Ⅱ. 研究成果の刊行に関する一覧表

書 籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
詳細は下記参照	詳細は下記参照	佐々木次雄	図 説 呼吸器系細菌感染症：疫学、診断、治療	株）じほう	東京	2006年5月	約300ページ

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II. 研究成果の刊行に関する一覧表

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Ⅲ. 研究成果の刊行物・別冊

JOURNAL OF BACTERIOLOGY, Mar. 2005, p. 1875–1877 Vol. 187, No. 5
0021-9193/05/$08.00+0 doi:10.1128/JB.187.5.1875–1877.2005
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Involvement of P1 Adhesin in Gliding Motility of *Mycoplasma pneumoniae* as Revealed by the Inhibitory Effects of Antibody under Optimized Gliding Conditions

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Received 15 September 2004/Accepted 30 November 2004

To examine the participation of P1 adhesin in gliding of *Mycoplasma pneumoniae*, we examined the effects of an anti-P1 monoclonal antibody on individual gliding mycoplasmas. The antibody reduced the gliding speed and removed the gliding cells from the glass over time in a concentration-dependent manner but had only a slight effect on nongliding cells, suggesting that the conformational changes of P1 adhesin and its displacement are involved in the gliding mechanism.

Mycoplasma gliding. Mycoplasmas are parasitic, occasionally pathogenic, small-genome bacteria lacking a peptidoglycan layer (20). Several mycoplasma species, including *Mycoplasma pneumoniae*, *M. genitalium*, *M. pulmonis*, *M. gallisepticum*, and *M. mobile*, have distinct cell polarity and exhibit gliding motility in the direction of the tapered end (2, 10, 13). The mechanisms underlying gliding motility are intrinsically different from those of other motility systems and are not well understood (8, 12–15, 26).

***M. pneumoniae* and P1 adhesin.** *M. pneumoniae*, a human pathogen, forms a membrane protrusion, an attachment organelle, at a cell pole (11, 13). The cell surface of the attachment organelle exhibits clustering of a 170-kDa transmembrane protein, P1 adhesin, which is responsible for binding to animal cells and glass surfaces (4, 6, 19, 24). It shares structural similarities with the adhesion proteins of other mycoplasma species, such as MgPa of *M. genitalium* (7) and GapA of *M. gallisepticum* (5), but not with Gli349 of *M. mobile*, the fastest species (26). It is known that an antibody raised against P1 can block the binding of *M. pneumoniae* to animal cells and glass (4, 6, 19, 24), but the effects on glass binding or gliding have not been observed for individual cells (4, 6). Here we analyzed the effects of such an antibody on individual cells under conditions optimized for gliding.

Optimizing conditions for gliding. In previous work, we found that a greater proportion of *M. pneumoniae* cells glided at higher speeds in a phosphate-buffered saline (PBS) solution containing serum than in the growth medium (9). Accordingly, in the present study we examined the effects of medium on gliding. *M. pneumoniae* M129 cells grown in Aluotto medium (1, 17) were suspended in fresh medium, dispersed as previously described (21, 22), put on a clean coverslip, and incubated at 37°C for 60 min to let the cells bind to the glass. The coverslip with mycoplasma cells was then assembled into a tunnel slide, as previously described (12, 26). After incubation of the cells on a microscope stage chamber at 37°C for 10 min, the growth medium was replaced by PBS containing 10% horse serum or by a fresh medium. The microscopic images were recorded and analyzed (15–17, 26). Since all cells are not always gliding (9), we examined both the proportion of gliding cells in relation to the total cells and the gliding speeds to evaluate the effects of the various conditions. The gliding activity presented by the two parameters did not change when the medium was replaced by fresh medium, but it increased in response to the replacement with PBS containing 10% serum. The proportion of gliding cells was 0 out of 406 cells at time zero but increased with time and reached 0.37 at 60 min, when the growth medium was replaced by PBS containing 10% serum. This proportion stayed at 0, however, when the growth medium was replaced with fresh medium. The gliding speed in PBS containing 10% serum also increased with time and plateaued at 0.93 μm/s at 15 min, although it did not change in the fresh medium. The average gliding speed of *M. pneumoniae* was originally reported to be as fast as 0.4 μm/s in a medium, comparable to the speed observed here in the PBS containing serum (3, 18). The content of the Aluotto medium used here was slightly different from that of the Hayflick medium used in the previous studies. We did try the Hayflick medium, but no difference in the gliding results was observed. These observations may suggest that the active gliding of *M. pneumoniae* is induced by starvation, which was unexpectedly achieved in the previous studies (3, 18).

We next examined the effects of serum concentrations, temperature, and gelatin. Once cells were bound to glass with 10% horse serum, gliding continued even in its absence but was better in concentrations ranging from 5 to 20%. The number of cells that glided was approximately the same over a temperature range of 27 to 42.5°C, but their speed increased linearly with temperature over this range from approximately 0.5 to 0.8 μm/s, as previously observed in the gliding of the fastest mycoplasma species, *M. mobile* (15). The addition of 1 to 5%

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gelatin did not prevent cells from leaving the glass during gliding (9, 18). Therefore, the effects of antibody were examined in PBS plus 10% horse serum without gelatin at 37°C.

Inhibition of gliding by anti-P1 adhesin antibody. We made a monoclonal antibody by immunizing mice with a recombinant protein comprising 1,160 to 1,518 amino acids of a whole P1 molecule of 1,627 amino acids, which is known to have a site responsible for cell and glass binding (19). The specificity of antibody was confirmed by immunoblotting, immunofluorescence microscopy of fixed cells with and without permeabilization, and immunofluorescence microscopy of living cells (12, 22, 23, 26).

The effects of the antibody on gliding of individual cells were examined (Fig. 1 and 2). Cultured mycoplasma cells were resuspended in PBS containing 10% serum and bound to a clean coverslip at 37°C for 70 min. Then, PBS containing 10% serum was replaced by PBS containing 10% serum and various concentrations of the antibody, ranging from 0 to 300 μg/ml at time zero, and cells bound to glass with and without gliding motility were counted separately, as presented in Fig. 1A and B, respectively. The addition of antibody removed the gliding cells from the glass over time in a concentration-dependent manner (Fig. 1A). However, the antibody affected the glass binding of nongliding cells only slightly (Fig. 1B). These observations indicate that the displacement of a cell along a glass surface during gliding is essential to cell removal by the antibody. The effects of antibody on the gliding speed were examined (Fig. 2). The average speed of gliding cells was found to be reduced by the addition of antibody in a concentration-dependent manner, an effect similar to that for the inhibition of glass binding, indicating that the binding of antibody reduces the gliding speed.

Involvement of P1 adhesin in gliding. The dependence of the antibody's ability to inhibit glass binding during gliding on its concentration indicates that P1 is responsible not only for static binding but also for that during gliding (Fig. 1A).

