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BRIEF REPORT

Clinical features of *Bacteroides* bacteremia and their association with colorectal carcinoma

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Abstract
Purpose We investigated the clinical features of *Bacteroides* bacteremia for 5 years to determine the risk factors for mortality and to ascertain whether bacteremia due to *Bacteroides* spp. is associated with colorectal carcinoma.
Methods This study comprised a review of all patients with *Bacteroides* bacteremia at a teaching hospital in Tokyo from April 2003 to March 2008. We also conducted a case–control study between *Bacteroides* bacteremia and bacteremia due to other pathogens.
Results During the study period, 25 cases of bacteremia were due to *Bacteroides* spp. *Bacteroides* bacteremia was associated with a high mortality rate (24%). Malignancy (76%) was the major comorbidity, followed by a history of surgery (40%). Colorectal carcinoma was the most frequent ($n = 8$, 32%) of the comorbid malignancies and was recognized as the primary infection site in six cases. Prevalence of colorectal carcinoma as comorbidity was significantly higher in *Bacteroides* bacteremia than in other bacteremia.
Conclusions In the *Bacteroides* bacteremia cases of this study, colorectal carcinoma was the major comorbidity and primary infection site. Colorectal carcinoma screening in *Bacteroides* bacteremia patients is potentially an important diagnostic marker for the early detection of this infection in the future.

Keywords *Bacteroides* spp. · Bacteremia · Colorectal carcinoma · Clinical features

Introduction

Bacteroides spp. are Gram-negative anaerobes that are the most commonly isolated pathogens from human infections [1]. *Bacteroides* spp. normally colonize the gut, yet some species are opportunistic human pathogens, causing infection of the peritoneal cavity, infection following gastrointestinal surgery, and appendicitis due to abscess formation [1, 2].

Mortality from bacteremia caused by *Bacteroides* spp. has been high due to the natural resistance of *Bacteroides* spp. to many antibiotic drugs [3–5]. In the 1990s, the introduction and wide-spread use of preoperative antibiotic prophylaxis and broad-spectrum antibiotic drugs resulted in the dramatic decrease the occurrence of anaerobic infections [6, 7]. However, recent reports indicate that anaerobic infections have reemerged as a serious health threat, and so anaerobes, such as *Bacteroides* spp., are once again being recognized as important pathogens [8].

An increased prevalence of enterotoxigenic *Bacteroides fragilis* (ETBF), which belongs to the genus *Bacteroides*, has been reported in the stools of colorectal cancer patients, and ETBF has also been found to promote colon tumorigenesis [9, 10]. In studies focusing on the association between carcinoma and bacteremia, *Streptococcus bovis* has been reported to be a pathogen associated with colon carcinoma and, therefore, screening of colon carcinoma is recommended when *S. bovis* bacteremia is diagnosed [11].

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However, there is as yet no published study on whether bacteremia due to *Bacteroides* spp. occurs in association with colorectal carcinoma.

In the study reported here, we reviewed the clinical backgrounds of cases of *Bacteroides* bacteremia to determine whether colon carcinoma as comorbidity or primary infection site was associated with the presence of *Bacteroides* bacteremia.

Methods

Study population

All patients with *Bacteroides* bacteremia from April 2003 to March 2008 at the University of Tokyo Hospital (a teaching hospital, 1,150 beds) in Japan were enrolled in this study. The ethical committee of the University of Tokyo approved this project.

Case definition

In this study, bacteremia was defined as at least one blood culture positive for *Bacteroides* spp. in the presence of signs and symptoms of infection. including fever, chills, disorientation, hypotension, and respiratory failure. Elevation of the serum C reactive protein level (>0.3 mg/dl) and white blood cell counts (>10,000/mm^3) in the peripheral blood was useful as an adjunct to the diagnosis of infection. The onset of bacteremia was defined as the day of the first positive blood culture. Bacteremia cases that occurred >30 days after the initial episode were defined as new cases.

Microbiology

Blood specimens were inoculated into BacT/ALERT blood culture bottles, and the blood culture was assessed to be positive automatically by BacT/ALERT 3D systems (bioMérieux, Marcy l'Etoile, France). When growth was detected, microorganisms were identified by the Vitek I system (bioMérieux). To identify the anaerobes, including *Bacteroides* spp., we used the Vitek ANI card and vitek software (ver. VTK-R07.01, bioMérieux). Antibacterial susceptibility assays to determine the minimum inhibitory concentrations of antibacterial agents were performed by broth microdilution methods according to the guidelines M11-A7 recommended by Clinical Laboratory Standards Institute [12].

Evaluation of clinical background

The clinical courses of the patients were retrospectively reviewed to determine the following demographic characteristics: age; gender; severity of illness at the onset of bacteremia; underlying factors (malignancy, diabetes mellitus, use of steroids or immunosuppressive agents, liver cirrhosis, a history of surgery, previous antibiotic use); primary infection site; bacteremia-attributable mortality. The definition of 'a history of surgery' included cases in which any surgery had been performed before the onset of *Bacteroides* bacteremia. The definition of 'previous antibiotic use' referred to the use of an antibiotic drug 14 days before the onset of *Bacteroides* bacteremia. The Sepsis-related Organ Failure Assessment (SOFA) score was used to measure the severity of each patient's infection [13]. The definition of bacteremia-attributable mortality was the number of patients whose death was due to bacteremia.

Case–control study

A case–control study was performed to evaluate the specific characteristics of bacteremia due to *Bacteroides* spp. compared to the characteristics of bacteremia due to bacteria other than *Bacteroides* spp. Case subjects were those patients with *Bacteroides* bacteremia. By random sampling the same numbers of case subjects, control subjects were selected from hospitalized patients who had bacteremia due to bacteria other than *Bacteroides* spp.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD) unless otherwise indicated. The Student's t test or Mann–Whitney U test for continuous variables and the Fisher's exact test were used when appropriate to compare proportions. All P values are two-sided and were considered to be statistically significant at $P < 0.05$.

Results

During the study period in our hospital, there were 2,307 cases of bacteremia, of which 25 (1.08%) were due to *Bacteroides* spp. The species of *Bacteroides* identified included the *B. fragilis* group (13 cases), *B. eggerthii* (1 case), and *B. uniformis* (1 case); in ten cases, the species could not be identified. Seventeen men and eight women were included in the study.

Of the 25 cases, six patients (24%) died of *Bacteroides* bacteremia, of whom all died specifically due to *Bacteroides* septicemia although one patient had end-stage pancreas carcinoma that may also have played a role in the death. Mean SOFA score (±SD) at the onset of presentation was 3.68 ± 2.79 and was statistically significant between surviving patients and those with a negative

outcome (2.79 ± 1.90 vs. 6.50 ± 3.54, respectively; $P = 0.046$). No other clinical factors were identified as being statistically significant between the surviving patients and those that died. Of the 25 patients with *Bacteroides* bacteremia, 14 (56%) had a prior history of antibiotic use. The major comorbid condition was malignancy ($n = 19$, 76%), followed by a history of surgery ($n = 10$, 40%). There were six cases (24%) of polymicrobial infections, including those caused by *Escherichia coli* (2 cases) and by *Enterococcus faecalis*, *Stenotrophomonas maltophilia*, *Veillonella*, and *Staphylococcus epidermidis* (1 case each). Resistance to clindamycin, aminobenzylpenicillin, and piperacillin was present in 12 (48%), 25 (100%), and 13 (52%) cases, respectively.

The major comorbid malignancy was colorectal carcinoma ($n = 8$, 32%). Of the cases where bacteremia occurred after surgery, the surgeries included hemorrhoidectomy (1 case), dialysis shunt (1 cases), liver transplantation (2 cases), radical operation in colon carcinoma (2 cases), and a radical operation in other types of carcinoma (3 cases).

In terms of treatment, carbapenem was administered to 21 patients (84%) and ceftriaxone, cefozopran, and ciprofloxacin were each administered to one patient. No antibiotic drug was administered in only one case. Surgical interventions included abscess drainage (5 cases), intraperitoneal lavage and sutural repair (5 cases), and abscess removal (3 cases).

The primary infection site was identified as peritonitis (6 cases, 24%), abscess [11 cases (44%): circumintestinal (4 cases, 16%; liver (4 cases, 16%); lymphocyst (1 case, 4%; vertebral (1 case, 4%); lung abscess (1 case, 4%)], and cholangitis (3 cases, 12%). In five cases, the primary infection site was unknown, including four cases (16%) of febrile neutropenia.

Taking into account that colorectal carcinoma was the major comorbid condition of *Bacteroides* bacteremia, we investigated whether *Bacteroides* bacteremia was associated with colorectal carcinoma as the primary infection site. Six cases (24%) were associated with colorectal carcinoma. These cases had comorbidity of colorectal carcinoma, with the primary site of bacteremia being an abscess around the colorectal carcinoma or peritonitis due to rupture of the colon by invasion of the colorectal carcinoma. Primary infection sites of the other two cases with colorectal carcinoma were cholangitis and liver abscess. During hospitalization of the patients for *Bacteroides* bacteremia, colorectal carcinoma was detected in four of the six patients.

