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Molecular characterization of carbapenemase-producing clinical isolates of *Enterobacteriaceae* in a teaching hospital, Japan

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We examined the molecular characteristics of 13 phenotypically confirmed carbapenemase-positive *Enterobacteriaceae* clinical isolates, including the relationships between plasmid-mediated quinolone-resistance genes (*qnr*), 6′-*N*-aminoglycoside acetyltransferase-encoding genes [*aac(6′)*] and AmpC-encoding genes (pAmpC). Twelve isolates were bla_{IMP-1} positive (92.3 %), while one was bla_{IMP-11} positive (7.7 %). We detected *qnr*, *aac(6′)* and pAmpC genes designated bla_{ACT-1}-like in 76.9 %, 100 % and 53.8 %, respectively, of the 13 isolates. Plasmids were transferred successfully for three of the 13 metallo-β-lactamase (MBL)-producing isolates, and the sizes of plasmids extracted from these donors and transconjugants were deduced to be 65 kb or 70 kb. OmpC or OmpF protein expression was reduced in all *Enterobacter cloacae*, and one *Klebsiella oxytoca* lacked OmpK36. We demonstrate what appears to be the first evidence that, in Japan, *Enterobacteriaceae* producing MBLs carry various plasmid-mediated resistance genes, which may cause a further decrease in carbapenem susceptibility through reduction of the expression of outer-membrane proteins.

INTRODUCTION

In *Enterobacteriaceae*-related infections, resistance to carbapenems, which are used to treat multidrug-resistant bacterial infections, is an increasingly serious worldwide problem (Hawkey & Jones, 2009). Carbapenem resistance involves production of acquired carbapenemases [metallo-β-lactamases (MBLs) such as IMP, VIM and NDM-1, and OXA- or KPC-related non-metallo-carbapenemases], AmpC β-lactamase hyperexpression, reduced permeation of antimicrobials due to loss of outer-membrane proteins (OMPs), and overexpression of the efflux pump (Hawkey & Jones, 2009; Kumarasamy *et al.*, 2010). Carbapenemase genes are mainly contained in integrons with resistance genes against various antibiotics, including quinolone or aminoglycosides. To characterize the molecular mechanisms of carbapenem-resistant *Enterobacteriaceae* in Japan, we investigated molecular characteristics of such clinical isolates, including the relative contributions of impermeability and the efflux system. We also examined genetic relationships between plasmid-mediated quinolone resistance (PMQR) genes, 6′-*N*-aminoglycoside acetyltransferase [AAC(6′)]-encoding genes and plasmid-mediated AmpC-encoding (pAmpC) genes.

Abbreviations: EPI, efflux pump inhibitor; MBL, metallo-β-lactamase; PMQR, plasmid-mediated quinolone resistance.

METHODS

Bacterial strains. We examined 13 phenotypically confirmed carbapenemase-positive isolates of 29 clinical isolates with imipenem (IPM) or meropenem (MEM) MICs $\geqslant 2$ mg l^{-1} among 1704 *Enterobacteriaceae* clinical isolates collected at the University of Tokyo Hospital (Tokyo, Japan), a 1210-bed teaching hospital, between April 2007 and March 2009. These isolates comprised 10 *Enterobacter cloacae*, one *Klebsiella oxytoca*, one *Citrobacter freundii* and one *Serratia marcescens*, obtained from various biological samples (sputum, throat swab, urine, wound, bile and venous catheter).

Susceptibility testing. Carbapenemase production was screened by the modified Hodge test (CLSI, 2011). MBL and extended-spectrum β-lactamase production was confirmed by the double-disc synergy test (Arakawa *et al.*, 2000; CLSI, 2011) using mercaptoacetic acid and clavulanic acid, respectively, as β-lactamase inhibitors. Class C β-lactamase production was confirmed as described previously (Yagi *et al.*, 2005). MICs of antibiotics were determined by the MicroScan WalkAway system (WA; Siemens Healthcare Diagnostics) and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2011). Etest (AB BIODISK) was also used to determine the MICs of IPM and MPM and to detect small decreases in levofloxacin (LVX) susceptibility. Quality control for the MICs was performed using the reference strains *Staphylococcus aureus* ATCC 21293, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

PCR and sequencing. PCR amplification was performed with Premix Ex*Taq* enzyme (Takara Bio) according to the manufacturer's

instructions. Both strands of purified PCR fragments were sequenced with an ABI PRISM 3100 DNA sequencer (Applied Biosystems) and a similarity search was conducted using the BLAST program (DDBJ).

Detection of carbapenemase genes, PMQR genes, AAC(6′)-encoding genes and pAmpC genes. The presence of carbapenemase genes (bla_{IMP}, bla_{VIM} and bla_{KPC}), PMQR genes [*qnrA*, *qnrB*, *qnrS* and *aac(6′)-Ib-cr*], AAC(6′)-encoding genes [*aac(6′)-Ib* and *-IIc*] and pAmpC genes was determined using published primer sequences in the 13 phenotypically confirmed carbapenemase-positive isolates (Ode *et al.*, 2009; Pérez-Pérez & Hanson, 2002). The genetic diversity of isolates possessing MBL genes was analysed by repetitive element sequence-based PCR (Rep-PCR) (Shannon & French, 2004). BioNumerics software (version 4.0, Applied Maths) was used to analyse the DNA patterns and determine their similarity.

Conjugation experiments, extraction of plasmids, structure analysis of gene cassettes and Southern hybridization experiments. Conjugation experiments were performed as described previously (Ode *et al.*, 2009). Whole plasmid DNA was extracted from donors and transconjugants by alkaline lysis miniprep. The content and order of the gene cassettes inserted between the 5′-conserved segment (CS) and 3′-CS were determined as described previously (Ode *et al.*, 2009). Southern hybridization experiments were performed using the DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche Diagnostics).

Analysis of outer-membrane proteins (OMPs). OMPs were isolated by the rapid procedure of Carlone *et al.* (1986). Concentrations of OMPs were determined by BCA protein assay kit (Thermo Fisher Scientific). OMPs (20 μg) were resolved using 12.5 % (*E. cloacae*) or 10 % SDS-PAGE (*K. oxytoca*, *C. freundii* and *S. marcescens*) and stained with Coomassie blue. OMP profiles were compared to those of the relevant IPM-susceptible strains. The band intensities of the OMPs were analysed using Image J (NIH).

