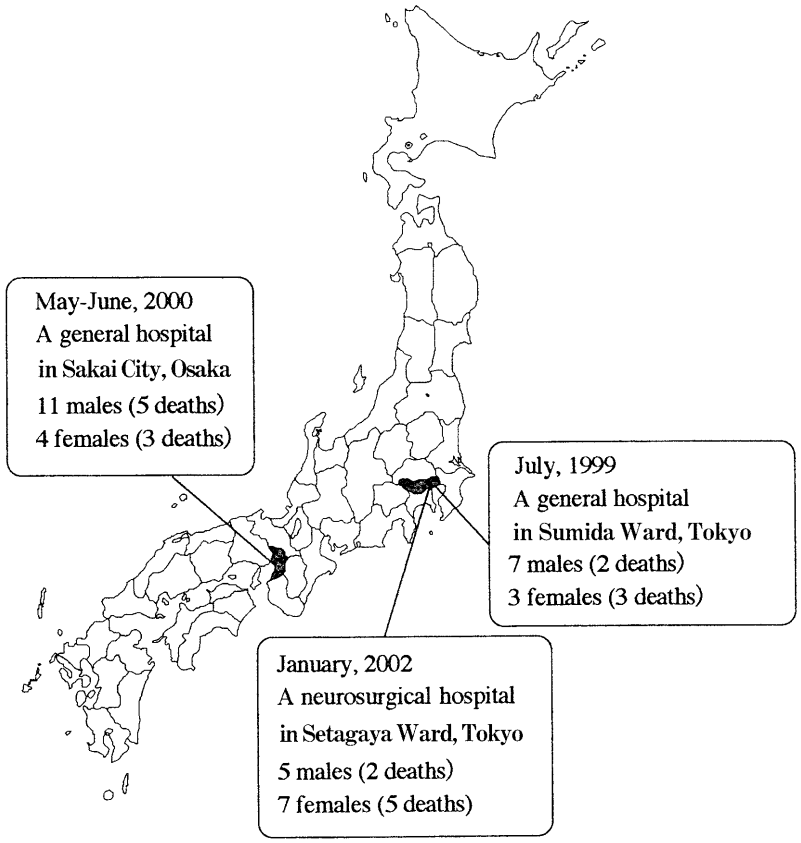

Fig. 1. Nosocomial outbreaks of *S. marcescens* blood-stream infection in Japan in the last three years

They are indistinguishable from each other by pulsed-field gel electrophoresis[8]. Carbapenem-resistant *S. marcescens* strains (strains TO1, TO2 and TO3), isolated in Tokyo, were kindly provided by Toyoko Oguri (Juntendo University, Tokyo). They were stored at −80℃ in 3% skim milk (Difco Laboratories, Detroit, Mich., USA) supplemented with 5% glucose (Difco). They were negative for the prodigiosin (a red pigment) production.

Media and bacterial growth

For bacterial growth, we used LB broth (Difco Laboratories, Detroit, Mich., USA) as the liquid media; this was inoculated and incubated at 37℃ for 12-18 h with agitation. Nutrient agar (Eiken Chemical, Tokyo) and Mueller-Hinton agar (Difco) were used as solid media.

Antimicrobial agents

Each of antimicrobial agents was a gift from their manufacturers. They included: ampicillin (Meiji Seika, Tokyo), amoxicillin (Fujisawa Pharmaceutical Co., Tokyo) and piperacillin (Toyama Chemical Co., Tokyo) as penicillins; cefazolin (Fujisawa Pharmaceutical Co.), cefaclor (Shionogi & Co., Osaka), cefotiam (Takeda Chemical Industries, Osaka), cefixime (Fujisawa Pharmaceutical Co.), ceftazidime (Glaxosmithkline K.K., Tokyo), ceftriaxone, (Nippon Roche, Tokyo), cefotaxime (Sigma, St. Louis, MO, USA), cefoperazone (Toyama Chemical Co.), cefepime (Bristol-Myers Squibb K.K., Tokyo), cefozopran (Takeda Chemical Industries) and cefpirome (Shionogi & Co.) as cefems; latamoxef (Shionogi & Co.) and flomoxef (Shionogi & Co.) as oxacephems; aztreonam (Eisai Co., Tokyo) and carumonam (Takeda Chemical Industries) as monobactams; imipenem (Banyu Pharmaceutical Co., Tokyo), panipenem (Sankyo Co., Tokyo) and meropenem (Sumitomo Pharmaceuticals Co., Osaka) as carbapenems; faropenem (Suntory pharmaceutical division, Osaka) as penems; amikacin (Meiji Seika), gentamicin (Schering-Plough K.K.,Osaka),

