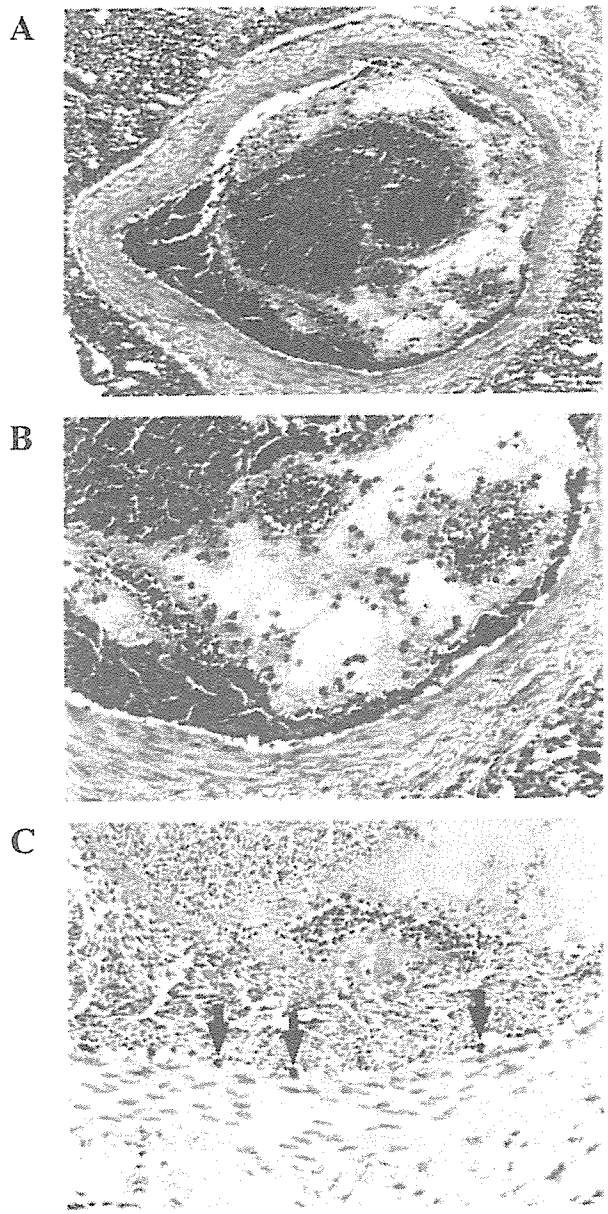

Fig. 2. Fibrin thrombus in the lung of HK156/836-infected chicken at 24 hr p.i. (A) Lower magnification photomicrograph of lung tissue in chicken with hematoxylin and eosin staining. Fibrin thrombus was found in the arteriole. (B) Higher magnification photomicrograph of fibrin thrombus with hematoxylin and eosin staining. The thrombus consists of thrombocytes, fibrin, red blood cells and heterophils. (C) Immunohistochemical analysis around the thrombus (higher magnification photomicrograph). Viral antigens were detected in the endothelial cells (arrows).

(Fig. 3A). We found that the number of thrombocytes significantly decreased in chickens infected with HK156/836 within 12 hr p.i. At 24 hr p.i., the number of thrombocytes dropped to approximately 18% of that collected before infection. By contrast, no appreciable decrease was observed in HK911-infected chickens. Prothrombin times of the peripheral blood were also significantly prolonged by HK156/836-infection (Fig. 3B). Prothrombin times of the sample collected at 12 and 24 hr p.i. were 5.9 and 7.8 sec longer, respectively than that of the sample collected before infection. On the other hand, HK911-infected chickens maintained baseline levels of prothrombin time during the experimental period. These results indicate that HK156/836 infection caused depletions of thrombocytes and coagulation factors in the peripheral blood, leading to severe coagulopathy in chickens.

Such acute depletions of thrombocytes and coagulation factors in the peripheral blood, as well as the formation of fibrin thrombi in vessels, implied that the acute and massive coagulation was induced in those chickens. Thus, to confirm whether HK156/836 indeed initiate blood coagulation, we examined the activation of tissue factor gene, the trigger of blood coagulation cascade, in the spleen, lung, and brain of virus-infected chickens by semiquantitative RT-PCR (Fig. 4). In most of the HK911-infected chickens, the transcripts of the tissue factor gene were under the level of detection in lungs and brains as was the case with mock-infected chickens. Although tissue factor transcripts were detected in the spleens of most of the chickens, HK911-infected chickens expressed slightly higher levels of the transcripts than mock-infected chickens. By contrast, HK156/836-infected chickens showed higher levels of the tissue factor expression in all organs tested than chickens of the other groups. These results suggest that increased level of tissue factor expression upon HPAI virus infection triggers tissue factor-mediated blood coagulation cascade, leading to consumptive coagulopathy in chickens.

Discussion

In this study, we showed that experimental infection of chickens with highly pathogenic HK156/836 caused coagulopathy in chickens, while avirulent HK911 did not. Since the only difference between these two viruses is HA cleavability, it was evident that the ability to undergo multiple replications in any organs is the critical determinant for their pathogenicity. As shown in Table 1, HK156/836 antigens were detected mainly in endothelial cells and monocytes/macrophages at the early stage of infection. Endothelial cells are thought to be one of the major targets for HPAI viruses in chickens as reported previously (3, 6, 19, 23, 30, 32). It is thus conceivable that the endothelial dysfunction by viral infection causes systemic hemorrhage manifestations in chickens.