Effects of efflux pump inhibitor. Susceptibility testing of IPM and MEM was performed by microbroth dilution with and without the efflux pump inhibitor (EPI) L-phenylalanine-arginine-*N*-naphthylamide (Sigma-Aldrich) as described previously (Kumita *et al.*, 2009). In comparison to MICs with and without the EPI, a twofold or greater decrease in MICs for each antibiotic indicated a significant change. Effects of the EPI were determined in triplicate.

RESULTS AND DISCUSSION

In the present study, the IPM or MEM MICs were ⩾2 mg l^{-1} for 29 isolates (1.7 %), and 13 isolates (0.8 %) were carbapenemase producers; this result was also phenotypically confirmed. Among these isolates, none produced extended-spectrum β-lactamases. Twelve (92.3 %) of the 13 carbapenemase-producing *Enterobacteriaceae* clinical isolates (10 *E. cloacae*, one *K. oxytoca* and one *C. freundii*) were bla_{IMP-1} positive. One (7.7 %) bla_{IMP-11}-positive *S. marcescens* isolate was also detected, similar to Takahashi *et al.* (2010), but no bla_{KPC}. Rep-PCR revealed four identical banding-patterns in the bla_{IMP-1}-positive *E. cloacae*, indicating the existence of four clonal genotypes (group I, E9 and E13; group II, E17; group III, E19, E20, E25, E26 and E29; and group IV, E22 and E23). MBL-positive *Enterobacteriaceae* isolates reportedly do not confer high-level resistance to IPM (Hirakata *et al.*, 1998). Since the IPM and MEM MICs found for three MBL-producing isolates were susceptible or intermediate with respect to interpretive criteria (MIC ⩽2 mg l^{-1}), our results also demonstrated the need to ascertain MBL status in *Enterobacteriaceae* isolates with such IPM MICs. Moreover, a previous study has shown that the carbapenem resistance rate of *Enterobacteriaceae* was approximately 1.2 %, and that the IMP-type MBL is the most commonly detected among MBL-producing *Enterobacteriaceae* in Japan (Nishio *et al.*, 2004). Although we tested only the carbapenemase genes bla_{IMP}, bla_{VIM} and bla_{KPC}, our finding was also in agreement with that of this previous study. However, we did not investigate whether the other carbapenemase genes, including bla_{NDM-1}, were present in the 29 clinical isolates with MIC ⩾2 mg l^{-1} for IPM or MEM.

Interestingly, *qnr* and *aac(6′)* genes were detected in 76.9 % and 100 % of the 13 isolates, respectively (Table 1). The pAmpC gene designated bla_{ACT-1}-like was detected in only seven *E. cloacae* (53.8 %); three *E. cloacae* (E17, E22 and E23) isolates did not possess this gene. In the bla_{IMP-1} cassette, *aac(6′)-IIc* was downstream of bla_{IMP-1} in nine *E. cloacae*, one *K. oxytoca* and one *C. freundii* (Table 1). This result was similar to that of a previous study (Cagnacci *et al.*, 2008). One *E. cloacae* (E17) had the cassette arrangement bla_{IMP-1}-*aac(6′)-Ib*, but the bla_{IMP-11} cassette array in one *S. marcescens* did not contain *aac(6′)* genes. Therefore, while the presence of genes encoding aminoglycoside resistance has not yet been fully examined, our results suggest a close genetic association between quinolone resistance and aminoglycoside resistance in clinical isolates of IMP-1-type MBL-producing *Enterobacteriaceae*.

Plasmids were transferred successfully for three of 13 MBL-producing isolates (K4, S7 and E19; Table 1). The sizes of

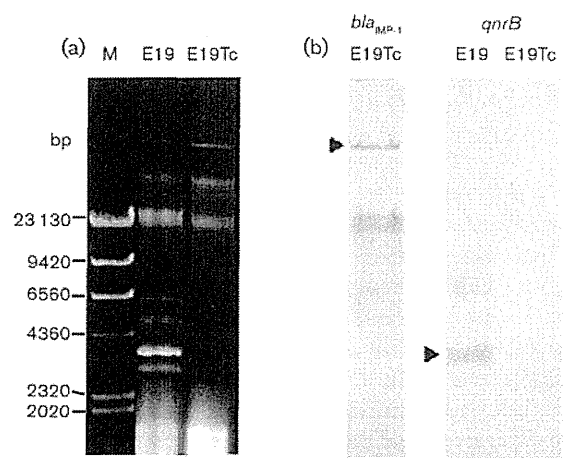

Fig. 1. Plasmid profiles and Southern hybridization analysis. Plasmids harbouring bla_{IMP-1} and/or *qnrB* in *E. cloacae* strains were separated by conventional electrophoresis on a 0.7 % agarose gel (a), and the corresponding Southern blot membranes hybridized with either bla_{IMP-1} or *qnrB* (b). Lanes: M, molecular mass standards; E19 and E19Tc, *E. cloacae* E19 and its transconjugant strain, respectively.

