**Microbiological examination.** Sputum or bronchoalveolar lavage fluid was used for smears and mycobacterial cultures according to standard methods (5). Any processed specimens that remained were stored at 2°C to 8°C for the duration of culturing in the study to allow the retesting of the specimens that showed a discrepancy in results between culture growth and preliminary identification by a DNA-DNA hybridization (DDH) mycobacterium kit (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan). Samples were cultured by using the Bactec MGIT 960 system or Ogawa solid medium, and the isolates were stored at −30°C to −80°C. *M. abscessus* species were preliminarily identified by microplate DDH technology using the DDH mycobacterium kit (21) at each hospital or institution. We collected all frozen isolates for multisequencing and susceptibility testing. Frozen isolates were recultured by using the MGIT 960 system and Ogawa solid medium and checked for contamination by growth on *p*-nitrobenzoic acid agar medium.

Further differentiation among *M. abscessus* species was performed at the Department of Mycobacteriology, Leprosy Research Centre, National Institute of Infectious Disease, and the Kobe Institute of Health. Sequences of clinical isolates which were previously identified as *M. abscessus* by DDH were compared with the reference *M. abscessus* (JCM 15300$^T$), *M. massiliense* (JCM 13569$^T$), and *M. bolletii* (JCM 15297$^T$) strains. The majority of the 16S rRNA gene, partial aspects of the *hsp65* and *rpoB* genes, and the 16S-23S rRNA internal transcribed spacer (ITS) region were amplified by PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and primers described previously (22). The PCR products were sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI Prism 310 genetic analyzer (Applied Biosystems). Sequences were analyzed for their similarity to sequences in the GenBank database by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Antimycobacterial susceptibility testing was performed at the National Hospital Organization Kinki-Chuo Chest Medical Center (Osaka, Japan). Susceptibility was determined by MIC methods using the Etest and broth microdilution.

**Etest.** Isolates of rapidly growing mycobacteria (RGM) were mailed on Ogawa slant medium and subcultured on 5% sheep blood agar at 35°C in ambient air for 72 h. Bacterial suspensions were prepared in cation-adjusted Mueller-Hinton broth (Eiken Chemical, Tokyo, Japan) to a 1.0 McFarland standard and plated onto cation-adjusted Mueller-Hinton agar. Etest strips (Sysmex bioMérieux, Tokyo, Japan) were placed onto Mueller-Hinton agar (Becton Dickinson, Fukushima, Japan) according to the manufacturer's instructions, and the results were read after 72 h of incubation. Final concentration ranges were 0.016 to 256 μg/ml for isoniazid (INH) and ethambutol (EB); 0.002 to 32 μg/ml for rifampin (RFP), ciprofloxacin (CPFX), and moxifloxacin (MFLX); and 0.064 to 1,024 μg/ml for streptomycin (SM). Susceptibility was evaluated according to Clinical and Laboratory Standards Institute (CLSI) breakpoint recommendations (8, 37) and those proposed previously by Woods et al. (36), Wallace et al. (33), Yang et al. (38), and Swenson et al. (27, 28).

**Broth microdilution.** Serial double dilutions of clarithromycin (CAM), kanamycin (KM), amikacin (AMK), and imipenem (IPM) were prepared at a concentration range of 0.015 to 64 μg/ml according to CLSI recommendations (37). Briefly, pure colonies were cultured in 7H9 broth with 0.2% glycerol in a tube for 3 to 5 days, vigorously vortexed, and then adjusted to a density equivalent of 0.5 on the McFarland scale. Bacterial suspensions in cation-adjusted Mueller-Hinton broth were transferred into the wells of dry microdilution plates containing the antimicrobial agents (Eiken Chemical). The inoculated plates were placed into plastic bags, incubated at 35°C in ambient air, and read after 72 h. The MIC was defined as the lowest concentration of drug that inhibited visible growth. Susceptibility was evaluated according to CLSI breakpoint recommendations (8, 37) and those proposed previously by Shen et al. (24).

**Data analysis.** The results are expressed as ranges and means or as numbers of patients. Categorical variables were analyzed by using the $\chi^2$ or Fisher's exact test. Continuous variables were analyzed by using the Mann-Whitney U test or *t* test. All *P* values are two sided; a *P* value of $<0.05$ indicates statistical significance. Statistical analyses were performed by using Excel 2011 (Microsoft) with the add-in software Statcel 3 (OMS Publishing Inc., Saitama, Japan).

## RESULTS

**Identification of clinical isolates.** Clinical isolates from 102 patients previously diagnosed with *M. abscessus* lung disease by DDH were identified by sequence analysis of the 16S rRNA, *hsp65*, *rpoB*, and ITS genes. Seventy-two (71%) isolates were identified as *M. abscessus*, 27 (26%) were identified as *M. massiliense*, and 3 (3%) were identified as *M. bolletii*.

**Patient characteristics.** We compared clinical characteristics and treatment outcomes among the 72 patients with *M. abscessus* and 27 patients with *M. massiliense* infections. Patients with *M. bolletii* infection were not included for further study because of their small number.

Table 1 summarizes the baseline characteristics of the patients. No significant differences were found between the *M. abscessus* and *M. massiliense* groups in any of the baseline characteristics, including demographic data, underlying conditions, and respiratory symptoms. The distributions of radiographic disease types were similar in both groups, except for bronchiectasis. Bronchiectasis was significantly more frequent in the *M. abscessus* group (73%; 40 of 55) than in the *M. massiliense* group (43%; 10 of 23) ($P = 0.014$). None of these patients tested positive for HIV.

**Drug susceptibility.** Table 2 shows the drug susceptibility results for 63 *M. abscessus* and 23 *M. massiliense* isolates, and Table S1 in the supplemental material shows the MIC distributions of the strains. The drug was determined to be effective against an isolate if the MIC of the antimicrobial agent was less than the susceptible concentration, as shown in Table 2. Of the parenteral antibiotics, KM and AMK were effective against most *M. abscessus* isolates (95% and 94%, respectively) and *M. massiliense* isolates (100% for both), with no difference between the species ($P = 0.388$ and 0.509, respectively). However, SM was ineffective against both *M. abscessus* (67%) and *M. massiliense* (61%) isolates ($P = 0.617$). Moreover, the rates of IPM drug resistance were significantly higher in *M. massiliense* than in *M. abscessus* isolates (48% and 19%, respectively; $P = 0.007$).

Of the oral antibiotics, CAM was effective against most *M. abscessus* and *M. massiliense* isolates (84% and 96%, respectively; $P = 0.145$). In contrast, MFLX, CPFX, and RFP were ineffective against most *M. abscessus* (92%, 95%, and 97%, respectively) and *M. massiliense* (96%, 91%, and 100%, respectively) isolates, with no difference between the species ($P = 0.488$, 0.883, and 0.534, respectively). We could not interpret the INH and EB susceptibilities because no breakpoints have been established for these drugs.

**Antimicrobial treatment and response.** Of the 102 patients, 42 (58%) with *M. abscessus*, 20 (74%) with *M. massiliense*, and 2 (67%) with *M. bolletii* infections received antimicrobial treatment for 3 to 178 months (mean = 33 months), 1 to 122 months (mean = 36 months), and 4 to 68 months (mean = 36 months), respectively. Forty-two patients with *M. abscessus* lung disease and 20 patients with *M. massiliense* lung disease were analyzed for treatment response (Table 3). Radiographic improvement rates were lower for patients with *M. abscessus* infection than for those with *M. massiliense* infection (29% and 48%, respectively; $P = 0.101$).

Microbiological responses also differed between the two

TABLE 1 Clinical characteristics of patients with *Mycobacterium abscessus* and *M. massiliense* lung disease

| Characteristic[a] | Value for group | | *P* value |
|---|---|---|---|
| | *M. abscessus* ($n = 72$) | *M. massiliense* ($n = 27$) | |
| Age (yr) [range (mean)] | 27–94 (68) | 44–84 (67) | 0.637 |
| No. of males/no. of females | 26/45 | 13/13 | 0.233 |
| Mean body mass index ($kg/m^2$) | 19.5 | 18.8 | 0.699 |
| No. of patients with symptom | | | |
| Cough | 25 | 9 | 0.931 |
| Sputum | 22 | 9 | 0.759 |
| Hemoptysis | 14 | 8 | 0.262 |
| Fever | 8 | 5 | 0.905 |
| Dyspnea | 4 | 5 | 0.057 |
| No. of patients with underlying disease | | | |
| Previous pulmonary tuberculosis | 12 | 7 | 0.297 |
| NTM (MAC) | 15 | 4 | 0.516 |
| Mycosis | 4 | 2 | 0.798 |
| Interstitial pneumonia | 4 | 2 | 0.803 |
| COPD | 3 | 2 | 0.879 |
| Diabetes mellitus | 3 | 3 | 0.196 |
| Steroid use | 3 | 3 | 0.196 |
| Malignancy | 2 | 1 | 0.823 |
| No. of smokers/no. of nonsmokers | 33/11 | 12/8 | 0.223 |
| No. of patients who consumed alcohol/no. of patients who did not consume alcohol | 30/6 | 11/3 | 0.490 |
| No. of patients with/no. of patients without radiological finding of: | | | |
| Cavitation | 28/28 | 14/9 | 0.379 |
| Centrilobular lesion | 38/19 | 14/11 | 0.355 |
| Infiltration | 35/16 | 12/11 | 0.173 |
| Bronchiectasis | 40/15 | 10/13 | 0.014 |
| Pleural effusion | 2/49 | 2/22 | 0.383 |
| No. of patients with positive AFB smear | 50 | 21 | 0.363 |

[a] Abbreviations: NTM, nontuberculous mycobacteria; MAC, *Mycobacterium avium* complex; COPD, chronic obstructive pulmonary disease; AFB, acid-fast bacillus.

groups. The initial sputum conversion rates were lower in patients with *M. abscessus* infection than in those with *M. massiliense* infection (31% and 50%, respectively; $P = 0.115$). The sputum acid-fast bacillus (AFB)-positive relapse rate after the initial conversion to a negative result was higher for patients with *M. abscessus* infection than for those with *M. massiliense* infection (65% and 30%, respectively; $P = 0.077$). Thus, the proportion of patients whose sputum converted and remained culture negative during the follow-up period was lower for patients with *M. abscessus* infection than for those with *M. massiliense* infection.

