exposures, suggesting that *Lb. casei* represents an excellent antigen delivery vehicle when cytotoxic mucosal immune responses to vaccine antigen are desired.

The E7-specific type1 immune response induced by LacE7 was directly dose-dependent over a range of 0.03–1.0 mg/head, but decreased when exposure exceeded a LacE7 level of 1.0 mg/head. These findings could be caused by an aggregation of the attenuated bacteria when levels approach 10 mg/head, resulting in interference with antigen translocation to GALT through M cells. In these investigations we chose LacE7 doses of 1.0 mg/head as optimal for oral immunization to induce E7-specific type1 immune response-related T cell activities and used this level of exposure for CTL detection and killer activity assays. E7-specific type1 immune cell numbers increased after boosting when compared to non-boost protocols, suggesting that mucosal lymphocyte populations include memory T cells that recognize E7.

The lack of an animal model with HPV E7-related mucosal neoplastic lesions hampers assessment of our therapeutic vaccination strategy for preclinical efficacy against CIN. Most previous studies on HPV therapeutic vaccines utilized murine models in which HPV16 E7-transformed TC-1 cells were injected subcutaneously to induce tumor formation [42,43]. This model can assess systemic, but not mucosal, immune responses to HPV-related tumors. In our study, TC-1 cell was used as target cells for mucosal E7-specific CTL in *in vitro* killer activity assays. The HPV-specific killer activity of mucosal lymphocytes was clearly demonstrated by the induction of granzyme B-producing $CD8^+$ T cells. Poo et al. have revealed that oral immunization of mice with *Lb. casei* expressing HPV16 E7 reduces the growth of subcutaneous TC-1 cell tumor and induces E7-specific type1 immune response-related splenic T cells [9]. We have shown that oral immunization with LacE7 preferentially elicits E7-specific type1 T cell responses in mucosal lymphocytes (2-fold higher) when compared to splenocytes. These data strongly suggest that the induced mucosal $CD4^+$ and $CD8^+$ T cells will have antitumor effects on mucosal HPV E7-related neoplastic lesions.

Oral routes of immunization offer many advantages: easy self-administration at home, reduction in hypersensitivity reactions, and decreased costs (no needles, syringes or trained personnel). Further, the production of lactic acid bacteria is also inexpensive. In this study the recombinant *Lb. casei*, LacE7, was heat-attenuated. Attenuation results in the destruction of the expression plasmid and prevention of self-replication. This negates the possibility for transfer of foreign genes to normal bacterial in the gut. The Rb-binding site of HPV16 E7 was mutated in the antigen-producing plasmid, thereby eliminating its oncogenicity but not its immunogenicity [44]. These modifications make the recombinant *Lb. casei* vaccine ensure drug safety. Unfortunately, we must await clinical trials on this promising therapeutic HPV vaccine to assess its actual antitumor effect on mucosal neoplastic lesions. Our data support the development of an initial clinical study on therapeutic vaccination of patients with CIN 2–3 patients using LacE7.

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ORIGINAL ARTICLE

Comparison Between Conventional Surgery Plus Postoperative Adjuvant Radiotherapy and Concurrent Chemoradiation for FIGO Stage IIB Cervical Carcinoma

A Retrospective Study

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Objective: To compare treatment outcome of conventional surgery followed by adjuvant postoperative radiotherapy (PORT) versus concurrent chemoradiation therapy (cCRT) for stage IIB cervical carcinoma.

Methods: A retrospective analysis was conducted of 59 patients with stage IIB uterine cervical cancer treated with radical surgery plus PORT (N = 34) or cCRT-alone (N = 25) from April 1996 to June 2008. The median follow-up time was 27 months (range, 3–150 months) in the cCRT group and 44 months (range, 4–134 months) in the PORT group. The median age was 59 years (range, 37–85 years) in the cCRT group and 49 years (range, 32–74 years) in the PORT group. All 34 patients in the surgery group underwent hysterectomy with pelvic lymph node dissection and received PORT. Twenty-five patients (42%) were assigned to the cCRT group.

Results: The 3-year overall survival rates for surgery plus PORT and cCRT-alone were 80.0% and 75.1%, respectively. The difference between these 2 treatments was not statistically significant (log-rank $P = 0.5871$). The late complication rate of grade 3–4 was 12% in the cCRT group and 16% in the surgery group.

Conclusion: This retrospective study suggests that survival results with cCRT and with conventional surgery plus PORT for patients with stage IIB cervical carcinoma are comparable.

