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●症 例

重症インフルエンザA（H1N1）2009 pdm肺炎の3例

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要旨：2011年1～2月にインフルエンザA（H1N1）2009 pdm重症肺炎の3例を経験した．内訳は40～45歳の男性2例と女性1例で，2例に肥満あり，2例は精神疾患治療中で1例は糖尿病であった．ワクチン接種済みは1例であった．発症から呼吸不全発現までは5～7日の経過で，鼻腔拭い迅速検査陽性は1例のみ．CT上はいずれも汎小葉性すりガラス影主体の重症肺炎で，1例は挿管管理を要した．診断は，2例で施行した咽頭拭いインフルエンザRNAの陽性，ほか1例でのペア血清AH1の有意上昇で行ったが，いずれの症例も初期の診断・治療に難渋した．

キーワード：インフルエンザA（H1N1）2009 pdm，重症肺炎，集学的治療
Influenza A (H1N1) 2009 pdm, Severe pneumonia, Combined modality therapy

緒 言

新型インフルエンザA/H1N1感染症は，我が国では2009年5月に最初の患者が確認されて以来，2009年度は累計2,061万人が外来受診をした．国立病院機構東京病院でも若年者を中心にインフルエンザ患者が増加し，インフルエンザを契機に呼吸器疾患を合併した症例を経験した[1]．2010年度は冬にインフルエンザ患者数のピークを迎え，例年の季節型インフルエンザと同時期の流行・患者数であり，また複数のウイルス型が混在していたことから，ポストパンデミック（pdm）の状態への移行が示唆された．これを受け，2010年度をもって新型インフルエンザA/H1N1は感染症法に基づく「新型インフルエンザ等感染症」とせず，インフルエンザA(H1N1) 2009 pdmと名称変更された[2]．

その一方で，インフルエンザA（H1N1）2009 pdmの重症患者が認められた．2011年初頭に国立病院機構東京病院において中年層の患者3名のインフルエンザA（H1N1）2009 pdm重症肺炎を経験したので，若干の文献的考察を加えて報告する．

症 例

【症例1】

患者：40歳，女性．無職．喫煙 なし．

主訴：咳嗽，呼吸困難．

既往歴：糖尿病．脂肪肝．統合失調症のため抗精神病薬を多数内服．

現病歴：2011年1月，入院9日前に感冒症状が出現した．2日前に38℃の発熱を認め近医を受診し，鼻腔迅速インフルエンザ検査を受けたが陰性のため，解熱薬が処方された．その頃から咳嗽・呼吸困難が出現した．入院前日夜間に症状悪化し，翌日近医にて室内気でSpO_2 65%と著明な低酸素血症が認められ，国立病院機構東京病院へ救急搬送された．当院での鼻腔迅速インフルエンザ検査も再度陰性であった．

入院時身体所見：身長157 cm，体重92 kg（BMI 37.3 kg/m^2），血圧154/77 mmHg，脈拍111回/min，呼吸数24回/min，体温37.4℃，SpO_2 90%（O_2 10 L/min吸入下）．両側下肺野でfine cracklesを聴取．皮疹・関節痛・筋肉痛認めず．四肢に浮腫軽度触知する．

検査所見：WBC 5,600/μl（Seg 79%，Band 6%，Mono 3%，Lym 12%），Hb 13.0 g/dl，Plt 10.9×10^4/μl，TP 6.6 g/dl，Alb 3.2 g/dl，T-Bil 0.43 mg/dl，AST 93 U/L，ALT 79 U/L，LDH 587 U/L，CPK 590 IU/L，BUN 8.1 mg/dl，CRE 0.56 mg/dl，CRP 19.3 mg/dl，Na 138 mEq/L，K 4.3 mEq/L，HbA1c（JDS）8.9%，KL-6 216 U/ml，SP-D 252 ng/ml，マイコプラズマPA 20倍未満，抗核

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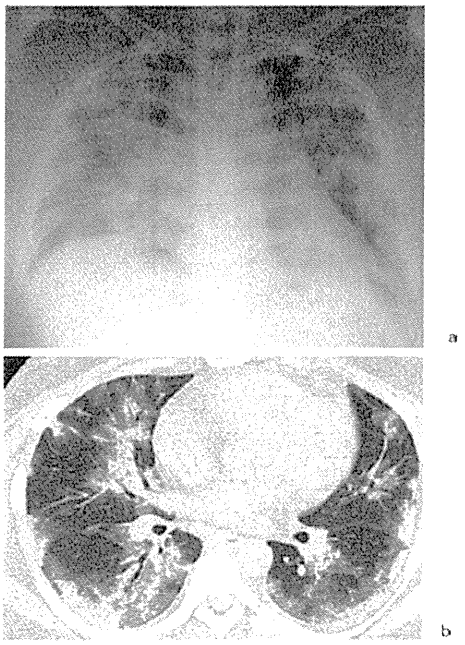

Fig. 1 (a) Chest X-ray on admission showed bilateral diffuse infiltration. (b) Chest CT showed diffuse panlobular ground glass opacity (GGO) and consolidation, along with the bronchovascular bundles.