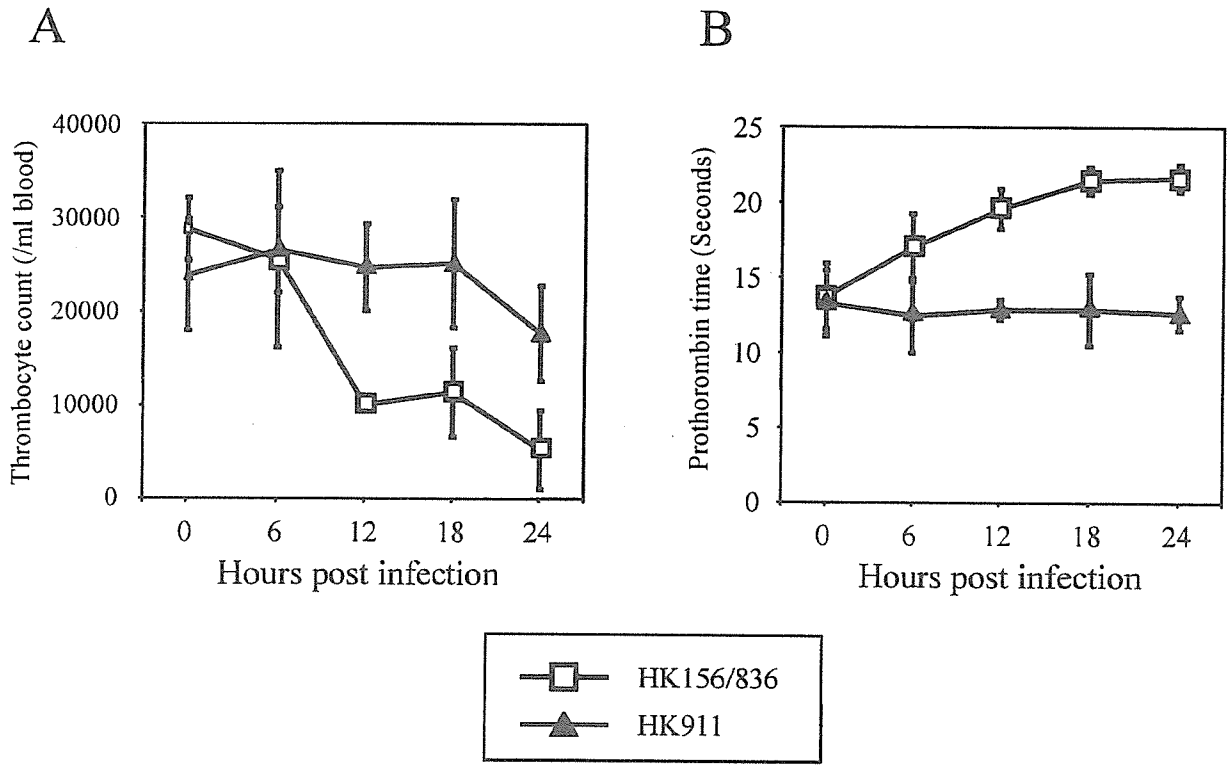

Fig. 3. Coagulopathy in chickens infected with HK156/836. (A) The number of peripheral blood thrombocytes in virus-infected chickens. Peripheral blood samples from three chickens infected with HK156/836 or HK911 were smeared on slide glasses and stained with anti-thrombocyte antibody. The number of thrombocytes was counted as described in "Materials and Methods." Averages and standard deviations of each group are shown. (B) Prothrombin time of peripheral blood in virus-infected chickens. Peripheral blood samples from three chickens infected with HK156/836 or HK911 were examined using an automated fibrometer. Averages and standard deviations of each group are shown.

Immunohistochemistry performed in this study could not clarify whether monocytes/macrophages were infected with the viruses, because the viral antigens detected might be the viral proteins phagocytosed. However, since it has been shown that influenza A viruses could infect chicken, human, and mouse monocytes/macrophages *in vitro* (2, 4, 10, 21, 38), it is conceivable that both endothelial cells and monocytes/macrophages are the major targets for HPAI viruses in chickens.

Interestingly, fibrin thrombi were found in arterioles at the lungs in HK156/836 virus-infected chickens, suggesting that the HPAI virus infection caused disseminated intravascular coagulation (DIC). DIC is a syndrome characterized by the excessive activation of coagulation cascade up to intravascular fibrin formation, accompanied by secondary fibrinolysis and coagulopathy for the consumption of platelets, coagulation factors, and fibrinogen. DIC is observed in a wide range of disease states including sepsis, burns, polytrauma, tumors, and viral infections. Some previous reports showed fibrin thrombi in HPAI virus infection in chickens, suggesting that DIC was induced in HPAI virus infections (1, 15, 17, 37). In the present study, the coagulopathy was indeed observed in the early stage of infection (12 hr p.i.), when virus replication was restricted mainly to endothelial cells and monocytes/macrophages. Thus, these findings suggest that replication of HPAI virus in endothelial cells and/or monocytes/macrophages triggers activation of coagulation cascade leading to DIC in infected chickens.

One possible mechanism underlying DIC in HPAI virus-infected chickens is the direct destruction of the large amount of endothelial cells by viral infection, which forms many clots to cover the large damaged regions in HPAI virus-infected endothelium, leading to the consumptive coagulopathy as suggested by Kobayashi et al. (17). The other possibility is that proinflammatory cytokines produced by HPAI virus infection may be involved in developing coagulation disorder. Proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 are the inducers of tissue factor, a trig-

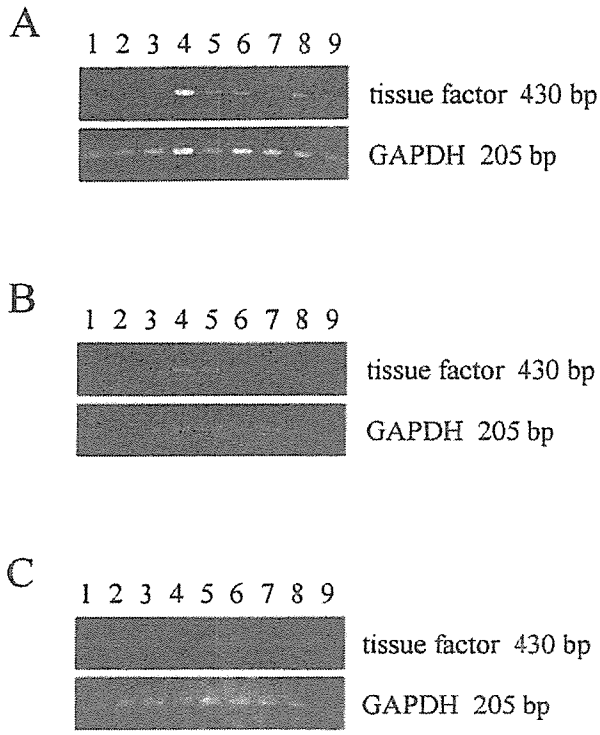

Fig. 4. Upregulation of the tissue factor mRNA in HK156/836-infected chickens. Three chickens from each group were sacrificed at 15 hr p.i. and spleens (A), lungs (B), and brains (C) were collected. Total RNA was extracted from each tissue and then tissue factor mRNA was detected by RT-PCR. RT-PCR for chicken GAPDH mRNA was also performed as a control. Lanes 1–3: mock-infected chickens, 4–6: HK156/836-infected chickens, 7–9: HK911-infected chickens.

ger of blood coagulation, on monocytes/macrophages and endothelial cells (9). It is also reported that these proinflammatory cytokines were secreted from human, rat, and mouse monocytes/macrophages infected with influenza A viruses *in vitro* (8, 10, 21). In addition, other *in vitro* studies showed that HPAI virus infection in human macrophages induced more intensive secretion of TNF-α than avirulent influenza A viruses did (4). Therefore, it is hypothesized that HPAI virus infection induces DIC by two independent mechanisms: 1) direct destruction of the endothelial cells, and 2) induction of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) from monocytes/macrophages to initiate massive coagulation.

