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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行物に関する一覧表

研究成果の刊行に関する一覧表 平成 25 年度

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In vivo efficacy of biapenem with ME1071, a novel metallo-β-lactamase (MBL) inhibitor, in a murine model mimicking ventilator-associated pneumonia caused by MBL-producing *Pseudomonas aeruginosa*

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ABSTRACT

ME1071, a maleic acid derivative, is a novel, specific inhibitor of metallo-β-lactamases (MBLs). In vitro, ME1071 can potentiate the activity of carbapenems against MBL-producing *Pseudomonas aeruginosa*. To confirm the clinical efficacy of ME1071 in ventilator-associated pneumonia (VAP) caused by MBL-producing *P. aeruginosa*, a mouse model that mimics VAP by placement of a plastic tube in the bronchus was used. Biapenem (100 mg/kg) or ME1071 plus biapenem (each 100 mg/kg) was administered intraperitoneally every 12 h beginning at 12 h after inoculation. Survival was evaluated over 7 days. At 30 h post infection, mice were sacrificed and the numbers of viable bacteria in the lungs and bronchoalveolar lavage fluid (BALF) were compared. Histopathological analysis of lung specimens was also performed. The pharmacokinetics of ME1071 was analysed after initial treatment. The ME1071 plus biapenem combination group displayed significantly longer survival compared with the control and biapenem monotherapy groups ($P < 0.05$). Furthermore, the number of viable bacteria in the lungs was significantly lower in the combination group ($P < 0.05$). Histopathological examination of lung specimens indicated that progression of lung inflammation was prevented in the combination group. Furthermore, total cell and neutrophil counts, as well as cytokine levels, in BALF were significantly decreased ($P < 0.05$) in the combination group. The percentage time above the MIC (%T > MIC) for biapenem without ME1071 was 0% in plasma; however, this value was elevated to 10.8% with ME1071. These results suggest that ME1071 is potent and effective for treatment of VAP caused by MBL-producing *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is an important cause of chronic lower respiratory tract infections and ventilator-associated pneumonia (VAP). VAP is a common nosocomial infection that occurs in 8–28% of all patients who receive mechanical ventilation [1]. Late-onset VAP in particular is more likely to be caused by *P. aeruginosa* and is associated with increased patient morbidity and mortality. VAP is difficult to treat because afflicted patients usually have serious concomitant diseases. VAP-associated mortality is estimated to be 25–40% for cases caused by *P. aeruginosa* [1]. *Pseudomonas aeruginosa* is intrinsically resistant to a variety of antibiotics and tends to become antibiotic-resistant due to antimicrobial treatments [2,3]. Of several resistance mechanisms, production of metallo-β-lactamases (MBLs) by *P. aeruginosa* is becoming a serious global concern [4,5]. MBLs confer resistance to all β-lactams except aztreonam [6]. Furthermore, the majority of MBL-producers exhibit a multidrug-resistant phenotype, including resistance to aminoglycosides and fluoroquinolones [7,8].

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MBL-producing *P. aeruginosa* strains are associated with a higher mortality rate than non-MBL-producing strains [9,10]. Thus, it is very important to investigate MBL inhibitors as a therapy for infections with MBL-producing strains.

ME1071, a maleic acid derivative, is a novel, specific MBL inhibitor that was discovered by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). In vitro, ME1071 can potentiate the activity of ceftazidime and carbapenems (especially biapenem) against MBL-producing *P. aeruginosa* [11]. In this study, the efficacy of ME1071 in combination with biapenem in a murine model that mimics VAP caused by MBL-producing *P. aeruginosa* was investigated. The pharmacokinetics of ME1071 and biapenem in mouse plasma and lungs was also examined using this model.

2. Materials and methods

2.1. Antimicrobial agents

Biapenem and ME1071 were kindly provided by Meiji Seika Pharma Co., Ltd. Both agents were dissolved in saline solution.

2.2. Bacterial strains

Animals were infected with *P. aeruginosa* NU125 strain, which is an IMP-type MBL-producer. This strain was clinically isolated from the sputum of patients at Nagasaki University Hospital (Nagasaki, Japan). Bacteria were stored at −80 °C in a Microbank™ system (Pro-Lab Diagnostics, Ontario, Canada) until use.

2.3. Laboratory animals

Male, ddY, specific pathogen-free mice (6-weeks-old; body weight, 30–35 g) were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University. Experimental protocols were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University.

2.4. Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) of the agents were determined by the broth dilution method with Mueller–Hinton broth (MHB) (Becton, Dickinson & Co., Franklin Lakes, NJ). MHB was added to a microtitre plate (Eiken, Tokyo, Japan) in the presence or absence of 32 mg/L (final concentration) of ME1071 [12]. Bacterial cultures were adjusted to an optical density of 0.5 McFarland standard and were diluted 1:10 in sterile saline. The final inoculum was ca. 5×10^5 CFU/well. Microtitre plates were incubated with the agents at 37 °C for 18 h. The lowest concentration of agent that prevented visible growth was considered as the MIC [11]. The effects of ME1071 on the MIC of biapenem towards *P. aeruginosa* were examined in vitro using five clinical isolates of *P. aeruginosa*, all of which had bla_{IMP}-type MBL. ME1071 alone did not have antibiotic effects.

2.5. Experimental model of ventilator-associated pneumonia

Disposable, sterile, plastic cut-down intravenous (i.v.) catheters with a 3-Fr (1-mm) outer diameter (Atom Co., Tokyo, Japan) were used for tracheal intubation. The tubes were 5.0 mm in length, with a few slits made at the proximal end to prevent blockage by oral secretions. To prepare inocula, *P. aeruginosa* was cultured on a Muller–Hinton II agar plate (Becton Dickinson, Le Pont de Claix, France) for 24 h. Bacteria were suspended in sterile saline and were adjusted to a concentration of 5×10^7 CFU/mL for the survival study and to 1×10^7 CFU/mL for other studies, as estimated by turbidimetry (DensiCHEK™ plus; bioMérieux, Hazelwood, MO). The intubation procedure was performed under pentobarbital anaesthesia (40 mg/kg delivered by intraperitoneal injection).

Infection was induced as described previously [13]. Briefly, the blunted end of the inner needle of an i.v. catheter (Angiocath™; Becton Dickinson Vascular Access, Sandy, UT) was inserted through the oral cavity with the outer sheath and the attached tube at the tip. The tube was advanced through the vocal cords into the trachea. The inner needle was retracted, after which a final gentle push of the outer sheath was used to place the tube in the main bronchus. Mice were then inoculated with *P. aeruginosa* suspended in saline solution (0.05 mL; 5×10^5 to 2.5×10^6 CFU/mouse) through the outer sheath and the tube.

2.6. Treatment protocol

Biapenem and ME1071 were injected intraperitoneally into the mice twice a day (each 100 mg/kg) beginning 12 h after inoculation [14]. In the control group, saline was injected into the mice instead of biapenem or ME1071. Treatment was continued for 7 days and mouse survival was evaluated for the same period. At 30 h post infection, each group was analysed by bacteriological and histopathological examination. Bronchoalveolar lavage fluid (BALF) was also analysed. The survival study and bacteriological study were performed separately.

2.7. Bacteriological and histopathological examination

Mice were sacrificed by cervical dislocation at 30 h post infection. The lungs were dissected under aseptic conditions and were suspended in 1 mL of saline. The organs were homogenised using a homogeniser (AS One Co., Osaka, Japan) and homogenates were quantitatively inoculated onto Muller–Hinton II agar plates using serial dilutions followed by incubation at 37 °C for 18 h. For histopathological examination, lung specimens were fixed in 10% buffered formalin (Sumitani, Tottori, Japan) and were stained with haematoxylin–eosin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan).