The obvious difference in resistance to the antibody between gliding cells (Fig. 1A) and nongliding cells (Fig. 1B) suggests that P1 induces conformational changes in gliding. In other words, the P1 molecules should be in a state where the accessibility of the antibody is significantly reduced when the cell is not gliding on glass, compared to the states occurring in gliding. This observation can be explained by an assumption that the P1 molecule itself is involved in a "power stroke" that propels a cell like a leg.

The binding of antibody to a cell was found to decrease the gliding speed (Fig. 2), consistent with the observation of *M. mobile* with anti-Gli349 antibody (26). The results of the present study can be explained by one of the following three hypotheses, based on the assumption of a power stroke of the P1 molecule. The first hypothesis is that the binding of antibody reduces the rate of release of P1 molecules from the glass, resulting in generation of a drag force, and also blocks rebinding after the release, as discussed for *M. mobile* (refer to Fig. 7 of reference 26). The second hypothesis is that only a fraction of P1 molecules are in the propelling cycle, while others are in a state of static binding, keeping the cells on the glass and also causing a drag force in normal gliding. In this case, the binding of antibody causes a decrease in the number of P1 molecules in the cycle, resulting in a shortage of propelling force for a cell with the normal speed. In the third hypothesis, a fraction of P1 molecules are in the propelling cycle, as proposed in the second hypothesis, but the drag force is not large enough to balance the propelling force exerted through a P1 molecule in the cycle. However, the duration of a stroke is short, and the speed of a cell depends on the sum of stroke durations. In this case, the decrease in the number of P1 molecules in the cycle directly reduces the cell's gliding speed.

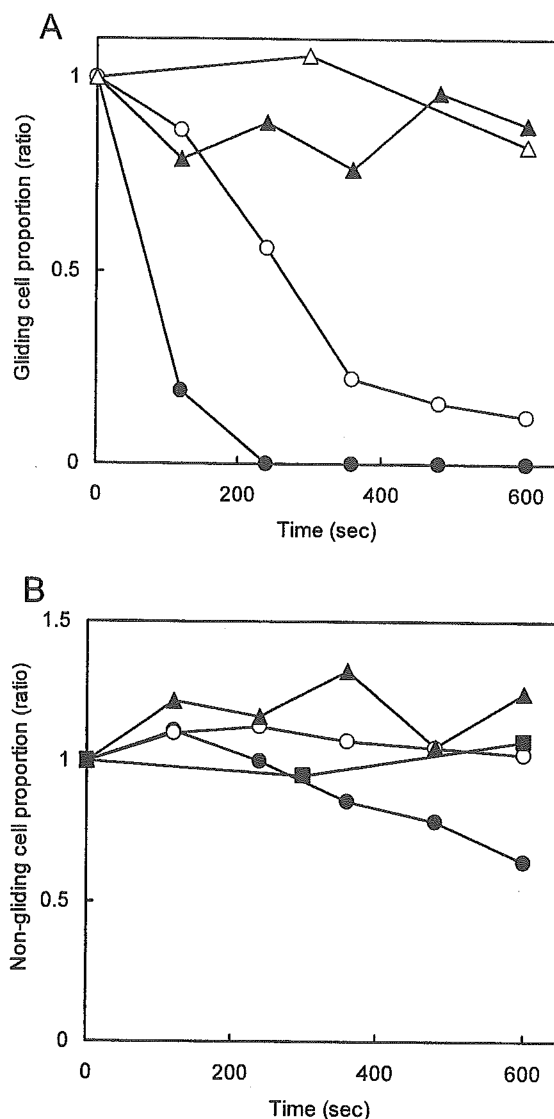

FIG. 1. Decrease in the number of bound cells after the addition of antibody. The number of bound cells relative to the initial number in a field of 9,600 μm^2 is shown. (A) The ratio of gliding cells remaining on the glass is shown for each time point after the addition of antibody to 300 (closed circles), 30 (open circles), 3 (closed triangles), and 0 (open triangles) μg/ml relative to the number of cells gliding at time zero. (B) The number of nongliding cells remaining on the glass is shown relative to the number of nongliding cells at time zero. The same symbols as those in panel (A) are used. More than 100 cells were analyzed to determine the total number of gliding and nongliding cells at the zero time point.

Conclusions. We showed that P1 is involved in the gliding motility of *M. pneumoniae*. This finding may suggest that the

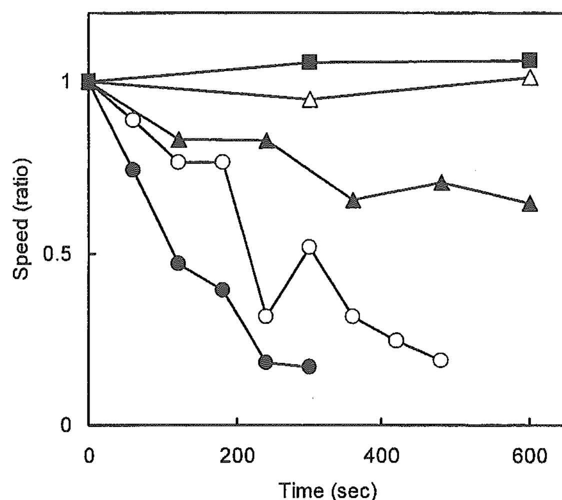

FIG. 2. Gliding speed after the addition of antibody. The gliding speeds normalized according to the initial speed are presented for each time point after the addition of antibody to 100 (closed circles), 30 (open circles), 10 (closed triangles), 3 (open triangles), and 0 (closed squares) μg/ml.

gliding of other mycoplasma species sharing an adhesion protein structure with P1 (5, 7, 25) also depends on their adhesion molecules.

This work was supported in part by Grants-in-Aid for JSPS Fellows to S.S. and for Scientific Research (C) to M.M. from the Japan Society for the Promotion of Science and by grants for Science Research on Priority Areas ("Motor Proteins, " "Genome Science, " and "Infection and host response") from the Ministry of Education, Science, Sports, Culture, and Technology to M.M.

