produced in both cell types following stimulation with *C. difficile* or *S. typhimurium* flagellin, but not with vehicle, in the presence of the control antibody (Fig. 4C, D). In contrast, CCL20 production by *C. difficile* or *S. typhimurium* flagellin was significantly inhibited in cells treated with the anti-TLR5 neutralizing antibody. These results demonstrated that *C. difficile* flagellin also induced CCL20 production that is mediated by TLR5.

C. difficile flagellin-induced production of IL-8 and CCL20 is mediated by p38

We next investigated the role of MAPK activation in *C. difficile* flagellin-induced production of IL-8 and CCL20. MAPKs are known to be activated by activation of TLR5 and to be important for mediation of their downstream signaling. Western blotting of Caco-2 cells indicated that p38 was phosphorylated after stimulation with *C. difficile* flagellin (Fig. 5A). In contrast, no detectable ERK phosphorylation was observed after *C. difficile* flagellin stimulation (Fig. 5B). The phosphorylation of p38 that was observed following stimulation of Caco-2 cells with either *S. typhimurium* or *C. difficile* flagellin was inhibited in cells treated with anti-TLR5 neutralizing antibody (Fig. 5C). These results indicated that stimulation with *C. difficile* or *S. typhimurium* flagellin leads to p38 phosphorylation via TLR5. We next examined whether p38 is involved in *C. difficile* flagellin-induced chemokine production. *C. difficile* flagellin stimulation of IL-8 (Fig. 5D) and CCL20 (Fig. 5E) production by Caco-2 cells was decreased by inhibition of p38 activation using the p38 inhibitor SB203580. These results indicated that p38 is partially involved in the production of IL-8 and CCL20 that is induced by *C. difficile* flagellin and is mediated by TLR5.

C. difficile toxin B promoted flagellin-induced production of IL-8 and CCL20 in proportion to the levels of TLR5 expression

We next investigated the potential role of *C. difficile* toxin B in flagellin induction of IL-8 and CCL20 production. We first determined if toxin B affects the epithelial barrier of the Caco-2 cell model epithelial system, which might regulate access of flagellin to TLR5 on the basolateral side of the cells. The time course of TEER was therefore examined in Caco-2 cells that were cultured as a tight, confluent monolayer and were stimulated with the *C. difficile* toxin B, *C. difficile* flagellin and vehicle for the indicated times over 24 h. TEER levels decreased rapidly after addition of toxin B (Fig. 6A), indicating that toxin B did disrupt the epithelial barrier of this model epithelial system. However, TEER levels did not change after addition of flagellin and vehicle. We next assayed the effect of toxin B on IL-8 and CCL20 production by Caco-2 cells stimulated with vehicle control, *C. difficile* toxin B, *C. difficile* flagellin, or *C. difficile* toxin B plus *C. difficile* flagellin. Cells treated with toxin B or flagellin produced a higher level of the chemokines IL-8 and CCL20 than control cells. Interestingly, stimulation with toxin B and subsequently with flagellin induced significantly higher levels of IL-8 and CCL20 compared with individual stimulation (Fig. 6B, C). Stimulation with toxin B and subsequently with flagellin induced high levels of IL-8 and CCL20 (4.73 and 3.75 folds, respectively) compared with flagellin stimulation.

Discussion

Little is known about the role of the *C. difficile* flagellin especially in the occurrence of CDAD. In this study, we therefore examined mechanisms by which *C. difficile* flagellin is involved in the activation

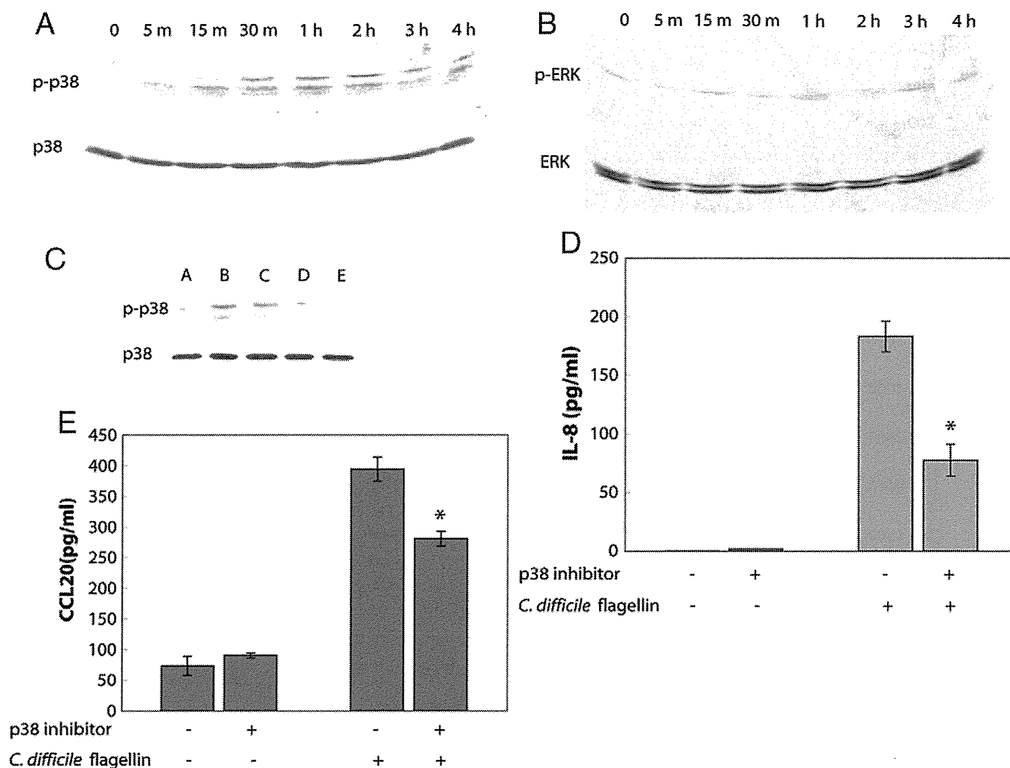

Fig. 5. *C. difficile* flagellin-induced production of IL-8 and CCL20 is partially mediated by p38. (A, B) Caco-2 cells were stimulated with 1.0 mg/ml of *C. difficile* flagellin for the indicated times, following which phosphorylation of p38 (p-p38) (A) and ERK (p-ERK) (B) was examined by Western blot analysis. The corresponding non-phosphorylated protein levels were also assayed. (C) Anti-TLR5 antibody (lanes D and E) or a control antibody (lanes A–C) was added into the culture medium of Caco-2 cells for 1 h. The cells were then stimulated with vehicle (lane A), 100 ng/ml of the positive control *S. typhimurium* flagellin (lanes B and D) or 1.0 mg/ml of *C. difficile* flagellin (lanes C and E) for 1 h. Phosphorylation of p38 was examined as in (A). (D, E) Caco-2 cells were pretreated with or without the p38 inhibitor SB203580 (10 μM) for 2 h. The cells were then stimulated with or without 1.0 mg/ml of *C. difficile* flagellin for 16 h. The concentration of IL-8 (D), or CCL20 (E) was then measured using an ELISA.

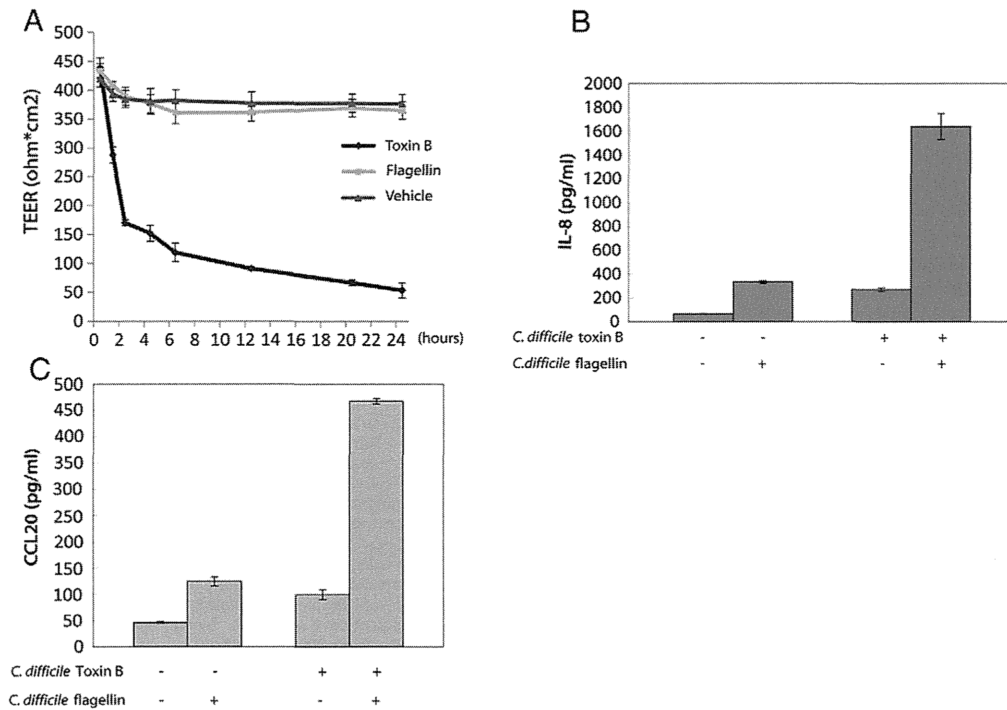

Fig. 6. *C. difficile* toxin B promoted flagellin-induced CCL20 production by increasing TLR5 expression. (A) Caco-2 cells were cultured as a confluent monolayer and stimulated with 0.25 μg/ml of *C. difficile* toxin B, 1.0 mg/ml of *C. difficile* flagellin and vehicle. Transepithelial electrical resistance (TEER) was measured at the indicated times. (B, C) Caco-2 cells were treated with vehicle or with 0.25 μg/ml of *C. difficile* toxin B for 1 h, and were then stimulated with vehicle or with 1.0 mg/ml of *C. difficile* flagellin for 16 h. The level of IL-8 (B) or CCL20 (C) production was measured using an ELISA.

of intestinal epithelial cells. We demonstrated for the first time that extracted *C. difficile* flagellin stimulated intestinal epithelial cells via TLR5 (Figs. 2–4). We also demonstrated that stimulation by *C. difficile* flagellin induced activation of NF-kappaB and p38 and the production of IL-8 and CCL20 similar to stimulation of cells by flagellin of other bacteria (Hayashi et al., 2001; Tallant et al., 2004; Yu et al., 2003). The production of these chemokines was mediated by p38 activation (Fig. 5), which is known to play an important role in TLR5 signaling. Our results indicated that *C. difficile* flagellin plays an important role in the pathogenesis of CDAD, similar to the role of *S. typhimurium* flagellin in *S. typhimurium* infection (Fournier et al., 2009).

Only a few reports have been published regarding the biological role of *C. difficile* flagellin (Delmee et al., 1990; Tasteyre et al., 2000), and a method for the extraction of *C. difficile* flagellin has not been fully established. Although a single 39 kDa band was observed by Coomassie Brilliant Blue staining following flagellin extraction (Fig. 1), we cannot exclude the possibility that the *C. difficile* flagellin used in this report might include other components of the bacterial body and that the cells might be activated through these contaminating components. Nevertheless, we showed that inflammatory cytokine production that was induced by our flagellin preparation was inhibited to basal levels in cells treated with anti-TLR5 antibody (Figs. 3, 4). These results indicated that the extracted *C. difficile* flagellin stimulated intestinal epithelial cells via TLR5.

In this report, cells were stimulated with 1 mg/ml of *C. difficile* flagellin. In our hand, 100 μg/ml and more of *C. difficile* flagellin were needed to induce chemokine production (Figs. 3, 4). It remains to be determined why high amount of *C. difficile* flagellin was needed for activation of intestinal epithelial cells. No reports have been published that describe concentration of *C. difficile* flagellin for cell activation. *C. difficile* does not usually have high virulence, and some of *C. difficile* are needed to increase much more in numbers to lead intestinal inflammation (van der Waaij, 1989). Although small amount of *C. difficile* always exist in the intestine, *C. difficile* does not usually develop diarrhea. *C. difficile* infection does not occur until *C. difficile* increases in number by antibiotic treatment. This might explain why higher amount of *C. difficile* flagellin was needed to stimulate intestinal epithelial cells.