2.8. Bronchoalveolar lavage (BAL) and cytokine enzyme-linked immunosorbent assay (ELISA)

BAL was performed as described previously [15]. Briefly, mice were treated and sacrificed at 30 h after inoculation. The chest was opened to expose the lungs and trachea and a disposable, sterile, plastic cut-down i.v. catheter was inserted into the trachea. BAL was performed three times sequentially using 1.0 mL of saline each time. The recovered fluid fractions were pooled for each animal. Total cell counts were performed following Turk staining. For differential cell counts, the cells were centrifuged at 850 rpm for 2 min onto slides, which were then stained with Diff-Quick stain (Sysmex, Tokyo, Japan). Differential cell counts were performed by counting 100 cells. The concentrations of interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNFα) in BALF were assayed using mouse cytokine ELISA test kits (R&D Systems, Minneapolis, MN).

2.9. Pharmacokinetic studies

At 12 h post infection, mice were treated with biapenem (100 mg/kg) or with ME1071 in combination with biapenem (each 100 mg/kg) and were then sacrificed by cervical dislocation at 5, 15, 30, 60, 90 and 120 min after treatment. Blood was collected by cardiac puncture. Four mice were used for each group. The blood was centrifuged and the isolated plasma was mixed with a one-fifth volume of (*N*-morpholino)-propanesulfonic acid (MOPS) buffer (pH 5.5). The lungs were homogenised using a homogeniser

after addition of 11 volumes of MOPS buffer (pH 5.5). After the homogenate was centrifuged, the supernatant and plasma were treated and were deproteinised with acetonitrile to quantify the concentration of biapenem and ME1071. The lung homogenate and plasma concentrations of biapenem were determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using an ACQUITY UPLC system with a BEH C18 column (2.1 mm ID × 50 mm; pore size 1.7 μm) and a Quattro Premier XE mass spectrometer (Waters, Milford, MA). A linear gradient of 0.1% (v/v) formic acid–acetonitrile was used for the mobile phase. The mass spectrometer was operated in the MRM mode using the ESI positive ion detection mode. MS/MS transition was performed at m/z 351 → 265 for biapenem and at m/z 456 → 396 for cefotaxime as an internal standard. The lung homogenate and plasma concentrations of ME1071 were determined by liquid chromatography with ultraviolet detection using a Waters 2690 System (Waters). A Capcell Pak Ph UG120 (4.6 mm ID × 250 mm; pore size, 5 μm; Shiseido, Tokyo, Japan) was used as the analytical column. A mobile phase consisting of 0.1% (v/v) formic acid–acetonitrile (50/50; v/v) was used with a flow rate of 0.7 mL/min and detection was monitored at a wavelength of 260 nm. In the assay of biapenem in the lung homogenate and plasma, good linearity of the calibration curve was obtained over the range of 0.1–100 μg/mL and the lowest limit of quantification was 0.1 μg/mL. Also, in the assay of ME1071 in the lung homogenate and plasma, good linearity of the calibration curve was obtained over the range of 0.2–100 μg/mL and the lowest limit of quantification was 0.2 μg/mL.

Plasma and lung concentration–time profiles of biapenem and ME1071 were analysed by fitting to a one-compartment model with first-order absorption using WinNonlin Professional software v6.1 (Pharsight Corp., Mountain View, CA). The best-fit model was obtained by the least-squares method. The percentage of time above the MIC (%T > MIC) of biapenem after administration of biapenem alone or in combination with ME1071 was calculated using Microsoft Excel 2003 (Microsoft Corp., Seattle, WA). The free %T > MIC (%fT > MIC) of biapenem was also calculated using the protein binding rate of biapenem at a value of 3.8% in mouse plasma [16].

2.10. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). Survival analysis was performed using the log-rank test, and survival rates were calculated using the Kaplan–Meier method. Statistical significance was determined by using the unpaired two-tailed t-test. P-values of <0.05 were considered statistically significant.

3. Results

3.1. Effect of ME1071 on the minimum inhibitory concentration of biapenem towards Pseudomonas aeruginosa in vitro

Addition of ME1071 together with biapenem reduced the MIC of biapenem 16–64-fold for all MBL-producing *P. aeruginosa* (data not shown). The strain for which the MIC reduced from 256 mg/L to 8 mg/L by ME1071 in combination with biapenem was used in the murine model study.

3.2. Survival

As shown in Fig. 1, the survival of mice over 7 days following infection with MBL-producing *P. aeruginosa* was significantly longer in mice treated with biapenem and ME1071 combination therapy. Biapenem monotherapy prolonged survival but not significantly. ME1071 alone did not have any effect on survival in this study.

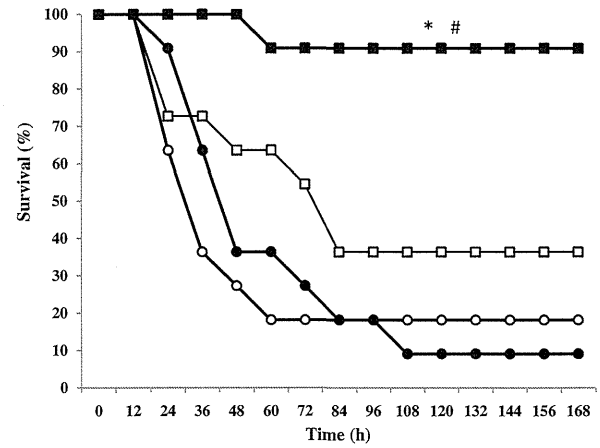

Fig. 1. Effect of biapenem and ME1071 therapy on the survival of mice in a ventilator-associated pneumonia (VAP) mouse model. Eleven mice in each group were treated with biapenem plus ME1071 (■), biapenem alone (□), ME1071 alone (●) (each 100 mg/kg) or saline solution (○). Survival was estimated at the indicated times and the results are displayed as a Kaplan–Meier plot. The survival times of the biapenem + ME1071-treated groups were significantly longer than those of the other groups as determined using a log-rank test: #P < 0.01 versus control and ME1071; *P < 0.05 versus biapenem.

3.3. Bacteriological examination

The numbers of viable MBL-producing *P. aeruginosa* in the lungs of the control group and the biapenem monotherapy and combination treatment groups of mice were (mean ± S.E.M., $\log_{10}$ CFU/mL of lung homogenate) 6.25 ± 0.43, 5.27 ± 0.34 and 4.40 ± 0.22, respectively (n = 6–7). The number of viable bacteria in the lungs of mice was significantly lower in the combination group than in the control or the biapenem groups (P < 0.05 for each comparison). There was no significant difference between the control group and the biapenem group (Fig. 2).

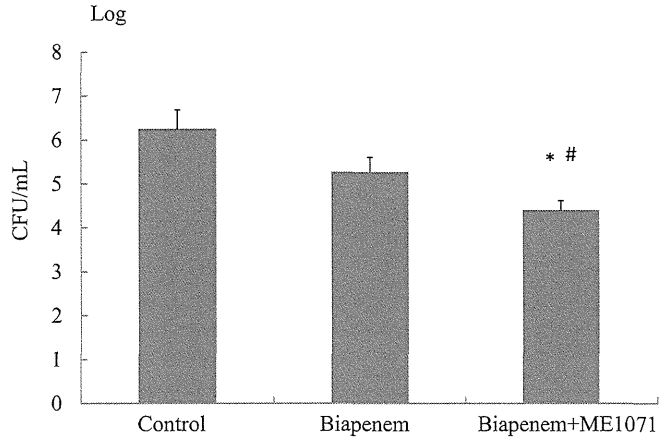

Fig. 2. Effect of biapenem monotherapy and biapenem plus ME1071 combination therapy on the number of viable bacteria in the lungs of a ventilator-associated pneumonia (VAP) mouse model. The number of viable bacteria in the lungs was calculated as CFU/mL (n = 6–7 in each group). #P < 0.01 versus control; *P < 0.05 versus biapenem. Bacteriological examinations were repeated three times and representative results are shown. Data are expressed as the mean ± standard error of the mean.