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J Infect Chemother (2005) 11:262–264
DOI 10.1007/s10156-005-0405-z
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NOTE

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In vitro activity of β-lactams, macrolides, telithromycin, and fluoroquinolones against clinical isolates of *Streptococcus pneumoniae*: correlation between drug resistance and genetic characteristics

Received: October 20, 2004 / Accepted: July 29, 2005

Abstract The in vitro activity of antimicrobial agents against *Streptococcus pneumoniae* was determined using 16 strains of penicillin-susceptible *S. pneumoniae* (PSSP) and 26 strains of penicillin intermediately resistant *S. pneumoniae* (PISP) + penicillin-resistant *S. pneumoniae* (PRSP) in Japan. The minimum inhibitory concentrations (MICs) of potent antibiotics, including eight β-lactams (benzylpenicillin, ampicillin, cefotiam, cefepime, cefditoren, faropenem, panipenem, and biapenem), three macrolides (erythromycin, clarithromycin, and azithromycin), telithromycin, and three fluoroquinolones (ciprofloxacin, levofloxacin, and gatifloxacin), were determined. Twenty-three strains exhibited genetic variations at *pbp1a* + *pbp2x* + *pbp2b*, which are genetic-PRSP (g-PRSP). g-PISP strains accounted for 62.5% (10/16) of the PSSP strains. The existence of an abnormal *pbp* gene conferred not only penicillin resistance but resistance to cephems; however, panipenem and biapenem had potent in vitro efficacy against alterations. Regarding the macrolide resistance mechanisms (*mefA* or *ermB*): 16 isolates had only *mefA*, 18 isolates had *ermB*, and 2 isolates had both *mefA* and *ermB*. There was no correlation between the existence of an abnormal *pbp* gene and the existence of the *mefA* gene or the *ermB* gene.

Key words PRSP · PBP · *mefA* · *ermB*

Streptococcus pneumoniae is an important pathogen in many cases of community-acquired pneumonia, acute otitis media, and purulent meningitis. In Japan, rates of penicillin-resistant *S. pneumoniae* (PRSP) are reported to be 30% to 46%.[1] Currently, the antibiotic resistance patterns of *S. pneumoniae* isolates vary widely. Using polymerase chain reaction (PCR) methods, mechanisms of penicillin resistance and macrolide resistance can be obtained. Determination of the *pbp* genotype or the existence of the *mefA* gene or the *ermB* gene is useful in assessing β-lactams and macrolide resistance.[2]

In the present study, we examined the in vitro activity of β-lactams, macrolides, telithromycin, and fluoroquinolones against 16 strains of defined penicillin-susceptible *S. pneumoniae* (PSSP) and 26 strains of penicillin intermediately resistant *S. pneumoniae* (PISP) + PRSP. The study was conducted at Saitama Medical School Hospital, a 1483-bed hospital in Saitama, Japan. We used clinical pneumococcal strains isolated from patients with infectious diseases between April and September 2002. The minimum inhibitory concentrations (MICs) of potent antibiotics against *S. pneumoniae*, including benzylpenicillin, ampicillin, cefditoren, and biapenem (Meiji Seik, Tokyo, Japan), cefotiam (Takeda Chemical Industries, Osaka, Japan), cefepime (Bristol Myers KK, Tokyo, Japan), faropenem (Yamanouchi Pharmaceutical, Tokyo, Japan), panipenem (Sankyo, Tokyo, Japan), erythromycin (Abbott Laboratories, Chicago, IL, USA), clarithromycin (Taisho Pharmaceutical, Tokyo, Japan), azithromycin (Pfizer Japan, Tokyo, Japan), telithromycin (Aventis Pharma, Tokyo, Japan), ciprofloxacin (Bayer Yakuhin, Osaka, Japan), levofloxacin (Daiichi Pharmaceutical, Tokyo, Japan), and gatifloxacin (Kyorin Pharmaceutical, Tokyo, Japan) were measured. The MICs were determined by the broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS).[3] Microtiter plates containing 5.0×10^4 CFU/well were incubated with antibiotic at 35° for 18h, and the lowest concentration of drug that prevented visible growth was considered the MIC. The medium used was Mueller-Hinton broth (BBL Microbiology System, Cockeysville, MD, USA), supplemented with 5% lysed horse blood, according to the recommendations of the NCCLS. Daily quality control testing was conducted with *S. pneumoniae* ATCC 49619 (American Type Culture

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Table 1. MICs of anti-pneumococcal agents

Anti-pneumococcal agent	MIC (μg/ml) of PSSP (*n* = 16)			MIC (μg/ml) of PISP + PRSP (*n* = 26)		
	Range	50%	90%	Range	50%	90%
Benzylpenicillin	≦0.06	≦0.06	≦0.06	0.13–2	1	2
Ampicillin	≦0.06–0.13	≦0.06	0.13	0.13–4	1	2
Cefotiam	≦0.06–4	0.25	0.5	0.5–16	4	8
Cefepime	≦0.06–1	0.25	0.5	0.5–2	1	1
Cefditoren	≦0.06–0.5	≦0.06	0.13	≦0.06–1	0.25	0.25
Faropenem	≦0.06	≦0.06	≦0.06	≦0.06–0.5	0.25	0.25
Panipenem	≦0.06	≦0.06	≦0.06	≦0.06–0.13	≦0.06	0.13
Biapenem	≦0.06	≦0.06	≦0.06	≦0.06–0.25	0.13	0.13
Erythromycin	≦0.125–>16	4	>16	≦0.125–>16	4	>16
Clarithromycin	≦0.125–>16	4	>16	≦0.125–>16	2	>16
Azithromycin	0.25–>128	128	>128	0.5–>128	16	>128
Telithromycin	0.013–0.06	0.06	0.06	0.013–0.25	0.06	0.06
Ciprofloxacin	0.25–2	0.5	1	0.25–1	0.5	0.5
Levofloxacin	0.5–1	0.5	1	0.25–1	0.5	1
Gatifloxacin	0.13–0.5	0.25	0.25	0.13–0.25	0.25	0.25

Table 2. Correlation between penicillin resistance and genetic characteristics

Genetic characteristic	PSSP (*n* = 16)		PISP + PRSP (*n* = 26)	
	g-PSSP (*n* = 6)	g-PISP (*n* = 10)	g-PISP (*n* = 3)	g-PRSP (*n* = 23)
No alteration	6			
pbp2x		8		
pbp1a + *pbp2x*		2	2	
pbp2x + *pbp2b*			1	
pbp1a + *pbp2x* + *pbp2b*				23
None	3	2	1	
mefA	2	2		12
ermB	1	5	1	11
mefA + *ermB*		1	1	

Collection). The results were interpreted according to the present NCCLS standards.[4] We reviewed these samples for alterations of penicillin-binding proteins and genetic transmission of macrolide tolerance. The genes reviewed were *pbp1a*, *pbp2x*, *pbp2b*, *mefA*, and *ermB*. Variations in the genes for penicillin-binding protein and macrolide resistance determinants were screened by PCR, using methods reported previously.[5] In the NCCLS recommendations, an MIC of penicillin of ≦0.06μg/ml is classified as PSSP, an 0.12 ≦ MIC ≦ 1.0μg/ml is classified as PISP, and an MIC of 2.0μg/ml ≦ is classified as PRSP. Regarding resistant gene abnormalities, we defined no alteration as genetic-PSSP (g-PSSP); abnormalities of *pbp2x* only, or *pbp1a* + *pbp2x*, or *pbp2x* + *pbp2b* as g-PISP; and abnormalities of *pbp1a* + *pbp2x* + *pbp2b* as g-PRSP.