抗体 40 倍未満，尿中肺炎球菌抗原（－），尿中レジオネラ抗原（－），鼻腔迅速インフルエンザ検査（－）．

画像所見：胸部単純 X 線写真（Fig. 1a）では両側肺びまん性すりガラス影，浸潤影を認め，胸部単純 CT（Fig. 1b）では末梢側と気管支血管束に沿った汎小葉性のすりガラス影・浸潤影を多発性に認めた．

入院後経過：経過としてインフルエンザ感染症も疑われたが，鼻腔迅速インフルエンザ検査は前医 2 回，国立病院機構東京病院で 1 回の計 3 回陰性であった．非定型肺炎，急性間質性肺炎，薬剤性肺炎が当初考慮され，入院日よりステロイドパルス療法，およびセフトリアキソン（ceftriaxone）＋シプロフロキサシン（ciprofloxacin）の投与が行われた．しかし呼吸状態は悪化し，第 3 病日に挿管・人工呼吸管理となった．第 4～5 病日に polymyxin-B direct hemoperfusion（PMX-DHP）療法を施行した．臨床的にはインフルエンザ肺炎も否定できなかったため，第 4 病日よりオセルタミビル（oseltamivir）も追加投与した．呼吸状態は PMX-DHP 療法 2 回目終了時点より徐々に改善に転じ，第 11 病日に抜管できた．その後再燃なく治癒した．ペア血清でインフルエンザ A H1 抗体価が 10 倍未満から 320 倍に上昇を認め，重症インフルエンザ肺炎と診断した．

【症例 2】

患者：41 歳，男性．職業　郵便局員．喫煙　なし．

主訴：発熱，咳嗽，呼吸困難．

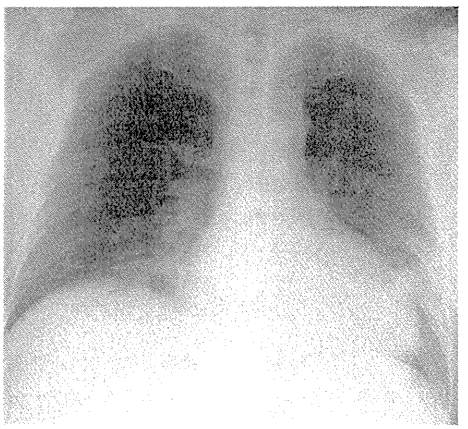

Fig. 2 Chest X-ray on admission showed dominant bilateral lower lung field GGO.

既往歴：慢性心不全（内服加療中）．

現病歴：2011 年 2 月，入院 6 日前より発熱・咳嗽が出現した．様子をみるも症状悪化し，5 日後近医を受診した．体温 40℃，SpO_2 90%（室内気），鼻腔インフルエンザ迅速検査（－）であった．肺炎の診断のもとに近医に入院したが，抗菌薬の効果は乏しく，翌日呼吸不全の急速な進行が認められ，同日国立病院機構東京病院に転院となった．

入院時身体所見：身長 178 cm，体重 83 kg（BMI 26.1 kg/m^2），血圧 140/80 mmHg，脈拍 110 回/min，呼吸数 21 回/min，体温 39.8℃，SpO_2 92%（O_2 10 L/min 吸入下）．両側下肺野で fine crackles 聴取．皮疹・関節痛・筋肉痛・末梢浮腫認めず．

検査所見：WBC 3,900/μl（Neu 88%，Mono 3%，Lym 9%），Hb 13.0 g/dl，Plt 12.8×10^4/μl，TP 6.8 g/dl，Alb 3.6 g/dl，T-Bil 0.43 mg/dl，AST 117 U/L，ALT 94 U/L，LDH 852 U/L，CPK 1,213 IU/L，BUN 22.7 mg/dl，CRE 1.13 mg/dl，CRP 15.3 mg/dl，Na 131 mEq/L，K 3.3 mEq/L，HbA1 c（JDS）6.0%，KL-6 230 U/ml，SP-D 106 ng/ml，マイコプラズマ PA 40 倍，抗核抗体 40 倍未満，尿中肺炎球菌抗原（－），尿中レジオネラ抗原（－），鼻腔迅速インフルエンザ検査（－）．