Table 1. Profiles of MBL-producing strains in this study

Strain	Genotype of:				Plasmid (kb)	Cassette array	MIC (mg l^{-1})*						Rep-PCR type	OMP†
	MBL	*qnr*	*aac(6′)*	pAmpC			IPM	MEM	AMK	GM	LVFX	CTX		
Enterobacter cloacae														
E9	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	bla_{ACT-1}-like	ND	[bla_{IMP-1}-*aac(6′)-IIc*]	4	2	⩽2	2	>4	>64	I	$OmpF^+$, $OmpC^-$
E13	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	bla_{ACT-1}-like	ND	[bla_{IMP-1}-*aac(6′)-IIc*]	1	1	⩽2	2	4	>64	I	$OmpF^+$, $OmpC^-$
E17	bla_{IPM-1}	*qnrA1*	*aac(6′)-Ib*	ND	65, 50, 6, 3.5	[bla_{IMP-1}-*aac(6′)-Ib*]	32	32	4	>8	4	>64	II	$OmpF^+$, $OmpC^{\pm}$
E19	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	bla_{ACT-1}-like	65, 45, 5.5, 5, 4.2, 3.5	[bla_{IMP-1}-*aac(6′)-IIc*]	4	4	⩽2	2	>4	>64	III	$OmpF^{\pm}$, $OmpC^+$
E20	bla_{IPM-1}	ND	*aac(6′)-IIc*	bla_{ACT-1}-like	5.5, 5, 4.2, 3.5	[bla_{IMP-1}-*aac(6′)-IIc*]	4	4	⩽2	⩽1	2	>64	III	$OmpF^{\pm}$, $OmpC^+$
E22	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	ND	3.5, 2.2	[bla_{IMP-1}-*aac(6′)-IIc*]	4	2	⩽2	2	2	>64	IV	$OmpF^+$, $OmpC^-$
E23	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	ND	3.5, 2.2	[bla_{IMP-1}-*aac(6′)-IIc*]	1	0.75	⩽2	2	0.38	>64	IV	$OmpF^+$, $OmpC^-$
E25	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	bla_{ACT-1}-like	ND	[bla_{IMP-1}-*aac(6′)-IIc*]	2	2	⩽2	2	>4	>64	III	$OmpF^{\pm}$, $OmpC^+$
E26	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	bla_{ACT-1}-like	5, 3.5, 2.5	[bla_{IMP-1}-*aac(6′)-IIc*]	2	4	⩽2	2	>4	>64	III	$OmpF^{\pm}$, $OmpC^+$
E29	bla_{IPM-1}	*qnrB6*	*aac(6′)-IIc*	bla_{ACT-1}-like	65, 5, 3.5, 2.5	[bla_{IMP-1}-*aac(6′)-IIc*]	4	1	⩽2	⩽1	>4	>64	III	$OmpF^{\pm}$, $OmpC^+$
Klebsiella oxytoca														
K4	bla_{IPM-1}	ND	*aac(6′)-IIc*	ND	65, 45, 3.5, 2.5	[bla_{IMP-1}-*aac(6′)-IIc*]	2	4	⩽2	⩽1	0.094	32	NT	$OmpK35^+$, $OmpK36^-$
Serratia marcescens														
S7	bla_{IMP-11}	ND	ND	ND	70	[bla_{IMP-11}]	4	2	4	⩽1	0.75	32	NT	$OmpF^+$
Citrobacter freundii														
C11	bla_{IPM-1}	*qnrA1*	*aac(6′)-Ib, IIc*	ND	ND	[bla_{IMP-1}-*aac(6′)-IIc*]	4	4	>16	>8	>4	>64	NT	38 kDa^+, 41 kDa^+
E. coli C600 (recipient)	ND	ND	ND	ND	ND	NT	0.25	0.16	⩽2	⩽1	0.032	⩽1	NT	NT
Transconjugants														
E19TC	bla_{IPM-1}	ND	*aac(6′)-IIc*	ND	65, 45	[bla_{IMP-1}-*aac(6′)-IIc*]	0.75	0.5	⩽2	4	0.032	32	NT	NT
K4TC	bla_{IPM-1}	ND	*aac(6′)-IIc*	ND	65, 45	[bla_{IMP-1}-*aac(6′)-IIc*]	1	0.5	⩽2	⩽1	0.032	32	NT	NT
S7TC	bla_{IMP-11}	ND	ND	ND	70	[bla_{IMP-11}]	2	2	⩽2	⩽1	0.032	32	NT	NT

ND, Not detected; NT, not tested.

*IPM, imipenem; MEM, meropenem; AMK, amikacin; GM, gentamicin; LVX, levofloxacin; CTX, cefotaxime.

†−, Loss of OMP expression; +, no reduction of OMP expression; ±, reduction of OMP expression.

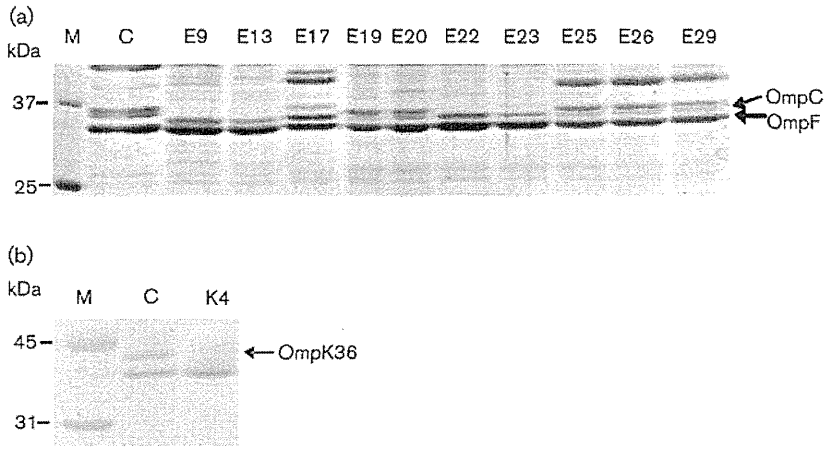

Fig. 2. SDS-PAGE analysis of OMPs in *E. cloacae* (a) and *K. oxytoca* (b). Lanes: M, size markers; C, control (relevant IPM-susceptible strain); other lanes labelled with strain number.

plasmids extracted from these donors and transconjugants (K4Tc, S7Tc and E19Tc) were deduced to be 65 kb (K4, E19, K4Tc and E19Tc) or 70 kb (S7 and S7Tc). Since K4Tc, S7Tc and E19Tc contained the same cassettes on each plasmid as K4, S7 and E19, respectively, our results also suggested that bla_{IMP}-harbouring plasmids in donors were able to transfer to transconjugants, as described previously (Ode *et al.*, 2009). On the other hand, since *qnrB* from E19 was present on plasmids other than those bearing bla_{IMP-1}, it was not transferred to E19Tc (Table 1, Fig. 1). Moreover, as the IPM and MEM MICs for transconjugants were lower than those for donors, factors other than MBL may increase carbapenem resistance. Therefore, to investigate further known carbapenem-resistance mechanisms, we assessed antimicrobial permeability, due to loss of OMPs, and overexpression of the efflux pump.