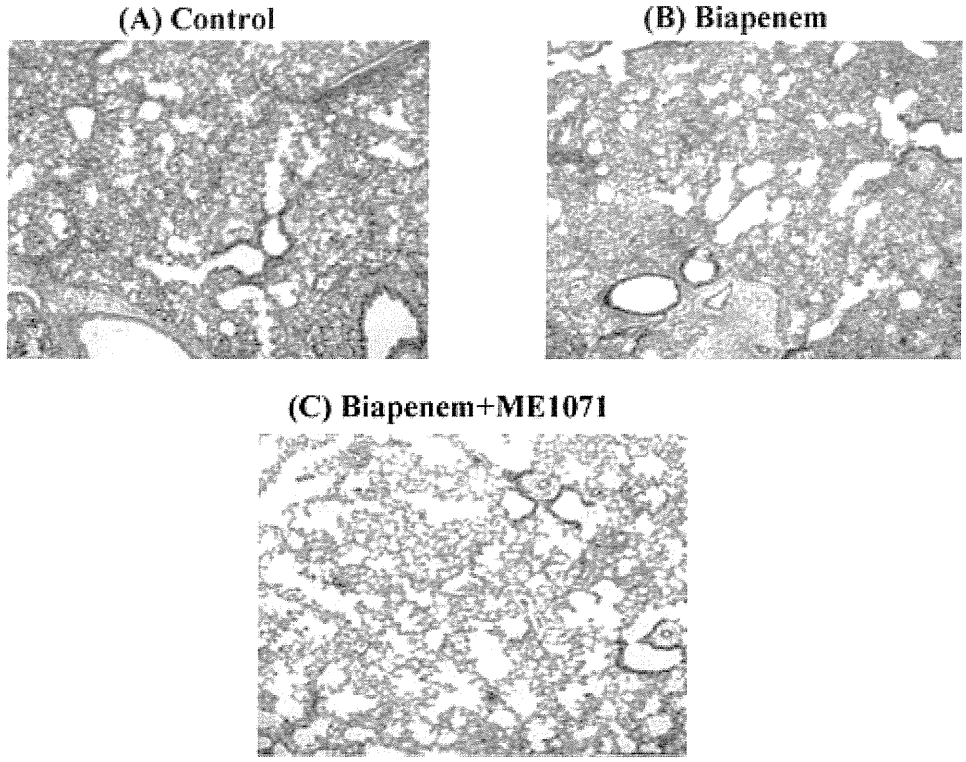

Fig. 3. Histochemical analysis of the lungs of infected mice treated with biapenem monotherapy and biapenem plus ME1071 combination therapy. Original magnification (40×; haematoxylin–eosin) of the lung at 30 h post infection. Representative photomicrographs of the lung tissues of the (A) control group, (B) biapenem group and (C) combination group.

3.4. Histopathological examination

Light microscopic analysis of the haematoxylin–eosin-stained lungs of the control group and the biapenem group at 30 h after inoculation revealed large numbers of inflammatory cells, particularly neutrophils, infiltrating the alveolar spaces. Conversely, only mild inflammatory changes were observed in the combination group (Fig. 3).

3.5. Analysis of bronchoalveolar lavage fluid

MBL-producing *P. aeruginosa* induced an increase in the total number of cells and of neutrophils in BALF. The numbers of total cells and neutrophils in BALF were significantly lower in the combination therapy group compared with the control group ($P < 0.01$) or the biapenem group ($P < 0.05$). To examine further the effects of ME1071, inflammatory cytokine levels in BALF were analysed. IL-1β, IL-6 and TNFα were all detected in BALF of the control and biapenem groups. Combination therapy significantly decreased the levels of IL-1β, IL-6 and TNFα compared with the levels in either of the other two groups ($P < 0.05$ for each comparison) (data not shown).

3.6. Lung and serum concentrations of biapenem and ME1071

The calculated pharmacokinetics of biapenem and ME1071 are presented in Fig. 4 and Table 1. The areas under the concentration–time curve from 0 to infinity (AUC_{0-inf}) of biapenem without or with ME1071, and of ME1071 when administered with biapenem, were 64.1, 50.6 and 165.3 mg h/L in plasma and 27.1, 23.3 and 83.6 μg h/g in the lungs, respectively. The half-life ($t_{1/2}$) of biapenem without or with ME1071 and of ME1071 when administered with biapenem was 0.26, 0.35 and 0.68 h in plasma and 0.31, 0.34 and 0.85 h in the lungs, respectively. The $\%T >$ MIC for biapenem without ME1071 was 0% both in the plasma and lungs. However, the percentage of biapenem with ME1071 was 10.8% in the plasma and 8.3% in the lungs.

4. Discussion

VAP is a leading cause of nosocomial infection-related mortality. VAP is difficult to treat because patients usually have serious concomitant diseases and sometimes cannot undergo an invasive examination. *Pseudomonas aeruginosa* is the predominant pathogen in VAP. Carbapenems are recommended as empirical treatment for VAP associated with *P. aeruginosa*. However, MBL-producing *P. aeruginosa* strains that are resistant to carbapenems are becoming a serious problem [4,5]. Furthermore, MBL-producing *P. aeruginosa* strains are associated with a higher mortality rate than non-MBL-producing strains [9,10]. Thus, treatment of VAP caused by *P. aeruginosa* is becoming more difficult. Colistin, which is an old antimicrobial agent belonging to the polymyxin family, has efficacy against MBL-producing Gram-negative bacteria [17,18]. However, colistin has renal toxicity and does not show a good distribution to the lungs following i.v. injection [19,20]. Furthermore, there are some reports regarding colistin-resistant Gram-negative bacteria [21,22]. Thus, it is very important to investigate novel therapies against MBL-producing Gram-negative bacteria.

ME1071 is a novel, specific inhibitor of MBLs. Ishi et al. reported that ME1071 has inhibitory activity against MBL-producing *P. aeruginosa* strains in vitro [11]. These authors indicated that the resistance of MBL-producers to biapenem (86%) showed the highest decrease (40%) in the presence of 32 mg/L ME1071. This result was the reason why we chose biapenem for combination therapy with ME1071. We also found that ME1071 in combination with biapenem had efficacy against bla_{IMP}-positive MBL-producing *P. aeruginosa* strains in vitro.