kanamycin (Meiji Seika), streptomycin (Meiji Seika) and isepamicin (Schering-Plough K.K., U.S.A.) as aminoglycosides; tetracycline (Wyeth Lederle Japan, Tokyo), doxycycline (Pfizer Pharmaceuticals Inc., Tokyo) and minocycline (Wyeth Lederle Japan) as tetracyclines; erythromycin (Shionogi & Co.), roxithromycin (Aventis Pharma, Tokyo), clarithromycin (Taisho Pharmaceutical Co., Tokyo) and azithromycin (Pfizer Pharmaceuticals Inc.) as macrolides; nalidixic acid (Daiichi Pharmaceutical Co., Tokyo), norfloxacin (Daiichi Pharmaceutical Co., Tokyo), ciprofloxacin (Bayer Yakuhin, Osaka), ofloxacin (Daiichi Pharmaceutical Co.) and levofloxacin (Daiichi Pharmaceutical Co.) as quinolones; chloramphenicol (Sankyo Co.); trimethoprim (Shionogi & Co.); sulfamethoxazole (Shionogi & Co.); and fosfomycin (Meiji Seika).

Reagents

Each of β-lactamase inhibitors, clavulanic acid (GlaxoSmithKline K.K.), sulbactam (Pfizer Pharmaceuticals Inc.) and tazobactam (Toyama Chemical Co.) was a gift from their manufacturers. Clavulanic acid was added to amoxicillin at the ratio of 1 : 2 (as in the combination drug), sulbactam was added to ampicillin and cefoperazone at the ratio of 1 : 2 and 1 : 1, respectively (as in the combination drug), and tazobactam was added to piperacillin at a concentration of 4 μg/ml, as described previously[10]. D-Glucose-6-phosphate was purchased from Wako Pure Chemical Industries, Osaka.

Susceptibility testing

Susceptibility testing of the bacterial strains was performed using the agar dilution method with Mueller-Hinton agar according to standard procedures[10,11]. The final concentrations of the antimicrobial agents ranged from 0.002 to 128 μg/ml. The test bacteria were grown for 18 h at 37℃ with agitation in LB broth, and diluted to approximately 10^6 colony-forming unit (CFU)/ml. Aliquots of the bacterial suspension (approximately 10^4 CFU of bacteria per spot) were inoculated on the surface of agar plates containing the antimicrobial agents. Incubation was for 18 h at 35℃. The MICs were determined as described previously[10,11]. *E. coli* NIHJ JC-2 was used as a reference strain for quality control[11]. When the susceptibility to sulfamethoxazole or trimethoprim was tested, Mueller-Hinton agar supplemented with and without 7.5% (vol/vol) defibrinated horse blood (frozen and thawed) was used[12]. When the susceptibility to fosfomycin was tested, Mueller-Hinton agar supplemented with glucose-6-phosphate (10 or 50 μg/ml) was used, in addition to Mueller-Hinton agar alone[13].

RESULTS

In vitro susceptibility of the nosocomial outbreak-derived *S. marcescens* strains

The MICs of the antimicrobial agents against the *S. marcescens* strains are summarized in Table 1. The

Table 1. MICs of antimicrobial agents for the nosocomial outbreak-derived *S. marcescens* strains and the carbapenem-resistant *S. marcescens* strains