Key Words: cervical carcinoma, surgery, chemoradiotherapy, high-dose-rate brachytherapy, stage IIB

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Cervical cancer is the most common gynecologic malignancy in Japan, with an estimated 5 new cases per 100,000 females every year. High dose-rate intracavitary brachytherapy (HDR-ICBT) in combination with external beam irradiation (EBRT) has become an acceptable treatment for carcinoma of the cervix.[1] HDR-ICBT has been widely used in treatment of uterine cervical cancer in Asia and Europe. Although some controversy exists in the United States over the use of HDR-ICBT,[2] an increasing frequency of its adoption has been noted.[3,4]

Recently published randomized clinical trials demonstrated a significant improvement in pelvic disease control and survival when concurrent chemotherapy consisting of cisplatin-containing regimens was added to radiotherapy (RT) in patients with locally advanced cervical cancer.[5–7] These results led to significant changes in the standard treatment of cervical cancer.

Radiotherapy has long been recognized as a successful treatment modality for all stages of carcinoma of the uterine cervix. In Japan, however, because patients present first at gynecologic clinics, gynecologists usually determine the treatment modality without additional inputs from radiotherapists. In general, Japanese gynecologists consider surgical treatment to be superior to RT, and, as a result, the majority of patients with stage IIB are subjected to radical hysterectomy plus pelvic, and with or without para-aortic lymphadenectomy followed by preventive postoperative RT (PORT). Consequently, other than this preventive postoperative RT, radiation oncologists in Japan have treated only stage IIB patients who refused surgery or who were not indicated for surgery because of other coexisting disease.

Although RT has been widely used in Western countries, there are only a few reports on definitive RT for early stages (stages I–II) of cervical carcinoma. Some studies indicated that RT for early stage patients was a feasible definitive treatment.[8–11] In Japan, no prior report has compared surgery and RT. We now report the results of a retrospective study in which the survival outcomes of surgery and RT were compared for stage IIB cervical cancer. The hypothesis was to be certified that definitive cCRT is not inferior to survival and less frequency about severe complications than radical hysterectomy plus PORT for stage IIB cervical cancer in this single institution.

PATIENTS AND METHODS

Patients

Between April 1996 and June 2008, a total of 59 consecutive patients were treated for FIGO (International Federation of Gynaecology and Obstetrics classification) stage IIB carcinoma of the cervix with conventional surgery plus adjuvant PORT or concurrent CRT at our institution. All patients with stage IIB treated during the 13-year period (1996–2008) were included in the study. Patients included were those previously untreated and who had a histologic diagnosis of squamous cell carcinoma, or adenocarcinoma in FIGO stage IIB. Patients with adenocarcinoma (n = 13) were also included in this study. Median age was 53 years (range, 32–85 years). Table 1 shows the patients' characteristics. Surgically treated patients comprised 58% (34/59) and cCRT-alone patients 42% (25/59) (Table 1). The patients submitting to definitive CRT were those with comorbidities or who refused surgery in our institution.

Patients were evaluated with a physical and pelvic examination without anesthesia, routine blood counts, blood chemistry profile, chest radiograph, intravenous urogram, and barium enema. Computed tomography (CT) scan and magnetic resonance imaging (MRI) were used only for detecting lymphadenopathy. Pelvic and para-aortic lymph nodes greater than 10 mm in minimum diameter

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TABLE 1. Comparison of Patients' Characteristics

	Surgery Plus PORT	cCRT	P
Total no. patients	34	25	
Age			
Median (range) (yr)	49 (32–74)	59 (37–85)	0.0001
Histopathology			
Squamous cell carcinoma	24 (71%)	22 (88%)	0.2026
Adenocarcinoma	10 (29%)	3 (12%)	
Pelvic nodal status			
Positive	17 (50%)	5 (20%)	0.0185
Negative	17 (50%)	20 (80%)	
Paraaortic nodal status			
Positive	3 (8%)	2 (8%)	0.9106
Negative	31 (92%)	23 (92%)	
Maximum tumor diameter (mm)			
>40	19 (56%)	14 (56%)	0.9928
≦40	15 (44%)	11 (44%)	
Median (range)	49.5 (18–100)	45.5 (30–80)	0.6153

detected by CT and MRI were considered to be positive for metastases. Neither lymphangiography nor surgical evaluation of lymph nodes was performed.