In order to investigate whether *Bacteroides* bacteremia frequently occurred in association with colorectal carcinoma, we compared *Bacteroides* bacteremia cases and other bacteremia cases as the controls (Table 1). The prevalence of colorectal carcinoma as the comorbidity was significantly higher with *Bacteroides* bacteremia than with other bacteremia (32 vs. 4%, respectively; $P = 0.02$), although the prevalences of other carcinomas were not significantly different between *Bacteroides* bacteremia cases and control cases. There were also significant differences in the frequency of colorectal carcinoma as the primary infection site between *Bacteroides* bacteremia and other bacteremia.

We then compared the characteristics of *Bacteroides* bacteremia cases which were associated with colorectal carcinoma and *Bacteroides* bacteremia cases which were not associated with colorectal carcinoma. We found no significant difference between colorectal carcinoma-associated cases and non-associated cases in *Bacteroides* bacteremia. Although without statistical significance, colorectal carcinoma-associated cases tended not to be associated with a history of surgery (0 vs. 53%, $P = 0.05$) or previous antibiotic use (1 vs. 68%, $P = 0.06$) compared to non-associated cases.

Discussion

Mortality attributable to *Bacteroides* bacteremia is known to be high, varying from 7.8% in the absence of *B. fragilis* up to 28% in cases of *B. fragilis* bacteremia [14]. There have also been other reports of a high mortality rate attributable to anaerobic bacteremia, including that caused by *Bacteroides* spp. [1, 15]. In our study, a high mortality rate (24%) was also attributable to *Bacteroides* bacteremia. The severity of the illness, susceptibility to the antibiotic drugs, and the appropriateness of treatment are commonly known factors that affect the prognosis of *Bacteroides* bacteremia [16]. However, in our patients, bacteremia-attributable mortality was associated with the severity of the illness—not with susceptibility to the antibiotics or surgical intervention. In our hospital, susceptibility to aminobenzylpenicillin, clindamycin, and piperacillin is routinely measured, although susceptibility to other antibiotic drugs, including carbapenem, is not routinely tested for. In most of the patients enrolled in this study, carbapenem was administered (84%). However, we were unable to obtain data on the rate of patients for whom the appropriate antibiotics were given. Resistant rates of *B. fragilis* to carbapenems have been reported to be less than 1% [17, 18]. It would therefore be reasonable to treat *Bacteroides* bacteremia with carbapenems in a hospital in which susceptibility testing of carbapenems is not available.

In this study, we demonstrated that patients with *Bacteroides* bacteremia tended to have colorectal carcinoma and that the colorectal carcinoma was the primary infection site of the *Bacteroides* bacteremia. Panwalker [19] reported that 87 (11%) of 793 patients with *Bacteroides* bacteremia

Table 1 Differences in clinical characteristics between *Bacteroides* bacteremia and non-*Bacteroides* bacteremia

	Bacteroides ($n = 25$)	Non-*Bacteroides*[a] ($n = 25$)	*P* value
Gender (male)	17 (68)	17 (68)	1.00
Age, years (mean ± SD)	62.6 ± 14.6	62.1 ± 16.5	0.91
Comorbid conditions			
Malignancy	19 (76)	17 (68)	0.75
Colorectal	8 (32)	1 (4)	0.02
Pancreatic	4 (16)	0 (0)	0.11
Hematological	3 (12)	5 (20)	0.70
Hepatobiliary	2 (8)	4 (16)	0.67
Urological	1 (4)	2 (8)	1.00
Bronchopulmonary	1 (4)	0 (0)	1.00
Craniocervical	0 (0)	3 (12)	0.24
Gastroesophageal	0 (0)	3 (12)	0.24
Others	1[b] (4)	1[c] (4)	1.00
A history of surgery	10 (40)	17 (68)	0.09
Immunosuppressive agents	6 (24)	4 (16)	0.73
Liver cirrhosis	2 (8)	3 (12)	1.00
Diabetes mellitus	3 (12)	1 (4)	0.61
Presence of other pathogens	6 (24)	2 (8)	0.25
Association with colorectal carcinoma	6 (24)	0 (0)	0.02
Bacteremia-attributable mortality	6 (24)	3 (12)	0.46

Data are present as the number (*n*) of cases, with the percentage in parenthesis, unless otherwise indicated

SD standard deviation

[a] Non-*Bacteroides* include the following species and numbers of each species: *Bacillus* spp., 1; *Enterobacter cloacae,* 1; *Enterococcus faecium,* 2; *Escherichia coli,* 1; *Klebsiella oxytoca,* 1; *Klebsiella pneumoniae,* 3; *Proteus mirabilis,* 1; *Pseudomonas aeruginosa,* 2; *Pseudomonas putida,* 1; *Serratia marcescens,* 1; *Staphylococcus aureus,* 4; *Staphylococcus* spp., 4; *Streptococcus* Group (*viridans*), 1; *Streptococcus* spp. (α),2

[b] Ovarian carcinoma

[c] Occult primary carcinoma

had colon carcinoma. However, the authors did not conclude that the colon carcinoma was caused primarily by the *Bacteroides* bacteremia because in most cases the bacteremia occurred after intra-abdominal surgery. In comparison, in our study, there was no history of surgery in six cases (24%) of colorectal carcinoma as the primary infection site. From this result, we conclude that *Bacteroides* bacteremia can occur frequently in relation to colorectal carcinoma. Indeed, in four of the six cases associated with colorectal carcinoma, hospitalization of the patient due to *Bacteroides* bacteremia led to the detection of colorectal carcinoma. We suggest, therefore, that in cases of bacteremia due to *Bacteroides* spp., screening for colorectal carcinoma should be considered.

We found that colorectal carcinoma-associated cases of *Bacteroides* bacteremia tended not to be associated with surgical history and previous antibiotic use, although the differences were not significant. Intra-abdominal surgery has been a popularly accepted risk factor of *Bacteroides* bacteremia, and previous antibiotic use may lead to several changes in the normal intestinal flora, such as increased *Bacteroides* spp. in the intestinal tract which would increase the risk of *Bacteroides* bacteremia based on reports that the increase in intestinal bacteria is associated with bacterial translocation [16, 20]. Therefore, surgery and previous antibiotic drugs are thought to be independent risk factors of *Bacteroides* bacteremia. We suggest that patients with *Bacteroides* bacteremia and no history of operation or previous antibiotic use be carefully examined for the presence of colorectal carcinoma.

There are several limitations to our study. First, this study is a retrospective study conducted in only one institute, and the enrolled patient population is small. Secondly, this study was cross-sectional and could not prove the cause and effect of the occurrence of *Bacteroides* bacteremia. In this case–control study, controls were selected from all bacteremia patients by random sampling due to the presence of pathogens other than *Bacteroides* spp. —not from all anaerobic bacteremia patients. We considered that these "other anaerobic bacteremia" cases were microbiologically adequate as controls, although the total number of such cases was much less than that of *Bacteroides*

bacteremia cases. For example, there were only four bacteremia cases due to *Peptostreptcoccus* spp. and seven cases due to *Fusobacterium* spp. during the study period in our hospital. Selecting cases of other anaerobic bacteremia may be biased in this study, and more cases of anaerobic bacteremia will be required to compare *Bacteroides* bacteremia cases. A large series of cases are needed to demonstrate that patients with colorectal carcinoma tend to be affected with *Bacteroides* bacteremia more than patients without colorectal carcinoma.

Despite the limitations to this study, this is the first study to demonstrate that bacteremia due to *Bacteroides* spp. is associated with colorectal carcinoma. We have presented colorectal carcinoma screening in cases of *Bacteroides* bacteremia, especially in those patients without a history of surgery or previous antibiotic use.

Conflict of interest None.

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NOTE

The usefulness of changing focus during examination using Gram staining as initial diagnostic clue for infective tuberculosis

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Abstract Gram staining is a useful technique for detecting bacteria but is highly questionable in detecting *Mycobacterium tuberculosis*. Its detection generally requires special staining, such as Ziehl–Neelsen staining. We experienced three cases in which tuberculosis was first suggested by Gram staining of sputum or pus, confirmed by Ziehl–Neelsen staining, and diagnosed by polymerase chain reaction or culture. To find colorless tubercle bacilli in clinical samples with various organisms, varying the focus to slightly longer and shorter during study of the slides is indispensable. We present criteria for detecting infective pulmonary tuberculosis in Gram staining. First, in the ordinary focus, weakly stained, thin, gram-positive bacilli are found; second, with a slightly longer focus distance, the thin, cord-like, conspicuous gram-positive bacilli can be observed; and third, with a shorter focus distance, the gram-positive bacilli have changed into the brightened, colorless, or ghost ones. Four laboratory technologists each evaluated 20 Gram-stained samples after being lectured on the criteria, with no prior information about the sample. They accurately evaluated the presence of the bacilli in Gram-stained preparations in more than 90% of samples containing 3+ bacilli on Ziehl–Neelsen staining. Gram staining is available as an easy and rapid initial clue to recognize highly infective tuberculosis.