In Hong Kong in 1997, highly pathogenic H5N1 avian influenza viruses, which were prevalent among the poultry, transmitted to humans (5, 31). Furthermore, since 2003 H5N1 HPAI viruses have been causing widespread disease not only in poultry but also in humans with many fatal cases in Asia. It was reported that some patients who progressed severe fatal influenza showed the following complications; coagulopathy, pulmonary hemorrhage, thrombocytopenia, elevation of TNF-α and so on (34–36, 40), although the H5N1 influenza virus replications were restricted in respiratory and gastrointestinal tracts in the patients (34, 36). The present results suggest a mechanism involved in pathogenesis of HPAI, which may have similarity to that of human H5N1 virus infection. Further studies are required to fully understand the pathogenesis of HPAI virus infection, which would facilitate the development of antiviral strategies against highly pathogenic influenza virus infections.

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ORIGINAL ARTICLE

Drug-resistant genes and serotypes of pneumococcal strains of community-acquired pneumonia among adults in Japan

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OISHI K, YOSHIMINE H, WATANABE H, WATANABE K, TANIMURA S, KAWAKAMI K, IWAGAKI A, NAGAI H, GOTO H, KUDOH S, KURIYAMA T, FUKUCHI Y, MATSUSHIMA T, SHIMADA K, MATSUMOTO K, NAGATAKE T. *Respirology* 2006; **11**: 429–436

Background: A high frequency of drug-resistant pneumococci has been reported in Asian countries. Few data on the drug-resistance or serotype of pneumococcal strains responsible for community-acquired pneumonia (CAP), however, are available for the past two decades in Japan.

Methodology: Susceptibility to antibiotics and the genotype of antibiotic-resistant genes and serotypes of *Streptococcus pneumoniae* isolates from 114 adult patients with CAP were examined in a nationwide study in Japan between 2001 and 2003.

Results: Most of the cases were non-bacteraemic pneumonia and the case fatality rate was 4.4%. The most frequent genotype of the *pbp* gene was *pbp1a+ 2x+ 2b* (gPRSP; 36.8%) followed by *pbp 2x* (28.1%) and of the macrolide-resistant gene, it was *ermB* (50.0%). The most common serotype was 19F (29.1%), followed by serotype 23F (13.2%), 6B (12.3%) and 3 (11.4%). The coverage of serotypes of isolates by a 23-valent pneumococcal polysaccharide vaccine (PPV) would be 82.5% in these patients with CAP. Most of strains with serotypes 19F and 23F were gPRSP. A cluster of serotype 3 strains associated with the *pbp 2x* and *ermB* gene was also noted.

Conclusion: A high frequency of altered *pbp* gene mutations or of macrolide-related genes and a high serotype coverage by the 23-valent PPV found in our study of pneumococcal pneumonia facilitates attempts to increase the coverage rate of the 23-valent PPV in adults older than 65 years in Japan.

Key words: community-acquired pneumonia, drug-resistance, pneumococcal polysaccharide vaccine, serotype, *Streptococcus pneumoniae*.

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INTRODUCTION

Pneumonia is currently the fourth leading cause of death in Japan and 94 900 persons (75 per 100 000) died of this disease in 2003.[1] *Streptococcus pneumoniae* (*S. pneumoniae*) is the most commonly identified cause of community-acquired pneumonia (CAP),

accounting for 16.5–38.9% of the CAP cases among adult patients in Japan.[2–5] An increasing prevalence of pneumococcal strains that are resistant to β-lactam antibiotics has been observed in both developing and developed countries during the last decade.[6] Recent studies have also shown high frequencies of penicillin- and macrolide-resistant pneumococci as a respiratory pathogen in several Asian countries, including Japan.[7–10] The resistance of *S. pneumoniae* to penicillin and other β-lactams has been shown to be associated with mosaic mutations in the *pbp1a*, *pbp2b* and *pbp2x* genes.[11,12] On the other hand, macrolide resistance in pneumococci is generally mediated by one of two possible mechanisms: (i) methylation of the 23S rRNA methylase encoded by the *ermB* gene; and (ii) macrolide efflux via the *mef A* gene.[13,14]

The increased rate of appearance of drug-resistant pneumococci in recent years emphasizes the need for preventing pneumococcal infections by vaccination with pneumococcal polysaccharide vaccine (PPV).[15] Although the effects of 23-valent PPV have been established for invasive pneumococcal diseases, such as bacteremia and meningitis, its effect on reducing the risk of pneumonia in adults remains a controversial issue.[16–19] Starting in 2001, the vaccination law in Japan permits the subsidization of the influenza vaccination of persons older than 65 years. Because of this, an additional preventive effect of the influenza vaccine and 23-valent PPV on reducing hospital mortality for pneumonia would be expected for persons older than 65 years.[20] The rate of vaccine coverage appears to be much lower in Japan (about 2%) than in the USA, where the proportion of subjects reporting ever having had the 23-valent PPV is 64.2% in adults older than 65 years.[21]

Few data on the drug-resistance or serotype distribution of pneumococcal strains responsible for CAP, however, are currently available in Japan.[4,7,8] This study was undertaken to examine the distribution of *pbp* genes or macrolide-resistant genes and serotypes that are responsible for CAP among adults in Japan.

METHODS

Patients

One hundred and fourteen patients with CAP and older than 16 years were enrolled at 20 hospitals nationwide in Japan between 2001 and 2003. Patients were enrolled only if all of the following findings were present: clinical symptoms and signs of pneumonia, the appearance of a new pulmonary infiltrative shadow on a CXR and isolation of *S. pneumoniae* from blood and/or lower respiratory tract specimens with compatible Gram stain findings.[22] After enrolment, the demographic data including underlying diseases, the severity of disease, the length of hospital stay and the outcome were evaluated. The severity of the disease was classified into the following three grades: mild, moderate and severe according to the Japanese Respiratory Society guidelines, based on a physical examination, CXR, white blood cell count, serum CRP value and arterial PaO_2.[5,23] Of the 114 patients, 89 (78.1%) were hospitalized. The other 25 patients (21.9%) were followed at outpatient clinics. All studies described here were approved by the Institutional Review Boards of our institutions and signed consent form was obtained from each subject.