## DISCUSSION

The *M. abscessus* group comprises ubiquitous environmental organisms frequently associated with nosocomial outbreaks and pseudo-outbreaks (18, 32, 34). The increasing availability of gene sequencing has tremendously influenced the taxonomy of bacteria, particularly mycobacteria, with many new species being described every year (30).

A new species related to *M. abscessus*, *M. massiliense*, was recently described (3). The species *M. massiliense* was proposed in 2004 based upon nonconventional phenotypic characterization and genotypic studies of 2 isolates recovered from the sputum and bronchoalveolar fluid of a patient in France (3). Since *M. massiliense* is closely related to *M. abscessus*, it is possible that *M. massiliense* infections have overlapped with *M. abscessus* infections in previous reports (3). Although several unique phenotypes that differentiate *M. massiliense* from *M. abscessus* have been identified, they are difficult to characterize using conventional clinical techniques because of the inability to discriminate between RGM due to their overlapping phenotypic patterns (9, 26). The molecular, biological, and clinical characteristics of these strains will help us to better understand and treat severe infections due to RGM (39). The proper identification of members of the *M. abscessus* complex has proven beneficial in both therapeutic management and epidemiological studies. *M. massiliense* is very closely related to *M. abscessus* but showed different susceptibilities to CAM, and their pathogenic potentials have been demonstrated by infections of immunocompetent and immunocompromised hosts (15, 18).

The proportions of *M. massiliense* strains among *M. abscessus* species vary according to geographical distribution. The prevalences of *M. massiliense* were 28% of 40 patients at the National Institutes of Health in the United States (39), 21% of 39 clinical isolates in the Netherlands (31), 22% of 50 patients with cystic fibrosis in France (23), 55% of 150 patients in South Korea (19), and 26% of 102 patients in Japan. There is currently no explanation for the large difference in the prevalences of *M. massiliense* between South Korea and Japan, as they are in the same Asian region.

No significant differences were found between the baseline clinical characteristics in the *M. abscessus* and *M. massiliense* groups, except for bronchiectasis in the radiological findings. Bronchiectasis was found significantly more frequently in the *M. abscessus* group than in the *M. massiliense* group (73% and 43%, respectively; $P = 0.014$). This result is almost consistent with the results of a Korean study (19).

Importantly, favorable microbiological response rates with similar combinations of antibiotic therapy were much higher for *M. massiliense* than for *M. abscessus* lung disease. This could be explained by the differences in CAM resistance. This study demonstrated a high level of resistance to CAM in *M. abscessus* isolates but not in *M. massiliense* isolates, indicating that treatment of *M. abscessus* lung disease may be more difficult. In fact, *M. abscessus* lung disease has been regarded as a chronic, incurable infection for most patients, given the current antibiotic options (4). The low MIC and absence of CAM resistance (except for one isolate) suggest that *M. massiliense* lung disease may be treated more effectively with a CAM-based antibiotic regimen. Recent studies showed that some RGM, such as *M. abscessus* and *M. fortuitum*, have an *erm* gene that induces macrolide resistance (4, 12). It is

TABLE 2 *In vitro* susceptibilities of 63 *Mycobacterium abscessus* and 23 *M. massiliense* isolates to different antimicrobials[a]

| Drug | Species | MIC (μg/ml) for categorization of susceptibility of: | | | Reference(s) | % resistant isolates (no. of resistant isolates) | *P* value |
|---|---|---|---|---|---|---|---|
| | | Susceptible | Intermediate | Resistant | | | |
| Clarithromycin | *M. abscessus* | ≤2 | 4 | ≥8 | 15 | 16 (10) | 0.145 |
| | *M. massiliense* | | | | | 4 (1) | |
| Kanamycin[b] | *M. abscessus* | ≤16 | 32 | ≥64 | 14, 20 | 5 (3) | 0.388 |
| | *M. massiliense* | | | | | 0 (0) | |
| Amikacin | *M. abscessus* | ≤16 | 32 | ≥64 | 15 | 6 (4) | 0.509 |
| | *M. massiliense* | | | | | 0 (0) | |
| Imipenem | *M. abscessus* | ≤4 | 8–16 | ≥32 | 15 | 19 (12) | 0.007 |
| | *M. massiliense* | | | | | 48 (11) | |
| Moxifloxacin | *M. abscessus* | ≤1 | 2 | ≥4 | 15 | 92 (58) | 0.488 |
| | *M. massiliense* | | | | | 96 (22) | |
| Ciprofloxacin | *M. abscessus* | ≤1 | 2 | ≥4 | 15 | 95 (60) | 0.883 |
| | *M. massiliense* | | | | | 91 (21) | |
| Isoniazid[c] | *M. abscessus* | NA | NA | NA | | NA | |
| | *M. massiliense* | | | | | NA | |
| Rifampin[b] | *M. abscessus* | ≤1 | 2 | ≥4 | 14, 18 | 97 (61) | 0.534 |
| | *M. massiliense* | | | | | 100 (23) | |
| Ethambutol[c] | *M. abscessus* | NA | NA | NA | | NA | |
| | *M. massiliense* | | | | | NA | |
| Streptomycin[b] | *M. abscessus* | ≤32 | NA | ≥64 | 14, 20 | 67 (42) | 0.617 |
| | *M. massiliense* | | | | | 61 (14) | |

[a] Drug susceptibility results are shown for 63 patients with *M. abscessus* and 23 patients with *M. massiliense* infections. NA, not available.
[b] The CLSI breakpoints (8) for *Staphylococcus* species have been substituted as the breakpoints of these drugs against *M. abscessus* and *M. massiliense* isolates.
[c] The breakpoints for *M. abscessus* and *M. massiliense* isolates have not yet been established.

unknown whether other RGM such as *M. massiliense* have an *erm* gene. Thus, species-level identification is important because antibiotic susceptibilities and therapies differ significantly depending on the RGM species (4, 16). Since the KM and AMK resistance rates were less than 10% for both the *M. abscessus* and *M. massiliense* groups in the present study, KM and AMK could be two key drugs for the treatment of *M. abscessus* and *M. massiliense* lung diseases.

The IPM resistance rate was much lower in the present study than in the Korean study (19) (19% and 44% for *M. abscessus* and 48% and 67% for *M. massiliense*, respectively). The MFLX, CPFX, and RFP resistance rates were >90% for both the *M. abscessus* and *M. massiliense* groups in the present study. These findings are compatible with the fact that the *M. abscessus* complex has been regarded as being fluoroquinolone resistant (7). However, moderate *in vitro* activities of some fluoroquinolones against members of the *M. abscessus* complex have been demonstrated (2, 6, 19, 25). The use of fluoroquinolones as alternative oral agents during combination antibiotic therapy for *M. abscessus* and *M. massiliense* infections should be studied further.

The present study showed poor activities of INH and EB in both the *M. abscessus* and *M. massiliense* groups ($MIC_{90}$ for INH of ≥512 μg/ml; $MIC_{90}$ for EB of ≥32 μg/ml), which are identical to findings reported previously (27). Thus, INH and EB seem to be ineffective against both the *M. abscessus* and *M. massiliense* groups. Another study found that MFLX was active against *M. abscessus* and that a combination of CAM and MFLX was effective against *M. abscessus* strains in *in vitro* models (10). Moreover, some *M. abscessus* isolates are susceptible to the oral drug linezolid (35, 38). However, linezolid was rarely used in our survey to treat *M. abscessus* species lung disease because of the high cost and moderate to severe side effects in Japan. Thus, further studies are required to evaluate active combinations of oral antibiotics and determine their clinical significance.

The present study has several limitations. First, the retrospective study design necessitates the use of medical records for data collection, leading to variations in each factor. Second, the number of sputum specimens collected over time was relatively small. Had samples been collected more frequently, more conversions to negativity and relapses after conversion may have been found. Third, treatment regimens cannot be optimized based solely on retrospective studies with limited follow-up data. Moreover, the use of IPM, cefoxitin, fluoroquinolone, and linezolid is not permitted for the treatment of NTM diseases under the Japanese social health insurance system. Thus, the combination therapy recommended by the ATS/IDSA (16) has not been applied in most cases. Treatment regimens were decided in practice by physicians. Therefore, it is difficult to evaluate the true treatment response in this study.