A lack of major porins (OmpK35 and OmpK36 in *Klebsiella* spp., OmpC and OmpF in *Enterobacter* spp., 41 and 38 kDa porins in *C. freundii*, and OmpF in *S. marcescens*) has been associated with carbapenem resistance (Doumith *et al.*, 2009; Suh *et al.*, 2010; Zhang *et al.*, 2008). Although the method in this study was subjective, OmpC or OmpF protein expression was reduced or deficient in all *E. cloacae* isolates (Table 1, Fig. 2a), while OmpK36 was absent in one *K. oxytoca* isolate, in comparison to the IPM-susceptible strains (Table 1, Fig. 2b). On the other hand, no reduction in OMP expression was observed in *C. freundii* or *S. marcescens* (data not shown). Moreover, the MICs of IPM and MEM did not differ by more than fourfold between isolates with and without EPI. Therefore, reduced expression or lack of OMPs may also contribute to decreased carbapenem susceptibility in *E. cloacae* and *K. oxytoca* isolates. However, the IPM and MEM MICs for isolate E17 with reduced OmpC expression were much higher than those of other strains. Although we could not clarify these results in the present study, they suggest the possibility that the high-level resistance to IPM and MEM in E17 may be related to reduced expression or lack of porin-like proteins other than OmpC and OmpF, or to the presence of an efflux pump which is not inhibited by the EPI used in this study.

Although few isolates were examined, we have demonstrated that IMP-type MBL-producing *Enterobacteriaceae* with low-level IPM MICs frequently harboured various plasmid-mediated resistance genes. Furthermore, deficient OMP expression may further decrease carbapenem susceptibility in *E. cloacae* and *K. oxytoca*. This is the first study, to our knowledge, to demonstrate that both IMP-type MBL production and reduced expression or deficiency of OMPs may lead to acquisition of carbapenem resistance in such clinical isolates in Japan.

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INTERNAL MEDICINE

□ CASE REPORT □

Dengue Hemorrhagic Fever in an Adult Traveler Returning to Japan

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Abstract

We report the case of a Japanese traveler who developed dengue hemorrhagic fever (DHF) with a probable secondary infection with dengue virus type 2 (DENV-2). DHF usually occurs in children, and rarely in adult travelers. Proper and timely interventions can markedly reduce the mortality rate of DHF patients. The expansion of endemic areas and increased frequency of travel to these areas may suggest increased incidence of DHF in non-endemic areas in the near future. Early recognition of reinfection with dengue virus and warning signs of circulatory failure are crucial to prevent a severe shock state.

Key words: dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), plasma leakage phase, antibody-dependent enhancement

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Introduction

Dengue virus (DENV) causes dengue fever (DF), characterized by the sudden onset of fever and nonspecific symptoms such as headache, myalgia, arthralgia, rash, and gastrointestinal symptoms, together with characteristic laboratory findings such as leukopenia, thrombocytopenia, and elevated levels of transaminase (1, 2). Cutaneous petechiae and mucosal hemorrhage such as epistaxis, gingival bleeding, and gastrointestinal bleeding may also occur in some cases. Dengue usually goes into spontaneous remission after approximately 1 week, whereas this disease can exhibit a wide range of clinical presentations—from asymptomatic or uncomplicated DF to severe manifestations known as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (3).

DENV, which is transmitted by *Aedes* mosquitoes (4), is a positive-sense, single-stranded RNA virus belonging to the genus *Flavivirus* of the family *Flaviviridae*, and has four serotypes: dengue virus types 1, 2, 3 and 4 (DENV-1, DENV-2, DENV-3 and DENV-4) (5). The regions in which the virus is endemic have extended from rural districts to urban areas, and the incidence of DF has been increasing rapidly over the past few decades owing to lifestyle changes after World War II and the development of transportation. Currently, the disease is endemic in more than 100 countries and regions, many of which are located in Southeast Asia, and approximately 50-100 million cases of DF occur worldwide each year (1).

DHF/DSS are usually seen in children of endemic areas (6) and rarely in adults, especially in adult travelers (3). However, a secondary infection, particularly with the Asian-genotype strain of DENV-2 (7, 8), may increase the risk of DHF/DSS even in adults (5). Here, we report a case of DHF in an adult traveler, who had repeatedly traveled to Southeast Asia, with a probable secondary DENV-2 infection. Although a revised dengue classification has recently been proposed by World Health Organization (WHO) and is now under evaluation (9), we mainly used the traditional classification (2) that allows comparison with the numerous previous reports.

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Table 1. **Laboratory Data on Admission**

Peripheral blood		Blood chemistry	
WBC	2.3×10^3 /μL	Alb	3.8 g/dL
Neutrophil	79.0%	AST	66 IU/L
Eosinophil	0.0%	ALT	31 IU/L
Basophil	1.0%	T-Bil	0.6 mg/dL
Monocyte	6.0%	BUN	19.7 mg/dL
Lymphocyte	14.0%	Cre	1.03 mg/dL
Others	0.0%	CRP	1.98 mg/dL
RBC	478×10^4 /μL		
Hb	14.2 g/dL	Coagulation test	
Ht	42.4%	PT-INR	1.29
Plt	8.3×10^4 /μL	aPTT	48.0 s
		Fbg	311 mg/dL

RBC, red blood cell; Ht, hematocrit; Plt, platelet; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin; BUN, blood urea nitrogen; Cre, creatinine; CRP, C-reactive protein; PT-INR, prothrombin time-international normalized ratio; aPTT, activated partial thromboplastin time; Fbg, fibrinogen

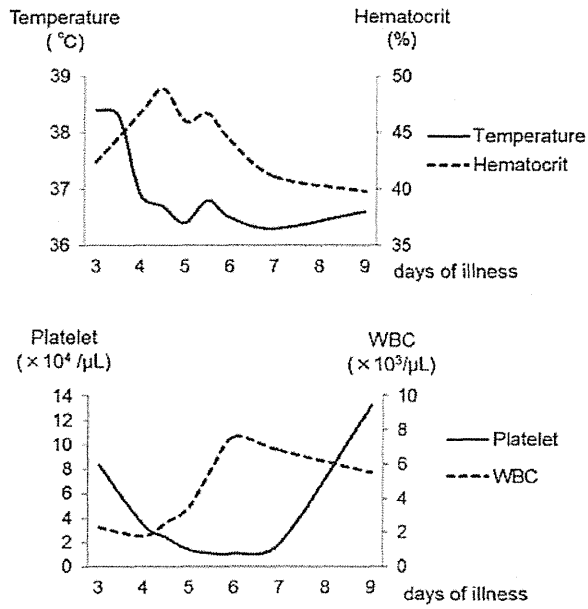

Figure 1. Clinical course of dengue illness in the present patient.