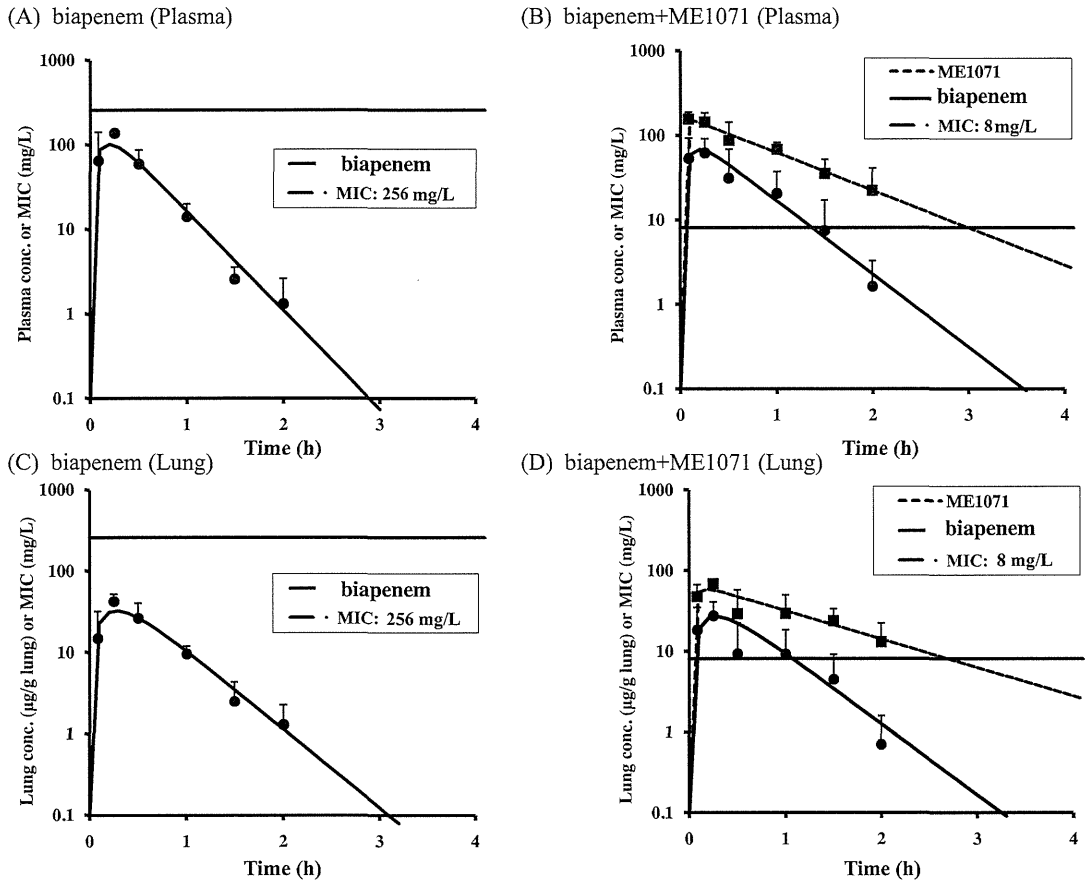

Fig. 4. Pharmacokinetics of biapenem and ME1071 in the plasma and lungs of infected mice. Pharmacokinetics of biapenem and ME1071 in plasma (A and B) or the lungs (C and D) of biapenem-treated (A and C) or biapenem + ME1071-treated (B and D) mice are shown. Each point represents the mean ± standard error of the mean from four mice. MIC, minimum inhibitory concentration.

In the present study, the in vivo activity of ME1071 against MBL-producing *P. aeruginosa* was investigated using a murine model of VAP. Because insertion of an endotracheal tube is a risk factor for infection in patients on mechanical ventilation, mice were intubated with a sterile tube through which MBL-producing *P. aeruginosa* suspended in saline was delivered [23]. This model is useful for investigating the efficacy of drugs against infectious agents [13,14].

In this study, biapenem plus ME1071 combination therapy significantly prolonged survival compared with controls, biapenem monotherapy and ME1071 monotherapy (Fig. 1). Furthermore, the combination therapy was significantly more effective than control and biapenem monotherapy at reducing the number of viable bacteria in the lungs (Fig. 2). These findings suggest that the ME1071 in combination with biapenem is effective at treating VAP caused by MBL-producing *P. aeruginosa*. Aoki et al. showed that calcium–ethylene diamine tetra-acetic acid (EDTA), which is a MBL inhibitor, in combination with imipenem had efficacy against pneumonia caused by MBL-producing *P. aeruginosa* [24]. The current study agrees with this report.

We further demonstrated, using histopathological examination and BALF analysis, that the combination therapy prevented inflammation in the lungs (Fig. 3). Previous reports indicated that inflammatory cytokines such as IL-1β and IL-6 can be good markers for the diagnosis of VAP [25,26]. Furthermore, other investigators showed that these cytokines are elevated in a murine model of *P. aeruginosa* infection and that IL-6 levels in particular correlated with the deterioration of lung function [27]. Therefore, combination

Table 1
Selected pharmacokinetic parameters estimated for biapenem and ME1071 in mouse plasma and lung.

Site/administration	Detection	AUC_{0-inf} (mg h/L of plasma or μg h/g of lung)	C_{max} (mg/L of plasma or μg/g of lung)	$t_{1/2}$ (h)	%*T* > MIC	%*fT* > MIC[a]
Plasma						
100 mg/kg biapenem	Biapenem	64.1	138.0	0.26	0.0	0.0
100 mg/kg biapenem + 100 mg/kg ME1071	Biapenem	50.6	62.0	0.35	10.8	10.8
	ME1071	165.3	156.6	0.68	–	–
Lung						
100 mg/kg biapenem	Biapenem	27.1	42.4	0.31	0.0	0.0
100 mg/kg biapenem + 100 mg/kg ME1071	Biapenem	23.3	27.4	0.34	8.3	8.3
	ME1071	83.6	68.6	0.85	–	–

AUC_{0-inf}, area under the concentration–time curve from 0 to infinity; C_{max}, maximum drug concentration; $t_{1/2}$, half-life; %*T* > MIC, time above the minimum inhibitory concentration (defined as the percentage of a 12-h period); %*fT* > MIC, free %*T* > MIC.

[a] The free fraction of biapenem (*f*) is 0.962.

therapy might contribute to prolongation of survival by inhibiting inflammatory cytokines that can result in deterioration of lung function.

The pharmacokinetics of ME1071 and biapenem in the plasma and lungs of this model mouse was analysed (Fig. 4 and Table 1). The values of pharmacokinetic parameters measured for biapenem without ME1071 (such as AUC and $t_{1/2}$) were similar to those measured for biapenem with ME1071. This result indicates that ME1071 did not affect the pharmacokinetics of biapenem.

The $\%T>$ MIC is an important pharmacodynamic parameter that influences the outcome of treatment with β-lactam antibiotics, including carbapenems [28]. In this study, the $\%T>$ MIC for biapenem with ME1071 was 10.8% in plasma. On the other hand, this value was 0% for biapenem alone. This result may be a major reason why combination therapy was more effective than biapenem monotherapy. In general, biapenem has a stronger short-term bactericidal effect and post-antibiotic effect (PAE) than meropenem [29,30]. In addition, in the present study, ME1071 was shown to have a very long $t_{1/2}$. The efficacy of biapenem plus ME1071 combination therapy might therefore be due not only to the difference in the $\%T>$ MIC of biapenem in the presence of ME1071, but also to the PAE of biapenem and to the length of $t_{1/2}$ of ME1071.

The $t_{1/2}$ of biapenem in this study was higher in plasma than in our previous mouse model study, whereas the $t_{1/2}$ in lungs was the same [14]. We cannot state clearly the reasons for the difference between this study and our previous study regarding the pharmacokinetics of biapenem. This result may be due to differences in the concentration of bacteria inoculated, the materials and methods for pharmacokinetic studies, and the extent of inflammation in lungs. Further studies will be needed to examine the most appropriate dosage for biapenem and ME1071 in animals. In addition, it will be necessary to investigate the efficacy of ME1071 against other MBL-producing Gram-negative bacteria. No significant concern was observed in non-clinical toxicological studies of ME1071. Thus, we should evaluate the clinical safety of ME1071 in the future.

In conclusion, the efficacy of biapenem with ME1071 was superior to that of biapenem monotherapy in the murine model of VAP associated with MBL-producing *P. aeruginosa*. These results suggest that ME1071 may be useful as a new strategy for treatment of the infections caused by MBL-producing bacteria.

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Competing interests: None declared.

Ethical approval: The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University [Project# 1003310842-4].