画像所見：胸部単純 X 線写真（Fig. 2）では両側肺びまん性すりガラス影を認め，胸部単純 CT では両側下葉を中心に気管支血管束に沿った汎小葉性のすりガラス影・浸潤影を多発性に認めた．

入院後経過：本症例でも鼻腔迅速インフルエンザ検査は前医 1 回，当院で 1 回の計 2 回陰性であった．しかし経過からインフルエンザ肺炎が疑われ，ペラミビル（peramivir）/パズフロキサシン（pazufloxacin）/シベレスタット（sivelestat）使用に加えステロイドパルス療法を 3 日間施行し，当初 NPPV（non-invasive positive

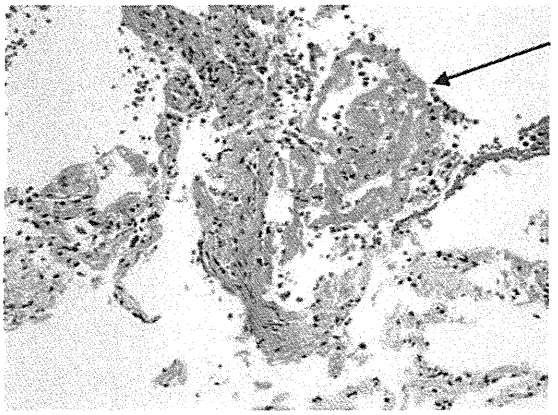

Fig. 3　The specimen of transbronchial lung biopsy (TBLB) showed diffuse thickening of the alveolar wall and hyaline membrane formation (arrow), namely, diffuse alveolar damage (DAD) (hematoxylin-eosin stain).

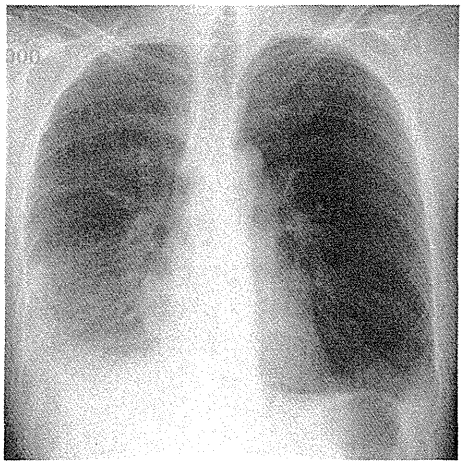

Fig. 4　Chest X-ray on admission showed bilateral GGO and consolidation.

pressure ventilation）も併用した．第3病日に入院初日に採取した咽頭拭い液におけるインフルエンザ（H1N1）2009 pdm RT-PCR 陽性と判明した．一方，入院後呼吸状態の改善は不十分であったため，第5～6病日にPMX-DHP 療法を施行，その後治癒した．本症例では第2病日に気管支鏡検査を施行し，右下葉の経気管支肺生検（Fig. 3）において胞隔のびまん性の肥厚・硝子膜形成を認めており，びまん性肺胞障害（diffuse alveolar damage: DAD）の所見であった．生検検体を用いたインフルエンザA (H1N1) 2009 pdm PCR は陽性であった．

【症例3】

患者：45歳，男性．職業　元板金屋．喫煙　20本×20年．

主訴：発熱．

既往歴：うつ病・パニック障害（内服加療中）．

現病歴：2011年1月，入院6日前に38℃の発熱が出現し，様子をみるも改善せず4日後近医を受診した．鼻腔インフルエンザ迅速キットA型陽性であり，インフルエンザと診断された．発症後時間経過していたため，抗インフルエンザ薬は投与されなかった．発熱が持続し抗菌薬の効果が乏しく，入院当日，近医での胸部単純X線写真にて両側肺炎像を認め，国立病院機構東京病院に紹介となった．

入院時身体所見：身長・体重　不測（明らかな肥満・痩せなし），血圧91/60 mmHg，脈拍106回/min，呼吸数20回/min，体温40.0℃，SpO_2 82%（室内気）．右下肺野で fine crackles 聴取．両下肢膝関節痛・筋肉把握痛あり．皮疹・末梢浮腫認めず．

検査所見：WBC 8,800/μl（Neu 79%，Mono 8%，Lym 13%），Hb 12.7g/dl，Plt 21.9×10^4/μl，TP 5.5 g/dl，Alb 2.7 g/dl，T-Bil 0.3 mg/dl，AST 136 U/L，ALT 91 U/L，LDH 635 U/L，CPK 148 IU/L，BUN 9.3 mg/dl，CRE 0.98 mg/dl，CRP 31.0 mg/dl，Na 135 mEq/L，K 5.3 mEq/L，HbA1c（JDS）5.4%，マイコプラズマPA 20倍未満，尿中肺炎球菌抗原（－），尿中レジオネラ抗原（－），鼻腔迅速インフルエンザ検査（－）．