Case Report

A 51-year-old man presented with a two-day history of a fever of 39.5℃, maculopapular rashes, diarrhea, malaise, nausea, and loss of appetite. He had stayed in Myanmar mainly in rural villages for 10 days, and the fever appeared just on returning to Japan. He had repeatedly traveled to stay in Myanmar for research, and had previously been diagnosed with DENV infection.

Blood examinations (Table 1) revealed bicytopenia (white blood cell count, 2,300/μL; platelet count, 8.3×10^4/μL), abnormal liver function (aspartate aminotransferase level, 66 IU/L; alanine aminotransferase level, 31 IU/L), coagulopathy (prothrombin time-international normalized ratio, 1.29; activated partial thromboplastin time, 48.0 s), and hypoalbuminemia (albumin level, 3.8 g/dL).

DENV-2 genome was detected in the blood and urine samples by reverse-transcription polymerase chain reaction assay. Anti-DENV IgM antibodies were negative (index 0.62), but anti-DENV IgG antibodies were positive (index 1.98) by an enzyme-linked immunosorbent assay (ELISA) at 3 days after the onset of illness. No other pathogens such as malaria, influenza virus were detected and bacterial blood cultures were negative.

His blood pressure was 100-110/75-80 mmHg with a heart rate of 90 beats per minute, but preshock-like symptoms such as momentary loss of consciousness were observed prior to hospitalization; therefore, intravenous fluid therapy (1-1.2 mL/kg body weight/hour) was conducted on admission to the hospital. Although the patient was unable to tolerate oral fluid, intravenous fluid therapy was started at the decreased maintenance rate compared with the recommended regimen (1.5-2.0 mL/kg/hour), and closely monitored vital signs and hematocrit, because circulatory disturbance observed was not so severe (9).

The fever had continued for 4 days and subsided on the second day of hospitalization. However, thrombocytopenia rapidly proceeded to 10×10^3/μL at the minimum, and even with fluid replacement, the hematocrit increased by 23% from his baseline (Fig. 1). Pleural effusion and ascites were also observed (Fig. 2) and the patient felt light-headed on standing. Bleeding tendencies such as epistaxis appeared. These findings indicated that the patient's condition had advanced to the plasma leakage phase. Persistent fever, symptoms of plasma leakage (hemoconcentration, pleural effusion and hypoalbuminemia), severe thrombocytopenia, and bleeding tendencies (petechiae and epistaxis) led to the diagnosis of grade II DHF (2).

Intravenous fluid therapy was stopped when the recovery of appetite, decrease in hematocrit, increase in urine output, and rise in blood pressure were observed. The white blood cell count exhibited a minimum of 1,800/μL on day 4 of his illness and began to rise after the fever subsided. The platelet count began to rise on day 7 of his illness after reaching 1.0×10^4/μL, which was consistent with the improvement of plasma leakage symptoms. By day 9 of his illness, the platelet count recovered to 13×10^4/μL. Although the platelet count fell to 1.0×10^4/μL, platelet transfusion was not given because serious bleeding symptoms (e.g., gastrointestinal bleeding) were not observed. After the transaminase level peaked on days 5-6 of his illness (aspartate aminotransferase level, 96 IU/L; alanine aminotransferase level, 41 IU/L), they remained at approximately 60 IU/L throughout the period of hospitalization. The patient was discharged without exhibiting any complication.

Discussion

DHF/DSS is reported in 1.0-3.0% of all cases of dengue (10, 11), 95% of which occur in children less than 15 years of age (6), and rarely in adult travelers (3). In Japan,

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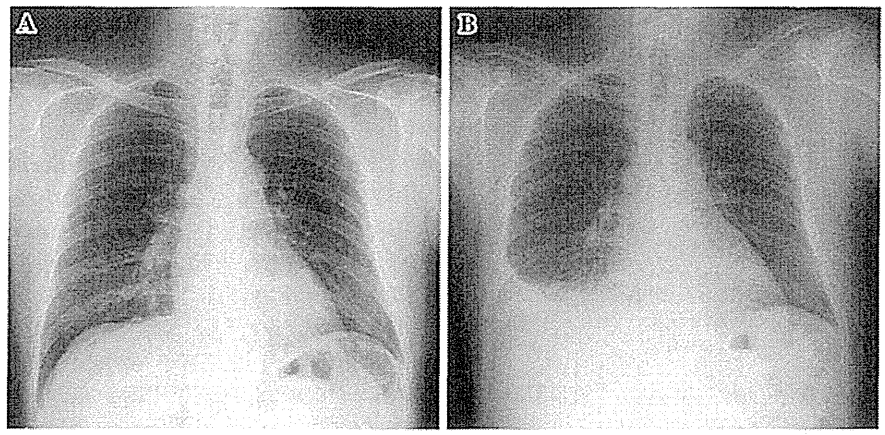

Figure 2. Chest radiographs. (A) Chest radiograph without pleural effusion on day 3 of his illness. (B) Chest radiograph with pleural effusion on the right side on day 5 of his illness.

as few as 50 to 100 cases of DF have been reported each year, whereas 245 cases in 2010. Travel-related dengue has been only seen in Japan ever since the epidemic between 1942 and 1945 (12). A case of DHF/DSS in a Japanese traveler was first documented in 1989 (13) and five adult Japanese cases of DHF/DSS have been reported to date in the literature.

The disease course of DHF is divided into three phases: febrile, plasma leakage (critical), and convalescent (recovery) phases (9, 14). Loss of plasma volume owing to an increased vascular permeability is the principal pathophysiology, and adequate fluid replacement results in a favorable outcome (2, 15). We predicted the disease progression from the patient's history of DF, the increased hematocrit and persistent thrombocytopenia. We could prevent the development of severe circulatory failure by early and effective fluid administration based on the frequent assessment of vital signs, urine output and hematocrit.