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Seroprevalence of Kaposi's Sarcoma-Associated Herpesvirus Among Men Who Have Sex With Men in Japan

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Kaposi's sarcoma-associated herpesvirus (KSHV), the etiologic agent of Kaposi's sarcoma, causes malignancies frequently in patients with acquired immunodeficiency syndrome. In the United States and Europe, KSHV infection is common among men who have sex with men. However, the seroprevalence of KSHV among men who have sex with men in Japan is unknown. In the present study, the seroprevalence of KSHV was investigated among 230 men who have sex with men and 400 age- and area of residence-matched men (controls) using a mixed-antigen (KSHV-encoded K8.1, open reading frame 59, 65, and 73 proteins) enzyme-linked immunosorbent assay and an immunofluorescence assay. Among the Japanese men who have sex with men, serological assays revealed that 27 (11.7%) were seropositive for KSHV; 20 (5%) of the men in the control group were also KSHV seropositive. The seroprevalence of KSHV among men who have sex with men was significantly higher than in the control group (odds ratio = 2.52, 95% confidence intervals = 1.38–4.62, $P = 0.0019$, Chi-square test). Infection with the human immunodeficiency virus, *Treponema pallidum*, or hepatitis B and C virus did not correlate with KSHV infection. Furthermore, the association of KSHV seropositivity with specific sexual activities was not statistically significant. In conclusion, a higher KSHV seroprevalence was found among Japanese men who have sex with men than among the controls, suggesting that the circulation of KSHV infection is more efficient among men who have sex with men in Japan than among men who do not engage in such sexual activities. ***J. Med. Virol. 85:1046–1052, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: KSHV; seroprevalence; men who have sex with men

INTRODUCTION

Kaposi's sarcoma (KS) is a malignancy observed frequently in patients with acquired immunodeficiency syndrome (AIDS). KS occurs not only in human immunodeficiency virus 1 (HIV-1)-positive men who have sex with men, but also in immunocompromised hosts like transplant patients, elderly people in the Mediterranean region, and young African patients [Antman and Chang, 2000]. Kaposi's sarcoma-associated herpesvirus (KSHV) has been detected in all cases of KS, and the serum of KS patients is positive for anti-KSHV antibodies [Antman and Chang, 2000; Ganem, 2005]. Thus, it is clear that KSHV is associated with the pathogenesis of KS, but its infection route and mechanism remain unknown. Among the general

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population, a high seroprevalence of KSHV has been shown in African countries; a medium seroprevalence in countries around the Mediterranean Sea; and a low seroprevalence in other regions, such as North America, Europe, and Asia, suggesting that the KSHV infections are spreading globally [Ganem, 2005]. Although serum antibodies to KSHV are detected in healthy individuals at various rates around the world, including 1.4% in the general Japanese population [Katano et al., 2000], they have been detected more frequently in men who have sex with men than in the general population in the United States and other countries. In previous studies, the seropositivity of KSHV in men who have sex with men ranged from 8% to 24% [Casper et al., 2002, 2006; Grulich et al., 2005; Engels et al., 2007]. Furthermore, there is a higher rate of KSHV seropositivity (i.e., >50%) in men who have sex with men and who are infected with HIV-1 [Katano et al., 2000; Casper et al., 2002]. These studies have argued that KSHV infection spreads effectively among men who have sex with men.

In Japan, the incidence of AIDS-KS has been increasing for several years. KS was found in 2.5% of AIDS patients in 1998, and increased to 5.6% in 2008. Similarly, the prevalence of individuals infected with HIV-1 has been increasing, with 70% of the total affected Japanese population being comprised of men who have sex with men (AIDS Surveillance Committee 2011, http://api-net.jfap.or.jp/status/index.html, Japanese). An earlier study reported that 60% of Japanese men who have sex with men infected with HIV-1 were also seropositive for KSHV [Katano et al., 2000]. However, the incidence of KSHV seropositivity among the total population of Japanese men who have sex with men is unknown. Despite the 1997 introduction of highly active antiretroviral therapy (HAART) in Japan, the number of KS cases has not decreased, due to the increasing number of men who have sex with men infected with HIV-1. In the present study, the seroprevalence of KSHV was measured and compared between Japanese men who have sex with men and age- and area of residence-matched control men; the investigation was conducted using enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs).

MATERIALS AND METHODS

Study Subjects

The study protocol was approved by the Institutional Review Board of the National Institute of Infectious Diseases (Approval Nos. 228 and 303). Sera were obtained during KSHV testing from participants at a free and anonymous HIV-1 test clinic for men who have sex with men. All participants in this study were also participants in the 2011 annual Nagoya Lesbian & Gay Revolution festival, one of the largest annual events for Japanese sexual minorities, held on June 4–5, 2011. The HIV-1 test was organized especially for the participants of the festival at a nearby public health center. A total of 257 individuals visited the public health center for the HIV-1 test; 237 agreed to provide informed consent and participate in the study. All participants completed questionnaires, including data on age, gender, area of residence, and sexual behavior. For the purposes of this study, men who have sex with men were defined as men who have insertive anal or oral sex with other men. Individuals who practiced both homosexual and heterosexual activities were also classified as men who have sex with men. Seven participants were excluded from the analysis: four were women, and three were men who described themselves as heterosexual in the questionnaire. Thus, 230 men who have sex with men were included in the study (Fig. 1).

Sera from 400 age-, gender-, and area of residence-matched individuals were collected as controls (Table I). The control sera were obtained from the World Health Organization and the National Serum Reference Bank/Tokyo, the National Institute of Infectious Diseases (http://idsc.nih.go.jp/yosoku/index-E.html). These sera were collected from healthy donors across all districts of Japan and across all age groups in order to survey the prevalence of various infectious diseases. Blood samples were collected in serum-separating tubes from individuals who visited public health centers for medical checks between 2008 and 2010. Collected sera were frozen, shipped to the serum bank, and stored at −80°C until use. There is no information regarding the sexual orientation of the control sera donors.

KSHV Serology

Serum KSHV antibodies were detected using both mixed-antigen ELISAs and IFAs, with a positive

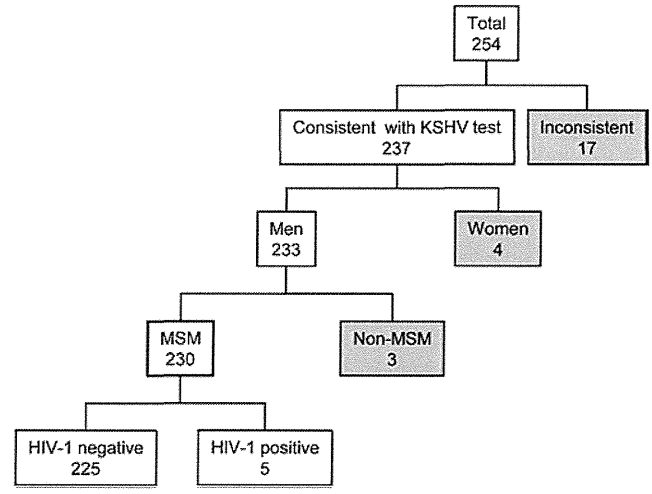

Fig. 1. Study flow diagram. Of the 257 individuals attending the free and anonymous HIV-1 test clinic, 237 agreed to participate in the study. According to the participants' responses to a questionnaire, three men who described themselves as heterosexuals and four women were excluded. Thus, 230 men who have sex with men were enrolled in the study. Five of them were HIV-1-positive.

TABLE I. Kaposi's Sarcoma-Associated Herpesvirus Seropositivity Among Men Who Have Sex With Men and Controls

	Men who have sex with men[a]	Control[a]	OR	(95% CI)	P^*
Total	27/230 (11.70%)	20/400 (5.00%)	2.52	1.38–4.61	0.003
ELISA	6/230 (2.61%)	2/400 (0.50%)	5.33	1.07–26.63	0.057**
IFA	26/230 (11.3%)	18/400 (4.50%)	2.70	1.45–5.05	0.001
Both	5/230 (2.17%)	0/400 (0.00%)	—		0.013**
Age					
18–29	5/75 (6.67%)	8/150 (5.33%)	1.23	0.39–3.90	0.957
30–39	11/81 (13.58%)	9/150 (6.00%)	2.46	0.97–6.22	0.087
40–60	6/46 (13.04%)	3/100 (3.00%)	4.85	1.16–20.35	0.048**
No answer	5/25 (20.00%)	—	—	—	—
Area					
Chubu	22/200 (11.00%)	16/319 (5.02%)	2.34	1.20–4.57	0.018
Other	5/30 (16.67%)	4/81 (4.94%)	3.85	0.96–15.46	0.105**

KSHV, Kaposi's sarcoma-associated herpesvirus; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; OR, odds ratio; CI, confidence interval.
[a]n/N (%): where n is the number KSHV seropositives, N is the total number of participants, and (%) is the percent of KSHV seropositive individuals in each category.
*Chi-square test for comparison of KSHV positivity between men who have sex with men and controls.
**Chi-square test with Yates correction was used because of sparse data.

result from either test indicating a positive serum sample. The mixed-antigen ELISA and IFA were performed as reported previously [Katano et al., 2000]. All of the serum samples were heat-incubated at 55°C for 30 min to inactivate any viruses in the serum. Mixed antigens, including K8.1 and open reading frames 59, 65, and 73 proteins, were employed as the immunogens in the ELISA. These proteins were identified as antigenic proteins encoded by KSHV using an expression library-based analysis [Katano et al., 2000]. These recombinant proteins were produced as glutathione *S*-transferase fusion proteins in *Escherichia coli*, as described previously [Smith and Johnson, 1988]. The cut-off value for the mixed-antigen ELISA was determined as the mean value plus 5× SD for 43 normal serum samples. The ELISA was validated by 100% (24/24) positivity in KS patients and 1.4% (14/1,004) in the general Japanese population [Katano et al., 2000]. Sera, diluted at 1:100, were used in the assay and all positive sera were tested in duplicate to confirm their positivity.