Keywords Gram stain · *Mycobacterium tuberculosis* · Ziehl–Neelsen staining

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Gram staining is a useful technique for detecting bacteria in infectious diseases. It is widely believed that the Gram stain can identify most bacteria, including mycobacteria [1]. Its role in the detection of mycobacteria, however, is highly questionable, as acid-fast stains are normally used for that purpose [1]. Special staining, such as Ziehl–Neelsen (Z–N) staining, is essential in detecting of *Mycobacterium tuberculosis*, which remains the most rapid, convenient, and least expensive method of directly detecting mycobacteria in specimens [2]. Subsequent confirmation by culture is also mandatory.

Major textbooks of microbiology, clinical pulmonary medicine, and clinical pathology generally fail to address in detail the staining characteristics of this organism with stains other than acid-fast techniques, either ignoring the Gram stain appearance of the tubercle bacillus or reporting a faint gram-positive quality [3].

In some textbooks, one and/or two patterns of Gram-stained tubercle bacilli have been described. *M. tuberculosis* often shows neutral staining [4], often appears as beaded gram-positive bacilli, or fails to stain at all [2]; it may appear to be gram positive, but the bacilli take up the stain weakly and irregularly and without requiring iodine treatment to retain it [1], or show weak gram-positive

staining and appears as colorless rods or "ghosts" [5]. In the literature, there are some reports of characteristics in Gram staining of *M. tuberculosis* as "gram-neutral" (neither positive nor negative) appearance. Hinson et al. reported a case in which Gram staining of sputum containing many mycobacteria resulted in a remarkable "gram-neutral" (neither positive nor negative) appearance of the mycobacteria. They first used the terms "gram-neutral" or "gram-ghost" to describe the failure of acid-fast bacilli in sputum to take up either crystal violet (gram-positive) or safranin (gram-negative). They have shown that the tubercle bacillus is neither gram-positive nor gram-negative on nonfixed slides of sputum [3]. Trifiro et al. [1] also reported the finding of ghost mycobacteria when slides were fixed with methanol and formalin. They have shown that purulent sputum without organisms on Gram stain should merit a search for acid-fast bacilli [3] and that observation of ghost bacilli on the initial Gram stain provides an early and helpful diagnostic clue before confirmation of mycobacteria is made by more specific stains [1, 3]. However, there is generally no description of Gram staining as a useful stain for *M. tuberculosis* in the textbooks. The reason why the usefulness of Gram stain as a helpful diagnostic clue has not been well recognized might be the absence of criteria or procedural searching for *M. tuberculosis* on Gram stain, especially in purulent sputum with various organisms.

We experienced three cases in which *M. tuberculosis* infection was first suggested by Gram staining of sputum or pus, confirmed by Z–N staining, and diagnosed by polymerase chain reaction (PCR) or culture. In one case, ghost bacilli in purulent pus without organisms were observed, as in the cases of Hinson and Trifiro. However, in the other two cases, observation of various amounts of gram-negative and/or gram-positive organisms with no conspicuous ghost bacilli in the purulent sputum led to understanding, at one glance, of bacterial pneumonia. In the ordinary procedure of focusing in slides of purulent sputa, neutrophils first are brought into focus to check the adequacy of the safranin stain and to investigate phagocytosed bacteria, in which focus gram-negative and/or gram-positive organisms are also brought into focus. After once setting a focus, there is no need to change it to detect causative organisms. In the focus, tubercle bacilli were weakly stained as unclear thin cord-like positive rods or sometimes inconspicuous neutral crystal-like fragments (Fig. 1). This technique may explain why tubercle bacilli are most likely to be missed in gram-stained purulent sputum with various organisms so far and that the stain has been considered useless in detecting pulmonary tuberculosis in spite of the positive observations by Hinson and Trifiro. However, with a slightly longer focal distance, the weakly stained gram-positive cord-like rod had changed into a clear conspicuous gram-positive thin bacilli, although the other organisms were out of focus (Fig. 2). In the next step, with shorter focus distance, brightened and colorless bacilli, "gram-neutral" or "gram-ghost," were revealed (Fig. 3). Our experience has shown that, when the sample contains various organisms, changing the focus of the microscope slightly during the examination of the slide is indispensable in searching for tubercle bacilli and that the staining characteristics of the bacilli in Gram stain is a biphasic stain pattern; by changing the focus, conspicuous thin, long, gram-positive bacilli appear to change into gram-neutral bacilli. Thus, we present the following criteria, a procedure for detecting infective pulmonary tuberculosis in Gram staining. (1) First, in ordinary focus, weakly stained, gram-positive long bacilli are found in samples (see Fig. 1); (2) then, with a slightly longer focus distance, the gram-positive, thin cord-like bacilli can be clearly observed (see Fig. 2); and (3) with a shorter focus distance, the gram-positive bacilli have changed into the brightened, colorless, or ghost bacilli (see Fig. 3).

The purpose of this study was to check whether the presented criteria are effective enough for technologists with various levels of analyzing skill to detect the bacilli in clinical samples. Thus, we taught the criteria before they analyzed their preparations. We also checked the level of smear positivity grade of the bacilli found in Gram staining by comparison with Z–N staining. Twenty samples obtained from inpatients or outpatients with tuberculosis at Teikyo University Hospital between April 2007 and September 2009 were analyzed. Samples included those of sputum ($n = 16$), bronchoalveolar lavage fluid (BALF) ($n = 2$), pleural effusion ($n = 1$), and pus ($n = 1$). *M. tuberculosis* was diagnosed by PCR or culture. The samples were subjected to Z–N staining, and scored for tubercle bacilli (none, 1+, 2+, and 3+) according to the criteria of the Japanese Society of Tuberculosis by one laboratory technologist: 1+ indicates one to nine bacteria per 100 hundred fields (×1,000); 2+, more than ten bacteria per 100 fields; and 3+, more than ten bacteria per field. Six, 6, 2, and 6 samples had a score of none, 1+, 2+, or 3+, respectively.

Four laboratory technologists who had been trained in bacteriology at our hospital analyzed each of the 20 Gram-stained preparations and assigned scores according to the specified grouping criteria. They had no prior information about the presence of bacilli in the samples. They accurately evaluated the presence or absence of the bacilli in the clinical samples in 91.7% of the cases (22/24) when no tubercle bacilli were present and in 48.2% of the cases (27/56) when bacilli were present in various numbers (1+ to 3+) according to Z–N staining. However, in samples with a tubercle bacilli score of 3+ on Z–N staining, they accurately evaluated the presence of the bacilli in 95.8%

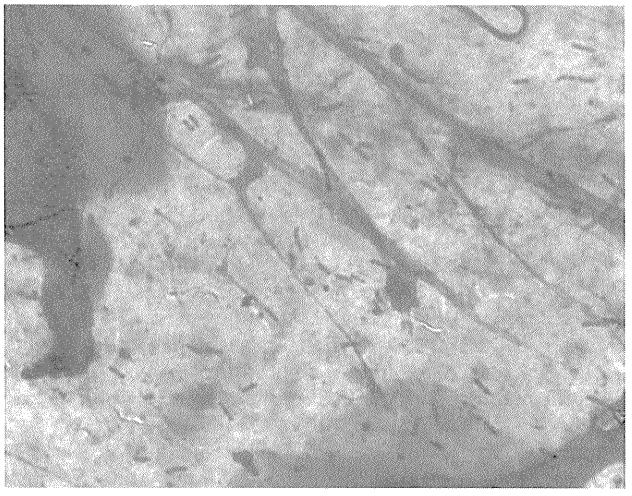

Fig. 1 Gram staining of sputum under ordinary focus (×1,000). There are many gram-negative rods. However, some weakly stained, thin, long gram-positive rods are scattered

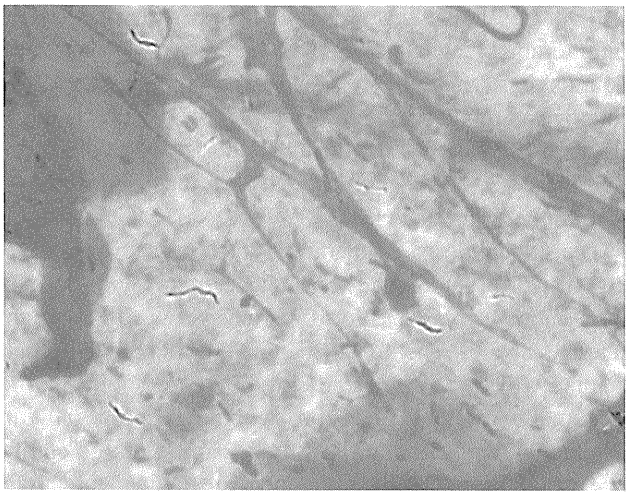

Fig. 2 Gram staining of sputum with slightly longer focus distance (×1,000). When weakly stained gram-positive rods are found, turning the dial to adopt a slightly longer focus distance will clearly show gram-positive cord-like bacilli

(23/24) of the cases, indicating the usefulness of Gram staining in detecting infective *M. tuberculosis*. By using the criteria, to confirm the ease of detection, three sixth-year medical students with no bacteriological training checked the samples after being lectured on the criteria, although both the number of samples (six samples; three 3+ and three negative on Z–N staining) and the number of fields they checked (10–20) were few. Two of the students showed 100% accuracy of detection in both groups, and the third student misread one sample each in both groups. Thus, they accurately evaluated the infective number of bacilli in 89% (8/9) of the cases.