Flagellin was known to be a ligand of TLR5 expressed in the intestinal tract. We thought that flagellin might play a role in occurrence of CDAD, probably collaborated with toxins. We therefore examined the effect of *C. difficile* flagellin on intestinal epithelial cells in the presence of *C. difficile* toxin B which was essential, and a key virulence determinant, for CDAD (Lyras et al., 2009). Previous reports showed that the Caco-2 model epithelial system used in our experiments closely resembles the membrane barrier of the large intestinal epithelium in vivo (Madara et al., 1988; Pontier et al., 2001). By analysis of TEER levels, we demonstrated that toxin B grossly disrupts tight junctions of the Caco-2 intestinal epithelial cell monolayer. This result suggested the possibility that toxin B disruption of tight junctions of intestinal epithelia might allow flagellin to reach the basolateral side of the epithelia, where it can stimulate the intestinal epithelial cells via TLR5. Almost all of the cell surface TLR5 is known to be distributed on the basolateral side of human colon epithelial cells, with only a few TLR5 distributed on the apical side (Gewirtz et al., 2001; Rhee et al., 2005). Consistent with our theory, stimulation of Caco-2 cells with toxin B and subsequently with flagellin induced significantly greater IL-8 and CCL20 production than stimulation with either toxin B or flagellin alone (Fig. 6B, C). These results demonstrated that collaboration of *C. difficile* flagellin with toxin B leads to enhanced production of inflammatory cytokines and therefore might lead to more intense inflammation at sites of infection.

Kuehne et al. (2011) demonstrated that *C. difficile* strains expressing either toxin A or toxin B alone are virulent. We purchased toxin A (List Biological Laboratories, Inc., Campbell, CA) and examined the function of toxin A in collaboration with *C. difficile* flagellin. However, toxin A did not promote cytokine productions induced by flagellin

(data not shown). These results might suggest that our results should adopt CDAD caused by toxin A negative/toxin B positive *C. difficile* only which is now spreading in clinical settings (Drudy et al., 2007).

Jarchum et al. (2011) recently showed by using a murine model that TLR5 stimulation protects mice from acute *C. difficile* colitis. In this study flagellin derived from *S. typhimurium*, not *C. difficile* flagellin, was administered one day before *C. difficile* infection, and examined how *Salmonella* flagellin had effect on *C. difficile* infection. This study is important when we consider the pathogenesis of *C. difficile* in the presence of many strains of bacteria in the intestinal tract, while our study is focus on the mechanism by which *C. difficile* toxin damage intestinal epithelial cells in collaboration with *C. difficile* flagellin.

Conclusion

In this study, we showed that *C. difficile* flagellin might have some roles in the pathogenesis of CDAD. However, further studies with the whole bacterium will be needed to reveal the role of flagellin in *C. difficile* infection.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. J Immunol 2005;174:4453–60.

Bandyopadhaya A, Sarkar M, Chaudhuri K. IL-1beta expression in Int407 is induced by flagellin of *Vibrio cholerae* through TLR5 mediated pathway. Microb Pathog 2008;44:524–36.

Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. Clin Infect Dis 2008;46(Suppl. 1):S12–8.

Delmee M, Avesani V, Delferriere N, Burtonboy G. Characterization of flagella of *Clostridium difficile* and their role in serogrouping reactions. J Clin Microbiol 1990;28:2210–4.

Donnelly MA, Steiner TS. Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5. J Biol Chem 2002;277: 40456–61.

Drudy D, Fanning S, Kyne L. Toxin A-negative, toxin B-positive *Clostridium difficile*. Int J Infect Dis 2007;11:5-10.

Du T, Alfa MJ. Translocation of *Clostridium difficile* toxin B across polarized Caco-2 cell monolayers is enhanced by toxin A. Can J Infect Dis 2004;15:83–8.

Fournier B, Williams IR, Gewirtz AT, Neish AS. Toll-like receptor 5-dependent regulation of inflammation in systemic *Salmonella enterica* serovar Typhimurium infection. Infect Immun 2009;77:4121–9.

Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol 2001;167:1882–5.

Harrison LM, Rallabhandi P, Michalski J, Zhou X, Steyert SR, Vogel SN, et al. *Vibrio cholerae* flagellins induce Toll-like receptor 5-mediated interleukin-8 production through mitogen-activated protein kinase and NF-kappaB activation. Infect Immun 2008;76: 5524–34.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410: 1099–103.

Jarchum I, Liu M, Lipuma L, Pamer EG. Toll-like receptor 5 stimulation protects mice from acute *Clostridium difficile* colitis. Infect Immun 2011;79:1498–503.

Kelly CP, LaMont JT. *Clostridium difficile*—more difficult than ever. N Engl J Med 2008;359: 1932–40.

Khan MA, Kang J, Steiner TS. Enteroaggregative *Escherichia coli* flagellin-induced interleukin-8 secretion requires Toll-like receptor 5-dependent p38 MAP kinase activation. Immunology 2004;112:651–60.

Kuehne SA, Cartman ST, Minton NP. Both, toxin A and toxin B, are important in *Clostridium difficile* infection. Gut Microbes 2011;2:252–5.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

Lee SE, Kim SY, Jeong BC, Kim YR, Bae SJ, Ahn OS, et al. A bacterial flagellin, *Vibrio vulnificus* FlaB, has a strong mucosal adjuvant activity to induce protective immunity. Infect Immun 2006;74:694–702.

Lyerly DM, Krivan HC, Wilkins TD. *Clostridium difficile*: its disease and toxins. Clin Microbiol Rev 1988;1:1-18.

Lyons S, Wang L, Casanova JE, Sitaraman SV, Merlin D, Gewirtz AT. *Salmonella* Typhimurium transcytoses flagellin via an SPI2-mediated vesicular transport pathway. J Cell Sci 2004;117:5771–80.

Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, et al. Toxin B is essential for virulence of *Clostridium difficile*. Nature 2009;458:1176–9.

Madara JL, Stafford J, Barenberg D, Carlson S. Functional coupling of tight junctions and microfilaments in T84 monolayers. Am J Physiol 1988;254:G416–23.

Murthy KG, Deb A, Goonesekera S, Szabo C, Salzman AL. Identification of conserved domains in *Salmonella* Muenchen flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. J Biol Chem 2004;279:5667–75.

Pontier C, Pachot J, Botham R, Lenfant B, Arnaud P. HT29-MTX and Caco-2/TC7 monolayers as predictive models for human intestinal absorption: role of the mucus layer. J Pharm Sci 2001;90:1608–19.

Redelings MD, Sorvillo F, Mascola L. Increase in *Clostridium difficile*-related mortality rates, United States, 1999–2004. Emerg Infect Dis 2007;13:1417–9.

Reichhart JM. TLR5 takes aim at bacterial propeller. Nat Immunol 2003;4:1159–60.

Rhee SH, Im E, Riegler M, Kokkotou E, O'Brien M, Pothoulakis C. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. Proc Natl Acad Sci U S A 2005;102:13610–5.

Sambol SP, Tang JK, Merrigan MM, Johnson S, Gerding DN. Infection of hamsters with epidemiologically important strains of *Clostridium difficile*. J Infect Dis 2001;183: 1760–6.

Schmeck B, N'Guessan PD, Ollomang M, Lorenz J, Zahlten J, Opitz B, et al. *Legionella pneumophila*-induced NF-kappaB- and MAPK-dependent cytokine release by lung epithelial cells. Eur Respir J 2007;29:25–33.

Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 2003;4:1247–53.

Steiner TS. How flagellin and toll-like receptor 5 contribute to enteric infection. Infect Immun 2007;75:545–52.

Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol 2007;77:1-14. [Chapter 14: Unit 14 2].

Tallant T, Deb A, Kar N, Lupica J, de Veer MJ, DiDonato JA. Flagellin acting via TLR5 is the major activator of key signaling pathways leading to NF-kappa B and proinflammatory gene program activation in intestinal epithelial cells. BMC Microbiol 2004;4:33.

Tasteyre A, Barc MC, Karjalainen T, Dodson P, Hyde S, Bourlioux P, et al. A *Clostridium difficile* gene encoding flagellin. Microbiology 2000;146(Pt 4):957–66.

van der Waaij D. The ecology of the human intestine and its consequences for overgrowth by pathogens such as *Clostridium difficile*. Annu Rev Microbiol 1989;43:69–87.

Voth DE, Ballard JD. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev 2005;18:247–63.

Yu Y, Zeng H, Lyons S, Carlson A, Merlin D, Neish AS, et al. TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism. Am J Physiol Gastrointest Liver Physiol 2003;285:G282–90.

Zhang Z, Reenstra W, Weiner DJ, Louboutin JP, Wilson JM. The p38 mitogen-activated protein kinase signaling pathway is coupled to Toll-like receptor 5 to mediate gene regulation in response to *Pseudomonas aeruginosa* infection in human airway epithelial cells. Infect Immun 2007;75:5985–92.

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Changes in the Mean Platelet Volume Levels after Bloodstream Infection Have Prognostic Value

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Abstract

Objective Thrombocytopenia is frequently observed during bloodstream infection (BSI); however, little is known about the trends in platelet size during BSI. The aim of this study was to investigate trends in platelet indices during BSI and to determine the relationship between the mean platelet volume (MPV) levels and the prognosis of BSI patients.

Methods We conducted a four-year retrospective study to assess the trends in the platelet indices and the clinical features of BSI. We enrolled 350 patients with positive blood cultures and measured the platelet indices during five periods: 30 to seven days before onset (1st period); within one day of onset (2nd period); three to five days after onset (3rd period); seven to 10 days after onset (4th period); and 14 to 19 days after onset (5th period). The end point was defined as 30-day mortality.

Results Among the BSI patients, the average platelet count decreased during BSI (29.4 $*10^9$/L to 24.0 $*10^9$/L, p<0.001), while the average MPV level increased (7.33 fL to 7.89 fL, p<0.001). The degree of MPV elevation in the nonsurvivors (n=25) was lower than that observed in the survivors (n=325) between the 1st and 2nd periods (0.00 fL vs. 0.35 fL, p=0.006), whereas between the 2nd and 3rd periods, the degree of MPV elevation in the nonsurvivors was higher than that observed in the survivors (0.74 fL vs. 0.19 fL, p=0.03). MPV elevation after BSI was identified to be a negative prognostic factor for BSI (odds ratio: 1.82; 95% confidence interval: 1.00-3.32; p=0.027).

Conclusion Changes in the MPV levels after BSI may therefore be a useful prognostic marker for BSI.

Key words: mean platelet volume, bloodstream infection, prognosis

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Introduction

Bloodstream infection (BSI) is a systemic infection that can lead to sepsis and high morbidity and mortality. The incidence of BSI increased from 147 to 168 per 100,000 people (average annual increase, 4.4%) in a population-based surveillance conducted in Finland from 2004 to 2007 (1). The mortality of nosocomial BSI caused by major pathogens is 24.5% in Japanese university hospitals (2). BSI often involves numerous organs and systems, including the hematopoietic system. Thrombocytopenia is frequently observed in BSI patients, particularly in severe cases (3-5). In contrast, thrombocytosis can occur after recovery from BSI (6). However, dysfunction of platelets in patients with BSI can only be determined by assessing the platelet count (7). The mechanism and degree of platelet dysfunction during BSI remain unclear.

Platelet indices, based on the platelet size, have been available to clinicians since the early 1970s (8). Among platelet indices, the mean platelet volume (MPV) and platelet distribution width (PDW), which approximate the coefficient of variation of the platelet population, are reportedly associated with the platelet function (9). Several studies have found that MPV plays a role in the diagnosis and management of patients with cardiovascular diseases, such as myocardial infarction and diabetes mellitus (10-12). Few studies have investigated the relationship between MPV and

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sepsis outcomes (13-16), and such studies have only been performed in small populations or with unclear timing of MPV measurement. Therefore, little is known about the trends in the platelet indices during BSI and the relationship between changes in the platelet indices and the outcome of BSI.

The aim of this study was to elucidate trends in platelet indices during BSI and to assess the relationships between these indices and the prognosis.