The predominant source of isolation was sputum from pediatric patients with pneumonia (*n* = 27), followed by sputum or nasopharynx specimens from adults with acute respiratory infection (*n* = 9), otorrhea from otolaryngology patients with acute otitis media (*n* = 3), cerebrospinal fluid from a pediatric patient with purulent meningitis (*n* = 1), and blood with sepsis from an internal medicine patient (*n* = 1).

Table 1 lists comparative antimicrobial activities against *S. pneumoniae*. In PISP + PRSP strains, the MIC_{90} of seven β-lactams (ampicillin, cefotiam, cefepime, cefditoren, faropenem, panipenem, and biapenem) were twofold or higher than those in PSSP strains. For macrolides and fluoroquinolones, however, the MIC_{90} values were almost equal between the two groups. Results of PCR investigation for gene alterations and the MIC distributions of benzylpenicillin and macrolide resistance determinants are shown in Table 2. Twenty-three strains exhibited genetic variations at *pbp1a* + *pbp2x* + *pbp2b*, which were g-PRSP. Detection of the *mefA* gene or the *ermB* gene is shown in the lower part of Table 2. Two strains exhibited genetic variations at *mefA* + *ermB*. There was no correlation between *pbp* mutations and the existence of the *mefA* gene or the *ermB* gene. Genetic resistance and the MIC ranges of β-lactams are shown in Table 3. The g-PRSP strains showed higher MIC values compared with the g-PSSP or g-PISP strains. The existence of a triple abnormal *pbp* gene affected drug resistance, not only in penicillin but also in cephems or carbapenems. Correlation between *mefA* or *ermB* and macrolide resistance was: only *mefA* was found in 16 isolates, for which the MIC range was 0.5–>16μg/ml for erythromycin and clarithromycin, and 2–>128μg/ml for azithromycin. Only *ermB* was found in 18 isolates, for which the MIC range was 2–>16μg/ml for erythromycin and clarithromycin, and 16–>128μg/ml for azithromycin. Two

Table 3. Correlation between *pbp* alterations and β-lactam resistance

Anti-pneumococcal agent	MIC range (μg/ml)			
	PSSP (n = 16)		PISP + PRSP (n = 26)	
	g-PSSP (n = 6)	g-PISP (n = 10)	g-PISP (n = 3)	g-PRSP (n = 23)
Benzylpenicillin	≦0.06	≦0.06	0.25–0.5	0.13–2
Ampicillin	≦0.06	≦0.06–0.13	0.13–0.5	0.5–4
Cefotiam	0.13–0.25	≦0.06–4	0.5–1	1–16
Cefepime	≦0.06–0.13	0.25–1	0.5–1	0.5–2
Cefditoren	≦0.06	≦0.06–0.5	0.13–0.5	≦0.06–1
Faropenem	≦0.06	≦0.06	≦0.06	≦0.06–0.5
Panipenem	≦0.06	≦0.06	≦0.06	≦0.06–0.13
Biapenem	≦0.06	≦0.06	≦0.06	≦0.06–0.13

isolates had both *mefA* and *ermB*, and the MIC ranges for these two isolates were ≧16μg/ml for erythromycin and clarithromycin, and >128μg/ml for azithromycin.

A limitation of this study is that there was a small number of pneumococcal strains within a short term, and the study took place in a single medical hospital. Under these conditions, we demonstrated that g-PISP and g-PRSP accounted for 11.5% (3/26) and 88.5% (23/26) of PISP + PRSP, respectively. Ubukata et al.[2] observed that genotypically proven g-PISP and g-PRSP had been isolated in 2002 at the rates of 33.0% and 54.9%, respectively. Pediatric strains have been shown, worldwide, to be more resistant than those from adults,[6] and household transmission of *S. pneumoniae* has been observed;[7] therefore, we should be cautious about the increase of drug-resistant *S. pneumoniae* in adults. The oral β-lactams cefditoren and faropenem showed favorable antibacterial activities, with MIC_{90} values of 0.25μg/ml against PISP + PRSP; therefore, they will be useful for empiric therapy against pneumococcal infections. The macrolide resistance mechanisms, *mefA* and *ermB*, were accurately detected and correlated with the MICs of erythromycin, clarithromycin, and azithromycin. The existence of both *mefA* and *ermB* confers strong resistance in macrolides. Telithromycin, however, was very effective against PISP + PRSP; therefore it will be useful for severe pediatric pneumococcal infection. Newer fluoroquinolones, such as gatifloxacin, have much greater in vitro activities with lower MICs against *S. pneumoniae*;[8] however, increasing resistance to fluoroquinolones has been documented in Hong Kong and Canada.[9,10] In Japan, the prevalence of fluoroquinolone-resistant *S. pneumoniae* is thought to be very low.[11] Yokota et al.[12] reported seven quinolone-resistant strains among 293 clinical isolates during 3 years. Although we did not find a quinolone-resistant strain in this study, we should follow strains for quinolone susceptibility for a longer period of time.

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ELSEVIER

Cytokines Involved in CNS Manifestations Caused by *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae **sometimes causes central nervous system manifestations, which may involve the host immune response, as the organism does not directly damage neural cells, or release toxins. Therefore we measured the levels of interleukin-6, interleukin-8, interleukin-18, interferon-γ, tumor necrosis factor-α, and transforming growth factor-β_1 in serum and cerebrospinal fluid samples from patients who manifested central nervous system manifestations during acute *M. pneumoniae* infection. The subjects were nine patients with early-onset encephalitis (central nervous system disease onset within 7 days from the onset of fever), four with late-onset encephalitis (onset at 8 days or later), three with encephalitis but without fever, and three with aseptic meningitis. Intrathecal elevations of interleukin-6 and interleukin-8 in all four types of central nervous system manifestations, and of interleukin-18 in late-onset encephalitis were observed. None of the cerebrospinal fluid samples contained detectable levels of interferon-γ, tumor necrosis factor-α, or transforming growth factor-β_1. In conclusion, interleukin-6, interleukin-8, and interleukin-18 might be involved in the inflammatory process leading to the central nervous system manifestations caused by *M. pneumoniae*.** **© 2005 by Elsevier Inc. All rights reserved.**

Narita M, Tanaka H, Togashi T, Abe S. Cytokines Involved in CNS Manifestations Caused by *Mycoplasma pneumoniae*. Pediatr Neurol 2005;33:105-109.