With regard to sensitivity, Gram staining is relatively insensitive. The specimen must contain about 100,000 organisms/ml before visualization is possible [6]. The sensitivity of the acid-fast stain has been estimated to be 5,000–10,000 bacilli/ml of sputum [4], indicating that Z–N staining is 10- to 20 fold more sensitive than Gram staining; this may explain, in part, why the sensitivity of Gram staining in finding tubercle bacilli was not high. However, when technologists were given information that the sample contained tubercle bacilli, they sometimes picked up the bacilli even when their level was only 1+ (data not shown). Therefore, with increasing skill, it may be possible to detect infective tuberculosis with greater sensitivity by Gram staining. The ease of the procedure was confirmed by both the technologists and the medical students, although the number of students participating in this study was small.

There are certain types of images to which attention must be paid to avoid misidentification on Gram staining. Crystal-like fragments are sometimes visualized as thin, brightened, neutral, and positive rods when changing the focus, which misled one of the students.

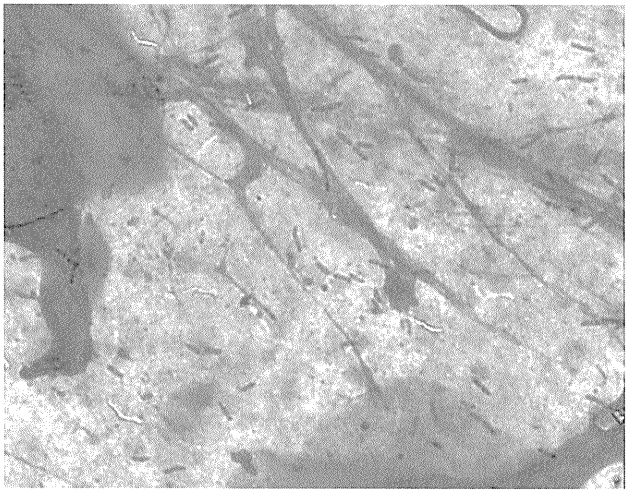

Fig. 3 Gram staining of sputum with slightly shorter focus distance (×1,000). The next step after detection of gram-positive cord-like bacilli is to turn the dial to a slightly shorter focus distance to reveal brightened rods and colorless bacilli

Hinson and Trifiro beneficially observed tubercle bacilli as the ghost type in Gram staining in clinical samples. In addition, we have shown that only with changing focus does *M. tuberculosis* have biphasic stain patterns, gram-positive staining and colorless rods or "ghosts" patterns, and that Gram staining can recognize highly infective *M. tuberculosis* with ease and rapidly for the first time, indicating its usefulness as an initial diagnostic clue for pulmonary tuberculosis. The delay in diagnosis of pulmonary tuberculosis includes the delay in Z–N staining of sputum, which is sometimes done after the therapy by the first antimicrobial agent has proven unsuccessful. This

study have shown that, on initial Gram staining before antimicrobial agent therapy, careful focus-changing examination is routinely needed as initial check for pulmonary tuberculosis, especially when patients have predisposing factors to active tuberculosis, such as diabetes mellitus, liver cirrhosis, hemodialysis, and administration of immunosuppressive drugs.

The tuberculosis epidemic is far from over and is aggravated by multi-drug-resistant tubercle bacilli and the even more dangerous form, extensively drug-resistant tubercle bacilli. The focus-changing technique of Gram staining will contribute as a helpful diagnostic clue before confirmation of tuberculosis for technologists, especially in developing countries. Further studies are needed to clarify the usefulness of Gram staining in finding infectious tuberculosis in various clinical fields or situations.

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CASE REPORT

Pseudomonas putida bacteremia in adult patients: five case reports and a review of the literature

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Abstract *Pseudomonas putida* belongs to the fluorescent group of *Pseudomonas species*, a group of opportunistic pathogens that primarily cause nosocomial infections. However, few cases of *P. putida* bacteremia in adult patients have been reported. We report five cases of *P. putida* bacteremia in adult patients and review 23 previously reported cases. Our five patients consisted of three cases of catheter-related bloodstream infection (CRBSI), one case of indwelling biliary drainage tube-related cholangitis, and one case of cholecystitis. Many of the 23 previously reported cases also included CRBSI. Of the clinical backgrounds, in all 28 reported cases including ours, 24 (85.7%) were immunocompromised. Of the clinical management, in CRBSI, devices were removed in almost all cases (92.9%). Antibiotic susceptibility data of our five cases and another previous case showed that patients with bacteremia had a high susceptibility of *P. putida* to anti-pseudomonal β-lactams. The prognosis for bacteremia with *P. putida* was good, as 26 (92.9%) of the total 28 cases were cured.

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Keywords *Pseudomonas putida* · Bacteremia · Antimicrobial susceptibility

Introduction

Pseudomonas spp. are aerobic Gram-negative bacteria. Due to their ability to metabolize a wide range of compounds, members of this species are able to colonize soil, fresh water and moist environments [1, 2]. These bacteria can act as opportunistic pathogens that primarily cause nosocomial infections. *Pseudomonas aeruginosa* is the most prevalent pathogen among the genus *Pseudomonas*, yet non-*aeruginosa* Pseudomonas have also been associated with clinical infections [3–5]. *Pseudomonas putida*, which belongs to the fluorescent group of *Pseudomonas* spp., has been recognized as a rare pathogen of bacteremia. Most reported cases of bacteremia with *P. putida* have been neonatal infections or outbreak infections due to transfusion of contaminated blood or fluid [6–8]. *P. putida* can acquire broad resistance to β-lactam antibiotics, and some isolates of this organism are capable of producing metallo-β-lactamases [9–12]. Despite this, there have been few reports about antimicrobial susceptibilities in bacteremia. Clinical courses of bacteremia with *P. putida* have not been precisely described due to the rarity of reported infections from *P. putida*, which comprises mostly immunocompromised patients and newborns [6–8, 13]. We report five adult cases of *P. putida* bacteremia occurring in our hospital between April 2003 and March 2007. Upon a brief review of the literature, we were able to identify clinical characteristics of *P. putida* bacteremia and antimicrobial susceptibility of *P. putida* in these bacteremic patients.

Cases

Case 1

A 79-year-old man was admitted for gastrectomy due to gastric cancer. After the surgery, cefmetazole was administered for 6 days because of postoperative prophylaxis. At 7 days post-operation, he became febrile. Peripheral catheter-associated bacteremia was suspected because he developed skin flare and pain at the insertion region with no other findings at the site of infection. The catheter was removed and cefmetazole was replaced with meropenem. *P. putida* was identified on blood culture. The patient fully recovered, and antibiotic treatment was continued for 10 days.

Case 2

A 76-year-old man was admitted for colectomy due to sigmoid colon adenocarcinoma. He had liver cirrhosis with hepatitis C virus and hepatocellular carcinoma. After the colectomy, cephazolin was administered for 5 days as a means of postoperative prophylaxis. At 6 days post-operation, he had a fever and diarrhea. As catheter-associated bacteremia and *Clostridium difficile*-associated diarrhea were suspected, the peripheral vein catheter was removed. Cephazolin was replaced with intravenous ceftazidime and oral vancomycin. *P. putida* was identified on cultures from blood and the catheter. The stool examination tested positive for *C. difficile* toxin. The patient fully recovered and antibiotic treatment was continued for 10 days.

Case 3

A 52-year-old woman with Burkitt lymphoma was admitted for induction chemotherapy. On day 15 post-chemotherapy, her neutrophil count reduced to 240/μl and she developed a fever of 39.5°C. For febrile neutropenia treatment, meropenem administration was initiated, and a central vein catheter was removed. *P. putida* was isolated on cultures from blood and the central vein catheter. She recovered from fever 3 days after antibiotic therapy, although meropenem was switched to ciprofloxacin on day 20 post-chemotherapy because the strain was resistant to almost all β-lactams. Antibiotic therapy with ciprofloxacin was continued for 10 days.