Materials and Methods

Patients

The subjects comprised all BSI patients ≥20 years of age admitted to the University of Tokyo Hospital (a tertiary teaching hospital with 1,150 beds) between April 2003 and March 2007. BSI was defined as positive blood cultures and clinical manifestations of infection (e.g., fever, chills and/or hypotension). The onset of BSI was defined as the time when positive blood cultures were collected. Patients who experienced more than one episode of BSI caused by the same pathogen within 30 days of the previous episode were regarded as having one episode of infection. The platelet indices were measured during each of the following five periods: between 30 and seven days before the onset of BSI (1st period); between zero and one day after the onset of BSI (2nd period); between three and five days after the onset of BSI (3rd period); between seven and 10 days after the onset of BSI (4th period); and between 14 and 19 days after the onset of BSI (5th period). Patients lacking platelet index data for the initial three periods were excluded. Patients with preexisting hematological diseases, chronic viral hepatitis, disseminated intravascular coagulation based on the criteria of the Japanese Ministry of Health and Welfare (17), myocardial infarction or diabetes mellitus were also excluded in order to prevent measurement inconsistencies. This study was approved by the relevant institutional review boards. Written informed consent was waived in this study due to the retrospective nature of the investigation.

Data collection

The platelet indices were determined in 2-mL blood samples in dipotassium ethylenediaminetetraacetic acid (EDTA) tubes using a hematology analyzer (XE-5000; Sysmex Corporation, Kobe, Japan). For the blood cultures, the specimens were inoculated into BacT/ALERT FA (bioMerieux), and the positivity of the blood cultures was assessed using the BacT/ALERT 3D system. To collect demographic and clinical information, medical records were reviewed retrospectively.

Statistical analysis

The relationships between categorical variables were analyzed using Pearson's chi-squared test. Fisher's exact test was used when the expected count was less than 5. Continuous variables were compared using Student's *t*-test or the Mann-Whitney U test. Pearson product moment correlation coefficients were determined in order to evaluate possible relationships among the platelet indices. A multivariate analysis was performed using a binary logistic regression analysis that allowed for adjustment of confounding factors. All p values were two-sided, and $p<0.05$ was considered to be significant. All analyses were performed using the SPSS software package for Windows (Ver.10.1).

Results

Demographic data of the patients with BSI

Of the 2,309 patients with positive blood cultures during the four years of surveillance, 350 met the inclusion criteria in that their platelet indices were measured in all of the three initial periods (Table 1). Among these 350 patients, 185 were men (53%) and 165 were women (47%). The average age of the patients was 67.2±15.6 years (range, 21-94 years). With respect to preexisting conditions, 137 (39%) patients had malignancies other than hematology and were treated without chemotherapy. Only in 104 patients were primary infections other than catheter-related BSI identified (30%).

Trends in the platelet indices during BSI

Trends in the platelet indices during BSI were examined. The platelet counts, MPV levels, plateletcrit levels and platelet PDW during BSI are shown in Table 2. The average platelet count decreased in the 1st and 2nd periods, then gradually increased thereafter to the baseline level. The average MPV level increased from the 1st period to the 3rd period and was negatively correlated with the platelet count (r^2=-0.327, -0.377, -0.446, -0.487, -0.460; $p<0.001$). These results indicate that the platelet indices change synchronously, then gradually normalize after infection.

Changes in the platelet indices in the survivors and nonsurvivors during BSI

Among the 350 patients with BSI, 25 patients (7.2%) died within 30 days of BSI onset. The nonsurvivors had a mean survival time of 17.5 days (range 5-30 days). The demographic characteristics of the survivors and nonsurvivors are shown in Table 1. The average age of the nonsurvivors was higher than that of the survivors, although the difference was not significant. The rates of preexisting diseases were similar between the two groups. The rates of fungal and polymicrobial BSI in the nonsurvivors were higher than those observed in the survivors, and the ratio of BSI pathogens between the two groups was significantly different ($p<0.01$).

The platelet indices in the measured periods were compared between the nonsurvivors and survivors (Fig. 1). In the 1st and 2nd periods, the average platelet count was similar between the two groups (Fig. 1A). In the 3rd period or

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Table 1. Demographic Data of Patients with BSI and 30-day Mortality

Factors	Number (%) of total patients (n = 350)	Number (%) of non-survivors (n = 25)	Number (%) of survivors (n = 325)	p
Age (years; mean ± SD)	67.2 ± 15.6	72.3 ± 11.3	66.8 ± 15.7	0.15[a]
Male	185 (53)	17 (68)	168 (52)	0.12[b]
Pre-existing diseases				0.66[c]
Malignancy	142 (41)	12 (48)	130 (40)	
Autoimmune diseases	30 (9)	1 (4)	29 (9)	
Cardiovascular diseases	62 (18)	3 (12)	59 (18)	
Others	116 (33)	9 (36)	107 (33)	
Pathogen				<0.01[c]
Gram-positive bacteria	166 (47)	11 (44)	155 (48)	
Gram-negative bacteria	138 (39)	5 (20)	133 (41)	
Fungi	25 (7)	6 (24)	19 (6)	
Polymicrobials	22 (6)	3 (12)	18 (6)	
Primary site infection				0.17[c]
Catheter related infection, Unknown	246 (70)	16 (64)	230 (71)	
Intraabdominal infection	59 (17)	4 (16)	55 (17)	
Urinary tract infection	24 (7)	2 (8)	22 (7)	
Pneumonia	6 (2)	2 (8)	4 (1)	
Skin, soft tissue infection, osteomyelitis	15 (4)	1 (4)	14 (4)	

Data are shown as numbers and proportions compared to total number of patients in each group. BSI: bloodstream infection. [a] Mann-Whitney U test. [b] Fisher's exact test. [c] Pearson's chi-squared test.

Table 2. Platelet Indices during BSI

Period (time from onset of BSI, days)	Platelet count ($*10^9$/L)	Mean platelet volume (fL)
1st (<-7)	29.4 ± 13.1	7.33 ± 0.91
2nd (0-1)	23.4 ± 11.7[a]	7.65 ± 1.06[a]
3rd (3-5)	24.0 ± 13.3	7.89 ± 1.21[a]
4th (7-10)	30.8 ± 15.8[a]	7.69 ± 1.19[a]
5th (14-19)	31.1 ± 15.1	7.54 ± 1.04[a]

Data are shown as mean and standard deviation. Periods represent each period in which platelet indices were measured, and days from onset of BSI are given in parentheses. [a]Significantly different from data in previous period by Student's t-test ($p < 0.001$). BSI: bloodstream infection.

later, the average platelet count in the nonsurvivors did not increase, whereas the average platelet count in the survivors gradually increased (5th period; 23.3 $*10^9$/L vs. 31.5 $*10^9$/L, p=0.04). The average MPV level was similar between the nonsurvivors and survivors in the 1st period, (7.42 fL vs. 7.33 fL, p=0.64) (Fig. 1B). In the 2nd period, the average MPV level in the nonsurvivors was lower than that observed in the survivors, although the difference was not significant (7.42 fL vs. 7.67 fL, p=0.26). In contrast, in the 3rd period or later, the average MPV level in the nonsurvivors was slightly higher than that observed in the survivors (4th period; 8.36 fL vs. 7.64 fL, p=0.08). These results indicate that nonsurvivors exhibit different trends in platelet indices than survivors, although the values of the indices did not differ significantly between the two groups.

Changes in the platelet counts and MPV levels during the three periods are shown in Fig. 2. The changes in the platelet counts in the nonsurvivors differed significantly from those observed in the survivors in the 1st and 2nd periods only (-3.56 $*10^9$/L vs. 1.08 $*10^9$/L, p=0.02) (Fig. 2A). The increases in the MPV levels were significantly lower in the nonsurvivors than in the survivors from the 1st period to the 2nd period, (0.00 fL vs. 0.34 fL, p=0.006) (Fig. 2B). In contrast, from the 2nd period to the 4th period, the increases in the MPV levels were significantly higher in the nonsurvivors than in the survivors (2nd period to 3rd period: 0.74 fL vs. 0.19 fL, p=0.03; 3rd period to 4th period: 0.29 fL vs. -0.20 fL, p=0.01). The changes in the platelet counts in the nonsurvivors were negatively correlated with the changes in the MPV levels in all but the final interval (r^2=-523, -0.721, -587 -0.104; p=0.007, p<0.001, p=0.004, p=0.72), and the changes in the platelet counts in the survivors were also negatively correlated with the changes in the MPV levels in all four intervals (r^2=-0.411, -0.579, -0.440, -0.308; p< 0.001). These results indicate that, although the changes in the platelet counts and MPV levels occurred synchronously

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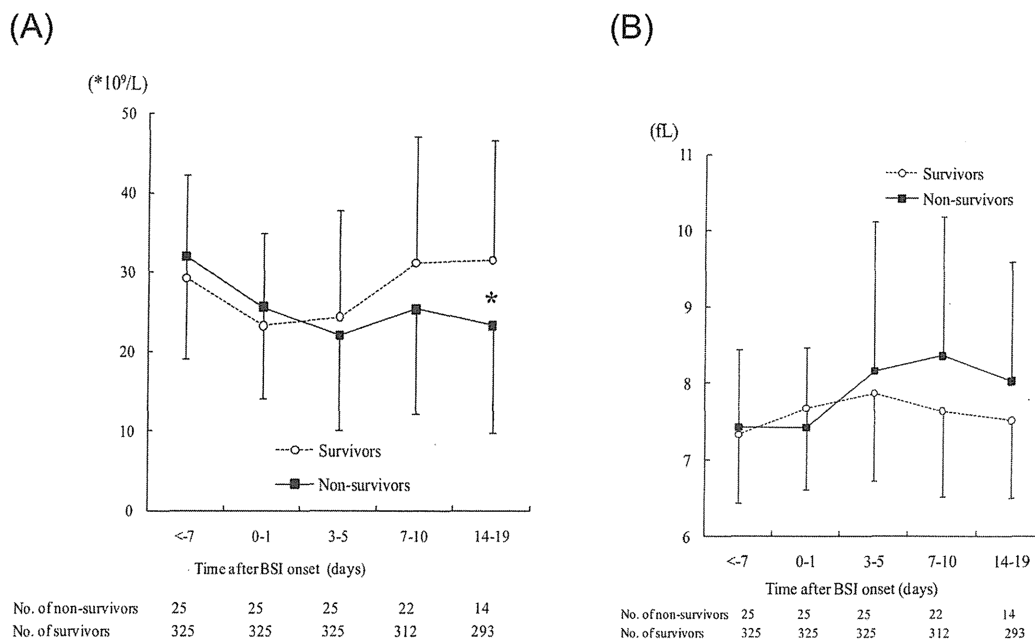

Figure 1. Trends in the platelet indices of the survivors and nonsurvivors during BSI. (A) Platelet count, (B) MPV. *: significant difference between the survivors and nonsurvivors (p<0.01), BSI: bloodstream infection, MPV: mean platelet volume

in both groups, changes in the MPV levels are an early discriminative parameter of the BSI prognosis.

Elevation of the MPV levels after BSI as a prognostic marker of BSI

A logistic regression analysis identified the following variables as being independently associated with mortality in BSI: age, Gram-positive bacilli pathogens and elevation of the MPV levels following BSI onset (Table 3). Independent covariates that were predictive of survival after BSI included the following: Gram-negative bacilli and elevation of the MPV levels at BSI onset. With respect to the changes in the levels of MPV, a receiver operator characteristic (ROC) analysis showed that the area under the ROC curve (AUC) was 0.80 ($p=0.04$) (Fig. 3). These results suggest that changes in the MPV levels after BSI onset are a prognostic marker in patients with BSI.

Discussion

Changes in platelet indices, such as the MPV levels, have been reported in the acute phase of cardiovascular diseases, including unstable angina and acute myocardial infarction (18). Thrombocytopenia is frequently observed following the onset of sepsis. In our study, we confirmed that, in addition to decreases in the platelet counts, the MPV levels also changed after the onset of BSI (Table 2). The average MPV level increased from onset to three to five days after onset, while the average platelet count changed inversely. With regard to the changes in the MPV levels at the onset of sepsis, only one illustrative case of MPV elevation was observed in a previous study (16). Other studies investigating the MPV levels during sepsis did not demonstrate changes in the MPV levels at onset (13-15); therefore, the causes of MPV elevation at BSI onset have not been thoroughly studied.