Antimicrobial agent	MIC (μg/ml)[a]	
	Outbreak-derived strains (n=10)	Carbapenem-resistant strains (n=3)
Penicillins		
Ampicillin	≥ 256	≥ 256
Ampicillin+Sulbactam[b]	≥ 256	≥ 256
Amoxicillin	≥ 256	≥ 256
Amoxicillin+Clavulanic acid[c]	128	128-≥ 256
Piperacillin	16	32-128
Piperacillin+Tazobactam[d]	8	8-16
Cephems		
(the 1st-generation)		
Cefazolin	≥ 256	≥ 256
Cefaclor	≥ 256	≥ 256
(the 2nd-generation)		
Cefotiam	≥ 256	≥ 256
(the 3rd-generation)		
Cefixime	8	≥ 256
Ceftazidime	0.5	≥ 256
Ceftriaxone	2	≥ 256
Cefotaxime	4	≥ 256
Cefoperazone	16	≥ 256
Cefoperazone+Sulbactam[e]	8	≥ 256
(the 4th-generation)		
Cefepime	0.13	128-≥ 256
Cefozopran	0.13	128-≥ 256
Cefpirome	0.06	64-128
Oxacephems		
Latamoxef	2	≥ 256
Flomoxef	1	≥ 256
Monobactams		
Aztreonam	1	0.25-1
Carumonam	0.5	0.13-1

Table 1. (continued)

Carbapenems		
Imipenem	0.25	128-≥ 256
Panipenem	0.25	≥ 256
Meropenem	0.03	64-128
Penems		
Faropenem	16	≥ 256
Aminoglycosides		
Amikacin	8	8-128
Gentamicin	1	8-64
Kanamycin	32	≥ 256
Streptomycin	2	4-128
Isepamicin	2	4-64
Tetracyclines		
Tetracycline	8	128-≥ 256
Doxycycline	4	16-64
Minocycline	2	4-32
Macrolides		
Erythromycin	≥ 256	64-≥ 256
Roxithromycin	≥ 256	≥ 256
Clarithromycin	≥ 256	128-≥ 256
Azithromycin	64	32-128
Quinolones		
(Older)		
Nalidixic acid	2	2-≥ 256
(Newer)		
Norfloxacin	0.06	1-32
Ciprofloxacin	0.03	0.25-8
Ofloxacin	0.13	1-16
Levofloxacin	0.06	0.5-8
Chloramphenicol	16	16
Others		
Trimethoprim	0.5	0.25-4
Sulfamethoxazole	32	≥ 256
Fosfomycin	≥ 256	≥ 256
Fosfomycin+G 6 P[f]	32	64-≥ 256
Fosfomycin+G 6 P[g]	32	32-≥ 256

a) Range; b) Ampicillin: Sulbactam=2 : 1[10]; c) Amoxicillin: Clavulanic acid=2 : 1[10]; d) Tazobactam at a fixed concentration of 4 μg/ml[10]; e) Cefoperazone: Sulbactam=1 : 1[10]; f) In the presence of 10 μg/ml of glucose-6-phosphate; g) In the presence of 50 μg/ml of glucose-6-phosphate.

MIC was essentially the same in each of the 10 nosocomial outbreak-derived strains. With respect to the penicillins, the strains were highly resistant to ampicillin and amoxicillin, and moderately resistant to piperacillin. Among the penicillins, the combination of piperacillin and tazobactam (β-lactamase inhibitor) showed the greatest activity, although the addition of tazobactam to piperacillin resulted in a decrease in the MIC values to some extent.

In the case of cephems, the strains were highly resistant to the 1st- and the 2nd-generation cephems and moderately resistant (or poorly susceptible) to many of the 3rd-generation cephems. Among the 3rd-generation cephems, ceftazidime showed the highest activity. The addition of sulbactam to cefoperazon resulted in no drastic improvement in susceptibility. The strains were susceptible to the 4th-generation cephems.

With respect to other β-lactam antimicrobial agents, the strains were moderately resistant to faropenem (penems), but were susceptible to the oxacephems, monobactams, and carbapenems.

The strains were resistant to some of aminoglycosides (e.g. kanamycin), macrolides, chloramphenicol, sulfamethoxazole and fosfomycin. They were very susceptible to newer fluoroquinolones and trimethoprim. The addition of glucose-6-phosphate to fosfomycin lowered the MIC values when compared with fosfomycin alone.

Among the antimicrobial agents tested, meropenem and ciprofloxacin showed the highest activity (MIC≤0.03 μg/ml). These were followed by cefpirome, norfloxacin, and levofloxacin (MIC≤0.06 μg/ml).