Bacterial strains and polymerase chain reaction (PCR) of drug-resistant genes

Each strain of *S. pneumoniae* was isolated from 114 patients with CAP. These strains were obtained from sputum ($n = 109$), BAL fluid ($n = 1$), blood ($n = 3$) and pleural effusion ($n = 1$). *S. pneumoniae* was identified by optochin sensitivity and bile solubility tests and confirmed by PCR for the autolysin (*lyt A*) genes.[11] Of these 114 strains, three penicillin-binding protein (PBP) genes (*pbp1a, pbp2x, pbp2b*) only in susceptible strains[11] or macrolide-resistant genes (*mefA* and *ermB*)[24] were amplified by means of PCR (Wakunaga Pharmaceutical Co., Hiroshima, Japan) according to the manufacturer's instructions. Amplification of DNA fragments were carried out with the oligonucleotide primer pairs: 5′-AAACAAGGTCGGACTCAACC-3′ and 5′-AGGTGCTACAAATTGAGAGG for *pbp1a*, 5′-CCAGGTTCCACTATGAAAGTG-3′ and 5′-CATCCGT CAAACCGAAACGG-3′ for *pbp2x* and 5′-CAATCTA GAGTCTGCTATGGA-3′ and 5′-GGTCAATTCCTGTCG CAGTA-3′ for *pbp2b* and 5′-CGTACCTTGGATATTC ACC and 5′-GTAAACAGTTGACGATATTG *ermB* for 5′-CTGTATGGAGCTACCTGTGG-3′ and CCCAGCTTAG GTATACGTAC-3′ for *mefA* as previously described.[11,24] The amplified regions of each PBP gene were positioned in highly divergent sequences of penicillin-resistant *S. pneumoniae* (PRSP).[11]

Antimicrobial susceptibility test

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards.[25] The susceptibilities of the 114 *S. pneumoniae* isolates to the following three β-lactam antibiotics, penicillin G (Meiji Seika Kaisha, Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical Co., Tokyo, Japan) and imipenem (Banyu Pharmaceutical Co., Tokyo, Japan) and clarithromycin (Taisho Pharmaceutical, Tokyo, Japan) and azithromycin (Pfizer Pharmaceutical) were tested. Standard quality control strains (gifts from Dr K Ubukata, Kitasato Institute for Life Science and Graduate School of Infection Control Sciences, Kitasato University) including the R6 strain (penicillin- and macrolide-sensitive), KM-99 and H69 strains (genotype *pbp 2x* and *ermB*), KK-133 strain (genotype *pbp1a + 2x + 2b* and macrolide-sensitive), KM-90 strain (genotype *pbp1a + 2x + 2b* and *mefA*) and H-29 and SHA-3 strains (genotype *pbp1a + 2x + 2b* and *ermB*) and T-197 strain (genotype *pbp1a + 2x + 2b*, mefA and *ermB*) were also examined in this study.

Serotyping

Pneumococci were serotyped on the basis of the quellung reaction observed microscopically after suspension in pneumococcal diagnostic antisera (Statens Serum Institut, Copenhagen, Denmark).

Statistical analysis

Statistical analyses were performed by the Krusukal-Wallis test or Mann–Whitney's *U*-test. Data were considered to be statistically significant, if the *P*-value was less than 0.05. All data are expressed as the mean ± SD.

RESULTS

The mean age (±SD) of the total 114 patients was 67.4 ± 15.8 years (range, 20–99 years) (Fig. 1). One small peak at 30 years and another large peak at 70 years were found in the age distribution. There were more male patients (59.6%) than female patients, except for the 80- and 90-year-old patients. A decreased number of patients between 80 and 90 is probably due to a decreased population in this age group in Japan.[1] Of these 114 patients, 82 (71.9%) had underlying conditions, such a chronic lung disease (39.5%), diabetes mellitus (12.3%), cerebrovascular accident (8.8%), hypertensive heart disease (7.9%), chronic liver disease (4.5%) or malignancy (4.5%). The severity of the disease for these patients was classified into three groups; mild (33.3%), moderate (42.1%) and severe (24.6%). The mean period (±SD) of hospital stay was 15.9 ± 11.5 days for patients with mild disease ($n = 20$), 24.1 ± 22.3 days for those with moderate disease ($n = 38$) and 34.3 ± 31.8 days for those with severe disease ($n = 24$). No significant difference in the length of hospital stay was found among these three groups. Of the 114 patients, 109 (95.6%) showed clinical improvement and five (4.4%) died within 30 days after the diagnosis of pneumonia. All of the fatal cases were associated with severe disease.

The MIC distributions for penicillin G, ceftriaxone and imipenem against the 114 *S. pneumoniae* isolates (one strain per patient) based on the PCR results are shown in Figure 2. The *in vitro* activity of penicillin G against these strains indicated that 26 patients (22.8%) were infected with penicillin-resistant strains (the MIC value was 2 μg/mL in 25 strains and 4 μg/mL in one strain of penicillin G) (Fig. 2A). Penicillin-resistant strains (MIC > 0.12 μg/mL for penicillin G) were isolated from 66 patients (57.9%). The pneumococcal isolates were classified into six groups according to the genotype of the *pbp* gene (Table 1). Only 13 strains (11.4%) were associated with no *pbp* gene mutation, which closely corresponded to penicillin-sensitive *S. pneumoniae* on the basis of the MIC distribution for penicillin G. The frequency of strains with genotype *pbp 1a+2x+2b*, closely corresponding to PRSP was the highest (36.8%). This genotype was named gPRSP in the present study.[26] Most strains (51.8%) possessed one or two *pbp* gene mutations with the highest frequency for the *pbp 2x* gene (28.1%). An alteration in the *pbp 2x* gene had no effect on the MIC value for penicillin G and imipenem (Fig. 2A,C). In contrast, the MICs for ceftriaxone were affected by alteration of the *pbp 2x* gene, consistent with a previous report (Fig. 2B).[26] As a result, a small cluster of antibiotic-susceptible strains, comprised of strains without a *pbp* gene mutation, was found for ceftriaxone.

Erythromycin-resistant strains (MIC ≥ 1 μg/mL) were isolated from 86 patients (75.4%). The pneumococcal strains were also classified into four groups according to the genotype of the macrolide-resistant genes (Table 1). The genotype of the *ermB* strains were highly resistant to erythromycin and the frequency (50%) was much higher than that for *mefA* (22.6%) in patients with CAP. Macrolide resistance in pneumococcal strains for CAP was mainly due to the *ermB* gene in Japan. Only seven strains (6.1%) possessed both gene mutations. While most strains with genotype *ermB* showed a high resistance to erythro-

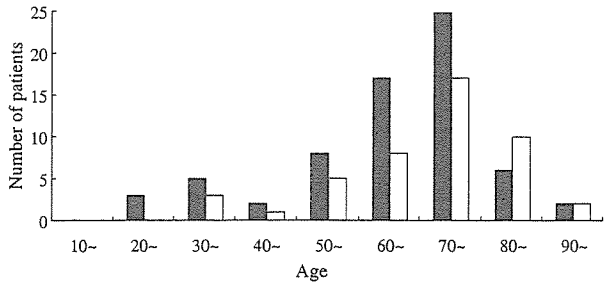

Figure 1 Distribution of gender and age of the 114 adult patients enrolled in this study with community-acquired pneumococcal pneumonia in Japan. Closed column is male and open column is female, respectively.