Larger platelets are known to be more active than smaller platelets and exhibit increased hemostatic capacity in a number of *in vitro* functional assays (9). In a study of septic animals, coagulation was activated in association with elevated MPV (19). Another study of an animal model in which endotoxin was administered reported that MPV increased at 0.5 hours and remained increased for 24 hours, while the platelet count and plateletcrit level changed inversely (11). Plasma obtained from septic patients sensitizes normal platelets to hyperaggregate and adhere to cultured endothelium (20). Based on the results of these studies and our findings, we suggest that a decrease in the platelet count after BSI implies that platelet consumption exceeds platelet production and that an increase in the MPV level after BSI implies an increase in the rate of platelet production due to overconsumption induced by inflammation. The trends in the platelet indices during BSI may therefore reflect the platelet function.

In this study, the nonsurvivors exhibited different trends in platelet indices, particularly with regard to the MPV levels, compared with the survivors (Fig. 1). Becchi reported that, in their study, the average MPV level in nonsurvivors increased, while that of the survivors decreased after sepsis (13), and van der Lelie reported that, in their study, among all of the patients who died of infection in the first week, the MPV level increased on the day of their death (16). These results are consistent with our findings. In our study, among the nonsurvivors, the trends in the MPV

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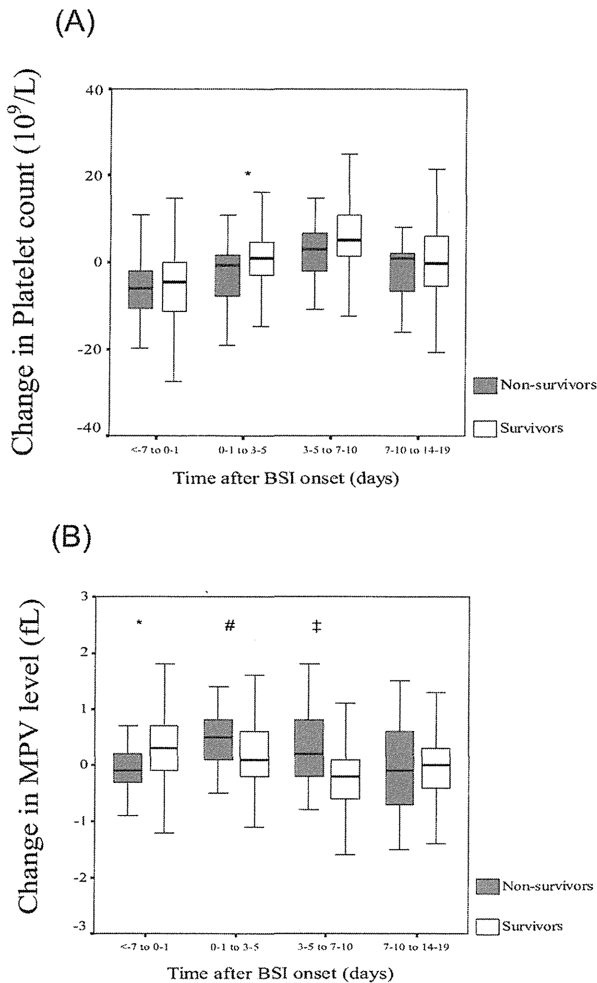

Figure 2. Differences in the changes in the platelet counts and MPV levels between the survivors and nonsurvivors during BSI depicted as box-plots. (A) Platelet counts, (B) MPV levels. *: significant difference between the survivors and nonsurvivors ($p<0.01$), #: significant difference between the survivors and nonsurvivors ($p=0.03$), ‡: significant difference between the survivors and nonsurvivors ($p=0.02$), BSI: bloodstream infection, MPV: mean platelet volume

levels from the onset of BSI included two phases: in the 1st phase, no elevation was seen at BSI onset, while in the 2nd phase, the MPV levels increased after onset. Platelet formation from megakaryocytes is an important determinant of the platelet size (21), although to the best of our knowledge, no studies have investigated changes in platelet formation in patients with severe sepsis. The release of platelet granules, another factor influencing the platelet size, was found to be elevated in severely septic patients with multiple organ failure (22), although that study included cardiovascular patients. We assume that changes in the MPV levels may be attributed to a combination of several factors, including decreases in platelet formation by megakaryocytes, platelet consumption in peripheral tissue and degranulation in larger platelets, and that the impact of inflammation on MPV elevation after BSI in nonsurvivors is stronger than that observed in survivors. To elucidate the mechanisms underlying the changes in the MPV levels observed in nonsurvivors, an analysis of platelet morphology and the megakaryocyte function in severely septic patients is required.

Based on our findings that the changes in the MPV levels during BSI in nonsurvivors are different from those observed in survivors, we hypothesized that MPV changes during BSI are predictive of the prognosis. A multivariate analysis identified MPV elevation at onset to be a protective prognostic factor, while MPV elevation after BSI onset was identified to be a prognostic factor (Table 3). We found that patients whose MPV level was elevated after the onset of BSI exhibited a 1.8-fold increase in death probability. Becchi reported that a logistic regression analysis showed a three-fold increase in death probability among patients with an MPV level <9.7 fL at the time of recruitment (13). To our knowledge, there are no studies investigating changes in platelet indices during BSI as a prognostic marker. Among values or changes in platelet indices during BSI, we believe that changes in the MPV levels are suitable for evaluating the prognosis, as the value of each platelet index changed within a short period in this study, while the platelet counts in the two groups exhibited similar trends in the early period after onset. Mortality is generally related to the number of acutely damaged organs (23). It is difficult to predict the prognosis of patients with sepsis using only a single indicator. The severity of illness when combined with several indices of the organ function is strongly associated with the prognosis of septic patients (24). The acute physiology and chronic health evaluation (APACHE) II, a universal scoring system, uses the platelet count as a platelet function index (25). In studies of myocardial infarction, however, the MPV level has been reported to be superior to the platelet count in predicting the prognosis (10, 26). Therefore, we believe that further prospective studies should be conducted using the MPV levels to evaluate the BSI clinical status.

Our study is associated with several limitations. The retrospective design of this study may have led to data loss. In order to assess the trends in the platelet indices from the pre-BSI period, patients whose platelet indices were not measured before BSI onset were excluded; thus, many patients with community-acquired BSI were not included as study subjects. Furthermore, patients with preexisting hematological diseases, who are frequently affected by nosocomial BSI, were also excluded due to abnormalities at baseline. To evaluate the platelet function in such patients, other assays of the platelet function in addition to measurements of the platelet size are required. For the blood sampling, we used tubes containing EDTA as an anticoagulant, which may have induced platelet swelling. The MPV levels measured in EDTA-preserved samples are reportedly higher than those measured in citrate-preserved samples, and MPV is known to increase on addition of EDTA to citrated samples at six hours after an analysis (27, 28). Most samples in our hospital were measured within one hour; thus, the influence of EDTA as an anticoagulant in our analysis is negligible.

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Table 3. **Independent Predictors of 30-day Mortality of BSI**

Factor	Odds ratio	95% CI	p[a]
Age	1.03	1.00-1.07	0.041
Pathogens			
Gram-positive bacteria	1.00		0.014
Gram-negative bacteria	0.12	0.01-0.34	0.006
Elevation in MPV levels in each intervals			
1st to 2nd (<-7 to 0-1)	0.50	0.23-1.09	0.037
2nd to 3rd (0-1 to 3-5)	1.82	1.00-3.32	0.027
3rd to 4th (3-5 to 7-10)	1.84	1.07-3.15	0.002

Elevation in MPV levels in each interval represents elevation in MPV levels from the previous period to the subsequent period, and days from the onset of BSI are given in parentheses. [a]Statistically significant on multivariate logistic regression. CI: confidence interval, MPV: mean platelet volume, BSI: bloodstream infection

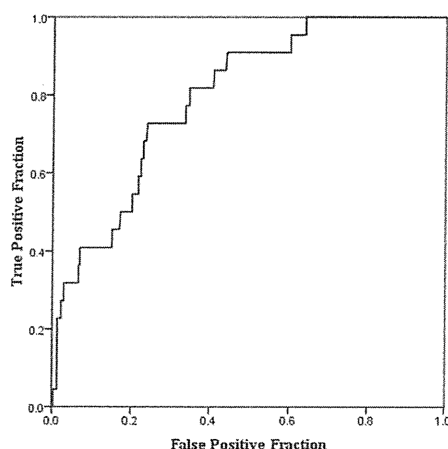

Figure 3. **Receiver operating characteristic (ROC) curves for prognosis prediction based on changes in the MPV levels after the onset of BSI.**

In conclusion, the platelet indices changed after the onset of BSI in this study. The degree of elevation of the MPV levels at onset was lower in the nonsurvivors than in the survivors; however, after elevation of MPV, the MPV levels remained higher in the nonsurvivors than in the survivors. Elevation of the MPV level after BSI onset was identified to be a prognostic factor for BSI. At present, in the management of sepsis, the administration of recombinant human activated protein C or platelet transfusion is indicated according to the severity of illness or the degree of thrombocytopenia, respectively (29). Monitoring the MPV levels as part of evaluating the hematopoietic and thrombotic status may provide more useful information for assessing the efficacy of antithrombotic treatment as well as therapies for regulating platelet consumption and activation.

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

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References

1. Skogberg K, Lyytikainen O, Ollgren J, Nuorti JP, Ruutu P. Population-based burden of bloodstream infections in Finland. Clin Microbiol Infect **18**: E170-E176, 2012.
2. Nagao M. A multicentre analysis of epidemiology of the nosocomial bloodstream infections in Japanese university hospitals. Clin Microbiol Infect 23 Nov 2012, DOI: 10.1111/1469-0691.12083
3. Vincent JL, Yagushi A, Pradier O. Platelet function in sepsis. Crit Care Med **30**: S313-S317, 2002.
4. Levi M. Platelets in sepsis. Hematology **10** Suppl 1: 129-131, 2005.
5. Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakynthinos S. Coagulation system and platelets are fully activated in uncomplicated sepsis. Crit Care Med **28**: 451-457, 2000.
6. Griesshammer M, Bangerter M, Sauer T, Wennauer R, Bergmann L, Heimpel H. Aetiology and clinical significance of thrombocytosis: analysis of 732 patients with an elevated platelet count. J Intern Med **245**: 295-300, 1999.
7. Michelson AD. Platelets San Diego. Academic Press, Calif., 2002.
8. Jackson SR, Carter JM. Platelet volume: laboratory measurement and clinical application. Blood Rev **7**: 104-113, 1993.
9. Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR. Platelet size as a determinant of platelet function. J Lab Clin Med **101**: 205-213, 1983.
10. Martin JF, Bath PM, Burr ML. Influence of platelet size on outcome after myocardial infarction. Lancet **338**: 1409-1411, 1991.
11. Yilmaz Z, Eralp O, Ilcol YO. Evaluation of platelet count and its association with plateletcrit, mean platelet volume, and platelet size distribution width in a canine model of endotoxemia. Vet Clin Pathol **37**: 159-163, 2008.
12. Hekimsoy Z, Payzin B, Ornek T, Kandogan G. Mean platelet volume in Type 2 diabetic patients. J Diabetes Complications **18**: 173-176, 2004.
13. Becchi C, Al Malyan M, Fabbri LP, Marsili M, Boddi V, Boncinelli S. Mean platelet volume trend in sepsis: is it a useful parameter? Minerva Anestesiol **72**: 749-756, 2006.
14. Bessman JD, Gilmer PR, Gardner FH. Use of mean platelet volume improves detection of platelet disorders. Blood Cells **11**: 127-135, 1985.
15. Dastugue N, Picheloup F, Sie P, Genestal M, Cathala B, Boneu B. Increase in mean platelet volume in shock-related thrombocytopenia. Nouv Presse Med **11**: 2899-2901, 1982.
16. Van der Lelie J, Von dem Borne AK. Increased mean platelet volume in septicaemia. J Clin Pathol **36**: 693-696, 1983.
17. Kaneko T, Wada H. Diagnostic criteria and laboratory tests for