In conclusion, we found clinically significant differences be-

TABLE 3 Antimicrobial treatments and treatment responses

| Characteristic[a] | Value for group | | P value |
|---|---|---|---|
| | *M. abscessus* ($n = 72$) | *M. massiliense* ($n = 27$) | |
| Treatment | | | |
| No. of patients with/no. of patients without operation | 3/69 | 3/24 | 0.201 |
| No. of patients receiving/no. of patients not receiving chemotherapy | 42/30 | 20/7 | 0.149 |
| No. of patients on chemotherapy regimen of: | | | |
| M, R, E | 8 | 5 | |
| M, C, A | 6 | 5 | |
| M | 8 | 0 | |
| M, F | 6 | 0 | |
| H, R, E | 2 | 2 | |
| Other | 12 | 8 | |
| Duration of treatment (mo) [range (mean)] | 3–178 (33) | 1–122 (36) | 0.723 |
| Result | | | |
| No. of patients with/no. of patients without radiological improvement | 17/41 | 12/13 | 0.101 |
| No. of patients with/no. of patients without sputum smear conversion to negativity | 17/38 | 11/11 | 0.115 |
| No. of patients with relapse/no. of patients without relapse after sputum smear conversion to negativity | 13/7 | 3/7 | 0.077 |
| Duration of positive sputum results (mo) [range (mean)] | 1–120 (25) | 1–62 (18) | 0.776 |

[a] Abbreviations: M, macrolides (clarithromycin, erythromycin, and azithromycin); A, aminoglycosides (streptomycin, amikacin, and kanamycin); F, fluoroquinolones (levofloxacin, moxifloxacin, garenoxacin, and gatifloxacin); C, carbapenems (imipenem and meropenem); H, isoniazid; R, rifampin; E, ethambutol.

tween *M. abscessus* and *M. massiliense* lung infections in Japan. Treatment responses rates with CAM-based antibiotic therapy were higher for *M. massiliense* than in *M. abscessus* lung disease. This difference in treatment responses may be explained by the difference in CAM susceptibilities between the two groups. Prospective clinical trials are needed to clarify these aspects.

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Data analysis of reviewed medical records was performed at the Hokkaido Social Insurance Hospital. The identification of *M. abscessus* species by microplate DDH technology was performed at each hospital or institution. Further differentiation among *M. abscessus* species was performed at the Leprosy Research Center, National Institute of Infectious Diseases, and the Kobe Institute of Health. Antimycobacterial susceptibility testing was performed at the National Hospital Organization Kinki-Chuo Chest Medical Center.

We have no potential conflicts of interest to report. All authors have submitted a International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest.

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INTERNAL MEDICINE

□ CASE REPORT □

# A Patient with Relapsing Polychondritis who Had Been Diagnosed as Intractable Bronchial Asthma

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## Abstract

A 62-year-old woman, diagnosed as bronchial asthma 3 years previously, was admitted due to acute severe dyspnea. Physical examination revealed saddle nose, flare/swelling of the ear auricles, and stridor. Computed tomography demonstrated thickening of tracheal/bronchial walls and stenosis of the lumen that deteriorated on expiration, suggesting tracheobronchomalacia. Auricle biopsy indicated cartilage destruction. Based on these findings, the patient was diagnosed as relapsing polychondritis. As demonstrated in this case, relapsing polychondritis involving airways might be misdiagnosed as bronchial asthma due to stridor and transient corticosteroid-related improvement. Early diagnosis is necessary to prevent irreversible airway stenosis and progression to tracheobronchomalacia.

**Key words:** intractable bronchial asthma, saddle nose, takotsubo cardiomyopathy, tracheobronchomalacia, relapsing polychondritis

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## Introduction

Relapsing polychondritis (RPC), considered to be an autoimmune disease (1), involves general cartilage and tissues containing a high concentration of mucopolysaccharides. This is a rare disorder with an estimated incidence of 3.5/1,000,000 persons/year (2), and the treatment has not been established. When tracheal/bronchial cartilages are affected, respiratory symptoms such as dyspnea and stridor may appear (3), which can be misleading, prompting an improper diagnosis as bronchial asthma (4). It could take long until correct diagnosis was made (5). Here, we report a patient with RPC who had been diagnosed as intractable bronchial asthma for a long period of time. Since RPC could be fatal, it is important to differentiate this disorder from bronchial asthma.

## Case Report

A 62-year-old woman was admitted to our hospital because of severe acute dyspnea, one month after she was referred to our hospital because of intractable bronchial asthma. Neither medical nor family history was contributory. She had a 22-year history of smoking (10 cigarettes/day). At the age of 59, she was admitted to another hospital with dyspnea, with no demonstration of saddle nose or flare/swelling of the ear auricles at that time. Based on elevated ST in an extensive area on electrocardiography and increases in serum and plasma biomarkers of cardiac injury, a tentative diagnosis of myocarditis was made in addition to bronchial asthma. Subsequently, asthma treatment with oral prednisolone was initiated. When prednisolone was decreased in dose or discontinued, her asthma condition was exacerbated until ventilator assistance was required. During this clinical

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Table 1. **Laboratory Findings on Admission**

| Hematology | | | Biochemistry | | | Serology | | |
|---|---|---|---|---|---|---|---|---|
| WBC | 23,000 | /μL | TP | 7.1 | g/dL | CRP | 0.91 | mg/dL |
| Neut | 88 | % | Alb | 4.2 | g/dL | ANA | <40 | |
| Lym | 11 | % | LDH | 303 | IU/L | RF | <10 | IU/mL |
| Mon | 1 | % | AST | 47 | IU/L | PR-3-ANCA | <3.1 | EU |
| Bas | 0 | % | ALT | 33 | IU/L | MPO-ANCA | <3.1 | EU |
| Eos | 0 | % | BUN | 23.5 | mg/dL | IgG | 871 | mg/dL |
| RBC | $474 \times 10^4$ | /μL | Cre | 0.56 | mg/dL | IgA | 167 | mg/dL |
| Hb | 13.6 | g/dL | Na | 141 | mEq/L | IgM | 112 | mg/dL |
| Ht | 43.5 | % | K | 4.0 | mEq/L | IgE | <35 | mg/dL |
| Plt | $36.3 \times 10^4$ | /μL | Cl | 103 | mEq/L | Troponin T | 0.038 | ng/mL |
| | | | CPK | 95 | IU/L | | | |

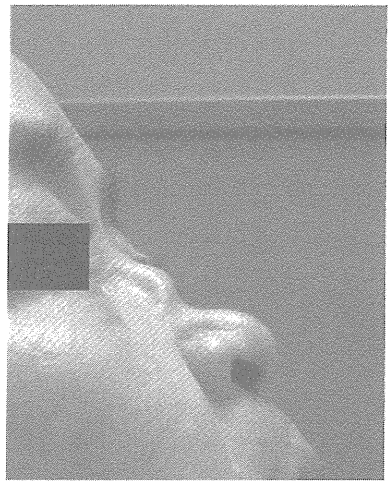

Figure 1. **Appearance of the affected part showing nasal chondritis (saddle nose).**

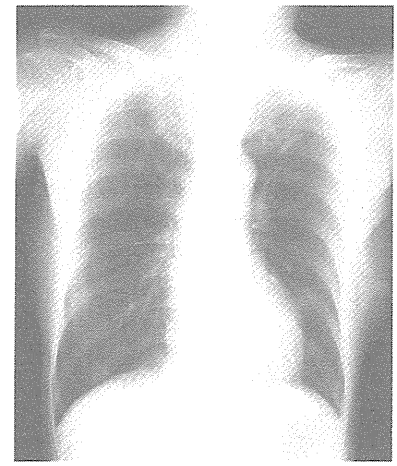

Figure 2. **Chest radiograph on admission showing narrowing of bronchial lucency. There is no abnormal finding in the lung fields.**

course several times she noticed flare/swelling of the ear auricles although she or her doctor did not realize that the symptom could be related with the dose of the corticosteroid. As for her saddle nose, she realized it when she was about 60 years old.

On admission, her height, weight and body temperature were 149.5 cm, 35.3 kg and 37.1℃, respectively. Her blood pressure was 154/92 mmHg with $SpO_2$ 80% under 12 L/min of oxygen flow by reservoir mask. Slight flare/swelling of the bilateral ear auricles as well as saddle nose (Fig. 1) was observed without abnormal findings in the palpebral or bulbar conjunctivae. By auscultation, stridor was audible on the bilateral sides with no abnormal heart sounds. Edema was not detected in either lower limb. Laboratory data on admission is listed in Table 1. The white blood cell (WBC) count was markedly increased to 23,000/μL while the C-reactive protein (CRP) level was 0.91 mg/dL. The patient's serum was negative for antinuclear antibody and antineutrophil cytoplasmic antibody (ANCA). Although the chest X-ray (Fig. 2) demonstrated no abnormalities in the bilateral lung fields, stenosis of the left and right principal bronchi was noted.

Due to respiratory failure, she was intubated and connected to a ventilator on the day of admission. Treatment with methylprednisolone at a dose of 500 mg/day for 3 days was initiated. After confirming improvement in respiratory condition, the dose of corticosteroid was gradually decreased. Extubation was conducted 7 days after admission. The pattern of a flow-volume curve (Fig. 3) recorded at an outpatient clinic before this admission indicated reversible intrathoracic stenosis, which became flat in the descending limb after a sharp peak associated with the collapse of central airways (6, 7), suggesting tracheobronchomalacia had been present. In addition, the swelling of the auricles and saddle nose lead to a tentative diagnosis of RPC.

Thoracic computed tomography (CT) (Fig. 4) performed after extubation revealed thickening of airway walls from trachea to bilateral principal bronchi with the luminal diameter reduced to approximately 5 mm, consistent with RPC. CT on expiration exhibited applanation of the lumen, more marked stenosis, and tracheobronchomalacia in comparison with findings on inhalation.

