculated into Madin-Darby canine kidney cells (MDCK), prepared in 48-well multiple well plates. The plates were kept at 34°C under a 5% CO_2 atmosphere for up to 10 days to assess cytopathic effects (CPEs). Fifty μl aliquots of supernatants of CPE positive samples were then passaged twice to obtain a sufficient virus titer to perform virus identification. Influenza isolates were typed and subtyped by hemagglutination inhibition (HAI) assay with commercially available influenza

Table 1. Demographic and clinical details, and

Patient (sample name)	Age (y.o.)	Sex	Date of onset (d/m/y)	Time to clinic visit	Prior medication	Medication at the Clinic
1 (N1)	5	F	16/9/2005	≦ 24 hours	No	Oseltamivir
2 (N2)	2	F	16/9/2005	1 day	No	Oseltamivir
3 (N3)	13	M	18/9/2005	3 days	No	Oseltamivir
4 (N4)	8	F	21/9/2005	≦ 24 hours	No	Oseltamivir

[1] Nested PCR for M2 gene of influenza A detection.

[2] Nested PCR for H3 subtype hemagglutinin (HA) gene of influenza A detection.

[3] Hemagglutinin inhibition test reacted with A/New York/55/2004(H3N2).

RT-PCR, reverse transcription-polymerase chain reaction; N/A, not addressed; S31N, mutation at poistion 31,

vaccine strain antisera for 2005/2006 season in Japan, A/New Caledonia /20/99 (H1N1), A/New York/ 55/ 2004 (H3N2), B/Shanghai/ 361/ 2002 (Denka Seiken Co., Ltd., Tokyo), using guinea pig red blood cells. An amantadine susceptibility test was conducted with two series of 10-fold dilution of viruses from CPE-positive cultures, plated in triplicate in a 96-well microplate confluent with MDCK cells with one dilution series containing 1.0 μg/ml of amantadine in the media as reported previously (Masuda et al. 2000). Amantadine resistant strains were identified when a less than 1.0 fold difference in log $TCID_{50}$/0.2 ml titer was observed between series of rows with and without the drug after incubation for 48 hrs at 37°C.

Polymerase chain reaction (PCR) and sequencing

RNA was extracted from 100 μl of nasopharyngeal swabs or influenza isolates using Extragen II kit (Kainos, Tokyo), according to the manufacturer's instructions. Reverse transcription (RT) to create complementary DNA (cDNA) was performed using an influenza A generic primer, Uni12, as reported elsewhere (Hoffmann et al. 2001). First, a nested PCR was performed using specific primers to amplify the M2 region, segment 7, of influenza A, as described previously (Masuda et al. 2000) and then the PCR products were sequenced to examine mutations at positions 26, 27, 30, 31 and 34 in the transmembrane region of the M2 protein, conferring amantadine resistance (Klimov et al. 1995). The templates were labeled by cycle sequencing reactions with fluorescent dye terminators (BigDye Terminator v 3.1 Cycle Sequencing Kit, Applied Biosystems), and the products were analyzed using an ABI 310 (Applied Biosystems) automatic sequencer following the manufacturer's instructions. Amplification and sequencing of the hemagglutinine (HA) gene, segment 4 of influenza H3N2 was performed with sets of nested PCR primers as reported elsewhere (Besselaar et al. 2004). Phylogenetic tree analysis was made with the obtained HA sequences, together with other recent H3N2 strains registered in Influenza Virus Resource (National Center for Biotechnology Information, *http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html*). Multiple alignment, phylogenetic analyses were performed using MEGA ver 3.1(Kumar et al. 2004). In order to avoid possible influence of laboratory contamination, RNA extraction, cDNA synthesis and PCR amplification from samples were repeated more than twice by different examiners. Both positive and negative controls were included along with the samples for every PCR reaction.

For references of sequencing of the HA gene and its phylogenetic tree analysis, 6 amantadine resistant H3N2 isolates carrying S31N mutation collected in Hanoi and Ho Chi Minh City, Vietnam in 2005 (Hanoi/HN 30602/05, Hanoi/HN30607/05, Hanoi/ISBM63/05, Hanoi/HN30720/05, Hochiminh/14/05, Hochiminh/16/05), and 4 sensitive (Hanoi/ISBM53/05, Hanoi/ISBM69/05, Hanoi/TB285/05, Hochiminh/2/05) were included. In addition, two sensitive isolates collected in Nagasaki, Japan in January in 2005 (2004-05 season) were added to the analysis as representatives of standard circulation (Nagasaki/ND2/05 and Nagasaki/ND8/05).