In vitro susceptibility of carbapenem-resistant *S. marcescens* strains

The carbapenem-resistant *S. marcescens* strains were resistant to most of the antimicrobial agents (Table 1). They were susceptible only to the monobactams among β-lactam antimicrobial agents (MIC≤1 μg/ml). The strains were also relatively susceptible to trimethoprim (MIC≤4 μg/ml). The MIC of piperacillin decreased more rapidly with the addition of tazobactam, compared with the case of the outbreak-derived strains.

DISCUSSION

There have been three large-scale hospital outbreaks of *S. marcescens* infection in Japan[8,9]. In the infected individuals who developed symptoms, the onset was a

spike-pattern fever accompanied by shaking, and *S. marcescens* was isolated during the fever period. In the past two hospital outbreaks (in 1999 and 2000), an analysis of isolates by pulsed-field gel electrophoresis showed an identical pattern[8,9]. The molecular DNA data of the *S. marcescens* strains in the third outbreak in 2002 have not been reported.

The mortality in the *S. marcescens* blood stream infection was high: 50% (5 of 10 patients) in the outbreak in Tokyo in 1999[8], 53% (8 of 15 patients) in the outbreak in Osaka in 2000[9], and 58% (7 of 12 patients) in the outbreak in Tokyo in 2002. In the outbreak in Tokyo in 1999, those patients with severe infection developed respiratory failure, disseminated intravascular coagulation (DIC), or shock, which progressed to multiple organ failure (MOF)[8].

With respect to the *S. marcescens* strains derived from the nosocomial outbreaks in Tokyo in 1999[8] and in Osaka in 2000[9], the in vitro susceptibility data were obtained with some antimicrobial agents by the disc-diffusion method. However, no precise MIC values for each antimicrobial agent obtained by the agar dilution method have been reported. In this study, we investigated the in vitro susceptibility of the outbreak-derived *S. marcescens* strains (Tokyo strains in 1999) to 43 antimicrobial agents.

For the treatment of *S. marcescens* infection, carbapenem is often recommended as for *Pseudomonas aeruginosa* infection. The nosocomial infection isolates (Tokyo strains in 1999) investigated in this study were highly susceptible to the carbapenems. The isolates were also susceptible to the 4th-generation cephems, monobactams, newer fluoroquinolones and trimethoprim, although they were resistant to some other antimicrobial agents such as penicillins and the 1st- and the 2nd-generation cephems. The addition of β-lactamase inhibitors to ampicillin, amoxicillin, piperacillin or cefoperazone did not drastically decrease the high MIC values.

Based on the MIC patterns for β-lactam antimicrobial agents, it was speculated that the outbreak strains possess class C β-lactamase (cephalosporinase)[14,15], which is characterized by a high level of resistance to ampicillin and the 1st- and the 2nd-generation cephems and a low level of resistance to piperacillin (this piperacillin resistance is only slightly lowered by the presence of tazobactam).

The fosfomycin activity estimated in vitro is influenced by the assay conditions[13]. For instance, the MIC of fosfomycin is lowered by the addition of sugar ester (such as gulucose-6-phosphate) in the medium or by incubation under unaerobic conditions. In this study, the addition of gulucose-6-phosphate to fosfomycin lowered the MIC values. Fosfomycin is transported into the bacterial cells via at least two transport systems, the sn-glycerol 3-phosphate transport (GlpT) system and the hexose phosphate transport (UhpT) system[13]. The latter is induced with glucose-6-phosphate. There is a possibility that fosfomycin is transported mainly by the UhpT system in the *S. marcescens* strains, as in e.g. *Escherichia coli* and *Staphylococcus aureus*[13], and therefore, the MICs of fosfomycin was lowered by the presence of glucose-6-phosphate. Fosfomycin reaches a serum concentration of 166 μg/ml following an intravenous injection at 50 mg/kg[16]. Further studies are necessary to evaluate the protective effect of fosfomycin against the *S. marcescens* infection in vivo.

In the outbreak in Tokyo in 1999, cefpirome (4th-generation cephem) or isepamicin (aminoglycoside) were administered to the three patients who exhibited the most rapid course, but their symptoms did not improve, and the patients died[8]. It is necessary to carry out a further epidemiological survey on the *S. marcescens* infection. In addition, it may be important to administer effective doses of antimicrobial agents such as carbapenems (and newer fluoroquinolones), which have been recommended in empiric therapy, during the early phase after infection.