Concurrent CRT

EBRT

All patients received EBRT using a linear accelerator with a photon-beam-energy of 10 MV to the whole pelvis with the 4-field box technique for a total dose of 30.6 Gy in 17 fractions (3.4 weeks, 1.8 Gy fractions from Monday to Friday). The irradiated volume was to include the whole uterus, the paracervical, parametrium and uterosacral regions, as well as the external iliac, hypogastric and obturator lymph node. Minimum margins were the upper margin of L-5 (superiorly), the lower margin of the obturator foramen or the lowest extension of the disease (inferiorly), and 2.0 cm beyond the lateral margins of the bony pelvis and its widest plane (laterally). For the lateral fields, the anterior margin was the anterior edge of the symphysis or 3 cm in front of the sacral promontory. The posterior margin was the S2–S3 interspace or the posterior border of the uterine cervix assessed by CT for treatment planning plus a 2 cm margin. After that, a midline block, 4 cm in width at midplane, was inserted with the anteroposterior parallel 2-field technique for a dose of 19.8 Gy in the last 11 fractions (ie, parametrial boost). This block extended to the top of the uterine.

Two patients who had para-aortal lymph node involvement received whole pelvic irradiation plus para-aortal irradiation using the 4-field box or conformal technique. Total dose to para-aortal lymph nodes was 50.4 Gy in 28 fractions.

ICBT

In our department, EBRT preceded ICBT. A midline block was inserted at the same time as the first application of ICBT. Four intracavitary iridium-192 (^{192}Ir) insertions were performed weekly, starting 3.4 weeks after starting EBRT. High-dose-rate intracavitary therapy was used. Brachytherapy was delivered using after-loading applicators placed in the uterine cavity and vagina. A Manchester system applicator (Nucleotron microSelectron HDR source) was used. The dose distribution was calculated for each individual patient and placement. Patients were treated in the dorsal lithotomy position. Point A was defined on radiographs as being 2 cm superior (along the tandem) to the flange abutting the external cervical os and 2 cm lateral from the axis of the tandem. Source loading corresponded to the Manchester System for uterine cervical cancer.[12] HDR-ICBT was performed once a week with a daily dose of 6 Gy at point A. The details of the method for ICBT were shown in our previous report.[13,14]

Total dose to the central area was 54.6 Gy and to the parametrium area was 50.4 Gy. When using biologically effective dose with α/β = 10 Gy, total dose to the central area was 74.5 Gy and to the parametrium was 59.5 Gy.

Chemotherapy

Concurrent CDDP-based chemotherapy combined with RT for stage IIB has been routinely performed in our department. All patients received platinum series-based chemotherapy combined with RT. All patients received CDDP (75 mg/m^2 in a bolus infusion on days 1, 22, and 43).

Surgery Plus Adjuvant RT

Radical hysterectomy with pelvic lymphadenectomy was performed on 34 patients. Radical hysterectomy at our institution includes resection of the uterus along with its attached parametrial soft tissue and a margin of the upper vagina, as in the world standard. Even when positive nodes were found, radical hysterectomy was continued without stopping. No radical hysterectomy was aborted. Para-aortic lymphadenectomy up to the level of the inferior mesenteric artery was performed for patients with adenocarcinoma or enlarged pelvic lymph nodes assessed by preoperative CT or MRI (N = 13). Because it is well-known that cases with positive pelvic lymph nodes are indicative of a very poor prognosis, lymphadenectomy up to the level of the inferior mesenteric artery was added for these high-risk cases in our institution. The median operation time was 390 minutes (range, 150–550 minutes) and the median quantity of operative blood loss was 1450 mL (range, 400–5600 mL).

All patients in the surgery group received postoperative adjuvant RT because of invasion to the parametrium. Adjuvant RT consisted of external pelvic irradiation (10 MV x-rays) with the 4-portal technique, one fraction of 1.8 Gy daily, with a total dose of 50.4 Gy over 5.6 weeks. The para-aortic region was irradiated with a dose of 50.4 Gy over 5.6 weeks with the conformal technique when metastases were detected in the surgical specimens of para-aortic nodes.

Follow-Up

Both radiation and gynecologic oncologists were involved in the follow-up the treated patients. The patients were seen every month for the first year, every 2 to 3 months for the next 2 years, and at least every 6 months thereafter. No patients were lost to follow-up. Follow-up procedures included pelvic examination, palpation of supraclavicular nodes, cervical Papanicolaou smear, and review of serum squamous cell carcinoma related antigen and cytokeratin 19 fragment antigen values. When central and/or parametrial recurrence was suspected by pelvic examination and/or Papanicolaou smear, a biopsy was taken for confirmation. Intravenously enhanced chest, abdominal, and pelvic CTs were performed annually. Other imaging studies, such as MRI, ultrasound and bone scintigraphy, were not routinely performed. Both acute and late complications were graded in accordance with the National Cancer Institute Common Toxicity Criteria Version 2.0.