Electrocardiography after admission (Fig. 5) revealed negative T waves and QT prolongation with I-, aVL-, II-, III-, aVF-, and $V_2$- to $V_6$-lead with a slight increase in the

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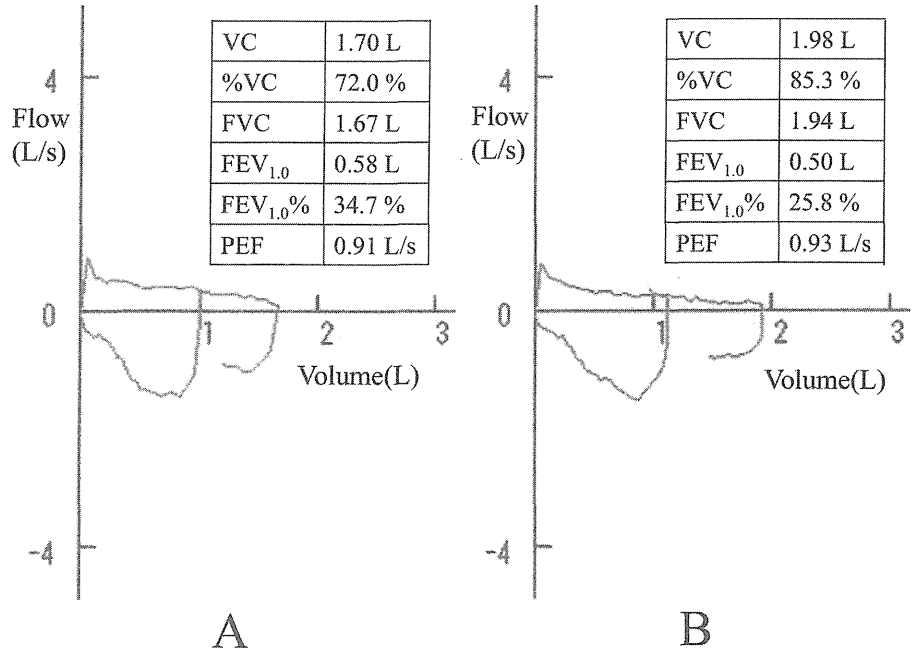


Figure 3. **Flow-volume curve before admission (A) and one month after admission (B) showing a constrictive pattern in the upper airway.**

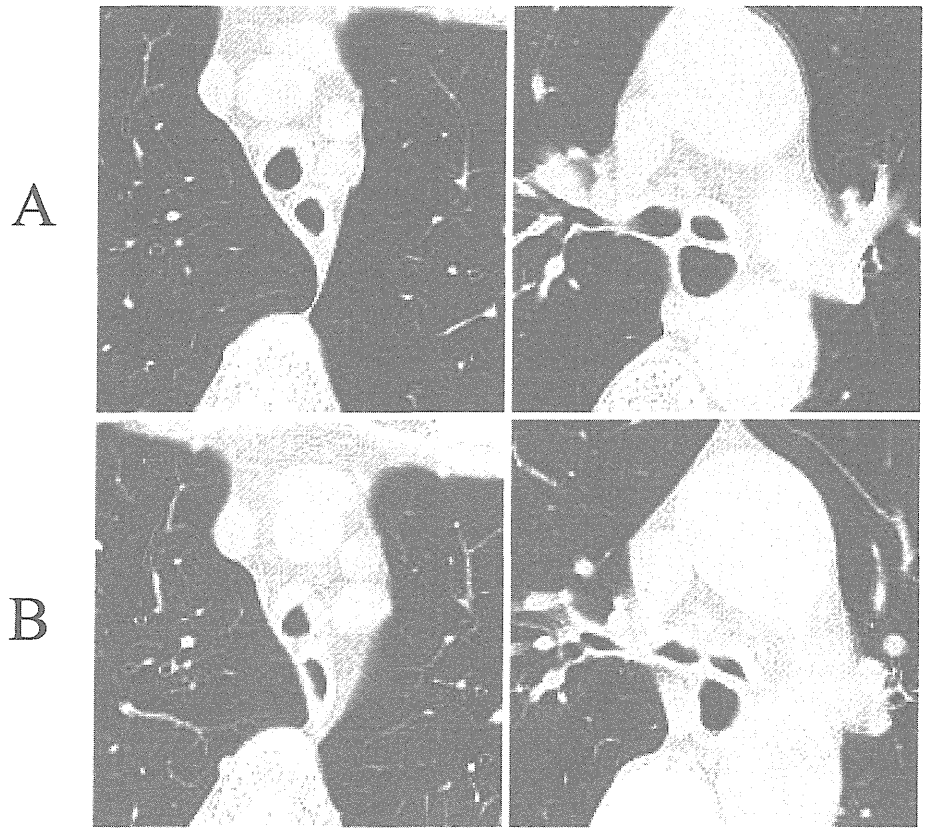


Figure 4. **Transverse (A) end-inspiratory and (B) expiratory CT scans. Chest CT scans showing thickening and edema of the tracheal and bronchial wall. Both main bronchi show severe stenosis in an expiratory CT scan.**

myocardial troponin-T level but without any increase in the other serum biomarkers of cardiac injury. In addition, echocardiography indicated akinesis of the left ventricular anterior wall and ventricular septum (intermediate to cardiac apex regions) and a decrease in the ejection fraction. Both the electrocardiographic and echocardiographic findings gradually and spontaneously subsided; echocardiography confirmed recovery of cardiac systolic function within a

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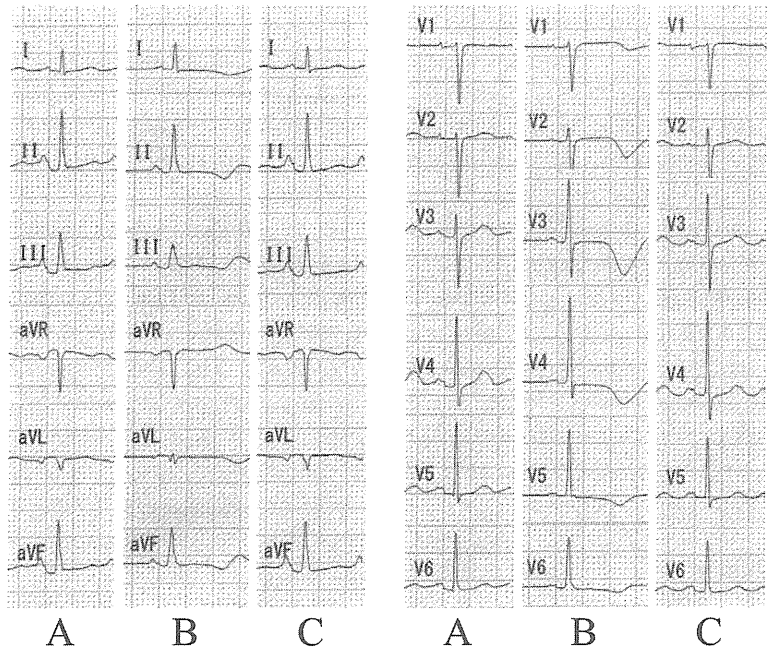


Figure 5. **Electrocardiography before admission (A), two days after admission (B), and two months after admission (C). (B): Negative T waves and QT interval prolongation in leads I, aVL, II, III, aVF, $V_2$-$V_6$. (C): Negative T waves improved.**

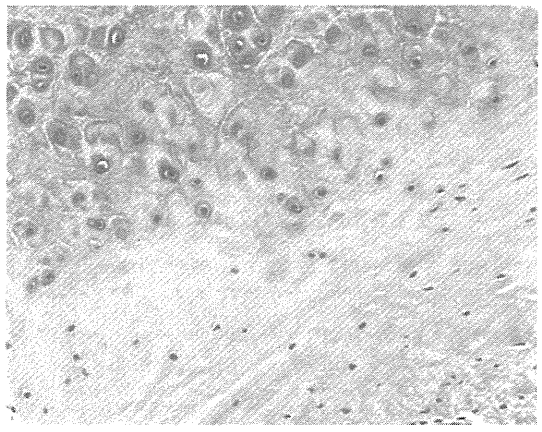

Figure 6. **Biopsy sample of the right auricle shows cartilage destruction and degeneration (Hematoxylin and eosin stain, ×40).**

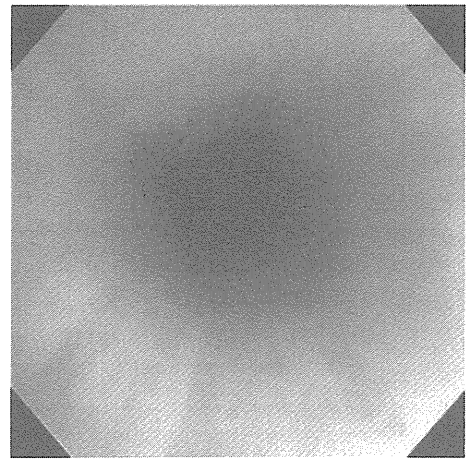

Figure 7. **Bronchoscopic finding of the trachea, showing the disappearance of cartilaginous rings.**

week, and negative T in electrocardiography persisted for about a month and was gradually normalized. Contrast-enhanced coronary CT revealed the absence of arteriosclerosis and stenosis in 3 vessels. Therefore, the cardiologists diagnosed she had takotsubo cardiomyopathy.

Since biopsy of the auricle (Fig. 6) demonstrated destruction of the cartilage and rupture of elastic fibers, a definitive diagnosis of RPC was made on 28th days after admission, based on the clinical and pathological findings. On the same day, the dose of oral prednisolone was increased to 30 mg combined with 100 mg of cyclosporine. The dose of prednisolone was decreased by 5 mg every 2 weeks until the maintenance dose was established as 15 mg.

The anti-type-II-collagen antibody was revealed to be negative at the concentration of 8.9 EU/mL (positive: >25 EU/mL) on the 62nd day after admission. Bronchoscopy (Fig. 7), performed on the 105th day after admission, did not indicate flare or swelling on the tracheal luminal surface although the disappearance of the tracheal cartilage rings was noted.