Case 4

A 48-year-old man with liver cirrhosis due to hepatitis B virus and hepatocellular carcinoma underwent liver transplantation. He was discharged with an indwelling biliary drainage tube and continuing to take 11 mg of tacrolimus and 12 mg of methyl prednisolone. Two months after discharge, he visited the emergency room with a fever and shivering. On the blood test, his C reactive protein level was 5.63 mg/dl, and white blood cell count was 18,100/μl. Hepatobiliary enzyme levels were not elevated. Indwelling drainage tube was clogged. Abdominal ultrasonography showed slight dilation of the biliary duct, which was suggestive of device related acute cholangitis. The biliary drainage tube was removed and endoscopic retrograde biliary drainage was performed. Intravenous administration of ceftazidime and vancomycin was initiated. *P. putida* was identified on blood culture, although no organism was cultured from bile. He fully recovered and antibiotic therapy was continued for 10 days.

Case 5

A 27-year-old man was admitted to hospital with fever that had persisted for 4 weeks and cough that had persisted for 5 days. He had common variable immunodeficiency after developing autoimmune hemolytic anemia at the age of 24 years and has been taking 15 mg of prednisolone daily. On chest CT, ground glass-like infiltrates were identified in the bilateral lung, and because the blood (1 → 3)-β-D-glucan level was 293.1 pg/ml, *Pneumocystis jirovecii* pneumonia (PCP) was suspected. After 2 weeks of treatment with trimethoprim–sulfamethoxazole, the patient recovered. On day 4 after the termination of PCP treatment, however, he became febrile again. On the blood test, the aspartate aminotransferase level was 131 IU/l, the alanine aminotransferase level was 310 IU/l, and total bilirubin was 3.3 mg/dl. Panipenem–betamipron was initiated. Abdominal CT showed thickening of the gallbladder wall and fluid around the gall bladder, which was indicative of acute cholecystitis. Percutaneous transhepatic gallbladder drainage was performed. *P. putida* and *Klebsiella oxytoca* were identified on blood cultures. No organism was cultured from bile. He fully recovered and treatment was continued for 18 days.

Discussion

P. putida has been recognized as a rare pathogen of bacteremia in adult patients. The five reported cases of *P. putida* bacteremia in our hospital accounted for 0.22% of the 2307 cases of bacteremia that occurred from April 2003 to March 2007. In the literature, only 23 cases have been reported, excluding paediatric and outbreak cases due to transfusion of contaminated blood or fluid (Table 1) [14–19].

Among the five cases reported herein, four had a medical device as the primary infection site. In 21 cases of

Table 1 Review of the literature, including our five cases

No.	Citation	Age/gender	Co-morbidities	Primary infection site	Indwelling device	Antibiotics	Outcome
1	[15]	65/female	Surgery for maxillary sinus carcinoma	Acute cholecystitis	VC	Kanamycin, cephalothin	Cured
2		30/female	Surgery for ectopic pregnancy	Unknown	VC	Nothing	Cured
3	[18]	19/female	ALL	CRBSI	CVC, removal	Moxalactam	Cured
4		45/female	CML	Unknown	CVC, removal	Ceftriaxone, amikacin	Cured
5		50/male	Lung cancer	Pneumonia	CVC	Cefoperazone, mezlocillin	Cured
6		72/male	Smoldering leukemia	Unknown	CVC	Piperacillin, ceftazidime	Cured
7		36/male	AML	CRBSI	CVC	Moxalactam, ticarcillin, tobramycin	Cured
8		25/male	AML	CRBSI	CVC, removal	Nothing	Cured
9–14	[14]	18–61[a]/male (3), female (3)	Lymphoma (4), AL (1), myeloma (1)	CRBSI (6)[b]	VC (6), removal (6)[c]	Nothing (5), antibiotic drug (1)[d]	Cured
15	[19]	18/female	APL	Thrombophlebitis	Unknown	Imipenem, amikacin	Cured
16		62/male	RHD, CHF	Unknown	Unknown	Ceftazidime	Died
17		32/male	Infective endocarditis	Unknown	Unknown	Imipenem, amikacin	Cured
18		70/female	Cerebral infarction	Acute tonsillitis	Unknown	Piperacillin	Cured
19		70/female	Carcinoma of cervix	Pneumonia	Unknown	Piperacillin, gentamicin	Cured
20		23/female	CHF	CRBSI	CVC, removal	Cefotaxime, oxacillin	Cured
21		23/female	Trauma	Unknown	Unknown	Cefoperazone, netilmicin	Cured
22		79/male	Gastric cancer	Unknown	Unknown	Cefazolin, gentamicin	Died
23	[16]	78/female	Nothing	Soft tissue infection	Nothing	Ceftazidime	Cured
24	Our cases	79/male	Post-gastrectomy (gastric carcinoma)	CRBSI	PVC, removal	Meropenem	Cured
25		76/male	LC, post-colectomy (colon carcinoma)	CRBSI	PVC, removal	Ceftazidime	Cured
26		52/female	Burkitt lymphoma	CRBSI	CVC, removal	Meropenem, ciprofloxacin	Cured
27		48/male	LC, post-liver transplantation (HCC)	Acute cholangitis	Biliary drainage tube, removal	Ceftazidime	Cured
28		27/male	CVID, AIHA	Acute cholecystitis	Nothing	Panipenem, betamipron	Cured

VC central venous catheter or peripheral venous catheter, *PVC* peripheral venous catheter, *CVC* central venous catheter, *CRBSI* catheter related blood stream infection, *ALL* acute lymphoblastic leukemia, *CML* chronic myelogenous leukemia, *AML* acute myelogenous leukemia, *AL* acute leukemia, *RHD* rheumatoid heart disease, *CHF* congestive heart failure, *LC* liver cirrhosis, *HCC* hepatocellular carcinoma, *CVID* common variable immunodeficiency, *AIHA* autoimmune hemolytic anemia

[a] Age range

[b] Definite (4) or probable (2)

[c] As primary (5) or secondary (1) treatment

[d] Antibacterial drug was administrated in one case as a primary treatment, but the name of the drug was not mentioned

Table 2 Antibiotic susceptibilities of *P. putida* in our five cases

Antibiotics	Susceptible	Intermediate	Resistant
Piperacillin	4 (80)	1 (20)	0 (0)
Ceftazidime	4 (80)	0 (0)	1 (20)
Cefotaxime	1 (20)	2 (40)	1 (20)
Aztreonam	1 (20)	2 (40)	2 (40)
Meropenem	4 (80)	0 (0)	1 (20)
Imipenem/cilastatin	3 (60)	0 (0)	2 (40)
Gentamicin	4 (80)	1 (20)	0 (0)
Amikacin	5 (100)	0 (0)	0 (0)
Ciprofloxacin	5 (100)	0 (0)	0 (0)

Numbers shown in parentheses represent the percentage of cases that are susceptible or resistant to the different antibiotics

Antibacterial susceptibility assays to determine the minimum inhibitory concentrations (MICs) of antibacterial agents were performed by broth microdilution according to guidelines recommended by the Clinical Laboratory Standards Institute (CLSI)

identified primary infection sites including our cases, 13 (61.9%) were device related, and among these cases, 12 were CRBSI (92.9%). Of the device-associated infections, the ability of microorganisms to adhere to materials and to promote the formation of a biofilm appears to be the most important feature of their pathogenicity [20]. *P. putida* are able to form biofilms [21]. For treatment of *P. putida* bacteremia, the medical device was removed in 12 (92.3%) of the 13 cases. Thus, when *P. putida* is isolated on blood culture of a patient with a medical device, device-related bacteremia should be mostly suspected and the device should be removed.

In addition to device-related infection, acute cholecystitis was evident in 2t cases (9.5%) and pneumonia in 2 cases (9.5%). *P. putida* has been reported to cause urinary tract infection as well as pneumonia in several cases [19]. We thought that like *P. aeruginosa*, *P. putida* could also colonize the respiratory tract and urinary tract in immunocompromised patients. Since *P. putida* can be detected in the normal oropharyngeal flora [22, 23], it may also cause cholecystitis by colonizing the intestinal tract.

Considering the clinical background, 24 (85.7%) of the 28 cases were in an immunocompromised state (from the use of immunosuppressive drugs, liver cirrhosis, malignancy or other immunosuppressive diseases, or post-operation). Although this factor might be biased as the previous cases comprised those from a cancer center only, limiting the clinical background to cancer patients. Our five cases were all in an immunocompromised state. *P. putida*, therefore, could cause bacteremia, particularly in an immunocompromised patient, though the overall incidence of bacteremia with *P. putida* still remains low.