画像所見：胸部単純X線写真（Fig. 4）では下肺野優位（右肺＞左肺）にすりガラス影・浸潤影を認め，胸部単純CTでは両側下葉を中心に胸膜直下優位の浸潤影・すりガラス陰影と小葉間隔壁の肥厚を認めた．

入院後経過：鼻腔拭い液によるインフルエンザ迅速検査は前医で陽性であったが，当院で2回施行した検査は陰性であった．インフルエンザ肺炎と細菌性肺炎の合併が疑われ，oseltamivir/ceftriaxone/ciprofloxacin を使用した．第3病日に第2病日採取の咽頭拭い液におけるインフルエンザA (H1N1) 2009 pdm RT-PCR 陽性と判明．上記治療にて改善不十分であったため，第5病日にperamivir を追加投与したところ，再燃なく治癒した．本症例では第6病日に気管支鏡検査を施行し，右下葉の経気管支肺生検において肺胞腔の滲出性・器質化病変を広範に認めた．なお，気管支肺胞洗浄液は血性で，インフルエンザ PCR は陰性であった．

今回我々が経験したインフルエンザA（H1N1）2009 pdm 重症肺炎3例の特徴を Table 1 に示す．

患者背景としては年齢40～45歳の男性2例と女性1例で，2例に肥満あり，基礎疾患として2例は精神疾患治療中で1例は糖尿病であった．ワクチン接種済みは1例，また発症から医療機関受診まで2～5日であった．

Table 1 Characteristics of the three patients

	Case 1	Case 2	Case 3
Age	40	41	45
Obesity	(+)	(+)	(−)
Psychiatric disorder	(+)	(−)	(+)
Vaccination	(−)	(−)	(+)
CT findings	diffused GGO and consolidation	lower lung dominant GGO	lower lung dominant GGO and consolidation
Diagnostic evidence	serological test	pharyngeal swabs RT-PCR TBLB specimen RT-PCR serological test	pharyngeal swabs RT-PCR serological test
Antiinfluenza drug (onset-administration days)	oseltamivir (12)	peramivir (6)	oseltamivir (7), peramivir (10)
PMX-DHP/ steroid	(+/+)	(+/+)	(−/−)
Mechanical ventilation	(+) IPPV	(+) NPPV	(−)
Outcome	cured	cured	cured

考 察

我が国の2010年9月～2011年3月上旬のインフルエンザの発生動向によると[2]，国民の12人に1人，累計1,030万人がインフルエンザで医療機関を受診し，受診者の2万人に1人が重症化したと推計された．重症者報告数は417例，平均年齢39.5歳，65歳未満の割合は76%であり，前年度より高年齢にシフトしたものの，依然比較的若年層に多かった．15歳以上に絞ると，何らかの基礎疾患をもつ患者が76%であった．また，海外では高度肥満を重症化因子とする報告が多い[3][4]．年齢層・基礎疾患や肥満の有無に関しては，当院症例においても重症例の特徴が少なからず認められた．

本症例におけるインフルエンザの診断はペア血清，咽頭拭い液インフルエンザRT-PCR，肺組織PCR検査によってなされた．当院における鼻腔拭い液迅速診断キット検査（ラピッドテスタ®FLUスティック使用）は3症例計4回すべて陰性であった．インフルエンザ迅速診断キットのインフルエンザA（H1N1）2009 pdm感染症における感度は報告によりばらつきがあるが，2009年のCDCの報告では40～69%[5]と低い．また，使用キット間による感度差の報告もあり，検出原理や抽出法の違いが指摘されている[6]．当院症例はいずれも発症から検体採取までの期間に開きがあること，検体採取部位がいずれも鼻腔であったことが，感度の低さにかかわっていた可能性がある．重症例においては，より感度が良いといわれる気管支洗浄液等の下気道検体を用いた診断も考慮される．また，インフルエンザA（H1N1）2009 pdmを従来のインフルエンザA型と分離して診断可能な迅速抗原キット（クリアライン®）や，迅速RT-PCRキット（新型インフルエンザA（H1N1）Real-Time Detection kit®）も今後使用が検討される．

Bautistaら[3]，Gomezら[7]は，本疾患の画像所見に関して，単純X線写真ではびまん性の間質性あるいは肺胞性陰影の混合，単葉性・多葉性の分布を示し，CTでは，多発性すりガラス陰影や肺胞浸潤影と気管支透亮像など多彩であると報告しており，細菌性肺炎の合併も相まって画像所見は複雑である．しかしながら，ウイルス性肺炎の画像所見としては，末梢の多発性すりガラス陰影，気管支血管周囲のすりガラス陰影が特徴的である[8][9]という指摘があり，当院の症例でも同様の所見が認められる．