## Discussion

RPC causes repetitive inflammation in the cartilage tissues of the whole body and in ocular/cardiovascular systems, which contain a high concentration of mucopolysaccharides, and it is likely to respond to steroids and immunosuppressive agents. Anti-type-II-collagen antibody was de-

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tected in approximately 30 to 50% of patients with RPC (8), suggesting an autoimmune disease.

McAdam et al. (9) established the diagnostic criteria in which patients with RPC were defined as having 3 or more of the following 6 items plus histological evidence of cartilage inflammation: 1) bilateral auricular chondritis, 2) non-erosive sero-negative inflammatory polyarthritis, 3) nasal chondritis, 4) ocular inflammation, 5) respiratory tract chondritis and 6) audiovestibular damage. In the present patient, auricular chondritis, nasal chondritis, and respiratory tract chondritis were noted in addition to cartilage destruction identified with the auricular cartilage biopsy, leading to a diagnosis of RPC. Although there was no increase in the anti-type-II-collagen antibody level, this could be because steroids had been frequently administered under a diagnosis of bronchial asthma.

Trentham and Le reported that the mean interval from the first visit to the diagnosis of RPC was 2.9 years (5). The present patient had been treated for bronchial asthma for about 3 years after her first visit at a local clinic with dyspnea at the age of 59 years. Since then, corticosteroid was administered for the treatment of suspected asthma attack and decreased and discontinued after symptoms subsided. The steroid dose-reduction or discontinuation had deteriorated not only her respiratory conditions but auricular swelling and saddle nose, which emerged during the course involving remission and exacerbation of her "asthma". As Segel et al. indicated (4), steroid administration to RPC patients might transiently improve a respiratory symptom that was related to RPC.

In the present case, the diagnosis may have been delayed for the following reasons: 1) auricular chondritis and saddle nose emerged after the onset of airway symptoms, 2) symptoms (auricular swelling/saddle nose) other than airway symptoms were underestimated, and 3) the patient had been diagnosed as bronchial asthma due to steroid therapy-related improvement. Previous case reports of relapsing polychondritis misdiagnosed as bronchial asthma (10-12) suggested similar reasons for the misdiagnosis. The present case exhibited saddle nose and flare/swelling of ear auricles, which was not connected with her airway symptoms by her doctor. Several studies reported that the incidence of airway symptoms in RPC patients ranged from 20 to 50%, and that airway symptoms were initially present in 10 to 15% (3, 9). Other common sites involved in RPC included the auricles, joints, and nasal cartilage although many patients might not show all symptoms at onset.

Clinical features of relapsing polychondritis, different from typical bronchial asthma, include the following: 1) inhaled bronchodilator and corticosteroid are ineffective and oral corticosteroid is required, 2) lung function test reveals upper airway obstruction, and 3) CT scan demonstrates stenosis and edema of large airways. Based on the present case report, we strongly suggest that relapsing polychondritis should be differentiated from intractable bronchial asthma by physical examination, lung function test, and imaging technique.

Concerning the prognosis of RPC patients, the 5- and 10-year survival rates were 74 and 55%, respectively (13). Airway involvement is considered to be a major prognostic factor (14). Inflammation and destruction of tracheobronchial cartilages caused airway edema, airway collapse (tracheobronchomalacia), and cicatricial stenosis of the airways. In patients without advanced cartilage destruction, treatment might normalize respiratory function (4). In the present case, repeated airway chondritis led to irreversible tracheobronchomalacia. The disappearance of the tracheal cartilage ring by bronchoscopy suggested advanced cartilage destruction, consistent with a flow-volume curve indicating the pattern of intrathoracic airway stenosis. Since common causes of death in RPC patients included respiratory failure and airway infection, insertion of a tracheobronchial stent must be considered (3, 14).

In the present case, the results of coronary CT, electrocardiography, echocardiography, and serum biomarkers of cardiac injury suggested the concomitant development of takotsubo cardiomyopathy. According to studies reported (15, 16), aortic regurgitation, mitral valve regurgitation, or pericarditis was detected in approximately 10% of patients with RPC while no study has reported the concomitant development of takotsubo cardiomyopathy. Physical/mental stress may be involved in the pathogenesis. In the present patient, severe dyspnea may have induced takotsubo cardiomyopathy. Furthermore, β2 stimulants administered before and after admission may also have been an etiological factor (17). This is the first report of takotsubo cardiomyopathy in the patient with RPC. Takotsubo cardiomyopathy should be considered when differentiating heart diseases in patients with RPC.

As described above, early diagnosis/drug therapy for RPC may prevent or delay progression to tracheobronchomalacia. On the other hand, RPC is easily misdiagnosed as bronchial asthma because of its response to corticosteroid. It is important to differentiate RPC from bronchial asthma based on physical examination, detailed imaging, and respiratory function test findings.

**The authors state that they have no Conflict of Interest (COI).**

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RESEARCH PAPER

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# Characterization and long-term persistence of immune response following two doses of an AS03$_A$-adjuvanted H1N1 influenza vaccine in healthy Japanese adults

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**Keywords:** adjuvant, AS03, H1N1, long-term, pandemic, persistence

**Abbreviations:** AESI, adverse event of special interest; ATP, according to protocol; CBER, Center for Biologics Evaluation & Research; CHMP, Committee for Medicinal Products for Human Use; CI, confidence interval; GMFR, geometric mean fold rise; GMT, geometric mean titre; HA, hemagglutinin; HI, hemagglutination inhibition; pIMD, potential immune-mediated disease; RBC, red blood cell; SAE, serious adverse event; SCR, seroconversion rate; SPR, seroprotection rate; TVC, total vaccinated cohort; VRR, vaccine response rate; WHO, World Health Organization



**Background:** Long-term persistence of immune response and safety of two doses of an A/California/07/2009 H1N1 pandemic influenza vaccine adjuvanted with AS03 (an α-tocopherol oil-in-water emulsion-based Adjuvant System) administered 21 d apart was evaluated in Japanese adults [NCT00989612].

**Methods:** One-hundred healthy subjects aged 20−64 y (stratified [1:1] into two age strata 20−40 y and 41−64 y) received 21 d apart, two doses of AS03-adjuvanted 3.75 μg haemagglutinin (HA) H1N1 2009 vaccine. Immunogenicity data by haemagglutination inhibition (HI) assay six months after the first vaccine dose (Day 182) and microneutralization assay following each of the two vaccine doses (Days 21 and 42) and at Day 182 are reported here.

**Results:** Persistence of strong HI immune response was observed at Day 182 that met the US and European regulatory thresholds for pandemic influenza vaccines (seroprotection rate: 95%; seroconversion rate: 93%; geometric mean fold-rise: 20). The neutralizing antibody response against the A/Netherlands/602/2009 strain (antigenically similar to vaccine-strain) persisted for at least up to Day 182 (vaccine response rate: 76%; geometric mean titer: 114.4) and paralleled the HI immune response at all time points. No marked difference was observed in HI antibody persistence and neutralising antibody response between the two age strata. The vaccine had a clinically-acceptable safety profile.

**Conclusion:** Two priming doses of H1N1 2009 pandemic influenza vaccine induced an immune response persisting for at least six months after the first vaccine dose. This could be beneficial in evaluating the importance and effect of vaccination with this AS03-adjuvanted pandemic influenza vaccine.

The H1N1 2009 epidemic in Japan started off as isolated outbreaks in small clusters between May and July 2009. The number of cases rose steadily from mid-August 2009 and peaked in November 2009.[1] An estimated 20 million cases (as of February 05, 2010)[2] and 202 deaths related to H1N1 2009 (as of the end of H1N1 2009 pandemic)[3] were recorded in Japan. The majority of infections were recorded in school children and young adults, with the hospitalization rates being highest in children aged 5−9 y.[4,5] Although, adults appeared to be less susceptible to the H1N1 clinical disease, H1N1 2009 related fatality peaked in adults aged 40−49 y in addition to children aged <10 y (as of August 10, 2010).[3,5]

Clinical effectiveness of neuraminidase inhibitors, zanamivir and oseltamivir has been reported.[6-8] These drugs are able to mitigate morbidity and mortality caused by an influenza pandemic. However, mass immunization is an effective intervention against pandemic influenza. Identifying the necessity to make available a large number of vaccine doses worldwide and the potential for cross-reactive immunity, the World Health Organization (WHO) supported the development of adjuvanted pandemic influenza vaccines in parallel with non-adjuvanted vaccines.[9,10] A H1N1 2009 pandemic vaccine utilizing 3.75 μg A/California/07/2009 (H1N1)v-like haemagglutinin (HA) antigen

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adjuvanted with AS03 Adjuvant System (an α-tocopherol oil-in-water emulsion-based Adjuvant System) was developed based on GlaxoSmithKline Biologicals' previous experience with the AS03-adjuvanted prepandemic H5N1 vaccine.[11-13] This H1N1 2009 vaccine has demonstrated strong immunogenicity (fulfilling the US and European regulatory guidance criteria for pandemic influenza vaccines) and a clinically acceptable safety profile in different populations.[14,15]

In an open-label, single group study (NCT00989612) in Japanese adults aged 20−64 y, two doses of this H1N1 2009 pandemic influenza vaccine administered 21 d apart was found to be well-tolerated and highly immunogenic (all subjects seroconverted/ were seroprotected 21 d after the second vaccine dose), achieving the US and European regulatory guidance criteria for pandemic influenza vaccines in adults.[16] This manuscript presents follow-up data from the same population (stratified into 20−40 y and 41−64 y) on the persistence of humoral immune response in terms of HI antibody titers against the vaccine-homologous strain six months after primary vaccination with two doses of this AS03-adjuvanted H1N1 2009 vaccine (at Day 182), as well as neutralising antibody titers against the vaccine-homologous strain following each of the two doses and six months later (Days 21 and 42; Day 182). Data on the safety profile of the vaccine up to Day 182 is also presented here.