In the IFA, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced TY-1 cells, a KSHV-infected cell line, were initially used as antigen cells. Positive sera were then examined in TPA-induced BCBL-1, a KSHV-infected PEL cell line, BJAB, a KSHV-negative B-cell line, and Raji, a KSHV-negative, EBV-positive B-cell line [Renne et al., 1996; Katano et al., 1999]. Sera, positive in BCBL-1 and TY-1 but negative in BJAB and Raji cells, were categorized as positive.

Human Immunodeficiency Virus, *Treponema pallidum*, and Hepatitis B (HBV) and C (HCV) Virus Infections

Serum HIV-1 RNA was measured by reverse transcription-polymerase chain reactions (COBAS AmpliPrep/COBAS TaqMan HIV-1 Test; Roche Diagnostics, Boehringer Mannheim, Germany). The presence of *T. pallidum* (TP) infection was determined using a Latex suspension (a rapid plasma regain, Sekisui Medical, Tokyo, Japan). HBV and HCV antigens were identified using Architect HBsAg QT and HCV (Abbott, Abbott Park, IL).

Statistical Analysis

Chi-square tests, with Yates correction, were used to compare KSHV seropositivity between men who have sex with men and controls. A multivariable logistic regression analysis, with a forced entry method, was performed to determine the independent role of the variables (answers in the participants' questionnaires). All of the statistical analyses were conducted using SPSS (IBM, Armonk, NY).

RESULTS

The median ages (mean, range) of the men who have sex with men and controls were 33.0 (33.1, 18–60) and 32.0 (33.4, 20–49) years, respectively. Twenty-seven (11.7%) of the 230 Japanese men who have sex with men were seropositive for KSHV, as determined by ELISA or IFA (Figs. 2 and 3, and Table I). Five serum samples were found to be positive by both ELISA and IFA, and one serum sample, positive by ELISA in the men who have sex with men group, was negative by IFA. In the control group, 20 (5%) of the 400 age- and area of residence-matched Japanese men were seropositive by ELISA or IFA; none of the ELISA-positive control sera were positive by IFA. Compared to the controls, the seroprevalence among men who have sex with men was significantly higher (odds ratio [OR] = 2.52, 95% confidence intervals (CI) = 1.38–4.61, $P = 0.003$, Chi-square test) than among the control men. In an examination of seroprevalence by age groups, 40–60 year-old men who have sex with men showed significantly higher positivity for KSHV than did the age-matched

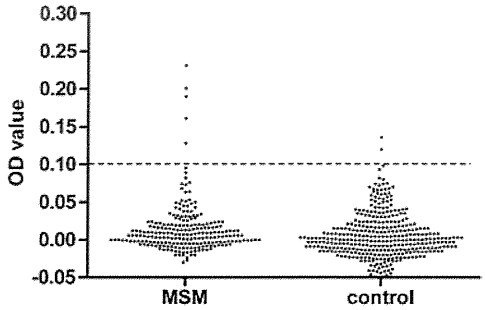

Fig. 2. Group scatter diagrams for enzyme-linked immunosorbent assay (ELISA) results. The scatter diagrams show the results of reactions of sera from men who have sex with men and controls in the mixed Kaposi's sarcoma-associated herpesvirus antigen ELISA. Optical density (OD) values were calculated as follows: (sample OD − negative control OD)/(positive control OD − negative control OD) [Katano et al., 2000]. A horizontal broken line indicates the cut off value.

controls ($P = 0.048$, Chi-square test with Yates correction), indicating a higher seroprevalence of KSHV among older men who have sex with men. Furthermore, men who have sex with men from the Chubu area showed significantly more prevalent KSHV positivity than was observed in controls ($P = 0.018$, Chi-square test), but did not in any other area. This may have been due to the small number of samples from other areas.

The presence of serum antibodies against HIV-1, TP, HBV, and HCV was also tested in all samples from men who have sex with men. Of the five men who have sex with men and who were also HIV-1-positive, KSHV antibodies were detected in one. HIV-1 positivity among KSHV seropositive men who have sex with men (1/27, 3.7%) was 1.91 (95% CI: 0.21–17.78) times higher than among KSHV seronegative men who have sex with men (4/203, 2.0%). Of the 12 test subjects with TP antibodies, three were KSHV seropositive. The rate of TP positivity among KSHV seropositive men who have sex with men (3/27, 11.1%) was 2.69 (95% CI: 0.68–10.64) times higher than that among KSHV seronegative men who have sex with men (9/203, 4.4%). However, there was no significant difference between HIV-1 or TP infection rates and KSHV seropositivity ($P = 0.14$ and 0.56, respectively, Chi-square test). Two HBV-positive and 1 HCV-positive men who have sex with men were negative for KSHV; there was no association between KSHV infection and the presence of these antibodies.

The association between the infections and sexual behaviors, determined using the participants' questionnaires, is shown in Table II. KSHV seropositivity was not correlated with the possibility of HIV-1 infection (subjects' perceived potential HIV-1 infection status) or with their sexual behaviors during the previous 6 months. There were no statistical differences between the use of condoms during anal sex and the rate of KSHV seropositivity, regardless of whether the subjects were performing or receiving