The classical WHO guideline has classified dengue into DF and DHF/DSS (2). However, the relevance of this guideline has been debated because the main criteria such as hemoconcentration may often be accessed only retrospectively or plasma leakage can occur without thrombocytopenia or hemorrhage, which leads to misclassification of apparently severe cases as not severe (16). Thus, WHO revised the guideline in 2009, which reclassifies dengue into dengue with or without warning signs and severe dengue (9).

The current patient presented with fluid accumulation, increase in hematocrit concurrent with rapid decrease in platelet count, and mucosal bleeding whereas circulatory disturbance, fluid accumulation, or bleeding was not so severe as to be fatal. In addition, he had no severe organ involvement. These findings led to diagnosis of 'dengue with warning signs' under the new guideline, whereas delayed intervention may possibly lead to progression into severe dengue.

It has been suggested that a secondary infection with a DENV serotype different from that causing the primary infection increases the risk of DHF/DSS through antibody-dependent enhancement (ADE) (17, 18) and so-called 'original antigenic sin' (19, 20). Infection with one serotype confers life-long protective immunity to that same serotype, but only temporary and incomplete immunity against other serotypes. The resultant non-neutralizing antibodies as well as cross-reactive CD4+ and CD8+ T cells might lead to progression into DHF/DSS (17, 20).

The present patient had repeatedly traveled to stay in Myanmar for research, and had previously been diagnosed with DENV infection. Thus, this was considered as a probable secondary infection. Secondary infections are characterized by high hemagglutination inhibition titers or elevated IgG levels by ELISA in acute specimen (21). The IgM/IgG antibody ratio is also a valuable diagnostic tool when the patient has become positive for IgM antibodies which can be sometimes low or absent in secondary infections (21). The present case had IgG antibodies against DENV without a detectable IgM response as early as 3 days after the onset of illness, which was additionally indicative of a DENV secondary infection. However, serological cross-reactivity across closely related flaviviruses is frequently observed. The patient had no history of infection with other flaviviruses such as Japanese encephalitis virus (JEV) or yellow fever virus (YFV), whereas he had been vaccinated against JEV during childhood (but never YFV), and a possible influence of the vaccination could not be denied.

It has also been indicated that the severity of the disease depends on the serotype of the infecting virus (1). A secondary infection with DENV-2, especially the Asian-genotype strain of DENV-2, may induce stronger cross-reactive responses than other serotypes (22), and is closely associated with DHF/DSS (7,, 8). The infection of DENV-2 in Myanmar suggested that the infecting virus might be the Asian-genotype strain. There was a possibility that this might be the reason why the present patient developed DHF even though he was an adult traveler. This patient has no comorbid conditions such as diabetes mellitus and renal failure that may make dengue more complicated.

Here, we reported the case of a Japanese traveler who de-

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veloped DHF with a probable secondary infection with DENV-2 and required inpatient management. Although only a few cases of dengue have been reported to date in Japan, considering the worldwide expansion of endemic areas and the increasing number of frequent travelers to these endemic areas, future increases in the number of patients in nonendemic regions such as Japan are predicted. Most cases of dengue can be managed on an outpatient basis. However, a mortality rate of 10-20% has been reported when DHF is left untreated, which can be reduced to 0.2-1.0% with appropriate inpatient management (1, 9). Deterioration of symptoms usually occurs around the time of defervescence (1). Appropriate management in accordance with the precise identification of the risk factors and early recognition of developing DHF/DSS is necessary.

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

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ORIGINAL ARTICLE

Chronic hepatitis B in patients coinfected with human immunodeficiency virus in Japan: a retrospective multicenter analysis

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Abstract A nationwide survey in Japan revealed that about 6 % of human immunodeficiency virus (HIV)-positive patients are coinfected with hepatitis B virus (HBV). To further analyze the features of liver disease in HIV/HBV-coinfected patients, we analyzed 252 patients from six hospitals in the HIV/AIDS (acquired immunodeficiency syndrome) Network of Japan. The mean age was 39.5 years, and the proportion of male patients was very high (243 of 252; 96 %). The main transmission route was male homosexual contact (186 of 252; 74 %), followed by heterosexual contact. The HBV genotype was determined in 77 patients. Among them, genotype A HBV was the most frequent (58 of 77; 75 %) and was detected almost exclusively in homosexual patients. Acute hepatitis B was documented in 21 patients (8 %). Three of the 252 HIV/HBV-coinfected patients developed advanced liver disease with the complication of ascites, hepatic encephalopathy, or hepatocellular carcinoma. A comparison between patients not treated and those treated with antiretroviral drugs including anti-HBV drugs revealed that the baseline liver function was worse in treated patients. However, the serum albumin levels and platelet counts in both groups increased after treatment and were similar. Liver disease-associated death was not observed. Here, we characterize the clinical features of liver disease in HIV/HBV-coinfected patients in Japan for the first time. The findings suggest that antiretroviral therapy with anti-HBV drugs may retard the progression of a liver disease and prevent liver disease-associated death in such patients.

Keywords Acquired immunodeficiency syndrome · Chronic liver disease · HBV DNA · Genotype

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Introduction

The number of human immunodeficiency virus (HIV)-positive patients is growing in Japan [1]. Although combination therapy with antiretroviral agents has made HIV infection itself somewhat controllable in many cases since its introduction in 1996, and mortality from opportunistic infection has decreased, existing comorbidities are the focus of current patient care. In fact, more than 50 % of deaths in HIV-1-infected patients are not related to acquired immunodeficiency syndrome (AIDS); the mortality from liver disease is second only to AIDS-related mortality [2]. Risk factors related to significant liver

diseases among HIV-positive patients include a diagnosis of viral hepatitis [3], nonalcoholic fatty liver disease [4], and excessive alcohol consumption [5]. Among these factors, hepatitis B and hepatitis C are of particular importance because they can often lead to life-threatening diseases such as cirrhosis and hepatocellular carcinoma by themselves.

The estimated prevalence of chronic hepatitis B virus (HBV) infection in Japan is less than 1 %, or 0.9 million carriers [6]. However, about 6 % of HIV-positive patients are coinfected with HBV [7]; this coinfection rate is more than six times higher than that in the non-HIV population. In the United States, the HIV/HBV coinfection rate is reported to be in the range of 6–14 % [8–10].