## Results

**Study population.** The six month follow-up phase of this study was completed on April 19, 2010 (up to Day 182).

All 100 subjects who were part of the primary assessment and had received two doses of the H1N1 2009 vaccine completed the study up to Day 182 and were included in the according to protocol (ATP) cohort for persistence. The median age of subjects at the time of enrolment was 40.5 y (range: 21 to 59 y); 64% of subjects were female and all were of Japanese heritage.

**Immunogenicity.** *HI antibody immune response.* The haemagglutination inhibition (HI) immune response against the H1N1 2009 strain after six months after the first vaccine dose (Day 182) is presented in **Table 1**. The seroprotection rate (SPR) was 95%, seroconversion rate (SCR) was 93%, with a corresponding geometric mean titer (GMT) of 175.1 and geometric mean fold rise (GMFR) of 20. These values still met and exceeded the Center for Biologics Evaluation and Research (CBER) and Committee for Medicinal Products for Human Use (CHMP) guidance criteria for pandemic influenza vaccines. There was no appreciable difference in HI antibody persistence between the two age strata (overlapping 95% confidence intervals [CIs]). It is to be noted that the samples from Day 0, Day 21 and Day 42 were tested at the same time, while the Day 182 samples were tested later without an assessment of variability from earlier time points. Due to potential assay variability, a comparative interpretation of the HI response at Day 182 with earlier time points should be done with caution.

*Neutralizing antibody response.* Prior to receiving vaccination, 51% of subjects were seropositive for neutralising antibodies against the A/Netherlands/602/09 strain and the corresponding geometric mean titers (GMT) was low (8.5). Twenty-one days after the first vaccine dose (Day 21), the GMT rose to 136.9, with a vaccine response rate (VRR) of 74%. Following the second vaccine dose, these values increased to 305.8 and 96%, respectively. Six months after the first vaccine dose, persistence of neutralizing antibody response against the A/Netherlands/602/09 strain was evident (overall, GMT of 114.4 and VRR of 76%). No difference in neutralizing immune response was observed between the two age strata at any of the time points (overlapping 95% CIs) (**Table 2**). The proportion of subjects with antibody titers equal or above different threshold of positivity have been presented. The reverse cumulative curves for neutralizing antibodies 21 d after each of the two vaccine doses and at Day 182 (**Fig. 1**) and the neutralizing antibody titers for all time points (**Table 3**) showed a large proportion of subjects with titers equal or above the thresholds of 1:8, 1:16, 1:32 and 1:64, for six months after the first vaccine dose.

**Safety and Reactogenicity.** Overall, at least one unsolicited symptom was reported in 46 subjects (20−40 y: 21 subjects; 41−64 y: 25 subjects) during the 84 d post-vaccination follow-up period, of which 18 were considered to be vaccine-related.

Diarrhea, nasopharyngitis and headache (five subjects each) were the most frequently reported unsolicited symptoms. Of these, four cases of diarrhea and one case of headache were considered to be causally related to vaccination. One subject reported an unsolicited symptom of Grade 3 intensity (urticaria) which required medical attention and was unrelated to vaccination.

**Table 1.** Immune response in terms of haemagglutination inhibition antibodies against the vaccine homologous A/California/07/2009 strain at Day 182 (ATP cohort for persistence)

| Age strata | Time point | Seroprotection rates | | Seroconversion rates | | Geometric Mean titers | | Geometric Mean Fold Rise | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | % (95% CI) | N | % (95% CI) | N | Value (95% CI) | N | Value (95% CI) |
| Overall | Pre-vaccination | 100 | 6 (1.9–13.6) | – | – | 100 | 8.8 (7.3–10.5) | – | – |
| | Day 182 | 100 | 95 (88.7−98.4) | 100 | 93.0 (86.1−97.1) | 100 | 175.1 (144.2−212.7) | 100 | 20.0 (16.8−23.8) |
| 20−40 | Pre-vaccination | 50 | 6 (1.3–16.5) | – | – | 50 | 8.9 (7.1–11.1) | – | – |
| years | Day 182 | 50 | 98.0 (89.4−99.9) | 50 | 96.0 (86.3−99.5) | 50 | 182.6 (141.1−236.4) | 50 | 20.6 (16.0−26.5) |
| 41−64 | Pre-vaccination | 50 | 6 (1.3–16.5) | – | – | 50 | 8.6 (6.8–10.9) | – | – |
| years | Day 182 | 50 | 92.0 (80.8−97.8) | 50 | 90.0 (78.2−96.7) | 50 | 167.9 (124.5−226.5) | 50 | 19.4 (15.1−25.1) |

N, number of subjects with available results; CI, confidence interval; ATP: according to protocol

**Table 2.** Immune response in terms of neutralising antibodies against the A/Netherlands/602/09 strain [antigenically homologous to the vaccine strain] (ATP cohort for immunogenicity)

| Age strata | Time point | Vaccine Response Rates | | Geometric Mean titers | |
|---|---|---|---|---|---|
| | | N | % (95% CI) | N | Value (95% CI) |
| Overall | Pre-vaccination | 100 | – | 100 | 8.5 (7.1–10.2) |
| | Day 21 | 100 | 74.0 (64.3–82.3) | 100 | 136.9 (97.0–193.3) |
| | Day 42 | 100 | 96.0 (90.1–98.9) | 100 | 305.8 (242.5–385.6) |
| | Day 182 | 100 | 76.0 (66.4–84.0) | 100 | 114.4 (89.3–146.5) |
| 20–40 y | Pre-vaccination | 50 | – | 50 | 8.7 (6.6–11.4) |
| | Day 21 | 50 | 78.0 (64.0–88.5) | 50 | 146.6 (90.1–238.6) |
| | Day 42 | 50 | 96.0 (86.3–99.5) | 50 | 336.6 (247.8–457.1) |
| | Day 182 | 50 | 84.0 (70.9–92.8) | 50 | 133.5 (97.3–183.1) |
| 41–64 y | Pre-vaccination | 50 | – | 50 | 8.4 (6.6–10.7) |
| | Day 21 | 50 | 70.0 (55.4–82.1) | 50 | 127.8 (77.1–211.9) |
| | Day 42 | 50 | 96.0 (86.3–99.5) | 50 | 277.7 (194.3–397.0) |
| | Day 182 | 50 | 68.0 (53.3–80.5) | 50 | 98.0 (66.6–144.3) |

N, number of subjects with available results; CI, confidence interval; ATP: according to protocol



No potential immune mediated diseases (pIMD) or adverse events of special interest (AESIs) were recorded during the study period. Three serious adverse events (SAEs) were reported in two subjects during the entire study period. One male subject aged 44 y presented with ureteric calculi, approximately four months after the second vaccine dose which was resolved within three days and a female subject aged 36 y had a viral infection and pharyngeal ulceration approximately four and half months after the second vaccine dose which resolved in seven and 11 d, respectively; none of the SAEs were considered by the investigators to be vaccine-related. No fatalities were reported.

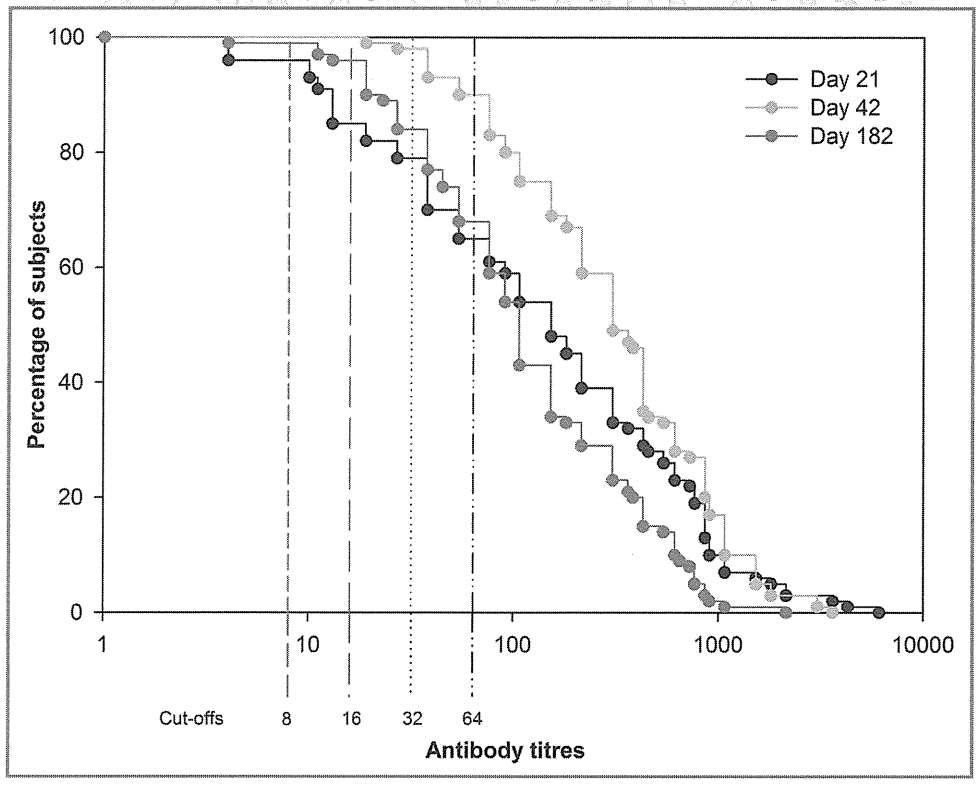


**Figure 1.** Reverse cumulative curves for neutralising antibody responses 21 d after each of the two vaccine doses (Days 21 and 42) and six months after the first vaccine dose (Day 182) (ATP cohort for immunogenicity). ATP, according to protocol.