Table 1 Genotype of drug-resistant genes and MICs distribution for penicillin G or erythromycin in 114 clinical isolates of *Streptococcus pneumoniae*

		MIC of penicillin G (μg/mL)		
Genotype	*n* (%)	Range	50	90
No mutation	13 (11.4)	0.01–0.13	0.03	0.13
pbp 2x	32 (28.1)	0.03–0.13	0.03	0.06
pbp 2b	2 (1.8)	0.06–0.13	0.06	0.13
pbp 2x+2b	15 (13.2)	0.03–1.0	0.25	1
pbp 1a+2x	10 (8.8)	0.06–0.25	0.13	0.25
pbp 1a+2x+2b	42 (36.8)	0.25–4.0	2	2
		MIC of erythromycin (μg/mL)		
Genotype	*n* (%)	Range	50	90
No mutation	24 (21.1)	0.03–0.13	0.06	0.13
mefA	26 (22.8)	0.25–4.0	1	2
ermB	57 (50.0)	0.5–128	128	128
mefA+ermB	7 (6.1)	8.0–128	128	128

MIC, minimum inhibitory concentration.

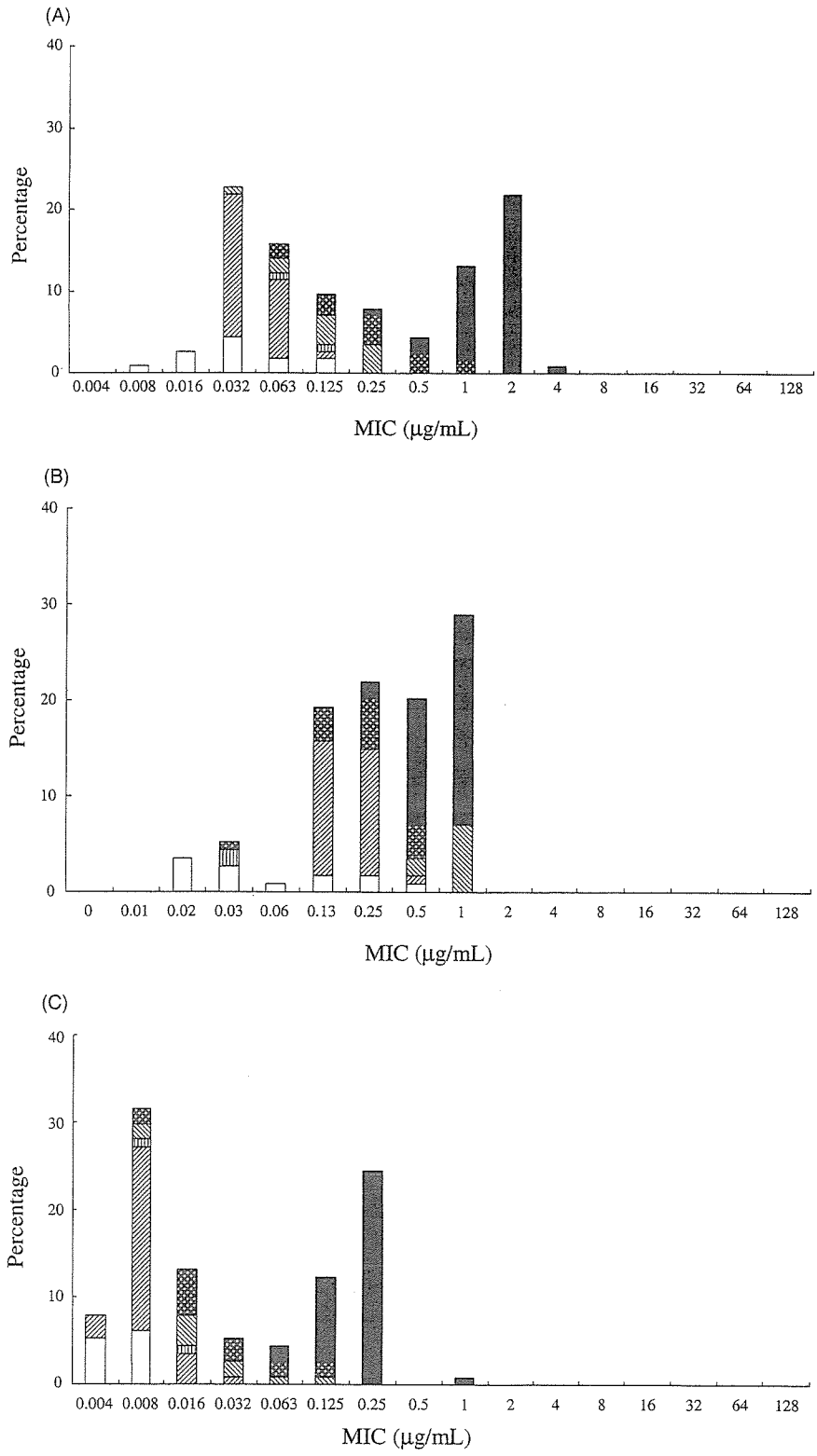

Figure 2 Relationship between MICs values for three β-lactam antibiotics: (A) penicillin G, (B) ceftriaxone and (C) imipenem; and the genotype of the *pbp* genes in 114 clinical isolates of *S. pneumoniae* from adults patients with community-acquired pneumonia. (□) No mutation; (▨) *pbp 2x*; (▥) *pbp 2b*; (▧) *pbp 1a+2x*; (▦) *pbp 2x+2b*; (■) *pbp 1a+2x+2b*.

mycin, in the case of the genotype *mefA* the resistance was low. The MIC range (μg/mL) and the MIC_{50} and MIC_{90} (μg/mL) against 114 isolates of *S. pneumoniae* were 0.02–128 and 16 and 128 for clarithromycin and 0.032–128 and 128 and 128 for azithromycin, respectively.