Intern Med 52: 1487-1493, 2013 DOI: 10.2169/internalmedicine.52.9555

disseminated intravascular coagulation. J Clin Exp Hematop **51**: 67-76, 2011.
18. Khandekar MM, Khurana AS, Deshmukh SD, Kakrani AL, Katdare AD, Inamdar AK. Platelet volume indices in patients with coronary artery disease and acute myocardial infarction: an Indian scenario. J Clin Pathol **59**: 146-149, 2006.
19. Taniguchi T, Takagi D, Takeyama N, Kitazawa Y, Tanaka T. Platelet size and function in septic rats: changes in the adenylate pool. J Surg Res **49**: 400-407, 1990.
20. Gawaz M, Dickfeld T, Bogner C, Fateh-Moghadam S, Neumann FJ. Platelet function in septic multiple organ dysfunction syndrome. Intensive Care Med **23**: 379-385, 1997.
21. Thompson CB, Jakubowski JA. The pathophysiology and clinical relevance of platelet heterogeneity. Blood **72**: 1-8, 1988.
22. Gawaz M, Fateh-Moghadam S, Pilz G, Gurland HJ, Werdan K. Severity of multiple organ failure (MOF) but not of sepsis correlates with irreversible platelet degranulation. Infection **23**: 16-23, 1995.
23. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med **29**: 1303-1310, 2001.
24. Vincent JL, de Mendonca A, Cantraine F, et al. Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. Working group on "sepsis-related problems" of the European Society of Intensive Care Medicine. Crit Care Med **26**: 1793-1800, 1998.
25. Knaus WA, Zimmerman JE, Wagner DP, Draper EA, Lawrence DE. APACHE-acute physiology and chronic health evaluation: a physiologically based classification system. Crit Care Med **9**: 591-597, 1981.
26. Huczek Z, Kochman J, Filipiak KJ, et al. Mean platelet volume on admission predicts impaired reperfusion and long-term mortality in acute myocardial infarction treated with primary percutaneous coronary intervention. J Am Coll Cardiol **46**: 284-290, 2005.
27. Dastjerdi MS, Emami T, Najafian A, Amini M. Mean platelet volume measurement, EDTA or citrate? Hematology **11**: 317-319, 2006.
28. McShine RL, Sibinga S, Brozovic B. Differences between the effects of EDTA and citrate anticoagulants on platelet count and mean platelet volume. Clin Lab Haematol **12**: 277-285, 1990.
29. Dellinger RP, Levy MM, Carlet JM, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. Intensive Care Med **34**: 17-60, 2008.

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Incidence of *Clostridium difficile*-associated diarrhea in patients using proton pump inhibitors: A Japanese study

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ABSTRACT

Objective: The incidence of *Clostridium difficile*-associated diarrhea (CDAD) has increased in many developed countries. In addition to previous use of antimicrobials, use of proton pump inhibitors (PPIs) is thought to increase the incidence of CDAD. However, most previous studies that showed a positive relationship between PPI use and CDAD were conducted retrospectively in Western countries. We investigated whether the use of PPIs increases the incidence of CDAD in Japan. Methods: The study was carried out with all the patients admitted to the department of internal medicine of Teikyo University Hospital from April 2009 to June 2009. Clinical data were obtained from medical records. CDAD was defined as detection of CD toxin from stool samples in diarrheal patients. PPI users were defined as patients that were prescribed with PPI for more than 30 days at the detection of CD toxin. The results of *Clostridium difficile* (CD) toxin were collected until April 2011. Results: A total of 793 patients were included, and PPIs were prescribed to 489 patients (59.8%). The average age of PPI users was higher than that of PPI nonusers (68.9 vs. 63.1 years). Among the 489 PPI users, 19 patients developed CDAD, while 4 developed CDAD among the 304 PPI nonusers. The relative risk of PPI use on the incidence of CDAD was 3.20 in univariate analysis (95% confidence interval, 1.10 to 9.32, p = 0.04), although the hazard ratio in multivariate analysis was 1.23 (95% confidence interval, 0.35 to 3.83, p = 0.82). Conclusions: There was no association between CDAD occurrence and PPI use in patients in Japan.

Keywords: *Clostridium difficile*-Associated Diarrhea; Proton Pump Inhibitors; Risk Factor

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1. INTRODUCTION

Clostridium difficile is a gram-positive anaerobe that causes a spectrum of diseases from asymptomatic carriage to mild diarrhea to severe pseudomembranous colitis. There has been a dramatic increase in the incidence of *C. difficile*-associated diarrhea (CDAD) in many developed countries. Development of CDAD in hospitalized patients is associated with length of hospital stay, resulting in higher health care costs [1]. The dominant risk factor is antibiotic use [2,3], but other postulated risk factors include advanced age, severe underlying illnesses [4], hospitalization [2], non-surgical gastrointestinal procedures [5], antineoplastic chemotherapy and immunosuppressant agents. In addition to these risk factors, long-term use of proton pump inhibitors (PPIs) has been suggested to be a risk factor for CDAD [6-10]. However, most previous epidemiological studies that showed a positive relationship between PPI use and CDAD were conducted retrospectively in Western countries. In this study, we investigated whether use of PPIs increases the incidence of CDAD at a single institution in Japan.

2. METHODS

2.1. Study Population

We enrolled all of the patients admitted to the Department of Internal Medicine of Teikyo University Hospital (teaching hospital, 1154 beds) in Japan from April 2009 to June 2009, and observed these patients from April 2009 to April 2011. This study design had hospital ethics committee approval; informed consent was waived.

2.2. Case Definition

PPI users were defined as patients prescribed PPIs for

more than 30 days. CDAD was defined as detection of CD toxin from stool samples in diarrheal patients. Usage of immunosuppressants was defined as use of more than 20 mg of prednisolone for more than 30 days. Long hospital stay was defined as a case of total hospitalization for more than 30 days. Comorbid diseases were defined as: renal failure, estimated glomerular filtration rate (eGFR) <30 ml/min; diabetes mellitus, fasting blood sugar levels of 126 mg/dl, or HbA1c ≥6.1%, or past treatment history; solid tumors, radiologically or pathologically verified masses; hematological malignancy, pathologically verified leukemia or lymphoma.

2.3. Evaluation of Clinical Background Data

Clinical data were obtained from medical records, and the results of C. difficile toxin analysis were collected until April 2011. Clinical courses of the patients were retrospectively reviewed to determine the following demographic characteristics: age; gender; comorbid diseases; usage of drugs; hospital stay and occurrence of CDAD.

2.4. Statistical Analysis

The student's t-test for continuous variables and chi-squared test were used when appropriate to compare proportions. For multivariate analysis, the logistic regression analysis was used. All p values were two-sided and were considered to be statistically significant when $p < 0.05$.

3. RESULTS

3.1. Clinical Factors in PPI Users and Nonusers

A total of 793 patients were included in this study. PPIs were prescribed to 489 patients (61.6%) (**Table 1**). The average age of PPI users was higher than that of PPI nonusers (68.9 ± 13.8 vs. 63.1 ± 17.8 years, p < 0.01), and the proportion of male patients among PPI users was also higher than that among PPI nonusers (male/female ratio, 2.26 vs. 1.36, p = 0.04). Among the 487 PPI users, 19 patients developed CDAD, while 4 developed CDAD among the 304 PPI nonusers. The relative risk of PPI use on the incidence of CDAD was 3.20 (95% confidence interval, 1.10 to 9.32, p = 0.04). The proportion of patients with ICU stay was higher in PPI users than that of PPI nonusers (8% vs. 4%, p = 0.03). Multiple logistic regression analysis showed that age, sex, use of H_2 receptor antagonists, long hospital stay, and ICU stay were associated with use of PPI (**Table 2**). We could not demonstrate association between use of antibiotics, CDAD and use of PPI (**Table 2**).

Table 1. Clinical characteristics of proton pump inhibitor users and nonusers.

Categories	PPI user (n = 489)	PPI nonuser (n = 304)	p
Age (years, mean ± S.D.)	68.9 ± 13.8	63.1 ± 17.8	<0.01
Sex (male)	338	189	0.04
Cormobid diseases			
Renal failure	14	8	0.85
Diabetes	41	32	0.31
Solid tumor	32	21	0.84
Hematological malignancy	25	10	0.22
Inflammatory bowel disease	2	2	0.53
Peptic ulcer	16	7	0.43
Collagen disease	15	12	0.51
Cirrhosis	7	3	0.59
Usage of drugs			
Antibiotics (duration; days)			<0.01
<4	229	183	
4 - 14	72	50	
>14	186	71	
H_2 receptor antagonist	21	66	<0.01
Laxative	78	54	0.51
Immunosuppresant	62	34	0.53
Anticancer drug	53	34	0.88
Hospital stay			
Long stay (>30 days)	246	71	<0.01
ICU stay	40	13	0.03
CDAD	19	4	0.04

Abbreviations: PPI, proton pump inhibitor; S.D., standard deviation; ICU, intensive care unit; CDAD, *Clostoridium difficile*-associated diarrhea.

Table 2. Clinical characteristics of proton pump inhibitor users and nonusers.

Categories	HR	95% CI	p
Age (≥65 years)	1.21	1.29 - 2.51	<0.001
Sex (male)	1.71	1.22 - 2.40	0.002
Usage of drugs			
Antibiotics (duration; days)			
<4	1.00		
4 - 14	1.26	0.72 - 1.77	0.61
>14	1.00	0.58 - 1.73	0.99
H_2 receptor antagonist	0.10	0.06 - 0.18	<0.001
Hospital stay			
Long stay (>30 days)	0.19	0.12 - 0.32	<0.001
ICU stay	2.86	1.42 - 5.78	0.006
CDAD	1.23	0.35 - 3.83	0.82

Abbreviations: HR, hazard ratio; ICU,CI, confidence interval; ICU, intensive care unit; CDAD, *Clostoridium difficile*-associated diarrhea.

3.2. Incidence of CDAD during the Study Period

The cumulative incidence of CDAD during the study period is shown in **Figure 1**. The onset of CDAD in more than half patients was within 200 days after admission in both PPI users and nonusers, although there were no significant differences between the two groups (12 cases in 19 PPI users, 2 cases in 4 PPI nonusers, p = 0.62).

4. DISCUSSION

PPIs are the major treatment for gastroesophageal diseases. From American College of Gastroenterology guidelines and a review article, indications for PPI use are as follows: gastroesophageal reflux disease, with complications such as Barrett's esophagus; presence of peptic ulcer disease (PUD) in the stomach or duodenum; use of non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin, and at least one other risk factor (among age over 70 years, medical history or complications of PUD, untreated *Helicobacter pylori* infection with a history of PUD, and/or medical history of gastric hemorrhage or perforation); and simultaneous use of antiplatelet agents, corticosteroids, anticoagulant drugs or selective serotonin reuptake inhibitors. In Japan, approximately half of physicians did not use international guidelines [11], and the most frequently chosen drug for comedication with NSAIDs was a mucoprotective drug with an approximately equal frequency of either PPI or H_2-receptor antagonist use [12]. In this study, the proportion of PPI use was 61.6%, which was higher than that in other reports in Japan. We believe that the reason for the higher proportion of PPI use is that patients with coronary disease are aggressively treated. Craig *et al.* noted that the majority of intravenous PPI prescriptions in hospital were inappropriate, particularly when initiated for non-upper gastroesophageal bleeding indications.

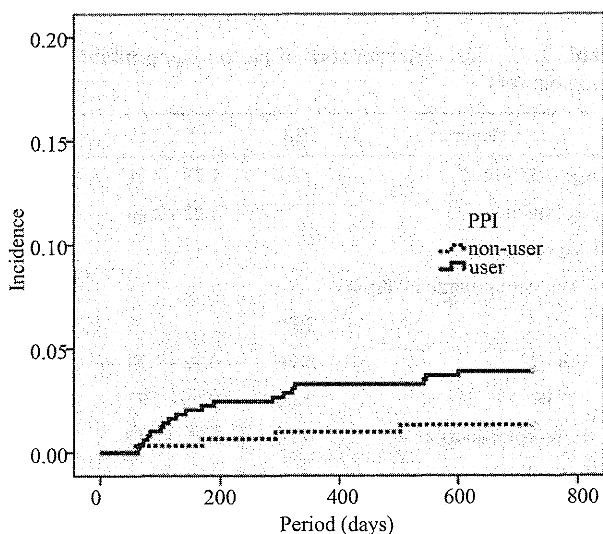

Figure 1. Cumulative incidence of *Clostridium difficile*-associated diarrhea (CDAD) during the study period. CDAD occurred continuously both in proton pump inhibitor (PPI) users and nonusers.