Several issues make the management of HIV/HBV coinfection complicated. HBV infection tends to be persistent in HIV-positive patients [9, 11, 12]. Chronic HBV infection may lead to hepatitis, cirrhosis, or hepatocellular carcinoma. The progression of a liver disease associated with chronic HBV infection is more rapid in HIV/HBV-coinfected patients than in HBV-monoinfected patients [13].

Combination regimens of antiretroviral therapy (ART) for coinfected patients should be carefully determined. Initial combination regimens of ART for HIV/hepatitis C virus (HCV)-coinfected patients are basically the same as those for HIV patients without HCV infection. However, because some nucleoside reverse transcriptase inhibitors (NRTIs) used in HIV treatment have activity against HBV, and some NRTIs mainly used in HBV treatment have partial activity against HIV [14], careful choice of treatment agents is necessary in HIV/HBV coinfection. Abrupt discontinuation of NRTIs that are active against HBV may aggravate viral hepatitis. Administration of entecavir, which has a weak activity against HIV, to HIV/HBV-coinfected patients without simultaneous effective HIV treatment may cause the accumulation of drug-resistant HIV strains [15–17]. In such cases, drug resistance of HBV may occur as well [18].

Drug-induced liver injury following ART is another concern. HIV/HBV-coinfected patients show an increase in transaminase level at a higher rate [19, 20]. However, it is often unclear whether this increase is caused by drug hepatotoxicity because the treatment of HIV infection causes immune reconstruction in patients, which alone could contribute to the transaminase level increase in viral hepatitis.

The objective of this study is to clarify the clinical features of HIV/HBV coinfection in Japan and to clarify the impact of ART on liver function among HIV/HBV-coinfected patients. The estimated prevalence of chronic HBV infection among the general population in Japan is decreasing yearly, but it remains much higher than that in the United States [21], where universal hepatitis B vaccination is introduced. Thus, the detailed analysis of HIV/HBV coinfection in Japan is of particular importance.

Patients and methods

We have conducted a multicenter retrospective study based on the data from a nationwide survey in 2006 conducted by sending questionnaires to 372 member hospitals of the HIV/AIDS network of Japan as of January 2006, and part of the results was reported earlier [7]. Following the survey, 6 of the 207 hospitals that responded to the survey—Hokkaido University Hospital (Hokkaido, Japan), University of Tokyo Hospital (Tokyo, Japan), Nagoya University Hospital (Aichi, Japan), International Medical Center of Japan (currently, National Center for Global Health and Medicine, Tokyo, Japan), Osaka National Hospital (Osaka, Japan), and Hiroshima University Hospital (Hiroshima, Japan)—were chosen for further studies because more than two-thirds of the HIV/HBV-coinfected patients identified in the survey went to these hospitals, and because both HIV experts and hepatologists were following up those patients there.

The questionnaire sent to the hospitals included items regarding the number of patients who visited the hospitals at least once between January and December in 2006 as follows: (1) the number of HIV-positive patients; (2) the number of hepatitis B surface antigen (HBsAg)-positive patients among (1); (3) the number of patients among (2) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/l; (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of HBsAg-positive patients among (4); (6) the number of patients among (5) who were determined at least once to have a serum ALT level higher than 100 IU/l; (7) the number of HIV-positive patients whose presumed transmission route is through homosexual contact; (8) the number of HBsAg-positive patients among (7); (9) the number of patients among (8) who were determined at least once to have a serum ALT level higher than 100 IU/l; (10) the number of HIV-positive patients who presumably contracted HIV through injection drug use; (11) the number of HBsAg-positive patients among (10); (12) the number of patients among (11) who were determined at least once to have a serum ALT level higher than 100 IU/l; (13) the number of HIV-positive patients whose transmission routes were classified as "others"; (14) the number of HBsAg-positive patients among (13); and (15) the number of patients among (15) who were determined at least once to have a serum ALT level higher than 100 IU/l.

We defined confirmed HIV infection with positivity for serum HBsAg as the criterion for HIV/HBV coinfection.

After identifying HIV/HBV-coinfected patients, medical records including laboratory data of these patients were reviewed between the date of the oldest available record for these patients and the final date of the record acquired by the end of the study. The laboratory data at the diagnosis or first recognition of HBV infection and the latest data in the study period were compared for analysis unless otherwise noted. HBV genotypes (A through D) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan) on the basis of the pattern of detection using monoclonal antibodies of a combination of epitopes on preS2-region products, each of which was specific for each genotype [22, 23].

Ethical issues

The respective ethics committees of the six hospitals approved the study. Informed consent was obtained from each study participant.

Statistical analyses

For the comparison of means of collected data, Student's *t* test (paired *t* test) was performed unless otherwise specified. The chi-square test was performed to determine the independence of clinical parameters.

Results

Two hundred and fifty-two patients were identified to have HIV/HBV coinfection. The mean age was 39.5 years, and the proportion of male patients was very high (243 of 252; 96.4 %). The main presumed transmission route of HIV was male homosexual contact (186 of 252; 73.8 %), followed by heterosexual contact. Among those HIV/HBV-coinfected patients, 21 of the 252 (8.3 %) acquired acute hepatitis during the study period (Table 1).

Table 1 Clinical background of HIV/HBV-coinfected patients

Number (male:female)	243:9
Age (year)	39.5 ± 9.6[a]
Presumed Transmission Route	
Transfusion	14
Homosexual contact	186
Heterosexual contact	24
Injection drug use	2
Others	4
Onset as acute hepatitis	21

[a] Mean ± standard deviation

The HBV genotype was determined in 77 patients. Among them, genotype A HBV was the most frequent (58 of 77; 75.3 %), followed far behind by genotype C (7 of 77; 9.1 %), which is the predominant genotype in the entire chronic hepatitis B population in Japan. Genotype B, which is also common in Japan, was found only in three patients (3.9 %). Genotype A was detected almost exclusively in homosexual patients (57 of 58; 98.3 %) (Fig. 1).