Relationships between the genotype patterns of the *pbp* gene (Table 2) or the macrolide-resistant gene (Table 3) among the 114 isolates are shown. The most frequent serotype was 19F (29.1%), followed by serotypes 23F (13.2%), 6B (12.3%) and 3 (11.4%) (Tables 2,3). Of the 114 strains, 94 (82.5%) possessed

Table 2 Genotype of the pbp genes and serotype distribution in 114 clinical isolates of *Streptococcus pneumoniae*

	No. of isolates with serotype (%)												
Genotype	19F	23F	6B	3	14	6A	11A	19A	9V	16F	15C	Other	Total
No mutation	1		1	1				1	1	1		7	13
pbp 2x	2	1	4	11	2	3	2	2	3		1	1	32
pbp 2b			1									1	2
pbp 2x+2b	4	1	3	1	3			1				2	15
pbp 1a+2x			1		4		2	1		1	1		10
pbp 1a+2x+2b	18	13	4			4	1		1	1			42
Total	25 (29.1)	15 (13.2)	14 (12.3)	13 (11.4)	9 (7.9)	7 (6.1)	5 (4.4)	5 (4.4)	5 (4.4)	3 (2.6)	2 (1.8)	11 (9.6)	114

Table 3 Genotype of the macrolide-resistant genes and serotype distribution in 114 clinical isolates of *Streptococcus pneumoniae*

	No. of isolates with serotype (%)												
Genotype	19F	23F	6B	3	14	6A	11A	19A	9V	16F	15C	Other	Total
No mutation	4	1	2	1		1	4	1	3	1		6	24
mefA	16	4	3						1	2			26
ermB	3	8	9	12	9	5	1	2	1		2	5	57
mefA+ermB	2	2				1		2					7
Total	25 (29.1)	15 (13.2)	14 (12.3)	13 (11.4)	9 (7.9)	7 (6.1)	5 (4.4)	5 (4.4)	5 (4.4)	3 (2.6)	2 (1.8)	11 (9.6)	114

serotypes that are involved in the 23-valent PPV. Non-covered serotypes in these strains were 6A (seven strains), 16F (three strains), 15C (two strains) and others (each one strain). The involvement of subtypes of the vaccine serotype as related serotypes led to a high vaccine coverage (94.7%). Most of strains with serotypes 19F and 23F were gPRSP (72% for serotype19F and 86.7% for serotype 23F). Interestingly, most of the type 3 strains were associated with the *pbp* 2x genotype (11/13) and the *ermB* genotype (12/13), respectively. Furthermore, all of nine serotype 14 strains contained the *ermB* mutation.

DISCUSSION

This is the first report demonstrating the clinical features and the frequency of an altered *pbp* gene or macrolide-resistant genes and serotype of pneumococcal strains responsible for CAP among adults in Japan. Although the number of patients examined was limited, the patients were enrolled at 20 hospitals which were widely distributed in Japan. The clinical and microbiological data shown in this study therefore might represent a trend for this disease in Japan. The rate of bacteraemic pneumonia in this study was lower than that reported in previous studies in Japan (4.7–9.3%) or in a study in the Asian Network (30.9%).[2,27,28] The case fatality in our study was similar to that reported in previous studies of pneumococcal pneumonia in Japan (0–3.4%),[2–4,8] but much lower than those reported in Asia and Europe (13.3–14.3%).[28,29] A low rate of mortality might be attributable to the low rate of bacteraemic pneumococcal pneumonia in this study. These rates are comparable to those reported for other Asian countries in recent years.[9,10] The grade of resistance was milder for third-generation cephems and imipenem than for penicillin G. A new breakpoint for ceftriaxone also defined all of the 114 pneumococcal strains, including gPRSP strains, as being susceptible.[30] Because the reduced affinity of penicillin to PBPs is much prominent by virtue of several mutations of *pbp* gene than that of third-generation cephem or imipenem, the resistance is more pronounced for penicillin than for third-generation cephem or imipenem.[12] On the other hand, the frequent use of macrolide antibiotics has resulted in an increasing incidence of macrolide resistance among pneumococcal isolates. The prevalence *mefA* gene and *ermB* gene mediated-mechanisms of macrolide resistance varies in different geographical areas. While strains with *ermB* genes are predominant in Europe and the Far East, a strain with the *mefE* gene is predominant in North America.[10,31–33] In this study, more than half of the pneumococcal strains possessed the *ermB* gene, which induced a high level of macrolide resistance. This rate was higher than that (39.1%) reported in an earlier study conducted between 1995 and 1997 in Japan.[24]

A nationwide study of the distribution of serotype of isolates from 590 patients with pneumococcal diseases was carried out between 1980 and 1984 and the rank order of isolates was reported to be serotype 3 (12.7%), 19F (9.3%), 23F (6.8%) and 6B (5.9%).[34] The coverage of these isolates by 23-valent PPV was 72.9% in this study. In the present study, the relative frequency of serotypes was higher by three times for serotype 19 and two times higher for serotype 23F and 6B and the vaccine coverage by 23-valent capsular polysaccharide was also increased, compared with a previous study in Japan.[34] The distribution of serotype in the present study was similar to the recent studies in Asian countries.[7–9] An association of serotype 3 strains with genotype *pbp2x* and genotype *ermB* was also found in the present study. A recent study by Japanese investigators suggested a clonal spread of serotype 3 strains which are susceptible to penicillin and positive for the *ermB* gene in a pilot study performed in a single institution in Japan.[35] Because an alteration in the *pbp 2x* gene did not affect the MIC for penicillin, strains with the *pbp 2x* gene were penicillin susceptible. Although the authors did not examine the *pbp gene* mutation in pneumococcal strains, their data are consistent with our results of a cluster of serotype 3 strains with genotype *pbp 2x* and genotype *ermB*. Collectively, our data are consistent with a clonal expansion of serotype 3 strains associated with altered *pbp 2x* gene and with *ermB* gene among adults across Japan.

A nationwide, prospective study in Japan demonstrated that the rates of β-lactam or macrolide-resistant pneumococcal strains responsible for CAP remain high. The coverage of serotypes of isolates by a 23-valent PPV would be 82.5% in these patients with CAP. The data reported on pneumococcal pneumonia should facilitate attempts to increase coverage by the 23-valent PPV in Japanese adults older than 65 years.