Statistical Analysis

Statistical analyses were performed using StatView Dataset File version 5.0 J for Windows computers (Cary, NC). OS, progression-free survival (PFS), and local (ie, within pelvic) recurrence-free survival (LRFS) were calculated from the first date of curative treatment. Survival time was plotted using the Kaplan–Meier

method. Differences in patients' characteristics were analyzed by the χ^2 test or Fisher exact test for 2 × 2 columns and unpaired t test for a succession of numbers. Differences in survival by treatment were evaluated using the log-rank test.

RESULTS

Patients and Tumor

The age and pelvic nodal status distributions were significantly different between the 2 groups of patients (Student t test or χ^2 test). The median age was 59 years (range, 37–85 years) in the cCRT group and 49 years (range, 32–74 years) in the PORT group (Table 1). The patient FIGO stage, follow-up time, para-aortic nodal status, and histopathology distributions showed no significant differences (Table 1). The Karnofsky Performance Status for all patients was more than 80%. The proportion of patients with tumors less than 4 cm in diameter was 41% (14/34) for the surgery group and 44% (11/25) for the CRT. The median size was 50 mm (range, 18–100 mm) for the surgery group and 45.5 mm (range, 30–80 mm) for the cCRT group. The positive rate of surgical margins for the surgery group was 21% (7 cases). In the surgery group, bilateral parametrial involvement was seen in only one patient, although it was unilateral in the other 33 patients. Of those with unilateral involvement, only one patient had more than half extension of parametrial involvement and the other 32 patients had less than half.

In the surgery group, the positive rate of pelvic nodal metastasis was 32% (11 cases) assessed by clinical method and 50% (17 cases) by histopathological method. The pelvic nodal status showed no significant difference between surgery and definitive CRT groups if assessed by same method (clinically) (χ^2 test, $P = 0.2916$).

Median follow-up time was 27 months (range, 3–150 months) in the cCRT group and 44 months (range, 4–134 months) in the PORT group. The proportion of the surviving patients was 74% (25/34) for the surgery and 76% (19/25) for the cCRT groups.

The first site of progression was local-alone (ie, within pelvis) in 8% (2 cases) for the definitive CRT group and in 12% (4 cases) for the surgery group. Additionally, it was distant-alone progression in 4% (1 case) for CRT group and in 18% (6 cases) for surgery group, and both local and distant in 8% (2 cases) for CRT group and 0% for the surgery group. In other words, the recurrent rate within the pelvis was 14% (8/59 cases).

Survival

When this analysis was closed, 44 of 59 patients were alive. Deaths resulted in all 15 patients with cervical cancer (6 patients for definitive CRT group and 9 patients for surgery group). No treatment-related deaths were encountered. Figure 1 shows a comparison of the OS curves for the definitive CRT and surgery groups. The 3-year OS rates were 75% for the cCRT group and 80% for the surgery, respectively; the difference between these 2 rates was not significant (log-rank $P = 0.5871$). The 3-year PFS rates were 79% for the cCRT group and 70% for the surgery, respectively ($P = 0.7247$, HR = 0.825, 95% CI = 0.281–2.422). The 3-year LRFS rate was 83% for the cCRT group and 86% for the surgery, respectively ($P = 0.4908$, HR = 1.622, 95% CI = 0.404–6.513). Clearly, none of these analyses was of any significance.

Complications

In the cCRT group, nonhematological acute toxicity and all late toxicity (complications that persisted or occurred for more than 60 days after treatment) of grade 3+ were noted in 3 patients (12%). Small bowel perforation without tumor recurrence (grade 4) and melena from radiation proctitis in 2 cases (both grade 3) were seen at 5, 8, and 11 months after the completion of CRT. Moreover,

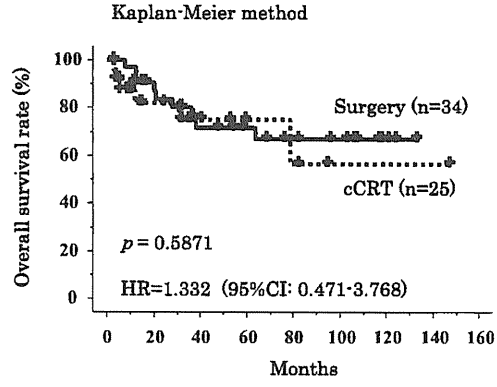

FIGURE 1. Overall survival curves in stage IIB patients comparing surgery plus postoperative radiotherapy (surgery) and concurrent chemoradiation (cCRT).

grades 1 or 2 melena and lymphoedema of the lower limbs were seen in each of 4 patients (16%).