We did not investigate the appropriateness of PPI use in PPI users. If patients with inappropriate use of PPI were excluded from this study, the incidence of CDAD in PPI users may have been higher, as CDAD patients used PPI more frequently than non-CDAD patients, and the patients met the indications for PPI use. Among other risk factors for CDAD incidence, short duration (<4 days) of antibiotic administration was pre-dominant in PPI users and PPI nonusers in this study (47% and 62%, respectively). Stevens *et al.* reported that cumulative antibiotic exposure appears to be associated with the risk of CDAD, although the ratio of short duration of antibiotic use was 9% and 22%, respectively [13]. The high proportion of patients with short duration of antibiotic use might be due to prophylaxis for coronary intervention.

Our study showed that PPI users tended to have multiple risk factors for CDAD occurrence when compared with PPI nonusers. A previous cohort study reported that in addition to a higher incidence of CDAD, PPI users tended to stay in surgical or medical wards longer and were exposed to more than one antibiotic, which was consistent with our study [14]. However, among our data, average hospital stay in PPI users was shorter than that of PPI nonusers. We speculated this tendency because patients with coronary diseases were included among PPI users for the purpose of coronary angiography. From this finding, we believe that it is difficult to evaluate the influence of PPI usage on all hospitalized patients prospectively because more PPI users tend to receive intensive care, which can be biased towards the relationship between PPI usage and CDAD. To lessen the influence of other risk factors on CDAD occurrence by PPI usage, subanalysis of more patients may be required.

On univariate analysis, usage of PPIs was a significant risk factor for the onset of CDAD, but no such relationship was seen on multivariate analysis in this study. Several reports have noted that usage of PPI was significantly related to CDAD [6-9]. In contrast, several other reports have found no relationship between PPI usage and CDAD occurrence [15,16]; however, these studies used cohorts of elderly subjects. Age is also a risk factor for CDAD [17,18]. In our study, the average age of the patients was 66.5 years, which is consistent with other studies. Yearsley *et al.* reported that CDAD was independently associated with acid suppression therapy with PPI in the case-control study. The average age of the cases in the study was higher than that of our study, and ratio of the cases who had received antibiotics was high

(92%) [19]. Patients using PPIs tended to develop CDAD, but the statistical significance was weak. A meta-analysis conducted by Janarthan *et al.* suggested that PPIs increased the incidence of CDAD [20]. This analysis was included in the retrospective hospital-based studies and population-based case-control studies. Clinical backgrounds of the subjects influence the results in the study about association between PPIs and the incidence of CDAD.

In our study, CDAD occurred both soon after admission and later during hospitalization. Acquisition of *C. difficile* and occurrence of CDAD are considered to occur by two routes. The first route is that *C. difficile* intrinsically colonizes the host intestine and proliferates under selective pressure such as antibiotic exposure. The second route is that *C. difficile* is transmitted exogenously by the medical environment and proliferates after reductions in host microflora. We believe that CDAD mainly occurs by exogenous transmission rather than intrinsic proliferation.

In conclusion, there were trends observed between CDAD incidence and PPI use in patients with advanced age, antibiotic usage and shorter hospital stays, although statistical association between CDAD incidence and PPI use was not demonstrated.

REFERENCES

[1] Kyne, L., Hamel, M.B., Polavaram, R., *et al.* (2002) Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile. Clinical Infectious Diseases*, **34**, 346-353. doi:10.1086/338260

[2] Bignardi, G.E. (1998) Risk factors for Clostridium difficile infection. *Journal of Hospital Infection*, **40**, 1-15. doi:10.1016/S0195-6701(98)90019-6

[3] Thomas, C., Stevenson, M. and Riley, T.V. (2003) Antibiotics and hospital-acquired *Clostridium difficile*-associated diarrhoea: A systematic review. *Journal of Antimicrobial Chemotherapy*, **51**, 1339-1350. doi:10.1093/jac/dkg254

[4] Kyne, L., Sougioultzis, S., McFarland, L.V., *et al.* (2002) Underlying disease severity as a major risk factor for nosocomial *Clostridium difficile* diarrhea. *Infection Control and Hospital Epidemiology*, **23**, 653-659. doi:10.1086/501989

[5] Pierce Jr., P.F., Wilson, R., Silva Jr., J., *et al.* (1982) Antibiotic-associated pseudomembranous colitis: An epidemiologic investigation of a cluster of cases. *Journal of Infectious Diseases*, **145**, 269-274. doi:10.1093/infdis/145.2.269

[6] Akhtar, A.J. and Shaheen, M. (2007) Increasing incidence of *clostridium difficile*-associated diarrhea in African-American and Hispanic patients: Association with the use of proton pump inhibitor therapy. *Journal of the National Medical Association*, **99**, 500-504.

[7] Aseeri, M., Schroeder, T., Kramer, J., *et al.* (2008) Gastric acid suppression by proton pump inhibitors as a risk factor for *clostridium difficile*-associated diarrhea in hospitalized patients. *American Journal of Gastroenterology*, **103**, 2308-2313. doi:10.1111/j.1572-0241.2008.01975.x

[8] Dalton, B.R., Lye-Maccannell, T., Henderson, E.A., *et al.* (2009) Proton pump inhibitors increase significantly the risk of *Clostridium difficile* infection in a low-endemicity, non-outbreak hospital setting. *Alimentary Pharmacology & Therapeutics*, **29**, 626-634. doi:10.1111/j.1365-2036.2008.03924.x

[9] Cunningham, R., Dale, B., Undy, B., *et al.* (2003) Proton pump inhibitors as a risk factor for *Clostridium difficile* diarrhoea. *Journal of Hospital Infection*, **54**, 243-245. doi:10.1016/S0195-6701(03)00088-4

[10] Pohl, J.F. (2012) *Clostridium difficile* infection and proton pump inhibitors. *Current Opinion in Pediatrics*, **24**, 627-631. doi:10.1097/MOP.0b013e328355a3e1

[11] Fujiwara, Y., Takahashi, S., Arakawa, T., *et al.* (2009) A 2008 questionnaire-based survey of gastroesophageal reflux disease and related diseases by physicians in East Asian countries. *Digestion*, **80**, 119-128. doi:10.1159/000226088

[12] Arakawa, T., Fujiwara, Y., Sollano, J.D., *et al.* (2009) A questionnaire-based survey on the prescription of non-steroidal anti-inflammatory drugs by physicians in East Asian countries in 2007. *Digestion*, **79**, 177-185. doi:10.1159/000211713

[13] Stevens, V., Dumyati, G., Fine, L.S., *et al.* (2011) Cumulative antibiotic exposures over time and the risk of Clostridium difficile infection. *Clinical Infectious Diseases*, **53**, 42-48. doi:10.1093/cid/cir301

[14] Dial, S., Alrasadi, K., Manoukian, C., *et al.* (2004) Risk of *Clostridium difficile* diarrhea among hospital inpatients prescribed proton pump inhibitors: Cohort and case-control studies. *Canadian Medical Association Journal*, **171**, 33-38. doi:10.1503/cmaj.1040876

[15] Beaulieu, M., Williamson, D., Pichette, G., *et al.* (2007) Risk of *Clostridium difficile*-associated disease among patients receiving proton-pump inhibitors in a Quebec medical intensive care unit. *Infection Control and Hospital Epidemiology*, **28**, 1305-1307. doi:10.1086/521664

[16] Lowe, D.O., Mamdani, M.M., Kopp, A., *et al.* (2006) Proton pump inhibitors and hospitalization for Clostridium difficile-associated disease: A population-based study. *Clinical Infectious Diseases*, **43**, 1272-1276. doi:10.1086/508453

[17] Karlstrom, O., Fryklund, B., Tullus, K., *et al.* (1998) A prospective nationwide study of *Clostridium difficile*-associated diarrhea in Sweden. The Swedish *C. difficile* Study Group. *Clinical Infectious Diseases*, **26**, 141-145. doi:10.1086/516277

[18] Brandt, L.J., Kosche, K.A., Greenwald, D.A., *et al.* (1999) *Clostridium difficile*-associated diarrhea in the elderly. *American Journal of Gastroenterology*, **94**, 3263-3266. doi:10.1111/j.1572-0241.1999.01534.x

[19] Yearsley, K.A., Gilby, L.J., Ramadas, A.V., *et al.* (2006) Proton pump inhibitor therapy is a risk factor for Clostridium difficile-associated diarrhoea. *Alimentary Pharmacology & Therapeutics*, **24**, 613-619.

doi:10.1111/j.1365-2036.2006.03015.x

[20] Janarthanan, S., Ditah, I., Adler, D.G., *et al.* (2012) Clostridium difficile-associated diarrhea and proton pump inhibitor therapy: A meta-analysis. *American Journal of Gastroenterology*, **107**, 1001-1010. doi:10.1038/ajg.2012.179

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Highly-Sensitive Allele-Specific PCR Testing Identifies a Greater Prevalence of Transmitted HIV Drug Resistance in Japan

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Abstract

Background

The transmission of drug-resistant HIV in newly identified infected populations has become an underlying epidemic which can be better assessed with sensitive resistance testing. Since minority drug resistant variants cannot be detected by bulk sequencing, methods with improved sensitivity are required. Thus, the goal of this study was to evaluate if transmitted drug resistance mutations at minority levels in Japanese patients could be identified using highly sensitive allele-specific PCR (AS-PCR).

Materials and Methods

Samples were taken from newly diagnosed HIV/AIDS cases at the National Nagoya Hospital from January 2008 to December 2009. All samples were bulk sequenced for HIV protease and reverse transcriptase. To detect minority populations with drug resistance, we used AS-PCR with mutation-specific primers designed for seven reverse transcriptase inhibitor resistance mutations, M41L, K65R, K70R, K103N, Y181C, M184V, and T215F/Y, and for three protease inhibitor resistance mutations, M46I/L and L90M.

Results

We studied 149 newly identified HIV cases. Bulk sequencing detected 8 cases with NRTI resistance mutations (one with A62V, one D67E, one T215D, one T215E, two with T215L and two T215S) and 15 with PI resistance mutations (one with N88D and 14 with M46I). Results obtained by AS-PCR and bulk sequencing demonstrated good concordance but the AS-PCR enabled the detection of seven additional drug-resistant cases (one M41L, two with K65R, two with K70R, and one M184V) in the RT region. Additionally, AS-PCR assays identified 15 additional cases with M46I, five with M46L and four cases with L90M in the protease region.

Conclusions

Using AS-PCR substantially increased the detection of transmitted drug resistance in this population from 15.4% to 26.8%, further supporting the benefit of sensitive testing among drug-naïve populations. Since the clinical impact of minority drug-resistant populations is not fully comprehended for all mutations, follow-up studies are needed to understand their significance for treatment.

Figures

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Introduction

The use of combination antiretroviral therapy of (cART) has resulted in sustained reductions in morbidity and mortality from HIV infection [1,2]. Five classes of antiretrovirals (ARVs) are currently available in clinical use in Japan. However, selection of drug resistance mutations during cART is still a major issue affecting the clinical efficacy of ARVs and prognosis of HIV infected individuals [3,4].

A United States Department of Health and Human Services (DHHS) guideline recommends drug resistance testing for patients before they begin cART to guide their therapy[5]. Conventional bulk sequencing is used to detect drug resistance mutations in viral RNA from patient plasma, but the method generally does not detect mutants that comprise less than 20% of the viral population in individuals [4-7]. This detection limitation is a concern, both because transmitted minority variants might persist at low frequencies and most newly diagnosed HIV infections are in persons who have been infected for several months to years, providing time for drug resistant viruses with reduced viral fitness to decay to levels that conventional testing is not able to detect [8-10]. Therefore, the ability to detect low-frequency variants below 20% would improve identification of infections involving drug-resistant viruses and better inform decisions on the selection of active ARVs, especially for persons initiating treatment with NNRTI regimens. To detect low-frequency variants, several methods were developed and used to analyze drug-naïve persons and drug-experienced persons [11-14]. Several studies have shown the advantages of highly sensitive drug resistance assays with women who received intrapartum single-dose nevirapine (SD-NVP) for the prevention of mother-to-child HIV transmission. These reports on testing for NVP resistance have found that drug resistance

emerges more frequently and persists for longer than previously demonstrated by bulk-sequencing. Persisting minority NVP-resistant viruses may result in poor virologic responses when subsequent regimens contain nevirapine-related drugs [15-19].. We previously reported that highly-sensitive drug resistance testing that is based on allele-specific real-time PCR can detect minority drug-resistant variants both in infections reported to be wildtype and infections involving other resistance mutations as determined by bulk sequencing. As with majority-level resistance, the amount of low-frequency resistance in new infections reflects both the prevalence of cART use in the region and behavior that is inconsistent with prevention practices for persons on therapy [20].