当院症例における経気管支肺生検所見は症例2でDAD，症例3は器質化肺炎の所見を呈していた．本疾患の病理所見としては，DAD，胞隔肥厚，気管気管支炎，壊死性細気管支炎，肺胞出血が観察されるとされており[10]，症例2はこれに合致していた．症例3は非典型的であったが，治療にて改善してきた時点の生検であり，治癒過程にある所見と判断した．

治療では，今回の症例はいずれも発症日から抗インフルエンザ薬投与まで6～12日経過しており，oseltamivirやperamivirが使用されていた．抗インフルエンザ薬開始の遅れは重症化と死亡率上昇に関与するといわれている[11][12]．Kelvinらによれば[13]，インフルエンザA（H1N1）2009 pdm感染症で入院した74人の患者のなかで，ARDSを呈した重症者37人と軽症者37人で臨床的特徴を比較したところ，重症者においては症状発現から入院までの日数が長く，高サイトカイン血症を呈し，抗インフルエンザ薬使用によるウイルス量低下が遅れたことを報告している．本症例でも医療機関受診の遅れと診断困難により抗インフルエンザ薬の開始が遅れ，重症化したことが示唆された．また，インフルエンザA（H1N1）2009 pdmにおいてoseltamivir耐性は1%程度との報告がある[14]．今回症例2，3においてperamivirが使用されたが，peramivirは経静脈的に投与可能であり，内服

困難な症例やoseltamivir耐性が疑われる症例での投与が考慮される[15]．重症例ではステロイド投与・PMX-DHP療法が使用された．インフルエンザA（H1N1）2009 pdm重症肺炎におけるステロイド治療に関しては見解が割れており[16][17]，またPMX-DHP療法の有用性に関する報告も現時点では少ないが[18]，いずれも炎症性サイトカインの過剰発現による「サイトカインストーム」状態の制御による病態改善が期待される．今回自験例2例において上記使用後の病態改善が認められ，重症呼吸不全を呈する例において，早期に，集学的治療の一環として考慮される治療手段と思われた．

今回の3症例は発症から治療開始までに時間を要しており，インフルエンザ流行期では，インフルエンザの早期受診の啓蒙と，重症肺炎症例では鼻腔拭い液迅速診断キット検査が陰性の場合でも，臨床的にインフルエンザ肺炎が疑われる場合は早期に抗インフルエンザ薬による治療を開始し，集学的治療を行うことが肝要と考えられた．

謝辞：本稿を終えるにあたり，本症例の病理診断で貴重な助言をいただいた国立感染症研究所感染病理部　佐多徹太郎先生に深謝いたします．

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Abstract

Three cases of influenza A (H1N1) 2009 pdm severe pneumonia

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In January and February 2011, we experienced on 3 cases of severe pneumonia caused by influenza A (H1N1) 2009 pdm. The patients were 2 males and 1 female: 2 were obese, 2 with psychiatric disorders, 1 was diabetic, and 1 had been vaccinated for the influenza virus. The disease progressed in the 5 to 7 days from the onset of symptoms to admission with acute respiratory failure. Although nasal swab rapid-diagnostic tests were negative except for one patient, the final diagnosis of the other pneumonia was later made. A detection of the other RNA in two cases and with elevation of anti-H1 antibody titer by paired serum in the other. HRCT images demonstrated panlobular ground glass opacities in all cases. A transbronchial lung biopsy, performed in two cases, detected diffuse alveolar damage in one case and organizing pneumonia in the other. As for treatment, along with oseltamivir and/or peramivir applied as antiviral drugs, steroid pulse therapy and/or polymyxin-B direct hemoperfusion (PMX-DHP) therapy were applied in two cases. One patient was intubated and mechanically ventilated, and the other was noninvasively ventilated. All cases were fully recovered and discharged, although we experienced difficulty in initial diagnosis and treatment.

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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.