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Significant Reduction of Nosocomial Pneumonia after Introduction of Disinfection of Upper Airways Using Povidone-Iodine in Geriatric Wards

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Key Words
Nosocomial pneumonia · Methicillin-resistant *Staphylococcus aureus* · *Pseudomonas aeruginosa* · Upper airways · Povidone-iodine · Geriatric long-term care ward · Pulsed-field gel electrophoresis · Respiratory infection

Abstract
We investigated the efficacy of disinfection of the upper airway using povidone-iodine against nosocomial pneumonia in geriatric wards. Cases of nosocomial pneumonia were retrospectively analyzed between January 1991 and March 1995 in geriatric wards (190 beds). Moreover, the relationship concerning methicillin-resistant *Staphylococcus aureus* (MRSA) isolates between patient and environment was investigated using pulsed-field gel electrophoresis (PFGE) with the *Sma*I restriction enzyme. The incidence of nosocomial pneumonia decreased significantly ($p < 0.05$). Major causative organisms of nosocomial pneumonia were MRSA and *Pseudomonas aeruginosa*, which significantly decreased. PFGE studies showed that the patterns of MRSA isolates show a strong association between patient and environment. Our study indicates that disinfection of the upper airways by povidone-iodine is very important in the prevention of nosocomial pneumonia in geriatric wards.

Introduction

Staphylococcus aureus, especially methicillin-resistant *S. aureus* (MRSA), is an important nosocomial pathogen. In particular, MRSA is of great concern in both hospitals and nursing homes [1–3]. Previous studies have shown that the phage groups of *S. aureus* in the hospital environment of a nursing home only infrequently matched the phage group that colonized the patients [1]. However, little information, including molecular analysis, is available regarding the relationship between colonization and environmental contamination of *S. aureus* in geriatric long-term-care institutions. In this study, we investigated the possible relationship between *S. aureus* types colonizing the respiratory tract and rectum, and *S. aureus* types isolated from the hospital environment.

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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 *Sma*I 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 *Sma*I 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.

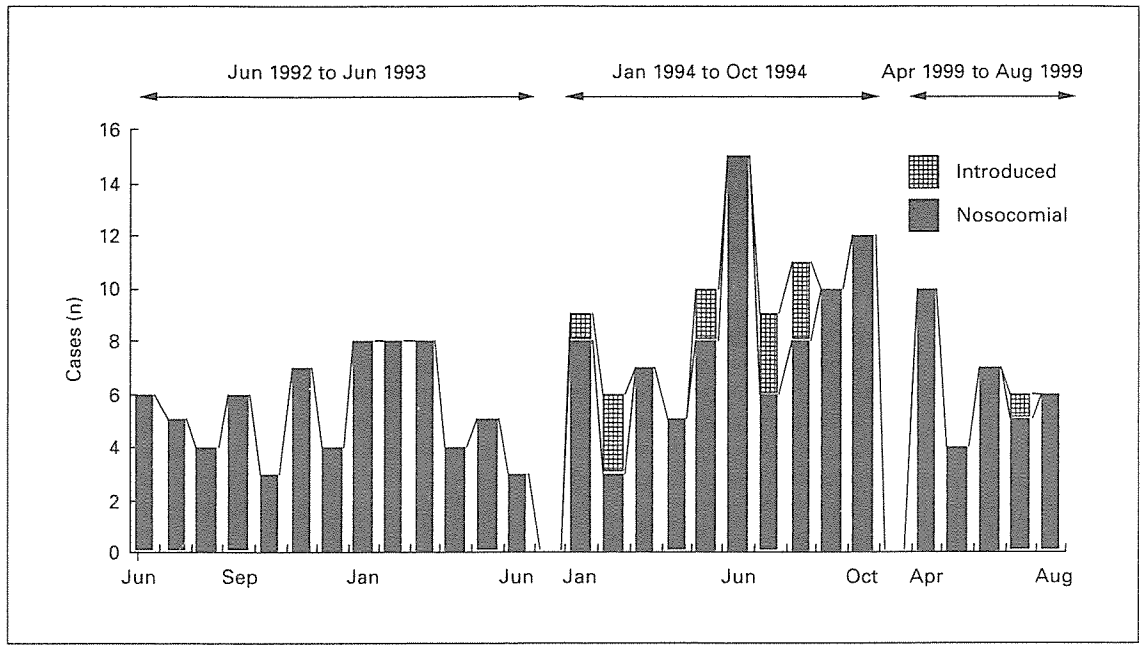

Fig. 1. The monthly new cases with MRSA colonization in geriatric wards. Several new cases were observed in hospital A each month.

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

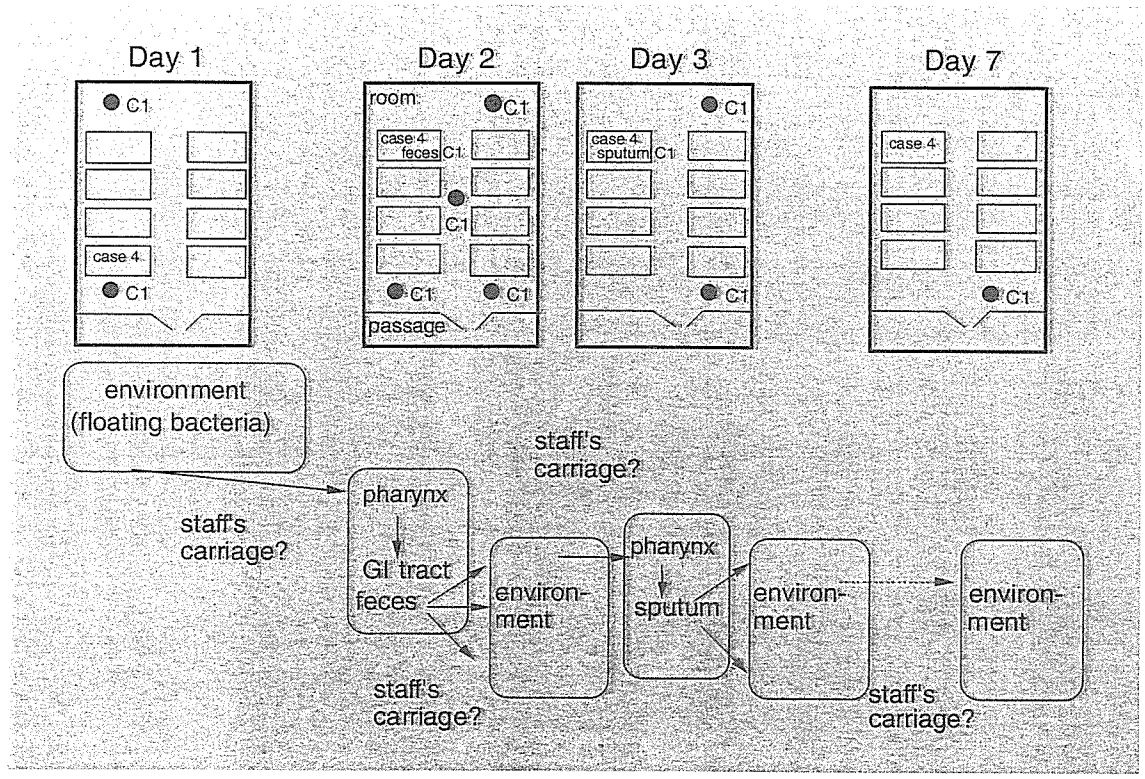

Fig. 2. The transmission of subtype C1 strain in a geriatric ward.