Nucleotide sequence accession numbers

The DNA Data Bank of Japan (DDBJ) accession numbers of the nucleotide sequences for 4 strains, A/Nagasaki/N1/05(H3N2) to A/Nagasaki/N4/05(H3N2), are AB262301 to AB262304.

laboratory findings of patients who had sample collection.

Outcome	Influenza rapid test	Influenza isolation	RT-PCR (M2)[1]	RT-PCR (H3-HA)[2]	M2 mutation
Recovered	A	Positive[3]	Positive	Positive	S31N
Recovered	A	Negative	Positive	Positive	S31N
Recovered	A	Negative	Positive	Positive	S31N
Recovered	A	Negative	Positive	Positive	S31N

serine to asparagine, in M2 transmembrane domain in influenza A.

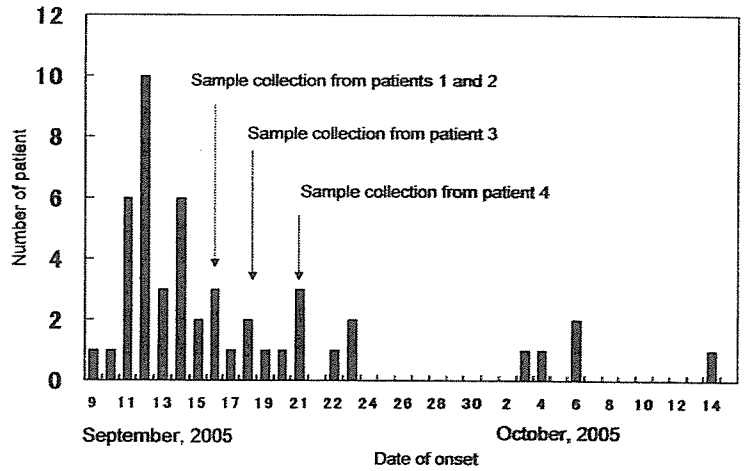

Fig. 1. Number of patients by date of onset, who visited Shimomura Clinic with influenza-like-illness, during September-October 2005 in Nagasaki, Japan.

Results

A total of 48 patients visited Shimomura Clinic as ILI with onset dates ranging from 9 Sep to 15 Oct (Fig. 1). The average patient age was 18.9 ± 16.8 years (0.5 to 82 years old), and a half of patients were children under age of 12. Rapid test was positive with influenza A in 27 (69.2%) of 39 patients. Nine patients were not tested because their symptoms were compatible for influenza without examination. Three quarter of ILI patients received oseltamivir treatment.

Four patients underwent nasopharyngeal swab collection, and they had no history of recent overseas travel or prior amantadine administration (Fig. 1 and Table 1). They received oseltamivir treatment and recovered from the illness without complications.

Influenza virus was recovered from one patient (Nagasaki/N1/05), and reacted with A/New York/55 /2004(H3N2) on HAI characterization (Table 1). It was conferred as amantadine resistant by the $TCID_{50}$ /0.2 ml susceptibility test, with a virus titer of 0.5 $\log_{10}$ in amantadine added medium, and 1.3 $\log_{10}$ in amantadine free medium.

All four nasopharyngeal swabs showed positive by RT-PCR with M2 protein primers, and carried amino acid substitution at position 31, serine to asparagine (S31N) in transmembrane domain in M2 protein (Table 1). HA gene PCR for A/H3 was positive with all, and sequencing results showed a motif with dual mutations at position 193, serine to phenylalanine (S193F), and at position 225, asparatic acid to asparagine (D225N), except for A/Nagasaki/N4/05 which possessed only S193F change (Fig. 2). Furthermore, six amantadine resistant strains in Vietnam also had a same motif with dual mutations. Those resistant strains formed a distinct cluster (named clade N) from other strains. However, 4 amantadine sensitive strains in Vietnam and 2 from Nagasaki did not have the motif and not clustered in the clade N.

Discussion

In 2005, several off-seasonal H3N2 influenza outbreaks occurred in Japan (Hirano et al. 2005; Inoue et al. 2005; Morikawa et al. 2005; Taira et al. 2005), but main causes remain unknown. We had a chance to study one of those outbreaks, in Nagasaki (Hirano et al. 2005). We could analyze only 4 samples, but all of them were resistant H3N2 viruses. Even with limited number of cases in a rather small geographic area, our results suggested high prevalence of resistant strains in the outbreak area. It is generally accepted that amantadine resistant viruses emerges under the pressure of the drug, but are less virulent or transmissible than susceptible viruses (Harper et al. 2005).