At the end of the study period, 113 patients (44.8 %) received some type of anti-HBV drug such as interferon, lamivudine, adefovir, or entecavir, not as part of anti-HIV treatment. Ninety-seven (38.5 %) patients were still taking anti-HBV drugs by the end of the study period. The median ALT level was 30.0 IU/l (5th percentile, 11.1; 95th percentile, 128.9), suggesting the existence of some liver injury. Liver function was normal in most HIV/HBV-coinfected patients. The mean serum albumin level was 4.1 ± 0.6 g/dl, and the median serum total bilirubin level was 0.8 mg/dl (5th percentile, 0.3; 95th percentile, 3.8). The mean platelet count was 21.0 ± 6.1 × 10^4/ml. The hepatitis B e antigen (HBeAg) was detected in 84 patients, and the HBV DNA level was high (higher than 100,000 IU/l) in 55 patients (Table 2). Three of the 252 (1.1 %) HIV/HBV-coinfected patients developed advanced chronic liver diseases, such as cirrhosis with the complication of ascites and/or hepatic encephalopathy, or hepatocellular carcinoma. Although we tried to retrieve information on alcohol consumption of the patients, it was available for only a limited number of patients (26 of 252); among the 26, only 2 patients had a habit of taking more than 60 g alcohol per day. The remaining 24 patients took alcohol only on social occasions. The antiretroviral agents used for these study patients are listed in detail in Table 3. Among those who had a known history of ART, 158 of 252 (62.7 %) received regimens that include anti-HBV drugs at least once previously, whereas 42 (16.7 %) did not, and no information is available for the remaining 52. The most common drug combination for HIV/HBV-coinfected patients was ATV/r + FTC/TDF (22 of 172; 12.8 %) (Table 4). FTC/TDF, composed of two drugs active against HBV, is recommended for HIV/HBV-coinfected patients

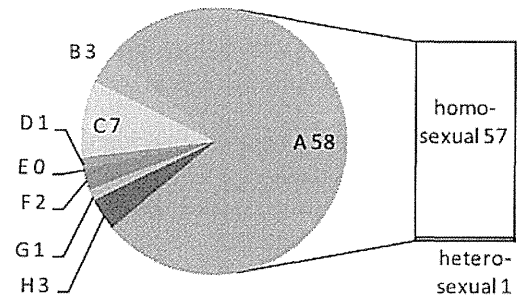

Fig. 1 Hepatitis B virus (HBV) genotype

Table 2 Liver function and related parameters of HIV/HBV-coinfected patients

Albumin (g/dl)	4.1 ± 0.6
Bilirubin[a] (mg/dl)	0.8 (5th percentile, 0.3; 95th percentile, 3.8)
ALT[a] (IU/l)	30.0 (5th percentile, 11.1; 95th percentile, 128.9)
WBC (× 10^3/μl)	5.2 ± 1.6
Platelet (× 10^4/μl)	21.0 ± 6.1
HBeAg (positive:negative)	84:68
HBV DNA (high:low)[b]	55:127

[a] Median and percentiles are provided instead of mean and standard deviation because of the nonnormality of the distribution

[b] HBV DNA level of 100,000 IU/l or higher is categorized as "high"

as one of the preferred NRTI backbones of the ART regimen [24].

We compared the clinical characteristics between patients who received the full ART and those who did not. Regarding the baseline statistical data, the observation period was longer for patients on ART, and there were more patients with AIDS in the ART group (10 of 64 vs. 52 of 162) (Table 5a). No significant difference was observed between the non-ART and ART groups in male/female ratio, age, transmission route, HBV markers, or advanced liver disease. Liver-related death was not observed, but hepatic failure with ascites and/or hepatic encephalopathy developed in 2 patients on ART and hepatocellular carcinoma developed in another patient.

Comparison between the ART group and the non-ART group revealed that the baseline liver function was worse in the ART group. At the beginning of the study period, the ART group showed a significantly lower CD4+ T-cell count than the non-ART group. The total white blood cell count and platelet count were also lower in the ART group. Although it is not statistically significant, the serum albumin level and prothrombin time (PT) index were lower in the ART group. However, at the end of the observation period, these parameters improved significantly in the ART group. The difference in CD4+ T-cell count between the ART and non-ART groups became marginal and became statistically insignificant (Table 5b).

Changes in the liver function of HIV/HBV-coinfected patients may not be fully explained by the changes in HBV activity because some parameters relevant to the estimation of liver function showed paradoxical changes. To clarify this observation, we compared the changes in liver function among HIV/HBV-coinfected patients on ART with respect to protease inhibitor (PI) use.

The mean serum total bilirubin level in patients on ART with PI use (PI group) at the beginning of the observation period was 1.1 mg/dl, whereas that in patients without PI use (non-PI group) was 0.8 mg/dl. The means at the end of the study period were 1.6 mg/dl in the PI group and 0.7 mg/dl in the non-PI group. Because the sample distribution of serum total bilirubin level did not follow the normal distribution by logarithmic transformation, we compared the means statistically. At the beginning, the difference in the mean between the PI group and the non-PI group was not significant ($p = 0.257$). At the end of the observation period, a statistically significant difference ($p = 0.001$) was observed. We then calculated the

Table 3 Antiretroviral treatment of HIV/HBV-coinfected patients

Antiretroviral drugs	Number of patients
NRTIs	
Zidovudine (AZT)	34
Didanosine (ddI)	9
Ddl / enteric coated	7
Zalcitabine (ddC)	1
Stavudine (d4T)	4
Lamivudine[a] (3TC)	84
Abacavir[3] (ABC)	38
Tenofovir[3] (TDF)	27
Emtricitabine (FTC) / TDF[a]	57
NNRTIs	
Nevirapine (NVP)	10
Efavirenz (EFV)	34
Delavirdine (DLV)	1
PIs	
Indinavir (IDV)	4
Ritonavir (RTV)	50
Nelfinavir (NFV)	8
Lopinavir (LPV)	3
Ritonavir-boosted LPV (LPV/r)	40
Atazanavir (ATV)	39
ATV/r	6
Fosamprenavir (FPV)	13

NRTI nucleoside reverse transcriptase inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor, *PI* protease inhibitor

[a] Agents with anti-HBV activity

Table 4 Antiretroviral regimens used for HIV/HBV-coinfected patients

Antiretroviral regimen	Number of patients
ATV/r + FTC/TDF	22
LPV/r + 3TC + TDF	8
LPV/r + FTC/TDF	7
EFV + FTC/TDF	6
ATV/r + 3TC + TDF	5