Introduction

It is well recognized that *Mycoplasma pneumoniae* can cause central nervous system manifestations, but the underlying pathomechanisms remain unclear. In this context, many studies have demonstrated that *M. pneumoniae* can be isolated, or its genome can be detected by polymerase chain reaction, in cerebrospinal fluid samples from patients with central nervous system manifestations. Therefore, direct invasion of the central nervous system by this organism seems to be a prerequisite for the development of central nervous system manifestations. On the other hand, there is no evidence that *M. pneumoniae* can directly damage neural cells, so immune-mediated pathomechanisms may be involved. If so, inflammatory cytokines are likely candidates for inflammatory mediators.

In previous studies, we found that the *M. pneumoniae* genome was detectable by polymerase chain reaction at a significantly higher rate in cerebrospinal fluid samples from patients with early-onset encephalitis (defined as central nervous system disease onset within 7 days from the onset of fever) than in cerebrospinal fluid from patients with late-onset encephalitis (onset at 8 days or later) [1,2]. Subsequent research confirmed this finding [3,4]. Thus, it seems that there may be distinct types of neurologic complications due to *M. pneumoniae* infection. The present study was conducted to determine whether various cytokines are specifically associated with the central nervous system manifestations caused by *M. pneumoniae*.

Patients and Methods

Patients

Characteristics of the patients, who were examined during the 5 years of 1999-2003, are summarized in Table 1. Diagnosis of *M. pneumoniae* acute infection was made serologically based on the following criteria: (1) a fourfold or greater rise in antimycoplasmal antibody titer measured by complement fixation or microparticle-agglutination test, or (2) a highest titer of 1:256 or more by complement fixation or 1:320 or more by microparticle-agglutination test. Cases were excluded when coinfection with another agent was apparent. The final decisions made by the attending physicians were respected concerning the diagnosis of encephalitis. Treatment was various, and antibiotics directed against *M. pneumoniae* were not used as an initial treatment in most of the cases. None of the patients died. Fifteen patients achieved full recovery within 28

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Received November 17, 2004; accepted March 7, 2005.

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doi:10.1016/j.pediatrneurol.2005.03.003 • 0887-8994/05/$—see front matter

Table 1. Characteristics of patients

Case No.	Age (yr)	Sex	Mp-antibody*	Pneumonia†	Type
1	10	F	1:5120	−	E, enc
2	15	F	1:1280	+	E, ADEM
3	9	F	1:1280	+	E, mg/enc
4	8	M	1:256	−	E, mg/enc
5	6	M	1:2560	+	E, enc/cerebellitis
6	7	M	>1:1280	−	E, mg/enc
7	12	M	1:10,240	−	E, ADEM
8	4	F	1:8 to 1:64	−	E, enc
9	2	M	1:2560	+	E, enc
10	7	M	1:4096	+	L, mg/enc
11	6	M	>1:20,480	+	L, mg/enc
12	2	M	1:1280	+	L, enc
13	6	F	1:320	−	L, ADEM
14	9	M	1:10,240	−	No, mg/enc
15	14	F	1:80 to 1:640	−	No, enc/GBS
16	10	F	1:2560	−	No, mg/enc
17	10	M	1:2048	−	mg
18	8	M	1:1024	−	mg
19	9	F	1:5120	+	mg

* *M. pneumoniae* antibody was measured by using a microparticle agglutination test, except in cases 4, 8, 10, 17, and 18, where a complement fixation test was used.

† +, present; −, absent.

Abbreviations:
ADEM = Acute disseminated encephalomyelitis
E = Early-onset encephalitis
enc = Encephalitis
GBS = Guillain-Barré syndrome
L = Late-onset encephalitis
mg = Meningitis
mg/enc = Meningoencephalitis
No = Encephalitis without fever

days. Four patients had minimal remaining neurologic deficits, which affected their daily activities to some degree, such as mild hearing loss, mild spasticity, or exacerbation of seizure activity in a patient with epileptic illness.

Cytokine Assays

Previous studies have revealed that various kinds of inflammatory and immunomodulatory cytokines are involved in the development of central nervous system manifestations caused by bacterial and other infections. Among them, we focused on interferon (IFN)-γ [5], tumor necrosis factor (TNF)-α [6], interleukin (IL)-6 [7,8], and IL-8 [9], because these cytokines have been reported to be involved in the pathogenesis of central nervous system manifestations by other organisms and can also be induced by *M. pneumoniae* [10,11]. In addition, we focused on IL-18 because we and our associates had recently found that local production of IL-18 and IL-8 in the lung plays a significant role in the pathogenesis of pulmonary disease caused by *M. pneumoniae* [12,13]. Moreover, transforming growth factor (TGF)-β_1 was included because recent studies have demonstrated that TGF-β_1 might work as a regulatory factor for central nervous system diseases through its antagonistic function, e.g., for IL-8 [14]. Levels of these cytokines were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits; the properties of the ELISA systems are summarized in Table 2.

Results

All results are presented in Table 3. Sequential serum or cerebrospinal fluid samples were obtained at intervals of 4 to 14 days from some patients. Because IL-18 and TGF-β_1 have wide physiologic concentration ranges between the detection limit and the normal upper limit (12.5 pg/mL to 260 pg/mL for IL-18, and 4.7 ng/mL to 74 ng/mL for

Table 2. Properties of ELISA kits for cytokine determination

Cytokine	Manufacturer	Detection Limit	Normal Range
IL-6	Fuji Rebio (Japan)	2.5	<10.0
IL-8	Amersham (UK)	5.0	<10.0
IL-18	MBL (Japan)	12.5	<260
IFN-γ	Amersham	0.1	<1.5
TNF-α	Amersham	4.5	<7.5
TGF-β_1	Amersham	4.7	<74

Detection limits are according to the manufacturer's instructions, and normal ranges are based on the manufacturer's instructions and our own previous results. These values relate to serum, and are here tentatively assumed to be applicable to cerebrospinal fluid. Values are expressed in pg/mL except TGF-β_1, in ng/mL.

Abbreviation:
ELISA = Enzyme-linked immunosorbent assay
IFN-γ = Interferon gamma
IL = Interleukin
TGF-β_1 = Transforming growth factor beta-1
TNF-α = Tumor necrosis factor alpha

Table 3a. Results of cytokine determination in serum and CSF samples from patients with CNS manifestations caused by *M. pneumoniae*