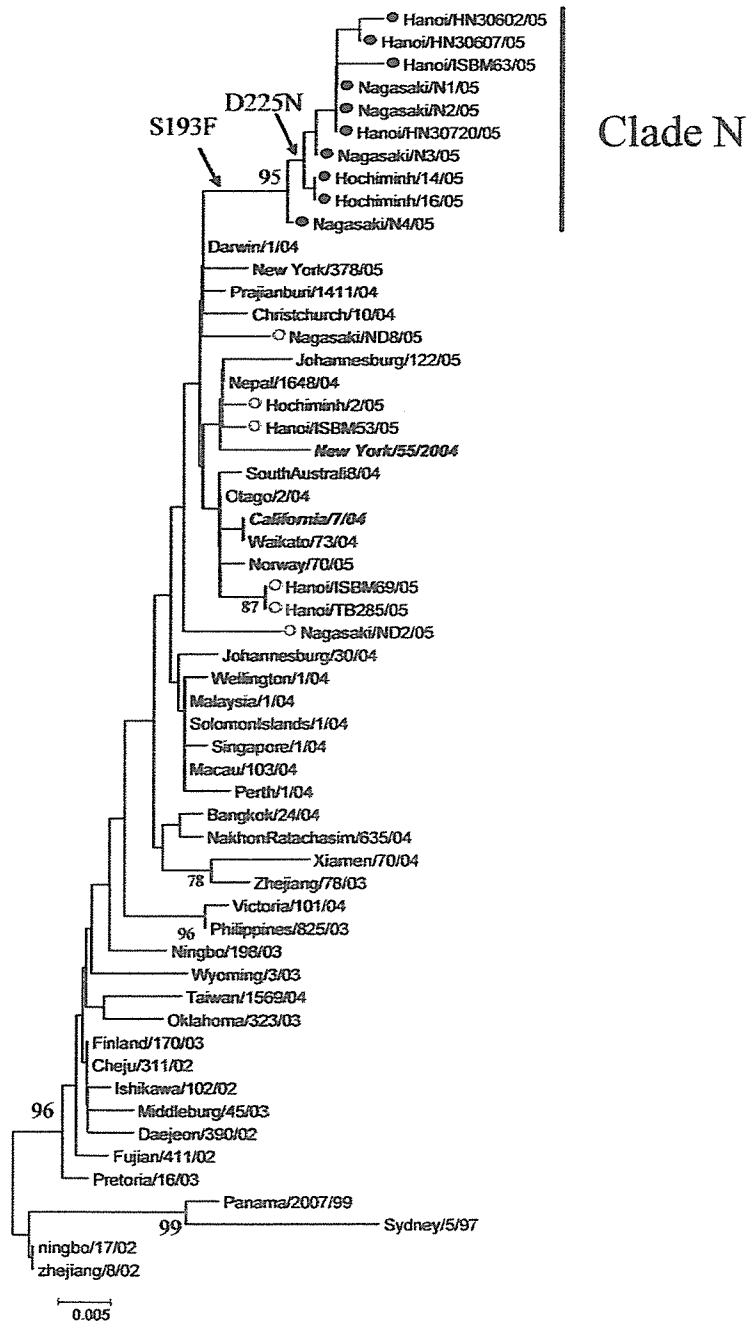

Fig. 2. Phylogenetic tree analysis among hemagglutin (HA) genes from human H3N2 viruses. Isolates and nasopharyngeal swabs collected in Nagasaki in Japan, Hanoi and Ho Chi Minh City in Vietnam in 2005, and selected strains in genetic database were included in the analysis. Strains labeled with closed circles indicated amantadine resistant strains possessing S31N mutation in M2 protein, and open circles sensitive. Presence of HA motif in amantadine resistant strains, mutations at positions 193, serine to phenylalanine (S193F), and 225, asparatic acid to asparagine (D225N), were shown by arrows, and this group was named "clade N". Virus names in italic boldface denote Japanese and WHO recommended influenza vaccine strains for H3N2 in Northern Hemisphere in 2005/06. Phylogenetic trees were inferred from 467 nucleotide sequences by the neighbor-joining method. Bootstrap values > 70% are shown.