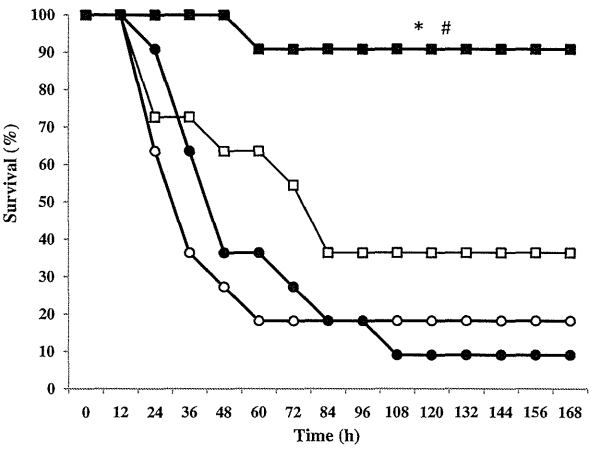

Fig. 1. Effect of biapenem and ME1071 therapy on the survival of mice in a ventilator-associated pneumonia (VAP) mouse model. Eleven mice in each group were treated with biapenem plus ME1071 (■), biapenem alone (□), ME1071 alone (●) (each 100 mg/kg) or saline solution (○). Survival was estimated at the 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).

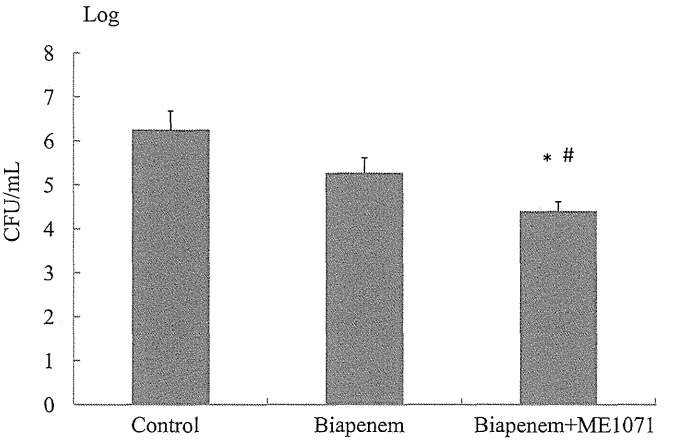

Fig. 2. Effect of biapenem monotherapy and biapenem plus ME1071 combination therapy on the number of viable bacteria in the lungs of a ventilator-associated pneumonia (VAP) mouse model. The number of viable bacteria in the lungs was calculated as CFU/mL (n = 6–7 in each group). $^{\#}P < 0.01$ versus 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.

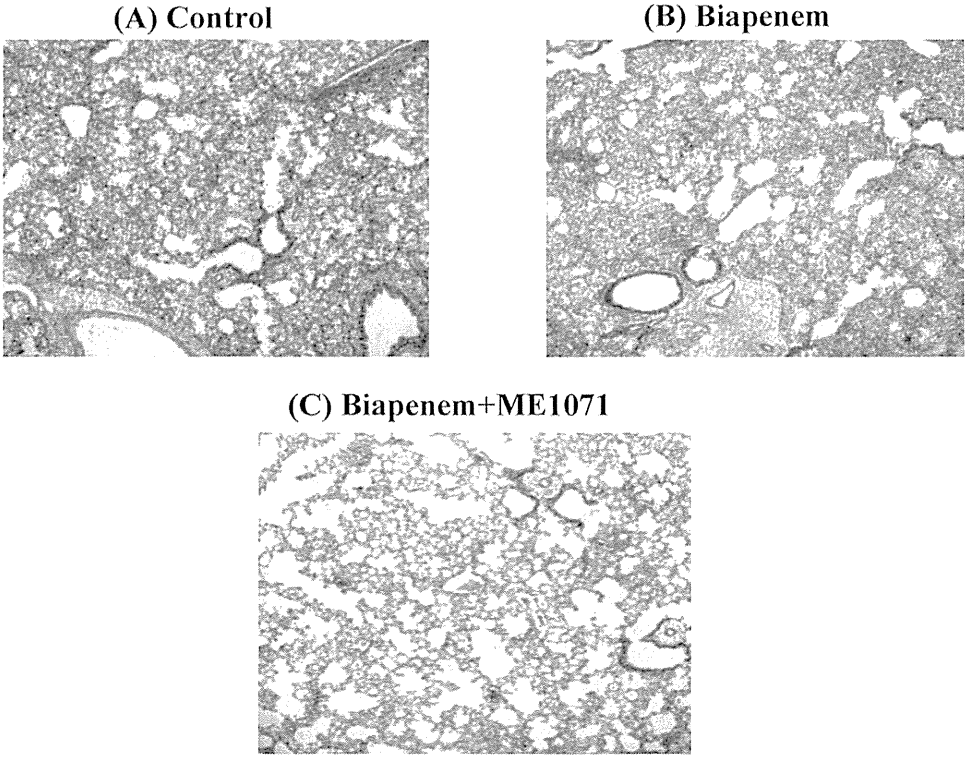

Fig. 3. Histochemical analysis of the lungs of infected mice treated with biapenem monotherapy and biapenem plus ME1071 combination therapy. Original magnification (40×; haematoxylin–eosin) of the lung at 30 h post infection. Representative photomicrographs of the lung tissues of the (A) control group,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