Case	Sample*	IL-6	IL-8	IL-18	IFN-γ	TNF-α	TGF-β1
1 (E, enc)	Serum-1	NT	NT	<260	1.5	<4.5	<74
	CSF-1	NT	NT	<260	<1.5	<4.5	<4.7
2 (E, ADEM)	Serum-1	12.8	<5.0	319	<1.5	<4.5	103.4
	CSF-1	91.7	155.8	<260	<1.5	<4.5	<4.7
3 (E, mg/enc)	Serum-1	8.9	<5.0	528	10.6	<4.5	<74
	Serum-2	597	1547	271	NT	<4.5	<74
	CSF-1	31.9	363.4	<260	<1.5	<4.5	<4.7
	CSF-2	7.3	<5.0	<260	<1.5	<4.5	<4.7
4 (E, mg/enc)	Serum-1	12.8	<5.0	<260	<1.5	<4.5	90.2
	CSF-1	7.7	6.5	<260	<1.5	<4.5	<4.7
5 (E, enc/cerebellitis)	Serum-1	8.0	NT	281	<1.5	<4.5	89.0
	Serum-2	7.0	NT	288	NT	<4.5	<74
	CSF-1	13.4	NT	<260	<1.5	<4.5	<4.7
6 (E, mg/enc)	Serum-1	9.6	<5.0	315	<1.5	<4.5	<74
	Serum-2	NT	NT	316	<1.5	<4.5	<74
	CSF-1	17.9	62.1	<260	NT	<4.5	<4.7
7 (E, ADEM)	Serum-1	11.2	<5.0	268	<1.5	<4.5	<74
	CSF-1	8.6	169.4	NT	<1.5	<4.5	<4.7
8 (E, enc)	Serum-1	NT	9.0	335	<1.5	<4.5	NT
	CSF-1	<2.5	42.6	<260	<1.5	<4.5	NT
9 (E, enc)	Serum-1	46.3	137.3	1180	16.9	<4.5	<74
	CSF-1	10.9	224.1	319	<1.5	<4.5	<4.7
10 (L, mg/enc)	Serum-1	7.0	21.3	357	<1.5	<4.5	<74
	CSF-1	7.7	<5.0	<260	<1.5	<4.5	<4.7
	CSF-2	7.7	<5.0	306	<1.5	<4.5	<4.7
	CSF-3	8.9	<5.0	<260	<1.5	<4.5	<4.7

Abbreviations as in Table 3b.

TGF-β_1), values within the physiologic range are reported as <260 pg/mL for IL-18 and <74 ng/mL for TGF-β_1 in Table 3.

Table 4 presents results in cytokine determination for cerebrospinal fluid, which can be considered essentially more important than results for serum, in the survey for the pathomechanism of central nervous system manifestations. In summary, elevations of IL-6 and IL-8 were frequently observed in all types of central nervous system manifestations, whereas IL-18 was rather specifically elevated in late-onset encephalitis. Otherwise, IFN-γ, TNF-α, and TGF-β_1 were not elevated in any sample.

Discussion

We and our associates previously demonstrated that IL-6 was only sporadically elevated in serum, whereas cerebrospinal fluid levels were highly elevated among patients with bacterial meningitis (49,017 ± 44,730 pg/mL), moderately elevated among patients with aseptic meningitis (1076 ± 1572 pg/mL), and somewhat elevated among patients with encephalitis (409 ± 835 pg/mL) [8]. In this context, the results of the present study indicate that the central nervous system manifestations caused by *M. pneumoniae* are rather similar to those caused by viruses rather than those caused by bacteria in terms of IL-6 response. Two of the three cases with aseptic meningitis exhibited moderately elevated levels of cerebrospinal fluid IL-6 (734 pg/mL in Case 18 and 1327 pg/mL in Case 19), and minimal elevations of cerebrospinal fluid IL-6 were found for the patients with encephalitis. Intrathecal production of IL-6 must be associated with central nervous system manifestations caused by *M. pneumoniae*, and might be a rather nonspecific phenomenon resulting from inflammation in the central nervous system rather than having a specific relationship to *M. pneumoniae*.

With regard to IL-8, previous studies have revealed that intrathecal production of this cytokine has a significant presence during the development of central nervous system diseases caused by various infections [9,15,16]. In this study, elevated cerebrospinal fluid levels of IL-8 were present in a total of 14 cases, that is, 82% of the cases tested. In accordance with previous studies [15,16], IL-8 was not detected or was detected at lower levels in serum in most of the cases. Furthermore in Case 3, an early rise of cerebrospinal fluid IL-8 in the absence of IL-8 in serum (acute phase) was followed by a later rise of serum IL-8 (convalescent phase, 10 days later) in the absence of IL-8 in cerebrospinal fluid. These observations strongly suggest that intrathecal production of IL-8 appears to be associated with the central nervous system manifestations caused by *M. pneumoniae*, although it may not be specific to *M. pneumoniae* as in the case of IL-6.

Slightly but consistently elevated levels of cerebrospinal fluid IL-18 were observed in all four patients with late-onset encephalitis. Although the increase of serum IL-18 levels during mycoplasmal infection was not specific to patients with central nervous system manifesta-

Table 3b. Results of cytokine determinations in serum and CSF samples from patients with CNS manifestations caused by *M. pneumoniae*

Case	Sample*	IL-6	IL-8	IL-18	IFN-γ	TNF-α	TGF-β_1
11 (L, mg/enc)	Serum-1	23.0	<5.0	1005	<1.5	<4.5	86.9
	CSF-1	64.5	430.6	331	<1.5	<4.5	<4.7
12 (L, enc)	Serum-1	NT	246.0	622	66	<4.5	<74
	Serum-2	NT	104.8	1240	38	<4.5	<74
	Serum-3	NT	10.5	305	<1.5	<4.5	78.2
	CSF-1	NT	114.0	285	<1.5	<4.5	<4.7
13 (L, ADEM)	Serum-1	19.2	7.0	506	<1.5	<4.5	121.7
	CSF-1	1259	147.9	282	<1.5	<4.5	<4.7
14 (No, mg/enc)	Serum-1	7.3	<5.0	<260	<1.5	<4.5	74.3
	CSF-1	<2.5	<5.0	<12.5	<1.5	<4.5	<4.7
15 (No, enc/GBS)	Serum-1	11.2	28.3	<260	<1.5	<4.5	102.3
	CSF-1	9.6	201.4	264	<1.5	<4.5	<4.7
16 (No, mg/enc)	Serum-1	8.9	<5.0	398	<1.5	<4.5	74.4
	CSF-1	142	327.4	336	<1.5	<4.5	<4.7
	CSF-2	226	NT	<260	<1.5	<4.5	<4.7
17 (mg)	Serum-1	8.3	119.3	<260	<1.5	<4.5	73.9
	CSF-1	30.7	219.3	<260	<1.5	<4.5	<4.7
	CSF-2	7.0	<5.0	<12.5	<1.5	<4.5	<4.7
18 (mg)	Serum-1	10.9	244.8	478	<1.5	<4.5	95.8
	CSF-1	734	252.2	<260	<1.5	<4.5	<4.7
19 (mg)	Serum-1	8.0	<5.0	NT	NT	<4.5	<74
	CSF-1	1327	1037	<260	<1.5	<4.5	<4.7

Values are expressed in pg/mL except TGF-β_1, in ng/mL.
* Samples with the same number were obtained on the same occasion in each case.