In Japan, the prevalence of resistance remained 0-3% in pre-treatment samples until the 2004-05 season in our survey (Suzuki et al. 2001, 2003), and less than 1% in USA and other countries (Ziegler et al. 1999; Tooley 2002). However, Bight and colleague's (Bright et al. 2005; Guan and Chen 2005) study recently highlighted a dramatic increase in the prevalence of resistant H3N2 strains in Asian countries, suggesting association with inappropriate drug administration. In the present study, all patients had neither prior amantadine administration nor history of recent overseas travel, under the situation amantadine is prescribed only by licensed clinicians in Japan. Our study also indicate the presence of resistant H3N2 strains in Vietnam, where amantadine is not available as either a prescriptive or a commercial drug. Recent report indicated that even in USA, resistant H3N2 was quite high (Bright et al. 2006). Thus, we support the assumption that the resistant viruses may not have arisen solely as a result of exposure to drugs, but naturally occurring resistant-associated mutations could have emerged (Guan and Chen 2005).

All resistant strains isolated from this outbreak and 6 isolates from Vietnam carried S31N mutation in transmembrane domain in M2 protein. The same mutation point was observed in recent resistant H3N2 in Asia and USA (Bright et al. 2005, 2006). Our resistant strains formed a distinct cluster (clade N) with dual mutations at positions 193 and 225, except for one strain had only S193F change, while amantadine sensitive strains in Vietnam and Nagasaki clustered in other clades. Although the origin of the clade N is unexplained, our findings suggest that clade N is circulated simultaneously in Japan and Vietnam. We need further study of the relationship between our strains and other resistant strains in China, Hong Kong, Taiwan, South Korea, and USA. The high incidence of resistant H3N2 viruses was found in almost the same geographic regions as H5N1 viruses. Resistant H5N1 viruses appeared to be largely limited to be in Thailand, Vietnam, and Cambodia, and are present in almost all viruses from the Vietnam/Thailand/Malaysia sublineage, while most H5N1 isolates from China and Indonesia are sensitive to amantadine and are present in other sublineage (Guan and Chen 2005; Cheung et al. 2006). The relationship between amantadine resistant H3N2 and H5N1 viruses and its HA lineage is quite noteworthy and may be strongly associated each other, but warrant examination.

Our findings suggest that an off-season community influenza outbreak with high prevalence of amantadine resistant H3N2 (clade N) occurred in Nagasaki, Japan. Additional investigation is needed to elucidate the origin and geographical spread of H3N2 strains of clade N, and its biological effect on the life cycle of the H3N2 viruses. The increase of resistant strains is a big problem on a global scale, and we need to stress international coordination for proper use of M2 inhibitors and increase antiviral surveillance to keep this cheap drug as one of strategic options for influenza control.

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Influenza vaccination in severely multiply handicapped persons/children

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Abstract

Background: Many reports about the preventative effects of inactivated influenza vaccine have been published, targeting persons with underlying medical conditions. However, the effectiveness for severely multiply handicapped persons/children (SMHPs) is not yet well established.
Methods: The study group consisted of 79 SMHPs (36 males and 43 females, aged 18–66 years), with long-term hospitalization in Niigata National Hospital. We compared serum antibody responses before and after two-doses vaccination.
Results: Before vaccination for the 2004–2005 season, SMHPs showed continuously high HAI titer in A/New Caledonia/20/99(H1N1)-strain from March to October in 2004. The seroprotection rates were increased after the first dose, but no remarkable change was seen after the second dose in all three strains. Subjects less than 30 years old ($\leq$29 group) had a high antibody titers against all three strains compared with subjects aged >40 years old. On the other hand, in the seroconversion rates, there were no significant differences in age, gender, and severity of symptoms.
Conclusions: According to our study, SMHPs are low responders except $\leq$29 group and the influenza vaccine effectiveness is more affected by their age than severity of symptoms. We suggest a recommendation for influenza vaccination especially in SMHPs; inactivated influenza virus vaccine (one dose) should be performed during the previous fall. In addition, further studies are needed about chemoprophylaxis, which can prevent influenza outbreaks in SMHPs.

Keywords: Influenza vaccine; Severely multiply handicapped persons/children; Antibody titer; Seroprotection rate; Chemoprophylaxis

1. Introduction

The benefits of inactivated influenza virus vaccine such as the significant preventative effects on influenza, the decrease of excess death rate, and its safety are documented [1–3]. Nevertheless, influenza illness and complication cannot be completely prevented. Epidemics of influenza virus infection occur worldwide every year. Influenza viruses can cause considerable numbers of deaths due to serious complications [4]. Recently, influenza virus encephalopathies were reported as mortal complication [5,6]. Centers for Disease Control and Prevention (CDC) recommends vaccination with inactivated influenza vaccine especially for patients who have chronic diseases, because they may quickly deteriorate into a condition [7].