Recently, it has been reported that the prevalence of drug-resistant HIV transmission among newly diagnosed patients analyzed by bulk sequencing is increasing in Japan, rising from 5.9% in 2003 to 8.3% in 2008 [21]. As the study concluded, this observation was seen not only for recently infected persons, but also chronically infected but recently diagnosed cases, raising concern over the amount of resistance detection lost due to reversion. Therefore, by use of a highly sensitive method in the current study we attempted to examine for the possibility and prevalence of transmitted drug resistant mutations hidden as minority populations.

Materials and Methods

Ethics statement

Specimens were anonymous residual diagnostic material from subjects who provided written consent for HIV testing. The Ethical Committee for Biomedical Science of the National Institute of Infectious Diseases determined that this testing did not involve identifiable human subjects and has approved the study.

Samples

The 192 plasma samples were collected from HIV/AIDS cases for drug resistance analysis from January 2008 to December 2009 in National Nagoya Hospital (Table 1). Among these, 149 cases of newly diagnosed HIV-1 subtype B-infected ART-naïve individuals were selected and analyzed in this study (Figure 1). All samples were collected as part of HIV surveillance studies under Institutional Review Board of National Institute of Infectious Diseases, and written informed consent was obtained from each patient. These samples were directly sequenced for HIV protease (PR) positions 1-99 and reverse transcriptase (RT) positions 1-240. Drug resistance mutations were defined according to the mutation list proposed by Bennett et al. 2009[22]. All testing was performed by the NIID AIDS Research Center in Tokyo, Japan[21].

		2008	2009	Total
Total		75	74	149
Gender	male	74	73	147
	female	1	1	2
	unknown	0	0	
Age	median(Q1, Q3)	39	38	39
Risk behavior	MSM	52	49	101
	Sexual	9	11	20
	MSM/Sexual	8	13	21
	Hemophiliac	1	0	1
	Unknown	5	1	6
VL	Median	9.70E+04	7.00E+04	7.90.E+04
	mode	1.10E+05	2.70E+04	1.10.E+05
CD4	average	199.7	225.4	212.0

Table 1. Demographics of samples.

CSV Download CSV

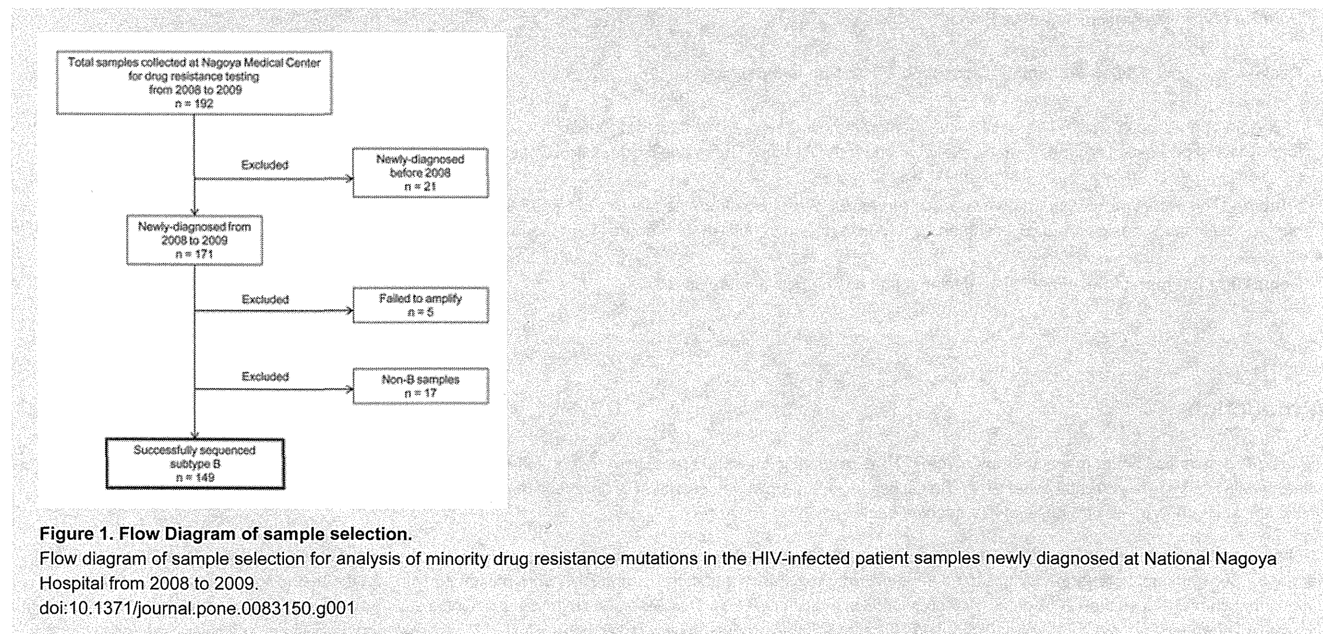

Figure 1. Flow Diagram of sample selection.
Flow diagram of sample selection for analysis of minority drug resistance mutations in the HIV-infected patient samples newly diagnosed at National Nagoya Hospital from 2008 to 2009.
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RNA extraction and virus template amplification

HIV RNA was extracted by Roche High Pure Viral RNA Kit from 200uL plasma samples. RNA was reconstituted in 100 uL of DEPC water and stored at -80°C until use. The HIV protease-reverse transcriptase (PR-RT) region was amplified by one-step RT-PCR (TAKARA One Step RNA PCR kit) with forward primer (DRPRO5 :

AGA CAG GYT AAT TTT TTA GGG A) and reverse primer (DRRT34 : GCT ATT AAG TCT TTT GAT GGG TCA TA). RT-PCR amplification conditions were 55°C for 40 minutes and 40 cycles of 95°C for 10 seconds, 52°C for 5 seconds, and 72°C for 90 seconds. In the case that the amplification of RT- PCR did not generate sufficient template, nested-PCR was performed using forward primer (PROFWD1F : CAG ATC ACT CTT TGG CAA CGA CC) and reverse primer (GEN4R : ATC CCT GGG TAA ATC TGA CTT GC)[23]. Nested-PCR amplification condition was 94°C 1 minute and 30 Cycles of 94°C for 10 seconds, 55°C for 4 seconds and 74°C for 15 seconds.

Real-time PCR (AS-PCR)

To detect minority populations with drug resistance, we used highly sensitive allele-specific PCR validated for subtype B HIV as described [17,23]. Briefly, mutation-specific primers were designed for seven reverse transcriptase inhibitor resistance mutations, M41L, K65R, K70R, K103N, Y181C, M184V, and T215F/Y. Results of highly sensitive allele-specific PCR and population sequencing data were compared for concordance and presence of additional mutations. The HIV-1 total copy primers, Com2F and Com4BR, span n.t. 258–420 in RT and were used with the common probes, Com1P and 2P (Table S1)[17,23]. For multiple mutation screening, several resistance mutation-specific reactions can be performed simultaneously. The cycle number at which the fluorescence emission exceeds the background fluorescence threshold is the threshold cycle (CT) and is the unit of measure for comparing the differences in amplification signals (ΔCT) between the total copy and mutation-specific reactions. All samples were tested in duplicate with the means of the total copy and mutation-specific CTs used for the determination of the ΔCT. Each ΔCT cutoff value for interpreting the presence of drug resistance mutations was determined previously [23] and were between 8.5 from 10.5 cycles, for validated assay cut-offs ranging from 0.03% to 2.0% mutant, depending on the assay.

Real-time PCRs were initiated with a hot-start incubation at 94°C for 11 minutes before proceeding to 45 cycles of melting at 94°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 30 seconds. All reactions were performed in a total volume of 50 uL/well in 96-well PCR plates using iQ5 real-time PCR thermocyclers with optical units (Bio-Rad) and AmpliTaq Gold polymerase (2.5 U/reaction; Applied Biosystems). Final reagent concentrations were 320 nM for the forward and reverse primers, 160 nM probe(s), and 400 mM dNTPs.

M46I/L primers for real-time-PCR

For this study, new primers for the detection of M46I and M46L protease inhibitor mutations were constructed as described before [17,23,24]. As with the RT primers, the protease mutation-specific primers (Table S2) were designed to preferentially anneal with the targeted mutation nucleotide(s), thus having reduced affinity for wild-type sequences. Specificity was enhanced by creating designed mismatches at the -2 nucleotide position relative to the primer 3'-end for each primer. Furthermore, to compensate for the spectrum of polymorphisms present, mixtures of three uniquely designed forward primers were required to detect M46L. Mutation-specific primer mixtures were experimentally evaluated and the ratios that best balanced differences in primer avidities and minimized cross-interference in primer annealing were selected.

Site-directed mutagenesis and cloning

M46I and M46L mutant clones for plasmid development were constructed by site-directed mutagenesis using HXB2 as a template. These constructs were used as positive control to verify the M46I/L primers and probes. To insert M46I or M46L mutations into HXB2, PCR with KODplus was performed using a pair of complementary primers (M46I-forward primer : GAA GAT GGA AAC CAA AAA TaA TAG GGG GAA TTc GAG G, M46L(ttg)-forward primer : GAA GAT GGA AAC CAA AAt TGA TAG GGG GAA TTG GAG G , M46L(ctg)-forward primer : GAA GAT GGA AAC CAA AAc TGA TAG GGG GAA TTG GAG G), and reverse primer : CTG GCA AAC TCA TTT CTT CTA ATA CTG TAT CAT CTG CTC C). PCR amplification conditions were 94°C for 2 minutes and 35 cycles of 98°C for 10 seconds, 68°C for 2 minutes and 30 seconds.

Evaluation of the new protease assays on plasmids and clinical samples

HXB2-M46I, HXB2-M46L and HXB2 (wild-type) plasmids were used in the preliminary selection of primer mixtures that provided the greatest sensitivity and specificity. The absolute mutation detection limits for the primer mixtures, that is, the greatest ΔCT that was able to distinguish mutant viruses from wild-type, were estimated from triplicate testing of mutant clone serial dilutions. The assays evaluated mutation-containing sequences at frequencies between 100%-0.0001% in a wild-type background, with each dilution having same total plasmid copies. The ΔCTs generated from the mutant dilutions were compared to the ΔCTs generated with the wild-type plasmids alone. Solely for the purpose of comparing relative assay detection limits with finite virus sequences, the ΔCT within the linear dilution range (R^2>0.995) that was equivalent to a frequency increase of 0.5 $\log_{10}$ above the wild-type mean ΔCT was chosen as the absolute assay detection limit. Selecting the detection limit in this manner provided an adequate buffer against variability in wild-type sequences and also took into account the PCR efficiency of the assay (slope of the dilution curve).

To evaluate cutoff values of M46I and M46L in patient samples, PR-RT sequences derived from ART-naïve patients were analyzed by real-time PCR. Forty-two PR-RT region sequences derived from 16 patients were cloned by TA-cloning to serve as heterogeneous wild-type sequences. Fifty-five samples with protease M46I were obtained from 20 individuals and 22 samples with M46L were obtained from 12 individuals. To increase the stringency of assay evaluations, specimens with substantial numbers of polymorphisms in primer binding sites were also included.

Assessing mutation associations in mutation-specific amplicons

To evaluate whether additional information on resistance mutations could be gained from the real-time PCR assays, we performed bulk sequencing (BigDye reagent, Prism 3130xl analyzer, Applied Biosystems) of the products from M46I/L or L90M-specific reactions to assess mutation linkage. Mutation-specific amplicon sequences were compared to their respective sample bulk sequence for evidence of nucleotide differences. Any other resistance mutation(s) found in the mutation-specific amplicons would indicate that they were on the same viral strand(s) as the mutation that was specifically targeted by the primers.