The rate of susceptibility of *P. putida* to antibiotics in our five cases is shown in Table 2. In case 3, *P. putida* was found to be resistant to both ceftazidime, an anti-pseudomonal cephalosporin, and to carbapenem, while in the other four cases, *P. putida* was susceptible to those antibiotics. In case 4, *P. putida* was resistant to imipenem/cilastatin but not to meropenem. We speculate that a long duration of imipenem/cilastatin use in this case would be related to resistance to imipenem/cilastatin, as in the case of *P. aeruginosa* which develops resistant strains that survive during antibiotic use [24]. In previous reports, *P. putida* isolated from the urinary tract, tracheal aspiration, and areas other than blood of bacteremia patients, has been found to acquire metallo-β-lactamases and were resistant to most β-lactams, including carbapenems [10–12]. On the other hand, Anaissie reported that of most of the clinical isolates, *P. putida* in bacteremic patients was susceptible to ceftazidime, imipenem, and ciprofloxacin [18]. We considered that in order to investigate the cause of high-rate susceptible strains in the blood, comparing susceptibilities of *P. putida* in patients with pneumonia or urinary tract infections to that in patients with bacteremia is imperative.

Prognosis of *P. putida* bacteremia was good, whereby 26 (92.9%) out of 28 cases were cured. Yang reported that in one of two cases where the patient died, inappropriate antibiotic therapy to other co-pathogens was a possible contributing factor [19]. Bacteremia with another *Pseudomonas sp.*, *P. aeruginosa* is known to result in poor prognosis of the disease. Mortality of *P. aeruginosa* bacteremia has been reported at over 30% [25, 26]. We believe the reason for a better prognosis from *P. putida* bacteremia was that the major cause of the bacteremia was device related, and infected devices were removed in most cases. However, we cannot exclude the possibility of bacteriological factors causing bacteremia, such as pyocyanin, exotoxin A, or the type III secretion system that can arise from *P. aeruginosa* infection but not from *P. putida* [27].

In conclusion, we report five adult cases of *P. putida* bacteremia. These cases displayed clinical characteristics: device relatedness as a major cause of bacteremia, an immunocompromised host, a high susceptibility of strains to β-lactams, and a good prognosis overall.

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Change of Positive Selection Pressure on HIV-1 Envelope Gene Inferred by Early and Recent Samples

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Abstract

HIV-1 infection has been on the rise in Japan recently, and the main transmission route has changed from blood transmission in the 1980s to homo- and/or hetero-sexual transmission in the 2000s. The lack of early viral samples with clinical information made it difficult to investigate the possible virological changes over time. In this study, we sequenced 142 full-length *env* genes collected from 16 Japanese subjects infected with HIV-1 in the 1980s and in the 2000s. We examined the diversity change in sequences and potential adaptive evolution of the virus to the host population. We used a codon-based likelihood method under the branch-site and clade models to detect positive selection operating on the virus. The clade model was extended to account for different positive selection pressures in different viral populations. The result showed that the selection pressure was weaker in the 2000s than in the 1980s, indicating that it might have become easier for the HIV to infect a new host and to develop into AIDS now than 20 years ago and that the HIV may be becoming more virulent in the Japanese population. The study provides useful information on the surveillance of HIV infection and highlights the utility of the extended clade models in analysis of virus populations which may be under different selection pressures.

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Introduction

Whether human immunodeficiency virus type 1 (HIV-1) has reached peak virulence or has started evolving toward attenuation is controversial, with different studies suggesting that HIV-1 virulence has been increasing [1,2,3,4,5,6], stable [7,8] or decreasing [9,10]. Arien et al. [11] proposed a model in which the viral virulence can be either attenuated or increasing depending on the genetic diversity of the host population. In a human population with mixed HLA (Human Leukocyte Antigen) alleles and diverse host polymorphisms, the CTL (cytotoxic T lymphocyte) response of the recipient may recognize a different set of HIV-1 epitopes from the donor, so that new mutations in viral epitopes may be necessary for CTL escape, which may cause a reduced viral fitness and lead to HIV-1 attenuation. In contrast, in a homogenous human population with little HLA and genetic diversity, the virus with acquired escape mutations from the donor may escape the CTL response of the recipient as well, so that the virus may become even more virulent leading to rapid disease progression.

In Japan, the number of HIV-1 infected individuals is increasing in recent years, and the main route of infection has changed from blood transmission in the 1980s to homo- and/or heterosexual transmission in the 2000s. The change of transmission routes may affect the pathogenicity of the virus circulating in the population. However, the lack of early viral samples makes it difficult to study possible changes in viral pathogenicity. In this study, we sequenced the full-length *env* gene from HIV-1 samples collected in the late 1980s from six Japanese subjects. In order to investigate possible changes in viral diversity and pathogenicity, we also sequenced the *env* gene from HIV-1 samples collected in the 2000s from 10 Japanese subjects.

The *env* gene is the fastest-evolving gene in the HIV-1 genome [12,13,14,15]. While new mutations in the *env* gene may allow the virus to escape from host immune response, they may also disrupt the function of *env* as the viral envelope. Such conflicting selective pressures shape the evolutionary dynamics of the virus and its population diversity. In this study, we are interested in the extent by which the *env* gene has been able to diversify at the population and individual levels, and whether the virus has been undergoing adaptive evolution. Since CD4 counts and information on viral load were unavailable for the early samples, we employed computational approaches to infer diversity changes and to detect positive selection acting on the *env* gene. In particular, we are interested in whether positive selection pressure differs between the early and recent samples and between within-host and between-host evolution [16,17]. We are also interested in detecting sites in the *env* gene targeted by the human immune system.

Results

Sequences from seven early subjects and 10 recent subjects were successfully obtained

Information concerning the transmission routes and sampling times of all subjects are shown in Table 1. Since not all the early samples were preserved in ideal conditions, we sequenced only

Table 1. Clinical information for the 16 subjects.

Subject No.	Transmission route	Infection time	Sampling date
From 1980s			
02	Blood products	Unknown	1988
03	Blood products	Unknown	1988
31	Homosexual	Unknown	1989
33	Heterosexual	Unknown	1989
40	Blood products	Unknown	1989
60	Homosexual	Unknown	1989
From 2000s			
6657	Homosexual	2004	2005
6739	Heterosexual	2000	2005
6826	Homosexual	2000	2005
6871	Homosexual	2004	2005
6946	Heterosexual	2005	2005
7015	Heterosexual	2000	2005
7060	Homosexual	2000	2005
7259	Heterosexual	Unknown	2006
7353	Heterosexual	Unknown	2006
7374	Homosexual	2004	2006

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seven of the 16 early subjects. By using Subtype Reference Alignments [18], the viral samples from one of seven early subjects were identified as subtype C, while all other early subjects as well as all recent subjects were confirmed to be subtype B. We thus removed the subtype C samples from our analysis. Forty-four sequences for the early group were obtained from PBMC (peripheral blood mononuclear cell) samples as the blood plasma samples could not be amplified by PCR, while 98 sequences for the recent group were obtained from blood plasma samples. Only 2 sequences were collected from each of subjects 3 and 31 in the early group. In total, 142 full-length *env* gene sequences were obtained from the 16 subjects and used for further analysis. These sequences have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB588196-AB588337 (142 entries).

As the codon-based analysis assumes no recombination within the sequence, we run the program RIP 3.0 to detect possible recombinants. The results showed that none of the 142 subtype B sequences was recombinant with other subtypes. For co-receptor usage, WebPSSM program predicted all samples to be CCR5-using viruses.

No significant difference in within-host diversity between the two groups

The phylogenetic tree of 142 *env* sequences was reconstructed using the Neighbor-joining method in MEGA4 [19] under the K80+G model [20] (Fig. 1). This tree is used in the codon-based maximum likelihood (ML) analysis. The robustness of our results to the tree topology is examined later, by duplicating the analysis using the ML tree under the GTR+G model (PhyML) [21]. In both the NJ and ML trees, viral sequences from the same subject formed a distinct cluster, with the between-host branches to be much longer than the within-host branches. These results are compatible with a severe bottleneck at each new infection.

The viral diversity was calculated by measuring nucleotide diversity (π) implemented in MEGA4. The between-host diversity was inferred to be 0.062 for samples of the 1980s and 0.118 for samples of the 2000s. The recent group had larger between-host diversity than the early group, which may simply reflect the accumulation of new mutations over time. The within-host diversities for the early and recent samples almost remained the same (0.012 vs. 0.011).

Positive selection on the viral *env* gene has become weaker in the 2000s

We used the branch-site model [22,23,24] to detect positive selection operating on the HIV-1 *env* gene, with the selective pressure measured by the nonsynonymous/synonymous rate ratio ω [25]. In this model, branches on the phylogeny are partitioned *a priori* into two categories: the foreground branches which may potentially be under positive selection, and the background branches along which positive selection is assumed to be absent. On the background branches, some sites are strongly conserved with $0<\omega_0<1$ while others are evolving neutrally with $\omega_1 = 1$. On the foreground branches some of those sites become under positive selection with $\omega_2 \geq 1$. The parameters in the model representing the proportions of site classes and the ω ratios are summarized in Table 2. The branch-site test compares the null model which assumes $\omega_2 = 1$ against the alternative model with $\omega_2 \geq 1$, with one degree of freedom used. The test allows detection of positive selection affecting the foreground branches even though most codons in the gene are under purifying selection.