High-risk persons, included severely multiply handicapped persons/children (SMHPs) may obtain limited effectiveness of vaccination compared with healthy adults. Many reports about the effectiveness of influenza vaccination have been published, targeting elderly persons and persons with underlying medical conditions (e.g. chronic heart disease,

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chronic lung disease, diabetes, HIV infection) [3,8–10]. All of these reports conclude that high-risk persons can substantially benefit from influenza vaccination, and should receive it. SMHPs have also been recognized as debilitated and compromised [11–13]. Their severe multiple handicaps consisted mainly of cerebral palsy, epilepsy, and intellectual disorder. SMHPs are identified by their body functions and structure, activity and participation in International Classification of Functioning, Disability and Health [14], in addition to their diseases. To our knowledge, however, no reports have been published concerning the effects of influenza vaccination assessed in a large number of SMHPs. We examined the effect of inactivated influenza vaccine from many different aspects in SMHPs. In this study, we propose that influenza vaccination should be performed in SMHPs.

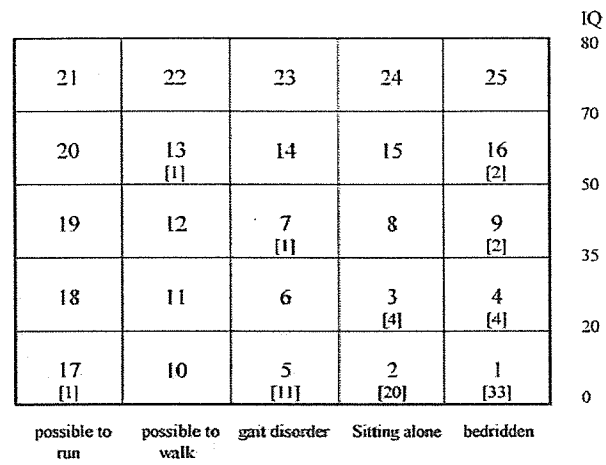

Fig. 1. Oshima's classification. The number of subjects is in parentheses [ ].

2. Methods

2.1. Subjects and study design

The study group consisted of 79 SMHPs (36 males and 43 females) under long-term hospitalization in Niigata National Hospital (**Table 1**). Their ages ranged from 18 to 66 years (mean 41.1 ± 11.2). Their severe multiple handicaps also consisted mainly of cerebral palsy, epilepsy, and intellectual disorder. Patients of Mucopolysaccaridosis, sequelae of encephalitis, Cornelia de Lange syndrome were also included. Oshima's classification (**Fig. 1**) is very useful and convenient to classify SMHPs according to severity of symptoms. Oshima's classifications 1–4 are true SMHPs in a narrow sense. Sixty-one cases (77%) were Oshima's classification 1–4 in this study. Oshima's classifications 5–25 are broadly SMHPs. The control group consisted of 49 healthy adults (9 males and 40 females, mean age 27.0 ± 5.3). All of them and/or their family members completed a form of informed consent to be included in this study. In addition, our hospital's Ethic Committee authorized this study.

We supposed that SMHPs be low responders, so they need two-dose vaccination for getting high antibody titers. All subjects were vaccinated with two doses of inactivated influenza vaccine in the 2004–2005 season. They were injected with the first dose in October 2004 and the second dose with 4–6 weeks later in November to December 2004. All subjects had been vaccinated with one or two doses annually from the 2000–2001 season. The control group members were vaccinated with one dose of inactivated influenza vaccine in the 2004–2005 season. Forty-two of them were vaccinated with one dose of inactivated influenza vaccine, the other seven were not vaccinated in the 2003–2004 season. We compared serum antibody responses before and after two doses of vaccination against influenza, according to interval of vaccine, age, gender, and severity of symptoms.

2.2. Vaccination

All of the subjects were administered with two doses of inactivated influenza vaccine (Denka Seiken Co., Ltd., Tokyo, Japan). The strains were A/New Caledonia/20/99-(H1N1), A/Wyoming/3/2003(H3N2), and B/Shanghai/361/2002, which were recommended for the 2004–2005 season by the World Health Organization. The vaccine contained at least 15 μg of hemagglutinin antigen in each 0.5 ml dose. According to the standard procedure in Japan,

Table 1
Characteristics of the subjects and age groups

	Control	All subjects	Age-group			
			≤29 group	30–39 group	40–49 group	≥50 group
No. of subjects	49	79	21	15	23	20
Age						
Range (mean)	20–39 (27.0)	18–66 (41.1)	18–29	30–39	40–49	50–66
Male/Female	9/40	36/43	11/10	5/10	11/12	9/11
Oshima's classification						
1–4/5–25	–	61/18	19/2	14/1	16/7	12/8
Body weight (kg)						
Range (mean)	–	16.1–54.6 (33.8)	16.1–48.9 (32.6)	17.1–42.9 (31.0)	22.7–54.6 (36.1)	22.1–46.2 (34.2)

subcutaneous vaccination with a regulated amount of vaccine (0.5ml) was performed.