Phylogenetic analysis

Protease sequences were aligned by means of the clustal-W program with a set of reference sequences recommended by the Los Alamos sequence database (http://www.hiv.lanl.gov/content/index). The results of the alignment were then analyzed by the neighbor-joining method using MEGA5 [24,25]. In order to analyze the relationship between M46I/L-positive amplicon sequences and bulk sequences, we extended the M46I/L-positive amplicon by using the PRO2L reverse primer (Table S1B), which allowed sequencing from PR codon 47 to RT codon 36 of these M46I/L-positive amplicons (270 bp). In the case of L90M amplicon analysis, 209 bp DNA fragments extending from amino acid 20 in PR to amino acid 89 in PR were represented in the phylogenetic tree. The phylogenetic relatedness of these mutation-containing amplicons excluding the resistance codon position were represented in trees constructed using Kimura 2-parameter model with a discrete gamma distribution [1 +G] and 500 bootstrap replications in MEGA5.

Statistics

The Mann-Whitney U test was used to test for differences in CD4 counts and VL between the groups with minority drug resistance mutations and those without minority drug resistance mutations.

Results

Minority M46I could be detected as low as 0.04% and M46L could be detected as low as 0.03% in site-directed mutant clones

Relative limits of detection were compared in a simple laboratory setting using serial dilutions of HXB2-M46I or HXB2-M46L in backgrounds of HXB2 wildtype plasmid. The ΔCT that was equivalent to a 0.5 log greater reactivity than the wild-type mean ΔCT on the dilution curve (M46I : ΔCT=15 cycles, M46L : ΔCT=17 cycles) was used to compare assay sensitivities (Figure 2). This approach yielded detection limits of 0.04% and 0.03% for M46I and M46L, respectively. As this was derived from cloned sequences this is a theoretical detection limit against which clinical specimens are evaluated.

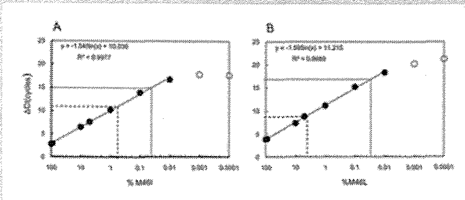

Figure 2. Mutation-specific assay reactivity on plasmids.
Cloned M46I (A.) and M46L (B.) mutant virus template was diluted 10-fold, from 100% to 0.0001%, in backgrounds of wild-type sequence to determine assay detection limits. Plotted are the mean ΔCT versus $\log_{10}$ of the mutant dilution series. The lower detection limit (lower dotted line) was placed at the ΔCT equivalent to 0.5 $\log_{10}$ below (0.5-log greater reactivity than) the wild-type ΔCT. For comparison, the mutant virus frequency equivalences for the established clinical cutoffs are also shown (dashed line).
doi:10.1371/journal.pone.0083150.g002

High sensitivity and specificity of M46I/L detection assays confirmed with clinical samples

Assay cutoff values intended for population-wide clinical screening were established using 42 cloned wild-type sequences derived from 14 patient-derived specimens collected by NIID from 2005-2007. The assay cutoffs selected based on plasmid sequences were evaluated against clinical specimens using a total of 55 samples with sequence-detectable M46I mutation and a total 22 samples with sequence-detectable M46L mutation. The resulting distribution of collated ΔCTs from the wild-type samples supported a ΔCT cutoff of 11 cycles for M46I clinical testing (ΔCTs ranged from 16.39–26.65 cycles) (Figure 3 and Table S3). Extrapolating from the dilution curve for cloned M46I sequences, this cycle difference corresponded to a frequency mean of 0.54% mutant virus (see Table S3). At this cutoff, all 55 genotyped M46I samples were positive (ΔCTs ranged from 1.39 to 10.1 cycles, Figure 3 and Table S3). For M46L assay, ΔCT cutoff was 9 cycles to avoid low-level amplification from spurious primer binding against clinical quasispecies specimens; this cutoff placement corresponded to a frequency mean of 4.01% mutant virus. All genotype M46L samples were positive (ΔCT ranged from 0.88 to 8.95 cycles) (Figure 3 and Table S3). Because of unusual polymorphisms, some samples comprised almost entirely of mutant virus produced ΔCTs near the cutoff. In these situations, elevated ΔCTs resulting from weak primer binding could be interpreted as mutant viruses present at low frequencies. Hence, this testing format is best-suited to provide highly specific population-level resistance screening and is not necessarily applicable to mutant virus quantitation.

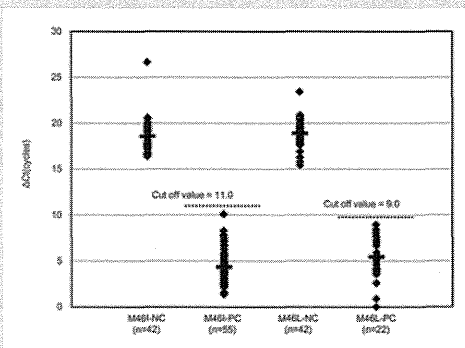

Figure 3. Assay reactivity with wild-type and M46I/L mutation clinical samples.
Dotted ΔCT values from clinical samples with sequence-detectable mutations and with wild-type sequences are shown. The range of reactivity for each assay is shown for wild-type and mutant samples. The mean of ΔCT (bar) for each group is indicated. Assay cutoffs (dotted horizontal line) were established to exclude all wild-type viruses from detection. PC; Positive clones with M46I/L, NC; Negative clone with no M46I/L(wild type).
doi:10.1371/journal.pone.0083150.g003

AS-PCR method identifies a greater prevalence of transmitted HIV drug resistance

Samples from a total of 149 subtype B cases were collected at Nagoya Medical Center for drug resistance testing. Drug resistance mutations were initially analyzed by bulk sequencing, and 23 cases were found to possess drug resistance mutations. As summarized in Table 2, all resistant mutations were found as sole mutation, one with A62V, one with D67E and six cases of intermediates at codon 215 (one with T215D, one with T215E, two cases of T215L and two cases of T215S), one with N88D and 14 cases of M46I mutation were detected by conventional bulk sequencing analysis, yielding a drug resistance mutation prevalence of 15.4% (23/149 cases) (Table 2). The sensitive screening detected an additional one case of M41L (0.67%), two cases of K65R (1.34%), two cases of K70R (1.34%), one case of M184V (0.67%), 15 cases of M46I (19.46%), 5 cases of M46L (3.36%) and 4 cases of L90M (2.68%) as minority-level drug resistance mutations (Table 2). The identified A62V, D67E, T215E, T215S and N88D mutations detected by bulk sequencing were not targeted by AS-PCR, and therefore were not included in determining changes in mutation frequency. All 17 mutations detected by bulk sequencing analysis that were also targeted by AS-PCR were likewise detected by the sensitive PCR method. The combined prevalence of drug resistance mutations in the total of 149 cases was 26.8% (40/149 cases) (Table 2). In one case, six mutations, M41L, K70R, M184V, M46I, M46L and L90M were detected as minority mutations by the highly sensitive assays (ID 29). These six mutations were undetectable by bulk sequencing analysis. In other cases, K70R and M46I were detected (ID 22), and M46I and M46L were detected in another case as minority drug resistance mutations (ID 5). Of those with minority drug resistance mutations, 11 cases were from 2008 and 12 cases were from 2009. The majority of patients with minority variants were MSM (90.9% in 2008 and 83.3% in 2009) and Japanese, and no significant differences were observed in viral load and CD4 counts by Mann-Whitney U test (p=0.17

and p=0.308, respectively) for persons with or without mutations. Though all of the samples from 2008 were Japanese patients, three cases from 2009 were non-Japanese patients (Table 2).

							Bulk-seq		AS-PCR	
ID	Gender	Risk behavior	Year	Nationality	VL	CD4	RT mutations	PR mutations	RT mutations	PR mutations
1	M	MSM	2008	Japan	2.0.E+04	402	A62A/V			
2	M	Heterosexual	2008	Japan	1.2.E+06	14		N88D/N		
3	M	Heterosexual	2008	Japan	3.1.E+05	38	T215L		T215F*	
4	M	MSM/Heterosexual	2008	Japan	5.8.E+05	222	D67D/E			M46I
5	M	MSM	2008	Japan	2.6.E+04	481				M46I, M46L
6	M	MSM	2008	Japan	1.7.E+06	10				M46I
7	M	MSM/Heterosexual	2008	Japan	4.1.E+05	39	T215S			
8	M	MSM	2008	Japan	2.2.E+05	14		M46I		M46I
9	M	MSM	2008	Japan	1.5.E+04	356				L90M
10	M	MSM/Heterosexual	2008	Japan	1.2.E+05	28	T215S			
11	M	MSM/Heterosexual	2008	Japan	1.3.E+05	348		M46I		M46I
12	M	MSM	2008	Japan	2.1.E+04	391			K65R	
13	M	MSM	2008	Japan	2.1.E+05	553		M46I		M46I, L90M
14	M	Heterosexual	2008	Japan	6.7.E+04	153		M46I		M46I
15	M	MSM	2008	Japan	2.2.E+05	10		M46I		M46I, M46L
16	M	MSM	2008	Japan	1.2.E+03	45	T215L		T215F*	M46I
17	M	MSM	2008	Japan	2.6.E+04	750		M46I		M46I
18	M	MSM	2008	Japan	1.1.E+05	146		M46I		M46I
19	M	MSM	2008	Japan	8.4.E+04	11				M46I
20	M	MSM	2008	Japan	1.4.E+05	86		M46I		M46I
21	M	MSM	2008	Japan	1.1.E+05	1050				M46I
22	M	MSM	2008	Japan	2.2.E+04	154			K70R	M46I
23	M	MSM	2009	Japan	7.2.E+03	319				M46L
24	M	MSM	2009	Japan	2.9.E+04	185				M46I

Table 2. Characteristics of HIV/AIDS patients with drug resistance mutations.

* T215F detection primers can detect T215L, T215I and T215V. **T215Y detection primers can detect T215D, T215H and T215N.

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Sequence analysis of M46I and M46L-specific amplicons showed that these mutations were not linked to L90M in the patient samples

To analyze the linkage between drug resistance mutations, we directly sequenced positive M46I/L or L90M-specific PCR products to ascertain whether additional genotypic information could be obtained from those amplicons. In ID 29, the I72V polymorphism observed in the bulk sequence was detected in M46L and L90M amplicons, but not in the M46I amplicon (Table 3). Additionally, M46I/L mutations were not detected in the minority L90M amplicon indicating these mutations were not linked. In ID 27, A71T was detected in the M46L amplicon, but this mutation was not found in the M46I amplicon or the bulk sequence (Table 3). In ID 22, the M46I amplicon matched the bulk sequence.

Sample	Sequences	Mutations
ID 29	Direct-sequencing	I62V, L63P, *I72V*, T74A, V77I, I93L
	M46I amplicon*	**M46I**, I62V, L63P, T74A, V77I
	M46L amplicon*	**M46L**, I62V, L63P, *I72V*, T74A, V77I
	L90M amplicon**	I62V, L63P, *I72V*, T74A, V77I, **L90M**
ID 27	Direct-sequencing	**M46I**, E21R, R41K, I62V, L63P, L89I, Q92K, I93L
	M46I amplicon*	**M46I**, I62V, L63P
	M46L amplicon*	**M46L**, I62V, L63P, *A71T*
ID 22	Direct-sequencing	E35D, M36I, L63P, H69K, V77I
	M46I amplicon*	**M46I**, L63P, H69K, V77I

Table 3. Genetic linkage of M46I/L or L90M and other mutations.

* M46I and M46L amplicons were spanned from M46 to N88.
** L90M amplicon was spanned from I15 to L90.

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Phylogenetic relatedness of minority and bulk sequence-detectable resistance mutations

Phylogenetic analysis conducted on all the protease sequences produced a pattern consistent with good separation of unrelated sequences even though they were not supported at the roots by significant bootstrap values due to somewhat short sequence lengths (Figure 4A). However, the branch tips show strong bootstrap support for the relatedness of minority variants to the patient bulk sequences from which they were derived. Moreover, some of the patients with bulk sequence-detectable M46I appeared to group together with relatively high bootstrap values (pairs X and Y, Figure 4A) and may represent infections linked within transmission clusters. The sequences within each X and Y pair were 100% identical in the 270 bp analyzed, with the exception of ≤3 mixed-base positions that included the nucleotide of the paired patient. In assessing the relatedness of the four detected minority L90M to infections that have the PR M46I mutation, three L90M were from patients that were wildtype at codon 46, the fourth was ID 13 which was also an M46I case (Figure 4B).