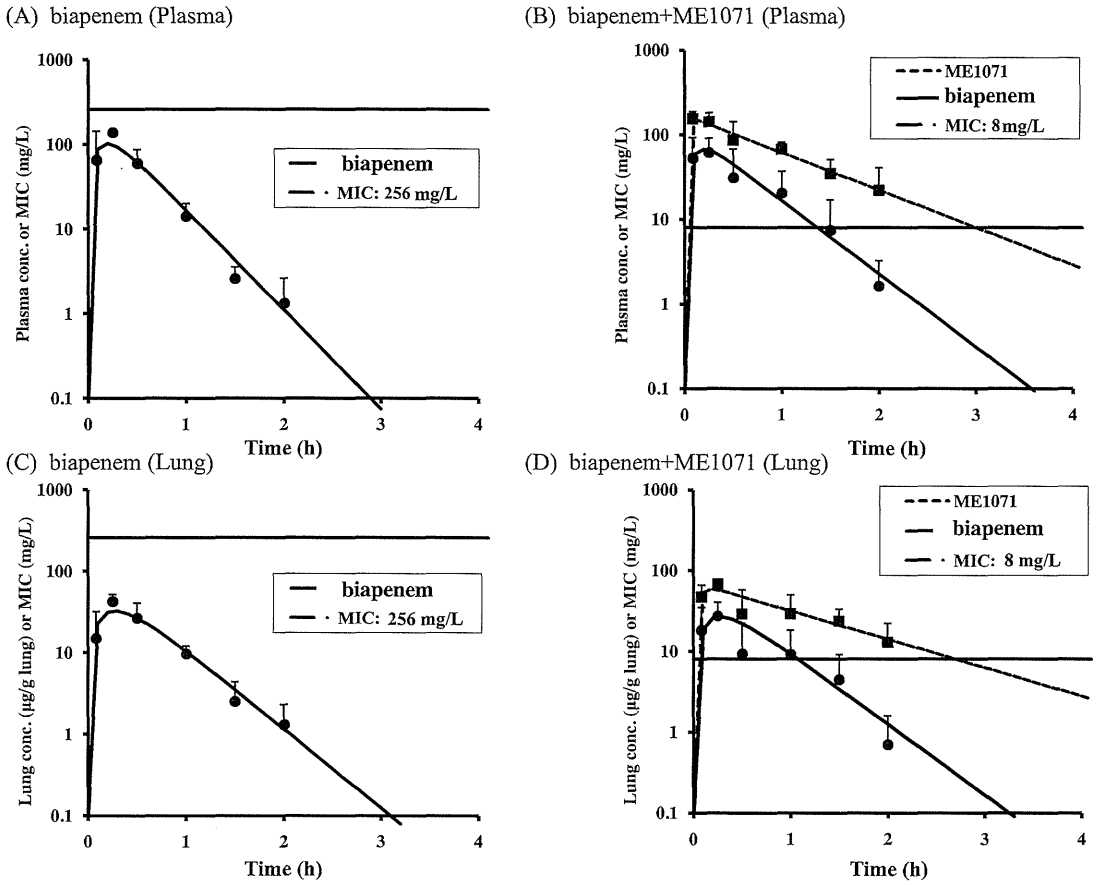

Fig. 4. Pharmacokinetics of biapenem and ME1071 in the plasma and lungs of infected mice. Pharmacokinetics of biapenem and ME1071 in plasma (A and B) or the lungs (C and D) of biapenem-treated (A and C) or biapenem + ME1071-treated (B and D) mice are shown. Each point represents the mean ± standard error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

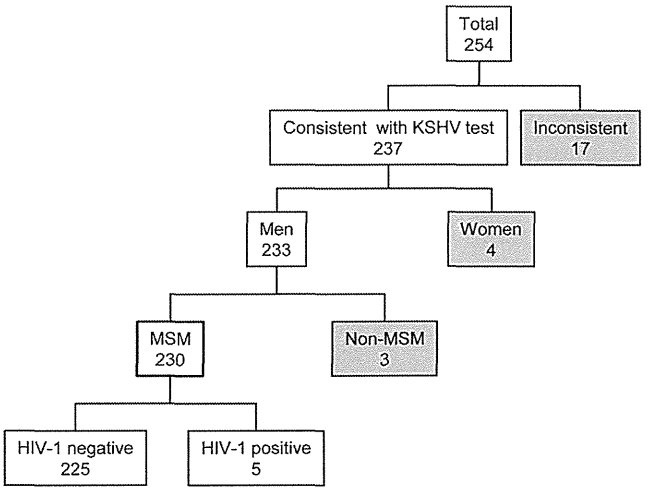

Fig. 1. Study flow diagram. Of the 257 individuals attending the free and anonymous HIV-1 test clinic, 237 agreed to participate in the study. According to the participants' responses to a questionnaire, three men who described themselves as heterosexuals and four women were excluded. Thus, 230 men who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