Side reactions were evaluated on all subjects. Subjects with any acute serious adverse events would be treated quickly and excluded from the study. They were carefully observed for anaphylactic shock for at least 30 min after vaccination. Other side reactions including local reactions such as redness, swelling, pain at the area of injection, and systemic reactions such as rising temperature over 37.5 °C and exanthema were checked at 48 h after vaccination. Other symptoms related to the vaccination were assessed at all times during this study.

2.3. Serologic studies

Serum samples were collected four times from each SMHP; before vaccination (March 2004, October 2004), and 3 weeks after each vaccination (first and second dose, respectively) according to previous reports [15]. Among control group, serum samples were collected two times (before and after vaccination). Each specimen was examined for antibody responses by analysis of hemagglutination inhibitation (HAI).

In our study, serum antibody titers for three strains, A/New Caledonia/20/99(H1N1), A/Wyoming/3/2003(H3N2), and B/Shanghai/361/2002 were measured. The influenza vaccines licensed in Japan (recommended by World Health Organization) in the last 5 years are as follows; A/New Caledonia/20/99(H1N1)-strain was selected in all five seasons. A/Wyoming/3/2003(H3N2)-strain was selected for the first time this season, although A/Panama/2007/99(H3N2)-strain was used from the 2000–2001 season to the 2003–2004 season. The strains for B are changed nearly every year. We also examined HAI titers of the March 2004-sample on the 2003–2004 season-strains; A/Panama/2007/99(H3N2)-strain and B/Shandong/7/97.

Geometric mean titer, seroconversion rate and seroprotection rate were examined in all subjects. The seroconversion rate is the percentage of subjects with a four-fold increase in HAI titer after vaccination. The seroprotection rate is the percentage of subjects with HAI titer of 1:40 or more. Seventy percent or more possession rates are considered effective immunity for that group.

Forty-eight subjects were also examined for serum IgG level at October 2004 with informed consent, to see their immunogenicity.

2.4. Statistical methods

The data analyses were performed using the Kruskal–Wallis test and the Mann–Whitney *U*-test. All statistical calculations were performed with Microsoft Excel 2002 (Microsoft Corp., Redmond, WA). We analyzed serum antibody responses against influenza with variables of interval of vaccine, ages, gender, and severity of symptoms. *P*-values <0.05 were considered significant.

3. Results

3.1. Safety

No acute serious adverse events were seen. Few side reactions occurred, but all of them were mild and transient; redness (two subjects), swelling (three subjects), pain at the area of injection (two subjects). No subject showed symptoms of rising temperature over 37.5 °C or exanthema.

3.2. Immunogenicity

Serum samples were collected four times from all subjects except one (male, 18 year old, Oshima's classification 2), whose sample was not collected in March 2004. There were no SMHPs with influenza illness during the 2000–2001 to the 2002–2003 season and the 2004–2005 season. In the 2003–2004 season, four SMHPs became infected with A/H3N2. They were excluded from evaluation of antibody response with A/H3N2.

Geometric mean titers, seroconversion rates and seroprotection rates in SMHPs are shown Tables 2–4, respectively. Compared with the two samples of before vaccination (March 2004 and October 2004), geometric mean titers were almost the same level in A/New Caledonia/20/99(H1N1) (Table 2). All three strains, there were no significant differences in the geometric mean titers between before and after vaccination in SMHPs. Control geometric mean titers were increased significantly ($P<0.01$).

The seroprotection rates of A/Wyoming/3/2003(H3N2) and B/Shanghai/361/2002 were significantly different between before and after vaccination ($P<0.01$). Similar to A/New Caledonia/20/99(H1N1) strain, these seroprotection rates were increased after the first dose, but showed no remarkable change after the second dose (Table 3). Subjects less than 30 years old ($\leq$29 group), which group is consisted significant many numbers of "true SMHPs", had high seroprotection rates to all three strains compared with subjects aged $\geq$40 years old ($P<0.05$) (Table 3). This data can mean that the influenza vaccine effectiveness is more affected by age than severity of symptoms. In the control group, the seroprotection rates were increased ($P<0.01$) to effective group immunity.