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.

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Antimicrobial susceptibility and serotype distribution of *Streptococcus pneumoniae* isolated from patients with community-acquired pneumonia and molecular analysis of multidrug-resistant serotype 19F and 23F strains in Japan

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SUMMARY

A nationwide study was undertaken to determine the susceptibility to penicillin and serotypes of *Streptococcus pneumoniae* in Japan. *S. pneumoniae* was isolated from 114 adult patients with community-acquired pneumonia over 22 months at 20 hospitals and medical centres in different regions in Japan. All but five isolates were from sputum. Forty-eight isolates (42·1 %) were susceptible, 40 (35·1 %) showed intermediate resistance (MIC, 0·12–1·0 μg/ml) and 26 (22·8 %) were resistant (MIC, ⩾2·0 μg/ml) to penicillin G. All isolates were susceptible to ceftriaxone (breakpoint 1 μg/ml), imipenem (4 μg/ml) and vancomycin (4 μg/ml). Most were resistant to erythromycin, clarithromycin and azithromycin; only two were resistant to levofloxacin. Differences were found in the distribution of serotypes among isolates showing susceptibility to penicillin (predominant types 3, 6B, and 19F), intermediate resistance (6B, 14, 19F, and 23F) and full resistance (19F and 23F). PFGE typing showed that 14 of the 25 strains of serotype 19F had a single DNA profile, pattern A, a pattern closely similar to that of the Taiwan multidrug-resistant 19F clone. Twelve pattern A strains were not susceptible to penicillin but carried the macrolide resistance gene *mef*(A). The DNA profiles of the 15 strains of 23F were also heterogeneous but six were highly similar (pattern b) yet distinct from the Spanish multidrug-resistant 23F clone although possibly related to the Taiwan multidrug-resistant 23F clone. The pattern b strains were not susceptible to penicillin and also harboured either *mef*(A) or *erm*(B). Our results indicate that multidrug-resistant pneumococci are spreading rapidly in Japan. Efforts to prevent the spread of the pandemic multidrug-resistant serotypes should be intensified.

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INTRODUCTION

Streptococcus pneumoniae is the major cause of community-acquired pneumonia (CAP) as well as otitis media, sinusitis, septicaemia, and meningitis [1]. Although susceptible to penicillin in the past, penicillin-resistant and multidrug-resistant pneumococci are now widespread in the world [1, 2]. In Japan, *S. pneumoniae* is the most common cause of CAP [3], and the proportion of multidrug-resistant strains is increasing at a dramatic rate [4, 5]. Nevertheless, there are few reports on the distribution of various serotypes and the genetic relatedness of pneumococci in this country [6]. In this study, we describe the results of a nationwide survey of the antimicrobial susceptibilities and the distribution of pneumococcal serotypes isolated from patients with CAP. We also examined the genetic relatedness of the predominant multidrug-resistant serotype 19F and 23F strains by pulsed-field gel electrophoresis (PFGE) and compared their DNA profiles with selected pandemic reference strains.

METHODS

Bacterial strains

A total of 114 isolates of pneumococci were recovered from 114 adult patients (mean age 67·4, range 20–99 years) presenting with CAP between November 2001 and August 2003 at 20 hospitals and medical centres located in different regions of Japan. Pneumonia was diagnosed if there was an appearance of a new abnormal shadow and likely infiltration on a chest roentgenogram and if at least two of the following clinical and laboratory findings were present: fever (temperature > 37·8 °C), cough, production of purulent sputum, dyspnoea, and leukocytosis (WBC count > 10 000/μl). The sources of the isolates were sputum ($n = 109$), blood ($n = 3$), pleural effusion ($n = 1$) and bronchoalveolar lavage ($n = 1$). Culture plates were incubated overnight in a 5% CO_2 atmosphere, and optochin sensitivity and bile solubility tests were performed to confirm *S. pneumoniae*.

Antimicrobial susceptibility test

MICs were determined by an agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards [7]. All isolates were tested for susceptibility to the following nine antibiotics: penicillin G (Meiji Seika Kaisha, Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical Co., Tokyo, Japan), cefditoren (Meiji Seika Kaisha), imipenem (Banyu Pharmaceutical Co., Tokyo, Japan), erythromycin (Dainippon Pharmaceutical Co., Osaka, Japan), clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan), azithromycin (Pfizer Japan Inc., Tokyo, Japan), levofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan), and vancomycin (Shionogi Co., Osaka, Japan).

Serotyping

Isolates were serotyped by the capsular swelling (quellung reaction) observed microscopically after suspension in pneumococcal typing antisera (Statens Serum Institut, Copenhagen, Denmark).

PCR

PCR was performed to detect alterations in penicillin-binding protein genes *pbp1a*, *pbp2x*, and *pbp2b* and macrolide resistance genes *mef*(A) and *erm*(B) by using a commercially available test kit (Wakunaga Pharmaceutical Co., Hiroshima, Japan) with primers modified as reported previously [8]. Briefly, a single colony from the blood agar medium was suspended in a microtube containing 30 μl of a lysis solution. The tube was placed in a thermal cycler, and bacterial cells were lysed at 60 °C for 10 min followed by 94 °C for 5 min. Next, 2 μl lysate was placed in a PCR tube containing 25 μl reaction mixture (1 ml reaction mixture contained 60 ng of a primer for each of the target genes, 80 μl of 10 mM dinucleoside triphosphate, 40 U *Tth* DNA polymerase, and 100 μl of 10 × PCR buffer). The PCR conditions were 94 °C for 20 s, 52 °C for 20 s, and 72 °C for 15 s for 30 cycles total.

PFGE

PFGE was performed on strains of serotype 19F, 23F. The Spanish multidrug-resistant serotype 23F clone (ATCC 700669), the Taiwan multidrug-resistant serotype 19F clone (ATCC 700905) and the Taiwan multidrug-resistant serotype 23F clone (ATCC 700906) were used as reference standards [9]. Strains were grown overnight in brain heart infusion broth at 35 °C, and PFGE of *Sma*I chromosomal digests was performed as described previously [10]. DNA banding patterns were interpreted according to the criteria of Tenover *et al.* [11] with a greater than three bands