We expect the human immune system to exert selective pressure on the virus, but the pressure may differ in the 1980s and in the 2000s or between within-host and between-host evolution. We conducted four analyses in which different branches in the reconstructed tree (Fig. 1) were designated as the foreground branches: (a) 1980s-within, (b) 2000s-within, (c) 1980s-between, and (d) 2000s-between (Fig. S2). The results are summarized in Table 3. The test result was significant with $p<1\%$ in all four analyses, indicating that positive selection most likely operated during both within-host evolution and between-host evolution and both in the 1980s and in the 2000s. Estimates of ω_2 under the model suggest that the selection pressure was stronger between hosts than within host. Interestingly, the early samples were under stronger selection even though the recent samples showed higher between-host diversity. We note that the use of ω_2 estimates in the branch-site model to measure the strength of positive selection may suffer from the strong correlation between estimates of p_2 and ω_2, as it is difficult to distinguish fewer sites under strong selection from more sites under weak selection. Thus we also calculated another heuristic measure of positive selection pressure: $p_2\omega_2$, with the expectation that both a higher ω_2 and a higher p_2 indicate stronger positive selection. Use of this measure instead of ω_2 leads to the same conclusions.

The branch-site model has the limitation that it allows only two types of branches (foreground and background) and positive selection is assumed not to occur on the background branches. For the HIV-1 *env* genes, it is possible that all branches on the tree have some sites under positive selection. The assumption of no positive selection on the background branches may affect the estimation of the strength of positive selection pressure along the foreground branches. Thus we extended the clade model C of Bielawski and Yang [26] to allow for more than two branch types (see Methods). The original model C allows for two branch types (clades) and assumes three site classes: site class 0 of conserved sites with $\omega_0<1$, site class 1 of neutral sites with $\omega_1 = 1$, and site class 2 with different selective pressures (ω_2 and ω_3) in the two clades. We

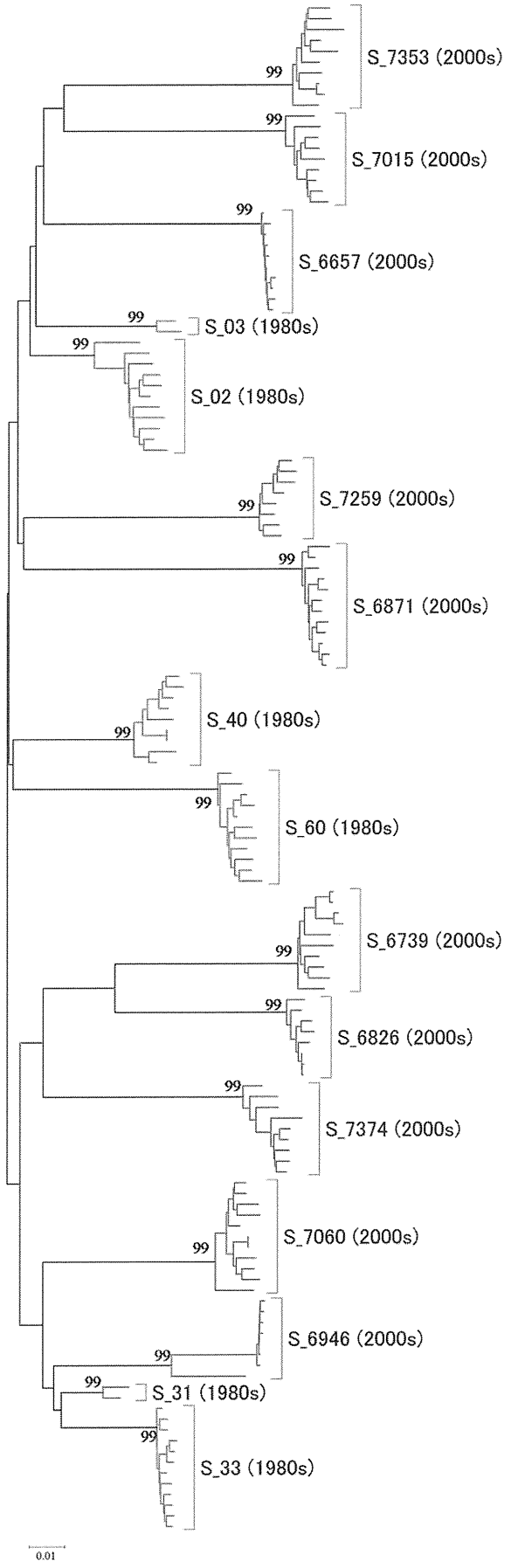

Figure 1. Unrooted phylogenetic tree of 142 *env* gene sequences from 16 Japanese subjects. The tree was constructed by using the neighbour-joining method (MEGA4). The 16 subjects are indicated as S_subject-number (Table 1). Viral samples from the 1980s are indicated in red while those from the 2000s are in blue. The higher bootstrap values (>70%) for major nodes were shown on the tree.
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extend this model to allow for more than two branch types, which have different rate ratios ω_2 and ω_3, … for site class 2 [27]. We specify five branch types in our analysis: 1980s-within, 2000s-within, 1980s-between, and 2000s-between, with all other branches grouped into one branch type. The results are summarized in Table 4. Estimates of the ω ratios for site class 2 for the five branch types (ω_2-ω_6) indicate positive selection for each branch type, but the selective pressure is weaker in the 2000s than in the 1980s. Indeed, estimates of ω_2-ω_6 under the clade model are in the same order as estimates of ω_2 under the branch-site model when the four branch types were individually designated as the foreground branches (Table 3). The results obtained from the two analyses are thus consistent.

We furthermore used the extended clade model to conduct two likelihood ratio tests to examine whether the positive selection pressure has changed between the 1980s and the 2000s. The null hypothesis for the first test is that the positive selection pressure for within-host evolution is the same in the 1980s and in the 2000s ($\omega_3 = \omega_4$). The log likelihood was calculated either with or without this constraint, producing $2\Delta\ell = 32.10$. The null hypothesis is thus rejected, with $p<1\%$. The null hypothesis for the second test is that the positive selection pressure for between-host evolution is the same in the 1980s and in the 2000s ($\omega_5 = \omega_6$). This is also rejected, with $2\Delta\ell = 22.06$, and $p<1\%$ and d.f. = 1.

Positively selected sites were detected in both early and recent samples

Amino acid sites inferred to be under positive selection by the BEB (Bayes Empirical Bayes) approach under the branch-site model at the $P = 95\%$ level are listed in Table 3. We compared those sites with antibody binding sites in the HIV Molecular Immunology Database [28]. Some inferred sites are located in indel-rich regions and are difficult to be identified in the reference sequence HXB2. These sites were excluded. All other inferred sites were identified within at least one of epitope regions for antibody, CTL/CD8 and T-Helper/CD4 (Table 5). Furthermore, each of the four branch-site analyses detected three sites under positive selection within one of the epitopes presented by HLA alleles commonly observed in the Japanese population (with frequency >10%) [29]. Importantly, two of the three sites in the V1-V5 regions, 204V and 420K, were detected to be under positive

Table 2. Parameters in the branch-site model.

Site class	Proportion	Background ω	Foreground ω
0	p_0	$0<\omega_0<1$	$0<\omega_0<1$
1	p_1	$\omega_1 = 1$	$\omega_1 = 1$
2a	$(1-p_0-p_1)p_0/(p_0+p_1)$	$0<\omega_0<1$	$\omega_2 \geq 1$
2b	$(1-p_0-p_1)p_1/(p_0+p_1)$	$\omega_1 = 1$	$\omega_2 \geq 1$

Note. This is the alternative model of the branch-site test of positive selection. The null model fixes $\omega_2 = 1$.
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Table 3. Log-likelihood values and parameter estimates under the branch-site models.