**Table 3.** Percentage of subjects aged 20−64 y with neutralising antibodies titers ≥ 1:8, ≥ 1:16, ≥ 1:32 and ≥ 1:64 against the A/Netherlands/602/09 strain [antigenically homologous to the vaccine strain] at all time points (ATP cohort for immunogenicity)

| Time point | | ≥ 1:8 | ≥ 1:16 | ≥ 1:32 | ≥ 1:64 |
|---|---|---|---|---|---|
| | N | % (95% CI) | | | |
| Pre-vaccination | 100 | 51.0 (40.8−61.1) | 20.0 (12.7−29.2) | 12.0 (6.4−20.0) | 6.0 (2.2−12.6) |
| Day 21 | 100 | 96.0 (90.1−98.9) | 85.0 (76.5−91.4) | 79.0 (69.7−86.5) | 65.0 (54.8−74.3) |
| Day 42 | 100 | 100 (96.4−100) | 100 (96.4−100) | 98.0 (93.0−99.8) | 90.0 (82.4−95.1) |
| Day 182 | 100 | 99.0 (94.6−100) | 96.0 (90.1−98.9) | 84.0 (75.3−90.6) | 68.0 (57.9−77.0) |

N, number of subjects with available results; CI, confidence interval; ATP: according to protocol

## Discussion

This is the first study assessing the persistence of immunological response against the A/California/07/2009 strain in Asian adults, six months after vaccination with the AS03-adjuvanted H1N1 2009 pandemic influenza vaccine.

Persistence of HI immune response against the A/California/07/2009 strain was observed for six months after the first vaccine dose (SPR: 95%; SCR: 93%); the CHMP and CBER guidance criteria for pandemic influenza vaccines were met and exceeded at Day 182. The observations from this study is in agreement with available data from studies in other adult populations that reported that the immune response induced by two doses of the 3.75 μg HA AS03-adjuvanted H1N1 2009 vaccine persists for six months after vaccination.[17,18] These observations are important for assessment of disease management strategies in the context of the WHO recommendations for the post-pandemic period which stresses on continuous vigilance, surveillance and disease management of circulating influenza strains.[19]

A previous head-to-head comparison study in UK between a similar AS03-adjuvanted H1N1 2009 vaccine and a non-adjuvanted whole-virion H1N1 2009 vaccine in adults (including those aged ≥65 y) reported that a single dose of the adjuvanted vaccine was sufficient to induce immune responses meeting the US and European regulatory criteria while two doses of the whole-virion vaccine were required. In addition, a large proportion of the participants were found to have protective levels of antibodies against the vaccine strain even six months after vaccination with two doses (although age-related decline was evident), indicating that pandemic influenza vaccines can potentially confer immunity against successive waves of the same virus.[20] This is in agreement with previous studies using the AS03-adjuvanted H1N1 2009 vaccine that have demonstrated substantial benefits in terms of induction of rapid, strong and long-lasting immune responses.

Theoretically, neutralization assays can capture a broad range of anti-influenza antibody activities and are able to interrupt several steps of the infectious life cycle of the virus. In contrast, HI assays are largely restricted to measuring the receptor-binding blocking activity of antibodies.[21] However, many different neutralising assays with different variations in protocols and expression of endpoints have been described[22] and it is likely that the biological activity of antibodies measured by these different assays is variable. The assay used in this study is characterized by a short incubation time between the virus and the tested serum. Although the extent of method-specific variation in neutralisation titer and its clinical significance is unknown, assay validation demonstrated good assay specificity (97% with 95% CI: 91.48–99.38%) and a good correlation with the HI assay (r = 0.64) (unpublished GSK data). The study demonstrated strong neutralising antibody response as evident from the high VRRs following each of the two vaccine doses and persistence of high VRRs for six months after the first vaccine dose. Neutralizing antibody responses observed in the study population confirmed the robust immunogenicity of the vaccine and persistence of anti-A/California/07/2009-like antibodies. Overall, the vaccine had a clinically acceptable safety profile in the study population.

The epidemiology and characteristics of the H1N1 2009 virus in Japan has been similar to that observed in other countries in the northern hemisphere and the trends in incidence mirrored those observed worldwide. Although there were fewer laboratory-confirmed H1N1 2009 cases after February−March 2010, and the last reported death due to H1N1 2009 in the pandemic period was in July 2010,[3] the virus continued to circulate in the post-pandemic phase, making it essential to investigate whether pandemic vaccination programs led to long-term persistence of immune response against the H1N1 2009 virus.

The present study advances information on the safety, immunogenicity and long-term immunological persistence of this AS03-adjuvanted H1N1 2009 pandemic influenza vaccine in an Asian population. Contrary to the observations made by Nicholson et al. using a similar vaccine in adults including the elderly,[20] no age-related declined immunological response was observed at Day 42 in the present study, and the data indicated that the immunological response was persistent up to Month 6 in both age strata (20–40 and 41–64 y). The safety profile of the vaccine in Asian adults was comparable to previous reports and no-Asia-specific safety concerns were reported. Thus, the data obtained from this study provides a holistic worldwide dimension to the safety and immunogenicity profile of the study vaccine observed across different populations, now including this Japanese population.

In conclusion, this study presents novel data on persistence of immunological response against the H1N1 2009 virus in adults and on neutralising antibody response induced by this H1N1 2009 pandemic influenza vaccine. It was established that following two doses of a 3.75μg HA AS03-adjuvanted H1N1 2009 pandemic influenza vaccine in adults aged 20−64 y, immune response against the vaccine homologous A/California/07/2009 strain persisted for at least six months after the first vaccine dose. The immunological response met the US and

European guidance criteria for pandemic influenza vaccines up to six months after the first vaccine dose. These results will be beneficial in evaluating the importance and effect of vaccination with this AS03-adjuvanted pandemic influenza vaccine.

## Materials and Methods

**Study design and subjects.** In the primary phase, 100 healthy Japanese adults aged 20−64 y without history of clinically-confirmed influenza infection or previous vaccination with a novel H1N1 2009 vaccine or any seasonal influenza vaccination within 14 d prior to study start were enrolled to receive 21 d apart, two doses of a monovalent AS03-adjuvanted 3.75μg HA A/California/07/2009 pandemic influenza vaccine. The subjects were further stratified by age (stratification ratio: 1:1) into 20−40 y and 41−64 y age strata.

Written informed consent was obtained from all subjects prior to conducting any study-related procedures. The study was conducted in accordance with the Good Clinical Practice guidelines, the Declaration of Helsinki and local regulations. All study-related documents were approved by Institutional Review Boards.

**Study vaccine.** The H1N1 2009 pandemic influenza vaccine was a monovalent, inactivated, split-virion antigen adjuvanted with $AS03_A$ (*Arepanrix*™, a trademark of the GlaxoSmithKline group of companies). The H1N1 viral seed for the vaccine was prepared from the reassortant virus NYMC X-179A (New York Medical College, New York) generated from the A/California/07/2009 strain, as recommended by the WHO.[23] $AS03_A$ is an oil-in-water emulsion-based Adjuvant System containing α-tocopherol (11.86mg tocopherol).

Both vaccine doses were administered intramuscularly at alternate deltoid muscles sides.

**Immunogenicity assessments.** Blood samples were collected before vaccination, 21 d after each of the two vaccine doses and six months after the first vaccine dose.

Serum samples collected six months after the first vaccine dose (Day 182) were tested at GSK Biologicals Central Laboratory using a validated in-house HI assay [cut-off: ≥ 1:10] that used chicken erythrocytes as previously described.[24]

The viral microneutralisation assay was performed on serum samples collected at all time points at Viroclinics Biosciences (Rotterdam, The Netherlands).[25] The sera were subjected to heat treatment at 56°C for 30 min and then tested in triplicate. The assay used a constant amount of A/Netherlands/602/2009 pandemic H1N1 Influenza virus (A A/California/07/2009-like virus) mixed with serial 2-fold dilutions of serum samples. The mixture of virus and serum was added to Madin-Darby Canine Kidney (MDCK) cell cultures ($10^4$ cells per well) and incubated for one hour at 37°C, following which the virus-antibody mixture was removed from the wells by aspiration, cells were fed with fresh culture medium and further incubated for 6 d at 37°C. After the incubation period, the well supernatants were transferred into 96 well plates and a suspension of turkey red blood cells (RBCs) was added to it; following an incubation for 60 min at 4°C, the culture supernatants (virus replication) were visualized by haemagglutination of RBCs. The 50% neutralisation titer of a serum was calculated by the Reed and Muench method.[26] The assay cut-off was 1:8.

The evaluation of outcome measures of immune response was based on the immunogenicity criteria for pandemic influenza vaccines in adults as required by the CHMP: point estimates for HI antibody SCR: > 40%, SPR: > 70% and GMFR: > 2.5 and CBER: lower bound of 95% CI for HI antibody for SCR: ≥ 40% and SPR: ≥ 70%.[27,28] SPR was defined as percentage of subjects with a post-vaccination titer ≥ 1:40, SCR as percentage of subjects with pre-vaccination titer < 1:10 and post-vaccination titer ≥ 1:40 or pre-vaccination titer > 1:10 and at least 4-fold increase in post-vaccination titer and GMFR as post-vaccination fold increase in GMTs for HI antibodies. For neutralising antibodies, immunological assessments were based on the VRRs defined as percentage of subjects with either a pre-vaccination titer < 1:8 and a post-vaccination titer ≥ 1:32, or a pre-vaccination titer ≥ 1:8 and at least a 4-fold increase in post-vaccination titer.