In the surgery group, the bladder damage was seen in one example for a complication during operation. Nonhematological acute toxicity of grade 3+ was seen in 9 patients (16%). Postoperative ileus (grade 4 in 1 patient and grade 3 in 3 patients), grade 3 pelvic lymphocyst in one patient, grade 3 pulmonary infarction in 2 patients, grade 3 bilateral hydronephrosis in one patient, and lymphangitis of lower extremities (grade 3) in one patient were seen. Moreover, grade 2 hypertension, urinary tract infection, unilateral functionless kidney, and pelvic lymphocyst were seen in each of a single patient (total was 16%). No ureteral stricture was seen in the neither PORT or surgery groups.

DISCUSSION

This is a retrospective analysis of 59 patients with FIGO stage IIB cervical cancer treated with surgery plus PORT (n = 34) or concomitant CRT-alone (n = 25). Some centers especially in Japan still consider both treatment modalities as a standard for FIGO stage IIB cervical cancer. In this study, the 3-year OS, PFS, and LRFS were the same for both groups, although the surgery cases had at least a debulking of lymph nodes when positive as compared with the definitive CRT group. Also, CRT patients received a slight low dose for tumor control: only 50.4 Gy with EBRT plus 4 × 6 Gy with HDR-ICRT. These techniques are quite different from those used to treat the same category of patients in the United States and Europe. Additionally, surgery plus PORT presented 16% of grade 3+ complications. Patients who received definitive CRT were at higher risk, when age and probable comorbidities were considered. This suggests that there is no treatment of choice with respect to local control of disease. Conceivably, the patient population might be too small to draw any conclusions about the superiority of either one of the 2 treatment modalities.

The type of radical hysterectomy performed was III and ureteral resection was performed, although we had 21% of positive margins. The median and mean number of excised nodes was 61 and 63.4 (range, 25–133).

Many previously published results[8–11,15,16] suggest that radical hysterectomy or definitive RT is standard treatments for IB–IIA. In the United States and Europe, definitive RT has been selected in many cases. Radical hysterectomy is not a world standard for stage IIB patients. In contrast, surgery is preferentially used over RT in Japan even for stage IIB cervical cancer, although those patients with stage IIB ideally should have been treated with concomitant

CRT. RT is usually selected only for the elderly or inoperable cases because of coexisting disease in Japan.

The age of the patients and pelvic nodal status were significantly different between those patients going to surgery and cCRT patients (Table 1). The mean age of the cCRT patients was significantly greater than that of patients in the surgery group (P = 0.0001). There was a tendency for cCRT to be performed for elderly patients who were fearful of surgery or general anesthesia. Regardless of these fears, the cCRT patients had survival rates comparable with the surgery patients.

For the surgery group, pelvic nodal status was pathologically assessed in the surgical specimen, whereas clinically assessed by CT or MRI in the cCRT group. The positive rate of pelvic nodal status for the surgery group was significantly higher than in the cCRT group (50% vs. 20%, P = 0.0185). In the surgery group, the positive rate of pelvic nodal metastasis was 32% (11 cases) assessed clinically. The pelvic nodal status showed no significant difference between surgery and definitive CRT groups if assessed by the same method (clinically) (χ^2 test, P = 0.2916).

Horn et al[17] concluded that tumor size, when bulky disease was defined as tumors larger than 4 cm, was also of prognostic importance in FIGO stage II cervical carcinomas. In this study, there was no significant difference in the maximum tumor diameter between the 2 groups (P = 0.6153), although stage IIB varied from minimal to medial and even to lateral parametrial invasions, just short of pelvic wall fixation. To determine the size of the tumor, pathologic evaluation was used in the PORT group and pretreatment MRI in the CRT group.

Rotman et al[18] concluded that pelvic RT after radical surgery significantly reduced the risk of recurrence and prolonged PFS in women with stage IB cervical cancer whereas PORT appeared to be particularly beneficial for patients with tumors comprised of adenocarcinoma or adenosquamous histologies. In this study, there was no significant difference in the number of adenocarcinomas between the 2 groups (P = 0.2026).

It was shown in a previous publication[19] that for the stage IIB patients with lateral parametrial involvement had significantly higher rates of pelvic failure and of survival in comparison with those patients with medial parametrial involvement.

In our experience, the low rate of major complications after cCRT suggests that this approach is well tolerated in most patients. Treatment-related toxicity of grade 3+ developed in 16% of the surgery patients and in 12% of the definitive CRT group. This difference was not significant, probably because of the small numbers of patients in both groups.