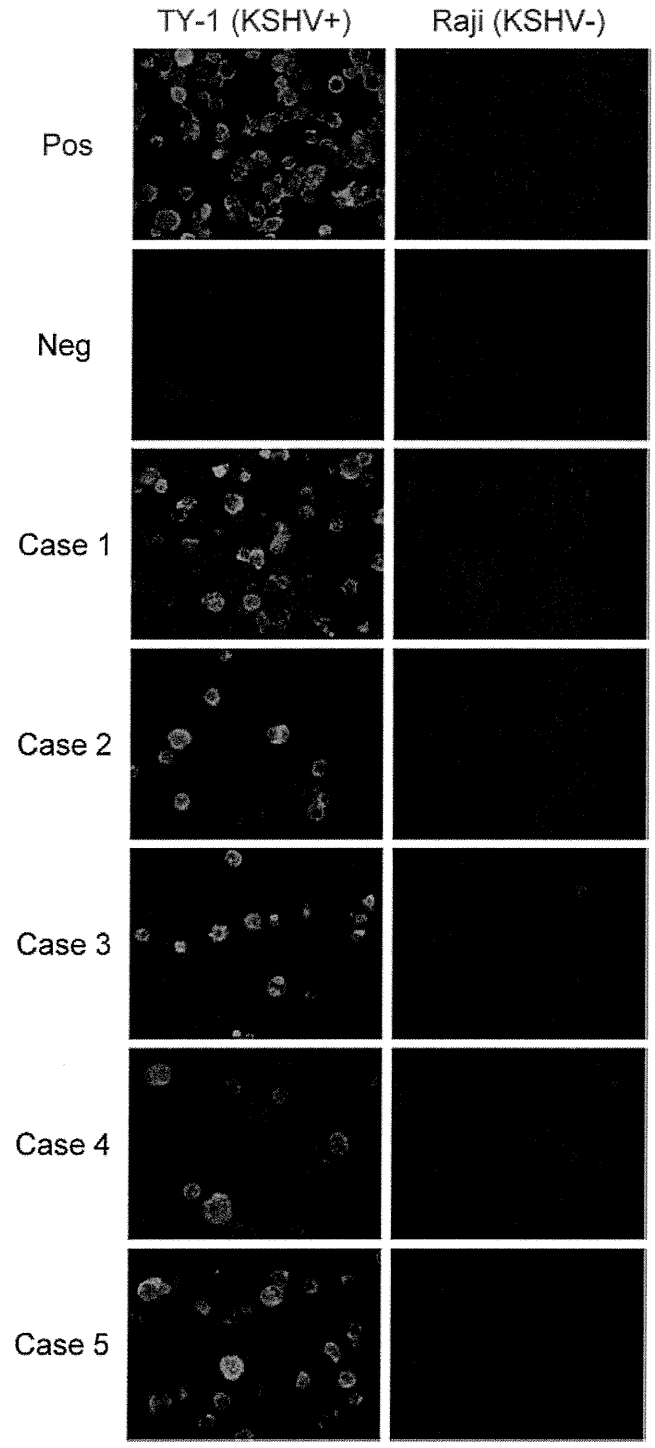

Fig. 3. Immunofluorescence images for Kaposi's sarcoma-associated herpesvirus (KSHV) immunofluorescence assay (IFA). Five positive samples from men who have sex with men are shown. The positive sera reacted with antigens in TY-1 (KSHV-positive, Epstein-Barr virus-negative lymphoma cell line), but not in Raji (KSHV-negative, Epstein-Barr virus-positive lymphoma cell line). Positive control serum from a Kaposi's sarcoma patient and negative control serum from a healthy individual are also shown.

TABLE II. Multivariate Model of Predictors of Kaposi's Sarcoma-Associated Herpesvirus (KSHV) Seropositivity in Sexual Behaviors

Question	Answer	KSHV+	Total	%	AOR (95% CI)*	P
Sexual orientation	Homosexual	25	196	12.76	Reference	
	Bisexual	2	34	5.88	0.431 (0.088–2.117)	0.300
Possibility of HIV infection	No	14	144	9.70	Reference	
	Yes	13	86	15.10	1.867 (0.767–4.544)	0.169
Sexual behaviors in last 6 months	No	2	10	20.00	Reference	
	Yes	25	216	11.60	0.356 (0.59–2.144)	0.260
Performance of insertive anal sex with main partner	Not wearing condom	4	30	13.30	Reference	
	Sometimes wearing condom	4	39	10.30	1.077 (0.141–8.224)	0.943
	While wearing condom	6	56	10.70	0.737 (0.095–5.724)	0.771
Receipt of anal sex with main partner	Partner not wearing condom	3	29	10.30	Reference	
	Partner sometimes wearing condom	2	30	6.70	1.467(0.123–17.574)	0.762
	Partner wearing condom	8	50	16.00	3.676 (0.365–36.975)	0.269
Performance of insertive anal sex with casual partner(s)	Not wearing condom	5	20	25.00	Reference	
	Sometimes wearing condom	1	31	3.20	0.117 (0.008–1.786)	0.123
	While wearing condom	8	68	11.80	0.346 (0.049–2.419)	0.285
Receipt of anal sex with casual partner(s)	Partner not wearing condom	4	14	28.60	Reference	
	Partner sometimes wearing condom	1	31	3.20	0.093 (0.005–1.699)	0.109
	Partner wearing condom	10	48	20.80	0.737 (0.085–6.400)	0.782

*AOR, adjusted odds ratio; CI, confidence interval.

anal sex or whether the anal sex was performed with the subject's main partner or with casual partners. However, condom use was associated with decreased (0.3–0.7 times less) KSHV positivity among subjects performing or receiving anal sex with casual partners than among those who did not use condoms.

DISCUSSION

This study showed that KSHV seroprevalence in Japanese men who have sex with men is 11.7%, which is similar to the seroprevalence among a similar population of men in the USA and Europe. The higher seroprevalence of KSHV among men who have sex with men, compared with controls, suggests that the circulation of KSHV infection among Japanese men who have sex with men is more efficient than among heterosexual males, as previously reported [Goudsmit et al., 2000; Casper et al., 2002, 2006; Grulich et al., 2005; Engels et al., 2007; Giuliani et al., 2007]. Although the transmission route of KSHV remains unclear, the higher seroprevalence of KSHV between men who have sex with men than that among the general population suggests that transmission likely occurs through homosexual behaviors in non-endemic areas, such as in the USA, Europe, and Asia [Goudsmit et al., 2000; Diamond et al., 2001]. In contrast, in KSHV endemic areas, such as Africa, a high seroprevalence of KSHV has been found even among children [Bourboulia et al., 1998; Butler et al., 2009]. Since high copy numbers of KSHV have been detected in the saliva of those infected with KSHV, vertical mother-to-child transmission may occur through saliva [Pauk et al., 2000; Mbulaiteye et al., 2006]. In addition, in KSHV endemic areas, sexual transmission has not been associated with KSHV infection [Shebl et al., 2011].

Of the 230 subjects in this study, 12 (5.2%) were positive for TP, suggesting that these were individuals with high levels of sexual activity. There were no significant associations between HIV-1, HBV, HCV, or TP and KSHV infections in Japanese men who have sex with men in the present study. A previous study with a large sample size, on individuals without HIV-1 infection but at high risk for sexually transmitted infections, demonstrated that the incidence of KSHV infection was different from that for HIV-1 and other sexually transmitted infections [Giuliani et al., 2007], suggesting that the routes of KSHV transmission and the opportunity for KSHV infection are different from other infections. The present study showed that the seroprevalence of KSHV is higher than that of the aforementioned sexually transmitted diseases in Japanese men who have sex with men, implying that KSHV infection can be an early marker of sexually transmitted infections in a certain proportion of study subjects.

Japanese men who have sex with men tend to use condoms less frequently for oral sex than for anal sex [Inoue et al., 2006]. Considering that the saliva of KSHV-infected persons contains high loads of KSHV, oral sex is possibly a transmission route of KSHV [Pauk et al., 2000]. There was no statistical difference in the incidence of KSHV positivity between those who did and those who did not use condoms during anal sex with their main partners (Table II). However, in subjects performing or receiving anal sex with

casual partners, the incidence of KSHV positivity was 0.3–0.7 times less among those who used condoms, compared with those who did not use condoms (Table II); this finding suggests that the risk of KSHV infection through anal sex can be reduced by condom use.

A gold standard for KSHV serology testing does not currently exist [Corchero et al., 2001; Pellett et al., 2003]. However, a combination of ELISA and IFA has been found to be more accurate for the detection of serum KSHV antibodies than any individual method. In the present study, 5% of the control sera were positive for KSHV in ELISA or IFA. A previous study demonstrated that by ELISA, alone, 1.4% of the Japanese general population was found to be positive for the KSHV serum antibody [Katano et al., 2000]. However, the findings in the present study are not directly comparable with those in that study as different serological assays were used in the present study and the control sera was obtained predominantly from 30- to 40-year-old men, most of whom resided in the Chubu area. Data, from the current study, using a combination of ELISA and IFA suggests that the seroprevalence of KSHV antibodies among the general, Japanese population is between 2% and 5%. Although information was not available on the sexual habits of those providing the control sera, 2% of adult Japanese men are estimated to have had sex with other men [Ichikawa et al., 2011]. Thus, in the present study involving 400 control subjects, there may have been up to eight participants who have engaged in homosexual sexual activity. If eight are excluded from 380 KSHV-negative controls, the seroprevalence of KSHV among men who have sex with men (11.7%) remains statistically higher than that among controls (OR 2.47, 95% CI 1.35–4.52, $P = 0.002$, Chi-square test), suggesting that the potential inclusion of a small number of men who have sex with men in the control group did not affect the conclusions. However, a more focused investigation, examining sexual orientation-matched samples, would be required to more accurately state the KSHV positivity among men in the control group.