**Safety and reactogenicity assessments.** Unsolicited adverse events were recorded up to 84 d following the first vaccine dose; pIMD (which are a subset of adverse events that include both autoimmune diseases and other inflammatory and/or neurologic disorders which may or may not have an autoimmune etiology), AESI and SAEs occurring during the entire study period were recorded.

**Statistical analyses.** The analyses of immunogenicity in terms of HI antibodies at Day 182 were performed on the per-protocol cohort for persistence, analyses of immunogenicity in terms of neutralising antibodies at all time points were performed on the per-protocol cohort for immunogenicity and the analyses of safety were performed on the total vaccinated cohort (TVC). The according to protocol cohort for immunogenicity included all subjects who received both vaccine doses and met all protocol-defined eligibility criteria and procedures and for whom data was available at Days 21 and 42. The according to protocol cohort for persistence included all subjects who received both vaccine doses and met all protocol-defined eligibility criteria and procedures and for whom data was available at Days 21, 42 and 182. The TVC included all vaccinated subjects for whom data was available. For the purpose of GMT calculations, antibody titers below the cut-off value of each assay were substituted by half of the cut-off value.

### Disclosure of Potential Conflicts of Interest

Drs. Hideyuki Ikematsu and Hideaki Nagai were the principal investigators of this study and disclose having received honoraria/paid expert testimony and travel grants from the commercial entity that sponsored the study. Drs. Masahiro Kawashima and Yasunobu Kawakami disclose having no conflict of interest. All participating institutions received compensation for study involvement. Drs. Paul Gillard, François Roman, Karl Walravens, Kazuyoshi Tenjinbaru and Ping Li are employees of GlaxoSmithKline Biologicals. P. Gillard and F. Roman report ownership of stock options.

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All authors participated in the implementation of the study including substantial contributions to conception and design, the gathering of the data, or analysis and interpretation of the data. All

authors were involved in the drafting of the article or revising it critically for important intellectual content, and final approval of the manuscript.

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### Trade mark statement


ClinicalTrials.gov Identifier: NCT00989612



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ORIGINAL ARTICLE

# Reevaluation of the Japanese guideline for healthcare-associated pneumonia in a medium-sized community hospital in Japan

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**Abstract** The Japanese guidelines for nursing- and healthcare-associated pneumonia (NHCAP) categorize patients by risk of resistant bacteria and defined antimicrobials to be used, similar to the healthcare-associated pneumonia (HCAP) guidelines of the United States. The data were collected in large-scale hospitals, possibly a cause of inconsistency with everyday practice in medium-sized community hospitals. To test the feasibility of this guideline based on a retrospective study performed in a medium-sized community hospital in Japan, the medical records of pneumonia patients were retrospectively studied [718 patients: NHCAP, 477, 66.4 %; community-acquired pneumonia (CAP), 241, 33.4 %). Factors related to patients' background, clinical and laboratory findings, treatment, and outcome were compared between NHCAP and CAP. The A-DROP system, scored by age, dehydration, respiratory failure, disorientation, and low blood pressure, evaluated the severity of pneumonia. In contrast to CAP patients, NHCAP patients included more elderly patients requiring nursing care and revealed higher rates of poor nutrition, dementia, aspiration, severe cases, detection of drug-resistant bacteria, and mortality. For NHCAP, the success rate did not differ between those receiving and not receiving proper initial treatment (76.9 vs. 78.5 %) nor did mortality rate within 30 days differ (13.1 vs. 13.8 %). Risk factors for mortality within 30 days for NHCAP were diabetes [adjusted odds ratio (AOR) 2.394, $p = 0.009$], albumin <2.5 g/dl (AOR 2.766, $p = 0.002$), A-DROP very severe (AOR 1.930, $p = 0.021$), and imaging showing extensive pneumonia (AOR 2.541, $p = 0.002$). The severity of pneumonia rather than risk of resistant bacteria should be considered, in addition to ethical concerns, in initial treatment strategy in NHCAP to avoid excessive use of broad-spectrum antimicrobials.

**Keywords:** Severity of pneumonia · Drug-resistant bacteria · Performance status · Healthcare-associated pneumonia · Nursing and healthcare-associated pneumonia

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## Introduction

Healthcare-associated pneumonia (HCAP) is considered to fall between community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP) [1, 2]. It has a poor prognosis and a high rate of detection of drug-resistant bacteria, as does HAP, according to reports from the United States [3, 4]. In contrast, the British Thoracic Society guidelines have documented that patients with nursing home-acquired pneumonia (NHAP), which is considered as a counterpart of HCAP, should be treated in the same way as CAP [5, 6]. In addition, a report from Spain indicated that HCAP more closely resembled CAP [7]. Differences in results might be derived from the differences in medical and healthcare systems among countries. In Japan, one report stated that more drug-resistant bacteria were detected as causative organisms for HCAP compared to CAP [8], whereas another report found that HCAP was pneumonia with a poor prognosis in the elderly rather than pneumonia caused by drug-resistant bacteria, and therefore closer to CAP [9].

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The healthcare system differs greatly between Japan and the United States, and it is difficult to apply the same definitions and analyses for HCAP. In Japan, many elderly and physically handicapped people receive care at home [10]. In addition, nursing homes are not yet popular, so a great many elderly patients are admitted to general hospitals and tend to stay longer [11]. Because more than half of cases referred to as HCAP in the United States would be defined as HAP in Japan, a divergence between actual medical practice and HAP guidelines has been observed. Taking those backgrounds into account, HCAP guidelines [called nursing- and healthcare-associated pneumonia (NHCAP) guidelines] were formulated in Japan based on data extracted on HAP patients in long-term convalescent or psychiatric wards and CAP patients who were elderly and physically handicapped, requiring nursing care or receiving intravascular treatment continuously on an outpatient basis.

There were only a few reports on HCAP in Japan [8, 9]; reports on HCAP in the United States were mainly analyses of severely ill patients in intensive care units (ICU) or in large hospitals. Therefore, sufficient data from medium-sized or smaller community hospitals were lacking. The present study was conducted to address the following two points in a medium-sized community hospital: first, to study clinical differences between NHCAP and CAP; and second, to determine which is more important, severity or resistant bacteria, to the prognosis of NHCAP.

## Patients and methods

### Patient data

Data were collected and analyzed retrospectively on pneumonia patients admitted between January 2008 and June 2010 to Sekishinkai Sayama Hospital, which is a community hospital designated as a secondary emergency hospital with 350 beds in Sayama City, Saitama Prefecture, Japan.

Pneumonia cases were divided into two groups according to guidelines from the Japanese Respiratory Society: NHCAP [12] and CAP. HAP and cases diagnosed as diseases other than infectious pneumonia during the course of the study were excluded.

For eligible patients, information on patients' background, clinical findings and laboratory data on admission, severity of pneumonia, detected bacteria, initial antibiotics administered and outcome were collected. The antibiotics were selected by the attending physicians, generally, but not strictly, based on the CAP guidelines. Failure of the initial treatment was defined as escalation of antibiotics within 48–72 h of hospitalization, poor clinical improvement (no defervescence, start of mechanical ventilation, use of vasopressor), or death. Adequacy of the initial treatment was determined such that, when the bacteria detected before treatment were sensitive to the initial antibiotics, the treatment was defined as appropriate and when not sensitive, the treatment was defined as inappropriate. For outcome, mortality within 30 days and total hospital mortality were examined.

Use of data for this study was permitted by the Information Systems Division of Sekishinkai Sayama Hospital. Also, this study was approved by the institutional review board of the National Hospital Organization Tokyo National Hospital.

### Criteria for NHCAP and CAP

Patients in the NHCAP group met one or more of the following criteria: (1) admitted to long-term convalescent ward or nursing home (including psychiatric wards); (2) discharged from hospital within preceding 90 days; (3) elderly and physically handicapped, requiring nursing care; or (4) receiving continuous intravascular treatment on an outpatient basis (dialysis, antibiotics, chemotherapy, immunosuppressive agents). The CAP was defined as pneumonia other than NHCAP (defined above) or HAP, which was defined as pneumonia occurring more than 48 h after admission to a hospital. Within these categories, patients requiring nursing care were higher than grade 3 performance status (PS) of the Eastern Cooperative Oncology Group (ECOG).

### Microbiological evaluation

Microbiological diagnosis was performed by cultures (sputum, blood, bronchial wash, pleural effusion) and Gram stain. Indigenous bacteria were excluded from culture-positive cases. We diagnosed pneumococcal pneumonia when *Streptococcus pneumoniae* was isolated from the sputum or urine antigen test was positive. *Mycoplasma* pneumonia (caused by *Mycoplasma pneumoniae*) and *Chlamydophila* pneumonia (*Chlamydophila pneumoniae*) were diagnosed only when there were significant findings in a single serum or paired serum test. *Legionella* pneumonia was diagnosed by a urinary antigen test that detects only serotype 1. Drug-resistant bacteria in NHCAP were defined as *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* spp., and extended-spectrum β-lactamase-producing *Enterobacteriaceae*, which have a risk of drug resistance according to the guidelines [12].

### Evaluation of severity

Severity of pneumonia was evaluated by the A-DROP system (old age, dehydration, respiratory failure, orientation