Our results confirm earlier findings that have suggested that cCRT is not inferior to surgery plus PORT for FIGO stage IIB cervical carcinoma regardless of age bias, although the 2 treatment groups were not similar (ie, the cCRT group was older and had more comorbidities) and the pelvic node status was different as well. The 3-year outcomes in both groups were also been shown to be compatible with previous reports.[2–8] Because the t test based on a sample of 59 is underpowered, clinical trails with more patients should be needed to further confirm the efficacy of cCRT or surgery plus PORT on FIGO stage IIB cervical carcinoma. By the power analysis, to detect the difference between 2 independent groups, when the input is assumed that tail = one, effect size r = 0.5 (large), 0.3 (medium), or 0.1 (small), α err probability = 0.05, power (1-β err probability) = 0.8, and allocation ratio N2/N1 = 1, total sample size is calculated as 102, 278, or 2476, respectively. This study is too underpowered to conclude whether such both techniques as radical hysterectomy plus PORT and definitive CRT are feasible.

In this study, it was to be certified that definitive cCRT is not inferior with regards to survival and less frequency about severe complications than radical hysterectomy plus PORT for stage IIB cervical cancer in this single institution. The limitations of our study included the retrospective nature of the study, heterogeneity of the patient population in the 2 treatment arms, shorter follow-up and physician's bias in the selection of the patients. A matched-pair analysis would be better to compare the 2 groups but could not be done in our study because of the small number of patients. Nevertheless, we hope that our experience will initiate further prospective studies especially in Japan.

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Spread of a Chromosomal Cefixime-Resistant *penA* Gene among Different *Neisseria gonorrhoeae* Lineages[▽]

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In *Neisseria gonorrhoeae*, the mosaic type of *penA*, which encodes penicillin-binding protein 2 (PBP 2), is associated with reduced susceptibility to oral cephalosporins. To investigate the relatedness of *N. gonorrhoeae* clinical isolates with reduced susceptibility, we sequenced the *penA* genes of 32 isolates. Five different amino acid sequence types of PBP 2 were identified, but all seemed to be derivatives of pattern X of PBP 2 (PBP 2-X). However, multilocus sequence typing of the isolates showed that the isolates belonged to six different sequence types. As PBP 2-X was identified in three different sequence types, horizontal transfer of the *penA* allele encoding PBP2-X was suggested. We demonstrated that the *penA* gene could be transferred from an isolate with reduced susceptibility to a sensitive isolate by natural transformation. Comparison of the sequence of the *penA*-flanking regions of 12 transformants with those of the donor and the recipient suggested that at least a 4-kb DNA segment, including the *penA* gene, was transferred. During horizontal transfer, some of the *penA* alleles also acquired variations due to point mutations and genetic exchange within the allele. Our results provide evidence that the capacity for natural transformation in *N. gonorrhoeae* plays a role in the spread of chromosomal antibiotic resistance genes and the generation of diversity in such genes.

Neisseria gonorrhoeae is one of the most common sexually transmissible infective agents. Humans are the only natural host for *N. gonorrhoeae*, and transmission is restricted to direct person-to-person sexual contact. As there is no vaccine for gonorrhea, the control of dissemination depends on timely identification and initiation of an appropriate antibiotic treatment for the infected person in order to prevent transmission.

N. gonorrhoeae strains that are resistant to various types of antibiotics have emerged, causing critical concern for public health around the world. Resistance to oral cephalosporins, such as cefixime, is emerging (2, 3, 10, 18), and approximately 30% of *N. gonorrhoeae* isolates in Japan now show reduced susceptibility to cefixime (20). The molecular mechanism of resistance has been elucidated as the formation of a mosaic structure of *penA*-encoded penicillin-binding protein 2 (PBP 2). The mosaic *penA* was generated by interspecies recombination with other neisserial species (3, 10), which is the same mechanism for chromosomally mediated penicillin resistance in *N. gonorrhoeae* (23). However, the precise junctions of recombination have not been fully elucidated.

penA-encoded PBP 2 proteins of *N. gonorrhoeae* are divided into several types on the basis of the amino acid sequence, and some of these types are associated with reduced susceptibility to cefixime (10, 14, 25, 27). Among these, the most common PBP 2 type is pattern X (PBP 2-X), implying the expansion of a single clone. According to the spread of isolates with reduced susceptibility to cefixime, the expansion of a single clone, which emerged at an early phase, is suggested (18). However, another possibility is that recombination of the *penA* gene occurred several times independently, followed by multiclonal expansion. Understanding of the mode of spread of antibiotic-resistant clones could help us construct a public health strategy for preventing the further spread of resistant clones.

To investigate the mode of dissemination of the newly emerged antibiotic-resistant *N. gonorrhoeae* isolates, we retrospectively characterized isolates with reduced susceptibility to cefixime (cefixime MIC ≥ 0.25 μg/ml, referred to hereafter as Cef^{Rs} isolates), using *penA* sequencing and multilocus sequence typing (MLST) with seven housekeeping genes. We also examined whether the horizontal transfer of *penA* occurred *in vitro*, resulting in the one-step emergence of Cef^{Rs} isolates from the susceptible isolate.