In A/New Caledonia/20/99(H1N1), their lower seroconversion rate (19.0% after second vaccination, Table 4) may have been due to the seroprotection rate already being high (51.9%, Table 3). There were no significant differences in age, gender, and severity of symptoms in the seroconversion rates (Table 4).

A/H3N2-strain and B-strain were changed in the 2004–2005 season, so these HAI titers could be little influenced by recent strains. We also examined HAI titers of the March 2004-sample on the 2003–2004 season-strains. Both A/Panama/2007/99(H3N2)-strain and B/Shandong/7/97 showed low seroprotection rates, 40.5% and 6.4%, respectively.

Table 2
The geometric mean titer

	Control ($N=49$) (95% CI)	All subjects ($N=79$) (95% CI)	Age-group			
			≤29 group	30–39 group	40–49 group	≥50 group
A/New Caledonia/20/99(H1N1)						
Before vaccination						
March 2004		32.6 (24.2–43.9)	54.6	26.4	34.4	21.4
October 2004	68.5 (45.6–102.8)	33.3 (24.3–45.4)	53.8	26.4	37.7	20.7
After vaccination						
First dose	218.4* (157.1–303.6)	49.4 (37.2–65.2)	82.7	43.9	49.4	31.4
Second dose		55.3 (44.9–68.2)	91.3	50.4	55.7	33.6
A/Wyoming/3/2003(H3N2)						
Before vaccination						
March 2004		9.7 (7.9–11.7)	11.6	10.5	7.2	7.3
October 2004	24.7 (18.1–33.9)	9.3 (8.0–11.5)	12.7	11.0	7.0	7.6
After vaccination						
First dose	53.1* (40.4–69.7)	19.5 (15.8–24.0)	37.3	16.4	14.4	15.2
Second dose		19.8 (15.9–24.5)	37.3	17.2	14.9	15.2
B/Shanghai/361/2002						
Before vaccination						
March 2004		14.0 (11.5–17.1)	18.0	23.0	12.0	9.0
October 2004	24.7 (18.3–33.3)	16.6 (13.5–20.4)	26.9	23.0	13.9	9.7
After vaccination						
First dose	78.9* (58.9–105.6)	28.6 (22.8–35.4)	50.4	38.2	20.6	18.0
Second dose		29.4 (23.4–37.0)	45.6	41.9	21.9	20.0

* $P<0.01$ for the comparison with the before vaccination (October 2004).

Table 3
The seroprotection rate (%)

	Control ($N=49$) (95% CI)	All subjects ($N=79$) (95% CI)	Age-group			
			≤29 group	30–39 group	40–49 group	≥50 group
A/New Caledonia/20/99(H1N1)						
Before vaccination						
March 2004		48.7 (37.6–59.8)	70.0	33.3	52.2	35.0
October 2004	69.4 (56.5–82.3)	51.9 (40.9–62.9)	71.4	33.3	56.5	40.0
After vaccination						
First dose	98.0* (94.1–100)	60.8 (50.0–71.5)	85.7**	46.7	56.5	50.0
Second dose		60.8 (50.0–71.5)	81.0	53.3	56.5	50.0
A/Wyoming/3/2003(H3N2)						
Before vaccination						
March 2004		9.5 (2.8–16.1)	15.8	14.3	13.0	5.0
October 2004	46.9 (32.9–60.9)	10.7 (3.7–17.7)	25.0	14.3	4.3	0.0
After vaccination						
First dose	79.6* (68.3–90.9)	33.3* (22.7–44.0)	60.0**	28.6	17.4	25.0
Second dose		33.3 (22.7–44.0)	60.0	28.6	21.7	20.0
B/Shanghai/361/2002						
Before vaccination						
March 2004		24.4 (14.8–33.9)	35.0	46.7	13.0	10.0
October 2004	44.9 (31.0–58.8)	30.4 (20.2–40.5)	57.1	40.0	21.7	5.0
After vaccination						
First dose	85.7* (75.9–95.5)	54.4* (43.4–65.4)	76.2***	73.3	43.5	30.0
Second dose		55.7 (44.7–66.7)	66.7	80.0	52.2	30.0

* $P<0.01$ for the comparison with the before vaccination (October 2004).
** $P<0.05$ for the comparison with 30–39, 40–49, and ≥50-group, respectively, after one-dose vaccination.
*** $P<0.05$ for the comparison with 40–49 and ≥50-group, respectively, after one-dose vaccination.