Abbreviation:

ADEM	= Acute disseminated encephalomyelitis	L	= Late-onset encephalitis
CSF	= Cerebrospinal fluid	mg	= Meningitis
E	= Early-onset encephalitis	mg/enc	= Meningoencephalitis
enc	= Encephalitis	No	= Encephalitis without fever
GBS	= Guillain-Barré syndrome	NT	= Not tested
IFN	= Interferon gamma	TGF-β_1	= Transforming growth factor beta-1
IL	= Interleukin	TNF-α	= Tumor necrosis factor alpha

tions [12], elevation of cerebrospinal fluid IL-18 was rather specifically associated with late-onset encephalitis in this study. Recent studies have revealed that IL-18 plays a significant role in the pathomechanism of murine experimental autoimmune encephalomyelitis [17]. It is possible that the increased level of cerebrospinal fluid IL-18 irrespective of whether it is produced intrathecally or not, plays a role in the pathomechanism of late-onset encephalitis, which is likely to be immune-mediated.

With regard to IFN-γ and TNF-α, the results of this study indicate that these cytokines do not play a role in the pathomechanism of central nervous system manifestations caused by *M. pneumoniae*. It also seems unlikely that TGF-β_1 has any role in the central nervous system manifestations.

We recently reported that the production of IL-18 in the systemic circulation and more importantly in the lung has a significant role in the pathomechanism of pulmonary disease caused by *M. pneumoniae* in children [12], as well as in adults [18]. The role of IL-18 in this case presumably involves induction of T helper type 1 cytokines and possibly IL-8 [13], whereas that of IL-8 may be to recruit neutrophils to the alveolar spaces. Although IL-8 may function to recruit neutrophils to the central nervous system in connection with the central nervous system manifestations, we found no correlation between the levels of cerebrospinal fluid IL-8 and the cerebrospinal fluid neutrophil cell counts (data not shown). That function of IL-8 might be restricted in a particular microenvironment in the central nervous system and thus might not be

Table 4. Summary of results in cytokine determination for cerebrospinal fluid

	IL-6	IL-8	IL-18	IFN-γ	TNF-α	TGF-β_1
Early-onset encephalitis	5/8	6/7	1/8	0/8	0/9	0/8
Late-onset encephalitis	2/3	3/4	4/4	0/4	0/4	0/4
Encephalitis without fever	1/3	2/3	1/3	0/3	0/3	0/3
Aseptic meningitis	3/3	3/3	0/3	0/3	0/3	0/3

No. of cases beyond the normal range/No. tested.
Abbreviations as in Table 3.

reflected in the cerebrospinal fluid cell counts. On the other hand, although the levels of IL-18 and IL-8 in the lung are closely associated with the pulmonary disease severity [12,13,18], none of the above factors appears to be closely associated with the clinical manifestations of central nervous system diseases, that is, the type of encephalitis, the severity of acute-phase clinical symptoms, or the neurologic outcome. In this respect, these cytokines may not play a central role in the central nervous system and there may be some other factors that are more closely associated with central nervous system manifestations.

Mycoplasmas have biologic activities which involve many aspects of host–pathogen interaction, and there has been a long-standing controversy about the use of corticosteroids to treat central nervous system manifestations caused by *M. pneumoniae* [19]. In the present study, unfortunately, treatment was not controlled, and we believe that not a few cases recovered with a self-limited course. The present study demonstrates that several cytokines are frequently expressed in the central nervous system in patients with *M. pneumoniae* infection. In this respect, recent accumulation of data has confirmed that macrolides have suppressive actions for these inflammatory cytokines in addition to their antimicrobial activity [20]. The anti-inflammatory activities are reported to work under lower concentrations of macrolides when compared with therapeutic ranges for antimicrobial activity. This fact may merit the use of macrolides of low permeability into the central nervous system for the treatment of central nervous system involvement. Taken together, our findings suggest that the routine antimicrobial treatment for *M. pneumoniae* using macrolides must be sufficient for most cases of central nervous system involvement associated with *M. pneumoniae* infection and lend support to the idea that the use of corticosteroids with concomitant administration of antibiotics would be effective to treat selected, severe cases of central nervous system manifestations.

This study was supported in part by a Grant for Studies on Emergency and Re-emergency Infectious Diseases from the Ministry of Health, Welfare and Labour of Japan (H15-Shinko-24).

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Microbiol. Immunol., 49(11), 959–970, 2005

Prospective Surveillance of Community-Onset and Healthcare-Associated Methicillin-Resistant *Staphylococcus aureus* Isolated from a University-Affiliated Hospital in Japan

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Received July 25, 2005. Accepted August 5, 2005.

Abstract: **We conducted a prospective comparative study of community-onset (CO) and healthcare-associated (HA) methicillin-resistant *Staphylococcus aureus* (MRSA) strains between 2000 and 2001 at Tokyo Women's Medical University Hospital (1,500 beds) in Japan. Of the 172 consecutive MRSA isolates analyzed, 13 (8%) were categorized as CO-MRSA. The mean age of patients with CO-MRSA was significantly younger than that of patients with HA-MRSA. Most CO-MRSA strains were isolated from skin and more likely to be susceptible to erythromycin, clindamycin, tetracycline, levofloxacin, and spectinomycin compared to HA-MRSA isolates. Pulsed-field gel electrophoresis (PFGE) analysis, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, and multi-locus sequence typing (MLST) revealed that CO-MRSA strains were divided into the following multi-clones: 3 clone A: II: ST5 (PFGE type: SCC*mec* type: MLST sequence type); 1 L: II: ST5; 1 H: IV: ST1; 1 I: IV: ST81; 2 D: IV: ST8; 1 B: IV: ST89; 1 B: IV: ST379; and 3 B: IV: ST91. Of the 159 HA-MRSA strains, 124 (78%) belonged to a single clone (PFGE clone A: SCC*mec* type II: *tst* and *sec* positive: coagulase type II: multi-drug resistance). Four CO-MRSA strains belonging to PFGE clone B: SCC*mec* type IV: MLST clonal complex 509 (ST89, 91, 379) had the exfoliative toxin B (*etb*) genes, but all CO-MRSA and HA-MRSA strains did not possess the Panton-Valentine leukocidin (*pvl*) genes. These results demonstrate that multiple lineages of CO-MRSA have the potential for dissemination in the community in Japan.**

Key words: **Community-onset methicillin-resistant *Staphylococcus aureus* (CO-MRSA), Healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA), Staphylococcal cassette chromosome *mec* (SCC*mec*), Multi-locus sequence typing (MLST)**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important nosocomial pathogens worldwide (5, 32). In Japan, the incidence (60–70%) of MRSA in inpatients continues to remain high (18, 19). Most healthcare-associated MRSA (HA-MRSA) isolates in Japan produce toxic shock syndrome (TSS) toxin-1 (TSST-1) and staphylococcal enterotoxin C (SEC), classified as the superantigen family, and belong to coagulase type II, *mecA*-Tn*554* polymorph I-A, staphylococcal chromosome *mec* (SCC*mec*) type II, and multi-locus sequence type (MLST) ST5 (2, 18–20). MRSA strains with TSST-1 are associated with TSS as