MATERIALS AND METHODS

Strains. The Kanagawa Prefectural Institute of Public Health is a reference laboratory for *N. gonorrhoeae* in Kanagawa Prefecture, Japan, where the primary isolation of *N. gonorrhoeae* from clinical specimens collected at nine hospitals was carried out. The *N. gonorrhoeae* isolates were identified and stored as described previously (11, 28). A total of 32 *N. gonorrhoeae* clinical isolates with reduced susceptibility to cefixime comprising 3 to 7 Cef^{Rs} isolates randomly selected from each year were examined (Table 1). *N. gonorrhoeae* isolates that belonged to sequence type (ST) 1901 (ST1901) were used for comparison of the sequences of the *penA*-flanking regions that we analyzed. Isolates NGON03-079, NGON03-092, NGON130-115, and NGON07-002 were collected at another hospital in Tokyo (Table 1). The MICs of cefixime and ciprofloxacin were determined by the agar dilution method (19).

Sequencing of *penA* and the *penA*-flanking region. To obtain genomic DNA, the clinical strains were suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and boiled for 10 min. After the cell debris was removed by centrifugation, the supernatant was used directly as the template DNA for PCR. The *penA* gene

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TABLE 1. Description of *Neisseria gonorrhoeae* isolates from Kanagawa Prefecture and Tokyo with reduced susceptibility to cefixime, 1998 to 2005

Isolate	Yr of isolation	Hospital	Sex[a]	Specimen[b]	Cefixime MIC (μg/ml)	MLST ST	PBP 2 type
NG9806	1998	Kanagawa, H1	M	U	0.25	7363	X
NG9811	1998	Kanagawa, H1	F	VD	0.25	7363	X
NG9812	1998	Kanagawa, H1	F	VD	0.25	7363	X
NG9911	1999	Kanagawa, H2	M	UD	0.25	7363	X
NG9912	1999	Kanagawa, H3	M	U	0.25	7363	X
NG9913	1999	Kanagawa, H3	M	U	0.25	7363	X
NG9914	1999	Tokyo, H9	M	U	0.25	7363	X
NG0002	2000	Kanagawa, H4	F	VD	0.25	1901	X
NG0003	2000	Kanagawa, H2	M	UD	0.25	7363	X
NG0008	2000	Kanagawa, H2	M	UD	0.25	7363	X
NG0109	2001	Kanagawa, H6	M	UD	0.25	7363	X
NG0110	2001	Kanagawa, H5	M	UD	0.25	1596	X
NG0111	2001	Tokyo, H9	M	U	0.25	1590	XXXI
NG0201	2002	Kanagawa, H7	M	UD	0.25	1596	X
NG0204	2002	Kanagawa, H7	M	UD	0.25	7363	X
NG0205	2002	Kanagawa, H2	M	UD	0.25	7363	X
NG0206	2002	Kanagawa, H5	F	VD	0.25	7363	X
NG0207	2002	Kanagawa, H5	M	UD	0.25	7363	X
NG0303	2003	Kanagawa, H3	M	—	0.25	7363	X
NG0304	2003	Tokyo, H9	M	U	1.0	7363	XXX
NG0311	2003	Tokyo, H9	M	U	0.5	7363	XXX
NG0312	2003	Tokyo, H9	M	U	0.5	7358	XXVI
NG0401	2004	Kanagawa, H7	M	UD	0.5	7363	X
NG0404	2004	Kanagawa, H8	M	U	0.5	7363	X
NG0410	2004	Tokyo, H9	M	U	0.5	7363	X
NG0503	2005	Kanagawa, H3	M	U	0.25	1901	XXXII
NG0508	2005	Kanagawa, H5	M	—	0.25	1596	X
NG0509	2005	Kanagawa, H3	M	UD	0.25	7363	X
NG0511	2005	Kanagawa, H3	F	VD	0.25	7363	X
NG0512	2005	Kanagawa, H3	M	UD	0.25	1588	X
NG0513	2005	Kanagawa, H3	F	VD	0.5	1901	XXXII
NG0514	2005	Kanagawa, H8	M	U	0.25	7363	X
NG0202[c]	2002	Kanagawa, H7	M	UD	<0.008	1901	V
NG0402[c]	2004	Kanagawa, H5	M	U	0.031	1901	V
NGON03-079[c]	2003	Tokyo, H10	M	UD	0.5	1901	X
NGON03-092[c]	2003	Tokyo, H10	M	UD	0.25	1901	X
NGON03-115[c]	2003	Tokyo, H10	F	U	0.5	1901	X
NGON07-002[c]	2007	Tokyo, H10	M	U	0.25	1901	X