Foreground branch	$\Delta\ell$	Parameter estimates	Detected sites
1980s-within	141.87	$p_0=0.616$ $p_1=0.323$ $p_2=0.061$ $\omega_0=0.076$ $\omega_1=1$ $\omega_2=$**6.598**	**3V** 151- 153A 158T **159I 161G** 163- 164- **166-** 174- **175-** 176- **178- 179- 181E 183A 184N 186T 229- 230- 234D 358N 373F** 476T **523H 527-** 530S **531T** 533V 712N **752I 798G**
2000s-within	38.05	$p_0=0.622$ $p_1=0.302$ $p_2=0.076$ $\omega_0=0.076$ $\omega_1=1$ $\omega_2=$**3.082**	12H 56K 203T **226P 228D** 231- 234D 366H **408E 415- 452N** **461-** 463- 466G 476T **523H 530S 692D** 708A 811D **903L**
1980s-between	64.73	$p_0=0.633$ $p_1=0.337$ $p_2=0.031$ $\omega_0=0.078$ $\omega_1=1$ $\omega_2=$**12.5**	**144D 145L 186T** 187N 188S **204V** 404K 411G **420K** 447L **459-** **461- 464V 470G 471S 474T** 526T 530S 708A 794P
2000s-between	58.58	$p_0=0.625$ $p_1=0.337$ $p_2=0.047$ $\omega_0=0.078$ $\omega_1=1$ $\omega_2=$**5.655**	40Q 95E 144D **145L 146R** 157S **184N** 186T **188S** 204V 341T **351Q 466G 470G 472N 473N** 530S 687L 708A **817R** 866W 907Y

Note. Positively selected sites are those with posterior probability $P>0.95$, and those with $P>0.99$ are shown in bold. Sites were numbered according to our alignment and the amino acids were from one of sequences sampled from subject 02 (02e02).

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selection along the between-1980s branches, whereas none of them was detected along the between-2000s branches.

Robustness of our results

Yap et al. ([30]; see also [31]) are concerned that estimation of the ω ratio may be sensitive to model assumptions concerning codon usage. The authors suggested alternative ways of accommodating nucleotide/codon frequencies in models of codon substitution. Those models, like most early ones, are not based on our understanding of the biological process but are instead mathematical constructs aimed at fitting the datasets empirically. Nevertheless the potential sensitivity of our results to model assumptions is a concern. We have examined the nucleotide frequencies in our dataset, and found that they were nearly identical between the recent and early viral samples. Our analysis has been conducted under the F3×4 model of codon usage, with three nucleotide frequency parameters used for each codon position [31]. To examine the robustness of the results to assumptions concerning codon usage, we repeated the analysis under the Fcodon model (CodonFreq = 3 in CODEML), which uses the 61 codon frequencies as parameters. All frequency parameters under the F3×4 and Fcodon models are estimated using the observed frequencies in the data. The results obtained under the Fcodon model are listed in Supplementary Tables S1 and S2 for the branch-site and clade models, respectively. They were very similar to those obtained in the corresponding analyses under the F3×4 model, indicating that our results may be robust to assumptions concerning nucleotide/codon frequencies.

Furthermore, we assessed the impact of the tree topology on our analysis. Instead of the NJ tree under K80+G, we also used the ML tree inferred using PhyML under GTR+G. Note that only the tree topology is used in the codon-based analysis, and branch lengths are re-estimated under the codon model. The results for the ML tree are included in Supplementary Tables S3 and S4 for the branch-site and clade models, respectively. These are highly similar to the results obtained using the NJ tree, suggesting that our conclusions may be robust to minor errors in the tree topology.

Table 4. Log-likelihood values and parameter estimates under the clade model.

	Class 0	Class 1	Class 2
Proportion	$p_0=0.591$	$p_1=0.316$	$p_2=0.094$
All others	$\omega_0=0.073$	$\omega_1=1$	$\omega_2=$**5.766**
1980s-within	ω_0	ω_1	$\omega_3=$**4.753**
2000s-within	ω_0	ω_1	$\omega_4=$**2.516**
1980s-between	ω_0	ω_1	$\omega_5=$**7.749**
2000s-between	ω_0	ω_1	$\omega_6=$**4.184**

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Discussion

In this study, we contrast the changes in genetic diversity and adaptive evolution of the HIV-1 *env* gene between samples collected in the 1980s, the beginning of HIV-1 pandemics, and those collected after 2000, when the virus had spread worldwide and multiple human to human transmissions had taken place.

We sequenced HIV-1 *env* genes from seven subjects sampled in the 1980s. The samples from six of seven subjects were identified as subtype B. This finding is consistent with the observation that subtype B has been the dominating subtype in Japan since 1980s [32]. Since the early blood plasma included heparin that inhibits PCR, none of the early samples could be amplified from blood plasma and we had to use PBMC instead. We were able to obtain full-length *env* genes from both early and recent samples in this study, which gave our analysis an advantage over most previous studies on the *env* gene, which usually used only partial *env* sequences [33,34,35].

No significant change in within-host diversity was found after nearly two decades of evolution. The reconstructed phylogenetic tree of 142 sequences demonstrated distinctly long internal branches and short external branches, suggesting that only a small number of viruses infected the new host cell at each transmission so that these founder viruses usually are quite different among hosts. Moreover, the viruses that successfully infected new host cells are under strong selective pressure from the host immune system, which limited within-host diversification, as indicated by those small clusters on the tree. Therefore, those individual-specific mutations harbored by founder viruses may have a large impact on the within-host evolution and affect the prognosis of HIV infection.

In between-host HIV evolution, the reset of viral fitness by a genetic bottleneck may play an important role, influenced by both viral and host factors. Arien et al. [11] described two different HIV transmission scenarios for human populations with either diverse or homogeneous genetic backgrounds. We note that Arien et al. 's argument does not yet constitute a quantitative model with precise mathematical predictions. For example, it is unclear what levels of host genetic variation should cause the HIV to become attenuated or more virulent. Nevertheless, a previous study, which examined

Table 5. Inferred positively selected sites with epitope information in HIV immunology database.

Foreground branch	*Detected sites*	*Numbering in HXB2*	*Functional region*	*Epitopes*		
				CTL/CD8+	**T-Helper/CD4+**	**Antibody**
1980s-within	**358N**	**N300**	V3	+ (A2)	+	+
	373F	**F317**	V3	+ (A2, A*0201, A11)	+	+
	533V	I467	V5	+	+	+
	712N	L645	gp41		+	+
	752I	**L684**	gp41	+ (A2, A*0201, A24)	+	+
	798G	**D728**	gp41			+
2000s-within	56K	K46	C1	+ (A2, A11, Cw7)	+	+
	203T	T163	V2	+ (Cw8)	+	
	226P	**P183**	V2		+	
	366H	R308	V3	+	+	+
	408E	**E351**	C3		+	+
	452N	**N392**	V4	+	+	+
	692D	**N624**	gp41		+	+
	708A	S640	gp41		+	+
	811D	D741	gp41		+	+
	903L	**V833**	gp41	+ (A2, A*0201, A33, A*3303)	+	+
1980s-between	**204V**	**S164**	V2	+ (Cw8)	+	+
	404K	S347	C3	+ (A*0201, A11)	+	
	420K	**I360**	C3			
	447L	S387	V4	+ (A2)	+	+
	708A	S640	gp41		+	+
2000s-between	40Q	K33	C1	+ (A2, A*0201, B44)	+	+
	95E	**V85**	C1	+	+	+
	296P	P238	C2	+	+	+
	341T	T283	C2	+	+	+
	351Q	**E351**	C2		+	+
	687L	L619	gp41		+	+
	708A	**S640**	gp41		+	
	817R	**R747**	gp41	+ (A2)		
	866W	**W796**	gp41	+		+
	907Y	C837	gp41	+ (A33, A*3303)	+	+

Note. +: site reported as an epitope for antibody, CTL/CD8+ and T-Helper/CD4+ in HIV immunology database. HLA alleles observed with higher frequency (>10%) in Japanese are shown in parentheses.

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adaptive HIV-specific immune responses and viral evolution in adult monozygotic twins simultaneously infected with the same virus, provided qualitative support for the model [36]. The study found that 15 out of 17 epitopes targeted by initial CD8 T cell response were identical in each twin, indicating the concordance of adaptive immune responses in the same genetic background.

In our analysis, the between-host selective pressures were inferred to be weaker for the recent samples than for the early samples, in spite of the fact that the recent samples had higher diversity between hosts. For the early group, three out of six subjects were infected by the blood products imported from a foreign country, and the others were most likely infected with the virus from overseas nationals, representing the transmissions between the populations with different genetic backgrounds. In contrast, all the 10 subjects from the recent group represent transmission within native Japanese population. Several studies have reported that the genetic diversity in the Japanese population is very small [37,38,39]. In a study of gene-based SNP discovery, Haga et al. [39] found one polymorphism per 807 bp in the Japanese population, much lower than one SNP got every 272 bp for the world average [40], indicating that the Japanese population appears to be more homogeneous. Accordingly, the new CTL escape mutation upon transmission to a new host in the 2000s will be less necessary than in the 1980s. Since the CTL escape mutations in viral epitopes usually exact a cost to viral fitness [41], the HIV transmission with fewer escape mutations will have a lower cost in viral fitness. In other words, in the 2000s, the HIV may have a higher viral fitness after the transmission to a new host. The viral fitness is a key factor affecting the viral virulence which measures the capacity of a virus to cause disease [42]. Thus, we speculate that the HIV circulating in the homogeneous Japanese population may have evolved to be more virulent.