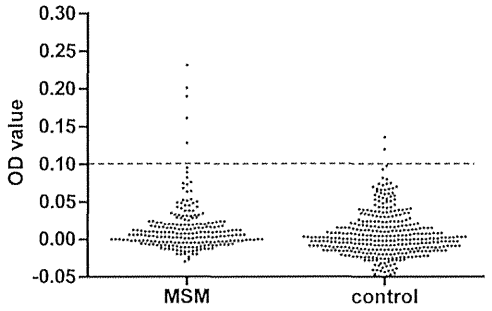

Fig. 2. Group scatter diagrams for enzyme-linked immunosorbent assay (ELISA) results. The scatter diagrams show the results of reactions of sera from men who have sex with men and controls in the mixed Kaposi's sarcoma-associated herpesvirus antigen ELISA. Optical density (OD) values were calculated 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

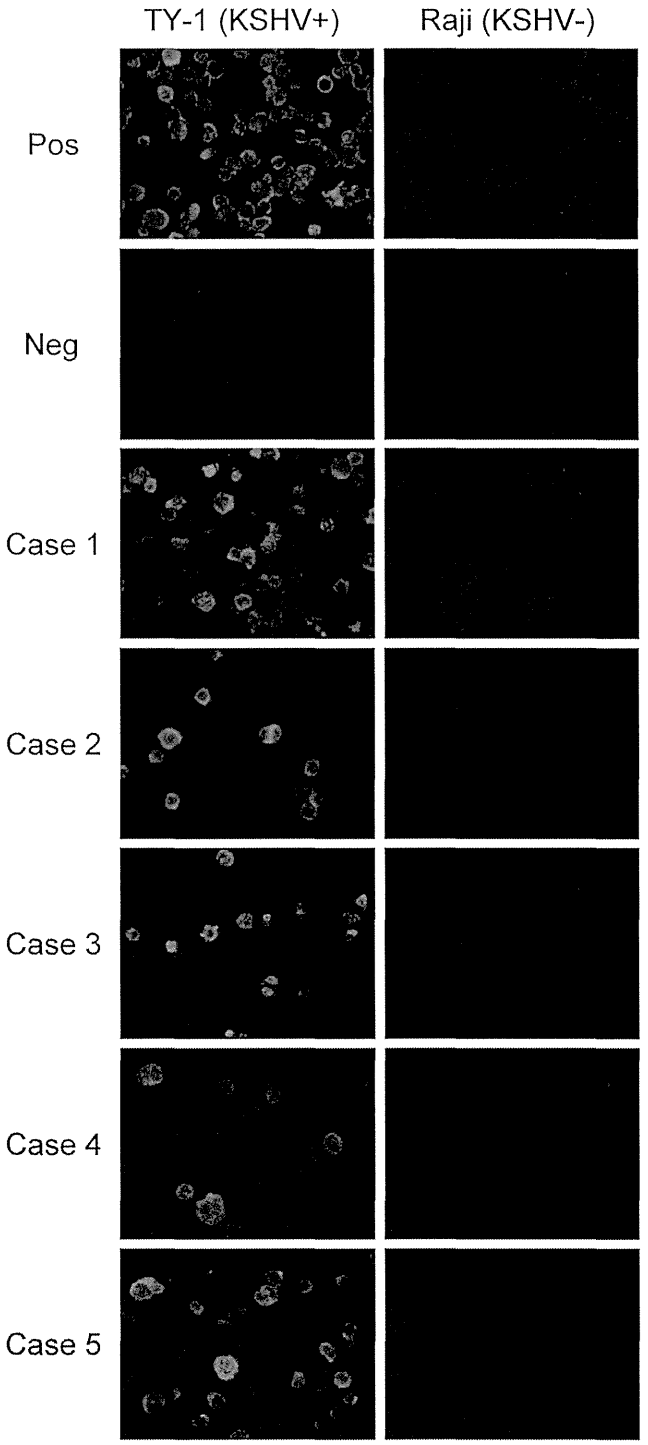

Fig. 3. Immunofluorescence images for Kaposi's sarcoma-associated herpesvirus (KSHV) immunofluorescence assay (IFA). Five positive samples from men who have sex with men are shown. The positive sera reacted with antigens in TY-1 (KSHV-positive, Epstein-Barr virus-negative lymphoma cell line), but not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