In conclusion, this study revealed that the seroprevalence of KSHV between Japanese men who have sex with men is 11.7%, which is higher than that among controls, suggesting that the circulation of KSHV infection among men who have sex with men in Japan is more efficient than among heterosexual males. In addition, the higher prevalence of KSHV antibodies than those for other infectious diseases that may be sexually transmitted suggests that the KSHV test may be an early maker for sexually transmitted diseases. Nonetheless, the transmission route of KSHV remains unclear. Further detailed studies on sexual behaviors and virus shedding in the saliva will be required to clarify the mechanism of KSHV infection among men who have sex with men.

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ORIGINAL ARTICLE

Prospective randomized comparison study of piperacillin/tazobactam and meropenem for healthcare-associated pneumonia in Japan

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Abstract Healthcare-associated pneumonia (HCAP) may have a more severe course than community-acquired pneumonia (CAP); hence, it is more likely to be caused by drug-resistant bacterial pathogens and anaerobes involved in aspiration pneumonia. We compared the efficacy and safety of initial empiric therapy with piperacillin/tazobactam (PIPC/TAZ, 13.5 g/day) with that of meropenem (MEPM, 1.5 g/day) as single broad-spectrum regimens with gram-negative and anaerobic coverage in patients with HCAP in Japan. The clinical cure rate was 75.9 % (22/29 cases) in the PIPC/TAZ group and 64.3 % (18/28 cases) in the MEPM group. The clinical efficacy rate was 87.9 % (29/33 cases) in the PIPC/TAZ group and 74.2 % (23/31 cases) in the MEPM group. The bacteriological eradication rate was 94.4 % (17/18) in the PIPC/TAZ group and 87.5 % (14/16) in the MEPM group. Adverse drug reactions were seen in 22.4 % (11/49 cases) of patients in the PIPC/TAZ group and 17.4 % (8/46 cases) of patients in the MEPM group. Although not statistically different, the PIPC/TAZ group had a slightly higher efficacy rate than the MEPM group. Both treatment regimens are tolerable and might be appropriate to use as initial empiric therapy for HCAP in Japan. To investigate the differences in efficacy profiles of those two regimens, a further confirmatory study with a larger cohort as determined by a power analysis is recommended.

Keywords Healthcare-associated pneumonia · Nursing and healthcare-associated pneumonia · Piperacillin/tazobactam · Meropenem · Antimicrobials

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Introduction

Pneumonia is the third leading cause of death in Japan, and mortality is especially high among elderly patients [1]. The Japanese Respiratory Society guidelines were established in August 2011 for the management of patients with nursing and healthcare-associated pneumonia (NHCAP) [2]. NHCAP differs from the healthcare-associated pneumonia (HCAP) that was described by the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA); its definition was modified to fit the Japanese healthcare system [3]. This study was started in 2009, and the definition of NHCAP had not been established at that time. Hence, patients with HCAP were recruited in this study. In Japan, general hospitals have extended-care wards, and patients in these wards tend to stay in hospitals longer as compared to those in Western countries. Therefore, in this study, patients who resided in extended-care wards were included as HCAP cases.

HCAP may have a more severe course than community-acquired pneumonia (CAP) and is more likely to be caused

by drug-resistant bacterial pathogens and anaerobes involved in aspiration pneumonia [4–8]. Inappropriate therapy is a major risk factor for mortality and leads to extended hospital stay [9]. ATS/IDSA guidelines for nosocomial pneumonia recommend that all such patients receive empiric therapy with a multidrug regimen directed against drug-resistant organisms [3]. Nevertheless, Kett et al. [10] reported that compliance with ATS/IDSA guidelines for dual gram-negative coverage in patients who are at risk from multidrug-resistant pathogens was associated with increased mortality, which can be explained by antibiotic-specific toxic effects such as acute deterioration of renal function or neurotoxic effects. Brito and Niderman [11] developed an algorithm for empiric therapy of HCAP that suggests that not all such patients require a broad-spectrum multidrug regimen to achieve appropriate and effective therapy. Patients at risk for multidrug-resistant pathogens included those with severe illness or those with other risk factors including hospitalization in the past 90 days, antibiotic therapy in the past 6 months, poor functional status, and immune suppression.

For HCAP treatment, piperacillin/tazobactam hydrate (PIPC/TAZ) and carbapenems such as meropenem hydrate (MEPM) are recommended. However, limited data are available for comparing the effects of these two antibiotics against HCAP. In this study, the efficacy and safety of initial empiric therapy with PIPC/TAZ was compared to that with MEPM in patients with HCAP in Japan.

Patients and methods

Patients

We enrolled patients with HCAP from Nagasaki University Hospital and 14 affiliated facilities in Nagasaki Prefecture from October 1, 2009, to May 31, 2011. The study was conducted with prior approval from the ethics committee of each of the participating medical facilities and was registered on a clinical trial registry (UMIN ID No.: UMIN000002269). The study protocol was explained thoroughly to the patients or their legal representatives before the start of treatment, and written informed consent was obtained from each patient.

Patients with HCAP and a pneumonia severity index score [12] in risk class III or IV were required to fulfill all four of the following criteria: (1) appearance of new infiltrates on chest radiography or computed tomography; (2) either (a) resided in a nursing home, long-term care facility, or extended-care ward (for more than 48 h), (b) been hospitalized for $\geq$2 days in the past 90 days, (c) receiving outpatient intravenous therapy, or (d) receiving home wound care; (3) positive findings of at least one sign of inflammation such as white blood cell (WBC) count $>10{,}000/mm^3$ or $<4{,}500/mm^3$, increased C-reactive protein level, or fever $\geq$37 °C; and (4) positive findings of at least one of the clinical symptoms or signs, such as cough, purulent sputum, moist rales, dyspnea, and tachypnea.

The following participants were excluded: (1) patients with bronchial obstruction or history of obstructive pneumonia; (2) those unable to receive treatment every 8 h; (3) those with severe hepatic dysfunction or renal dysfunction (creatinine clearance $\leq$30 ml/min); (4) those for whom evaluation of clinical efficacy was difficult (including patients with cancer or other underlying diseases); (5) those infected with methicillin-resistant *Staphylococcus aureus* (MRSA), including suspected cases; (6) those receiving corticosteroids (prednisolone >10 mg/day); (7) those with a history of hypersensitivity to carbapenems, penicillins, or other beta-lactam antibiotics with or without beta-lactamase inhibitors; (8) those who were pregnant or lactating; (9) those with pneumonia severity index score in risk class V; and (10) those who were judged as otherwise ineligible by the attending physicians.

For the safety analysis, all randomized patients who received at least one dose of the study medication were included. Among the full analysis set (FAS), which included all subjects who received at least one dose of the study medication during this study and had a valid baseline and at least one post-baseline follow-up assessment of the primary outcome measure, all patients who completely met the inclusion and exclusion criteria with no protocol violations (per-protocol set, PPS) were included for efficacy analysis.

Study design, dosage, and administration method

This study was a multicenter, randomized, exploratory study. The patients were randomly allocated to receive either PIPC/TAZ (4.5 g) every 8 h or MEPM (0.5 g) every 8 h. Randomization by the minimization method was performed at a centralized website by attending physicians after obtaining written informed consent from each patient. Minimization factors included age and gender. The treatment period was 3–14 days in principle, but could be extended to a maximum of 21 days. Concomitant use of other antimicrobial agents was not allowed.

Evaluation

The primary endpoint of this study was clinical cure rate at the test-of-cure visit. Clinical cure was evaluated as (1) cure, which indicated continued improvement or complete resolution of the symptoms and no requirement for additional antimicrobial agents 7 days after the end of treatment (EOT); (2) failure, which indicated the treatment was