Although its frequency is low, there have been cases of a multiple drug-resistant *S. marcescens,* which exhibits resistance even to carbapenems[3-7]. In this study, we also analyzed the carbapenem-resistant *S. marcescens* strains isolated in other hospitals in Tokyo. These isolates were resistant to most of the antimicrobial agents examined, and were susceptible to only limited antimicrobial agents such as monobactams and trimethoprim.

Integron, which is a resistance gene-joining system, is attracting attention for its genetic resistance mechanism[17,18]. Class III integron has been isolated from *S. marcescens,* and its genetic structure contains a gene encoding for resistance to carbapenems and a gene encoding for resistance to amikacin and gentamycin[3]. Class I integron encoding for resistance to carbapenems has also been reported[7]. Various resistance-genes are involved in *S. marcescens* resistance, and exhibit complicated resistance-phenotypes. For example, the carbapenem-resistant isolates analyzed in this study were susceptible to monobactams, while those in many other reports were resistant to monobactams[4-7].

The precise resistance-mechanism(s) of the present carbapenem-resistant *S. marcescens* strains is under investigation. However, based on the MIC patterns for β-lactam antimicrobial agents, it has been speculated that the carbapenem-resistant strains possess at least three β-lactamases: 1) class B β-

lactamase (metallo-β-lactamase)[14,19] which is responsible for resistance to carbapenems, cephems and oxacephems; 2) class A β-lactamase (penicillinase)[14,19] which is responsible for a high level of resistance to piperacillin (this piperacillin resistance is markedly lowered by the presence of tazobactam); and 3) class C β-lactamase[14,15], similar to the outbreak-derived strains. Fortunately, no large outbreak of hospital infection by the multiple drug-resistant *S. marcescens* has ever been reported in Japan.

In the outbreak in Tokyo in 1999, the use of 50% isopropanol -- which is easily degraded -- to disinfect the drip infusion port was suggested to be the cause of the hospital infection[8].

It has been thought that the *S. marcescens* infection in the three nosocomial outbreaks in 1999, 2000 and 2002, which may have been transmitted via a catheter, was an opportunistic infection and that the deaths were due to endotoxic shock. However, bacteriologically speaking, there are many questions: 1) Why was *S. marcescens* a causative agent of the hospital infection in the three outbreaks? Why was not it, (e.g. *P. aeruginosa* or *Enterobacter* spp.) which was considered to have a similar contamination route in hospitals? Does *S. marcescens* possess a specific defense system against humoral immunity? 2) Was the main cause of the deaths actually endotoxic shock? In the outbreak in Tokyo in 1999, serum endotoxin was measured in five of 10 patients[8]. In one death, the serum endotoxin level was higher than the cutoff value -- 10 pg/ml -- and consistent with the clinical symptoms, but the level was lower than the cutoff value in the other four patients.

The mortality rate was extremely high, with respective tolls of two patients (20%), three patients (20%), and three patients (25%) dying within two days after the spike-pattern fever in the outbreaks in Tokyo in 1999[8], Osaka in 2000[9], and Tokyo in 2002. It is possible that the deaths may have been caused by systemic inflammatory response syndrome (SIRDS)[20] followed by bacteremia and by endogenous anandamide[21-23]. It is also possible that *S. marcescens* produces an unknown virulent factor (lethal factor) or has a virulent mechanism. For example, *S. marcescens* produces a metalloprotease highly homologous to the elastase produced by *P. aeruginosa*. A detailed investigation of the pathogenicity of *S. marcescens* in the circulation (blood stream infection mechanism) is necessary.

In conclusion, the *S. marcescens* nosocomial outbreaks in Tokyo in 1999 were not primarily due to bacterial multiple drug resistance. Although there are points to be clarified in the mechanism of *S. marcescens* blood stream infection, for treatment, it is important to administer effective doses of antimicrobial agents -- e.g. the carbapenems (and newer fluoroquinolones) which have been recommended in empiric therapy -- during the early phase after infection, and for medical staff to make greaters efforts to prevent *S. marcescens* contamination of (e.g.) infusion systems.

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