Table 4
The seroconversion rate (%)

	Control ($N=49$) (95% CI)	All subjects ($N=79$) (95% CI)	Age-group			
			≤29 group	30–39 group	40–49 group	≥50 group
A/New Caledonia/20/99(H1N1)	40.8 (27.0–54.6)	19.0 (10.3–27.6)	23.8	20.0	8.7	20.0
A/Wyoming/3/2003(H3N2)	24.5 (12.5–36.5)	33.3 (22.7–44.0)	60.0	7.1	31.8	20.0
B/Shanghai/361/2002	46.9 (32.9–60.9)	25.3 (15.7–34.9)	19.0	20.0	21.7	40.0

Serum IgG levels were normal (>700 mg/dl) in all 48 subjects (data not shown). There was no significant difference between Oshima's classifications 1–4 and 5–25 in serum IgG level.

4. Discussion

It is generally accepted that the antibody titer is related to protection against influenza [16], and the majority of vaccinated children and young adults develop high serum antibody responses after vaccination (seroconversion rate >65%) [17–19]. The rates of morbidity and mortality due to influenza diseases are high among persons with underlying medical conditions [20]. They develop lower post-vaccination antibody titers than healthy young adults. The seroconversion rates were 35.7% with lung diseases, 33.3% elderly, and 26.5% with HIV infection compared with 58.8% of healthy volunteers [9].

SMHP is a clinical concept concerning patients with underlying medical conditions. In Japan, many SMHPs are cared for at chronic-care institutions. In other countries, they may live in their houses with familial care. Patients in chronic-care facilities are considered to be more appropriate for a study about the immunoreaction because they have limited exposure to infectious diseases. In addition, they can be carefully observed and easily scheduled for vaccination according to protocols for the prevention of serious illness. The present study was limited to SMHPs because no comparable reports have previously been published. For SMHPs and care-staff, our study offers one guideline to prevent influenza illness.

The recommended effective vaccination interval is >1 month among previously unvaccinated children aged <9 years old [7]. In the present study, administered two-dose vaccination with SMHPs in spite of aged >9 years old, we could not find out any differences in antibody titers according to the vaccination interval; the second dose did not enhance antibody responses, regardless of 4–6 weeks interval. Our result support the assertion that one dose of influenza vaccine is sufficient for satisfactory antibody responses among adults [21–23]. We conclude that one dose of influenza vaccine should also be recommended for SMHPs.

Varying levels of crossprotection have been reported [8]. In our study, antibody responses were different for A/H3N2-strain and B-strain, which was a changed strain. A/Panama/2007/99(H3N2)-strain showed a low seroprotection rate, in spite of five seasons of continuous selection. Similarly, B/Shandong/7/97 had a low seroprotection rate. B/Shanghai/361/2002-strain showed good responses from the first season. It is thus possible that the antibody responses are influenced by selected strains and their immunogenicity.

Generally, HAI titers last only 5–6 months. According to our study, SMHPs had continuous HAI titers from post-vaccination to the next fall, therefore HAI titers kept same level during approximately 10 months or more. This valuable data shows that influenza vaccine efficacy can continue until the next fall in SMHPs. We will schedule 2005–2006 season study including examination of 2004–2005 strain's HAI titers to confirm their durability. But SMHPs vaccinated with two doses still had low seroprotection rates and may experience serious illness if an outbreak occurs in an institution. It seems that SMHPs had low antibody responses to vaccination, although their HAI titers were not so low in the pre-season compared with control.

To prevent influenza endemics, SMHPs should be administered influenza vaccination annually. Care-staff should also be administered influenza vaccination. All staff including doctors, nurses, health-care workers, cleaning-staff receive free influenza vaccine coverage in our institution. Nevertheless, influenza endemics can occur in any institution, at any time, regardless of whether patients and staff have received influenza vaccinations. Influenza endemics can induce many numbers of serious complications and deaths. Moreover, we should consider that protection ability of SMHPs is not certain owing to their low immunoresponse. Antiviral drugs were effective for influenza prevention [24,25] and control of influenza outbreaks [26,27]. Oseltamivir has been approved for prophylaxis in both familial and institutional cases. In consideration of their debilitation, compromise, and low rate of seroprotection, chemoprophylaxis should be administered to SMHPs as soon as possible when influenza infection occurs around SMHPs. Antiviral medications are not a substitute for vaccination [7], but further studies are needed concerning chemoprophylaxis for SMHPs.

5. Conclusions

Among SMHPs, inactivated influenza vaccine can induce and maintain antibody responses during the influenza season. This means that the vaccine can decrease influenza-related mortality and complications among SMHPs. According to