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Abbreviations: BAL, broncho-alveolar lavage; CC, clonal complex; CLI, clindamycin; CNS, coagulase-negative staphylococci; CO-MRSA, community-onset methicillin-resistant *Staphylococcus aureus* (MRSA); ERY, erythromycin; ETA, exfoliative toxin A; ETB, exfoliative toxin B; GEN, gentamicin; HA-MRSA, healthcare-associated methicillin-resistant *Staphylococcus aureus* (MRSA); LVX, levofloxacin; MDR, multi-drug resistance; MET, methicillin; MIC, minimal inhibitory concentration; MLST, multi-locus sequence typing; MSSA, methicillin-susceptible *Staphylococcus aureus*; NCCLS, National Committee for Clinical Laboratory Standards; NICU, neonatal intensive care unit; NTED, neonatal TSS-like exanthematous diseases; OXA, oxacillin; PFGE, pulsed-field gel electrophoresis; PVL, Panton-Valentine leukocidin; SCC*mec*, staphylococcal cassette chromosome *mec*; SEA to SEE, staphylococcal enterotoxin A to E; SPT, spectinomycin; ST, sequence type; SXT, trimethoprim/sulfamethoxazole; TEC, teicoplanin; TET, tetracycline; TSA, Trypticase-Soy agar; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin-1; TWMUH, Tokyo Women's Medical University Hospital; VAN, vancomycin.

puerperal infection (25) and neonatal TSS-like exanthematous diseases (NTED) (37, 38). Recently, NTED has been reported in France (41). This major, new clone spread during the 1990s in Japan, and nationwide dissemination of this MRSA clone, especially in neonatal intensive care units (NICU), has been associated with pandemic NTED in Japan (18, 19).

Recently, MRSA has emerged in patients without established risk factors in the community (4–6, 10, 26, 32, 34). Community-onset MRSA (CO-MRSA) is predominantly associated with skin and soft tissue infections (10, 23, 26, 32), although life-threatening pneumonia has been reported (23). CO-MRSA strains are characterized by lack of multi-drug resistance (4–6, 8, 9, 26, 32), predominance of SCC*mec* type IV (4, 6, 8, 9, 26, 28, 30, 42, 43) and frequent possession of the Panton-Valentine leukocidin (*pvl*) genes (22, 23, 26, 42, 43).

The incidence of MRSA in outpatients has increased to 20–40% of *S. aureus* strains (18, 19). The isolation rate of MRSA among *S. aureus* in outpatients in our hospital has increased to 30% of all *S. aureus* isolates (18). There have been few reports on the clinical characteristics of CO-MRSA infections as well as phenotypic and genetic characterization of CO-MRSA strains as compared with HA-MRSA in Japan. From a global control and prevention perspective, it is important to study the molecular epidemiology of CO-MRSA strains in Japan. In the present study, we conducted a prospective surveillance of CO-MRSA and HA-MRSA strains in our university-affiliated hospital. In addition, a phenotypic and genetic characterization of these strains was performed.

Materials and Methods

Study design. We prospectively collected 200 consecutive MRSA strains (100 strains from inpatients and 100 strains from outpatients) isolated from various sources between May 24, 2000 and Feb. 19, 2001 at Tokyo Women's Medical University Hospital (TWMUH, 1,500 beds). Only the first isolate from each patient was analyzed. The medical record of each patient with MRSA was reviewed to determine whether it met the case definition of community-onset or healthcare-associated. The case definition of CO-MRSA was a patient who had no history of hospitalization in the previous 6 months or admission to a long-term care facility, surgery, dialysis, endotracheal intubation, placement of an indwelling intravenous or urinary catheter, injection drug use, diabetes, neoplasm, chronic renal failure, chronic liver diseases, corticosteroid therapy and anticancer chemotherapy (34, 43). A community-onset case was defined if MRSA was isolated from cultures of specimens collected within 72 hr prior to admission to our hospital (34, 43). MRSA was considered to be healthcare-associated in all cases that were not community-onset.

Bacterial strains. S. aureus was identified by standard microbiological methods including Gram staining, the catalase test, the latex-slide agglutination test for clumping factor and protein A (PS test, Eiken Chemistry Co., Ltd., Japan), and the tube coagulase test (18, 19). Coagulase typing (19) was carried out using a coagulase typing kit (Denka Seiken, Niigata, Japan). The strains were stored in 10% skim milk at −85 C until use, and maintained on Trypticase-Soy agar (TSA) with 5% sheep blood (BD Japan, Tokyo).

Antimicrobial susceptibility testing. Susceptibility testing was performed on the Walkaway System SI using the Pos Combo Panel Type 41J (Dade Behring, Inc., West Sacramento, Calif., U.S.A.). The antimicrobial agents tested were oxacillin (OXA), gentamicin (GEN), erythromycin (ERY), clindamycin (CLI), levofloxacin (LVX), vancomycin (VAN), teicoplanin (TEC) and trimethoprim/sulfamethoxazole (SXT). Minimal inhibitory concentrations (MICs) were interpreted as sensitive or resistant based on National Committee for Clinical Laboratory Standards (NCCLS) guidelines (27). With respect to CO-MRSA strains, supplemental MIC testing for OXA and methicillin (MET) using Etest (AB Biodisk, Solna, Sweden) was performed. Resistance to spectinomycin (SPT) and tetracycline (TET) was determined by plating strains on TSA containing 500 μg/ml of SPT, and 40 μg/ml of TET as previously described (19). In addition to resistance to OXA, multi-drug resistance (MDR) was defined as resistance to 4 or more different antimicrobials among the 9 drugs of GEN, ERY, CLI, LVX, VAN, TEC, SXT, SPT, and TET.

Pulsed-field gel electrophoresis analysis. Preparation of chromosomal DNAs and pulsed-field gel electrophoresis (PFGE) analysis was done as described previously (7). PFGE profiles were visually analyzed in addition to analysis using the Lane Multi Screener software version 3.0 (ATTO Co., Tokyo). The PFGE patterns were interpreted according to the criteria of Tenover et al. (40). Isolates showing six or fewer fragment differences were considered to be subtypes of a pulse type (40).

Detection of enterotoxins, exfoliative toxins, TSST-1 and the Panton-Valentine leukocidin (PVL) genes. Crude DNA extraction for PCR analysis was performed as previously described (19). Genes coding for staphylococcal enterotoxins SEA (*sea*), SEB (*seb*), SEC (*sec*), SED (*sed*), and SEE (*see*); exfoliative toxin A, B (ETA;