[a] M, male; F, female.
[b] U, urine; VD, vaginal discharge; UD, urethral discharge; —, no information.
[c] ST1901 isolates used for analysis of *penA*-flanking region.

was amplified and sequenced by using primers penA_F and penA_R (Table 2). The PCR mixtures were incubated for 2 min at 96°C, followed by 30 cycles of 10 s at 96°C, 10 s at 65°C, and 2 min at 72°C. Purification of the PCR products was done with an ExoSAP IT kit (GE Healthcare). Sequencing was carried out with the appropriate sequencing primers and an ABI BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems), followed by purification of the termination products. Both strands of the products were sequenced by use of an ABI 3130 xl sequencer. The translated amino acid sequences were compared with known PBP 2 amino acid sequences. Newly identified types were designated XXX to XXXII, as described by Ito et al. (10) and Whiley et al. (27).

A neighbor-joining tree with 33 PBP 2 amino acid sequences was generated by using the MEGA program (version 4) (22, 26). The reliability of the inferred relatedness was evaluated by the use of bootstrap tests (1,000 replicates) (7).

Amplification of the *penA*-flanking DNA was done by using primer set penA_3′F and dcaA_R and primer set penA_5′R and mraW_F (Table 2). The PCR mixtures were incubated for 2 min at 96°C, followed by 30 cycles of 10 s at 96°C, 10 s at 63°C, and 2 min at 72°C. The primers listed in Table 2 were used to sequence each PCR product.

MLST. PCR amplification and sequencing of the seven *N. gonorrhoeae* housekeeping genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*) were undertaken by using a previously described protocol (12). All nucleotide sequences were determined directly from the purified PCR products. After end trimming of the data obtained and editing by using Sequencher software (gene codes), the allele numbers of the STs were assigned by querying the *Neisseria* MLST database (http://pubmlst.org. /neisseria/) (13).

Pulsed-field gel electrophoresis (PFGE). Agarose plugs into which DNA was embedded were prepared as described previously (10), and the samples were digested with SpeI. The SpeI-digested genomic DNA was analyzed on a 1% agarose gel with 0.5× Tris-boric acid-EDTA buffer at 14°C by using a CHEF Mapper apparatus (Bio-Rad). The run time was 19.5 h at 6 V/cm, and the initial and final switch times were 0.5 and 35 s, respectively. The gel was stained with ethidium bromide.

***In vitro* genetic exchange of the *penA* allele during cocultivation.** An *in vitro* interstrain genetic exchange experiment was performed with strain NG0003 (cefixime MIC, 0.25 μg/ml; ciprofloxacin MIC, 0.031 μg/ml) and strain NG0202 (cefixime MIC, 0.004 μg/ml; ciprofloxacin MIC, 8 μg/ml). NG0202 was selected from 58 isolates susceptible to cefixime (cefixime MIC ≤ 0.125 μg/ml) and on the basis of the ciprofloxacin MICs. The strains were grown on GC agar plates with 5% CO_2 for 16 h and then suspended in GC broth. After adjustment of the optical density at 600 nm (OD_{600}) of the culture to 0.02 with GC broth, suspensions of strains NG0003 and NG0202 (500 μl each) were mixed and statically incubated for 16 h. One hundred microliters of sample was placed onto GC agar plates containing both cefixime (0.031 μg/ml) and ciprofloxacin (2 μg/ml) (Cef+Cip GC agar plate) in duplicate. Neither NG0003 nor NG0202 is able to form colonies on this medium. The plates were incubated for 20 h at 37°C with 5% CO_2, and the number of colonies on each plate was determined. The viable

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)	Primer use
penA_F	CGGGCAATACCTTTATGG TGGAAC	Amplification of *penA*[a]
penA_R	ACAACGGCGGCGGGGAT ATAAC	
penA_SF1	CAAAGATAGAAGCAG CCTG	Sequencing of the *penA* region
penA_SF2	GATATTGACGGCAAA GGTC	
penA_SF3	CTTTGGATGTGCGCGGC	
penA_SR1	GCCGTCGGTATATTCGC	
penA_SR2	CCAAAGGGGTTAACTTGC	
penA_SR3	TTCTCAACAAACCTGCAG	
penA_SR4	CTTTGCCGTTTTGCGGGG	
penA_5′R	GCCATCAGGACGAAGCT AATCC	Amplification of the region upstream of *penA*[b]
mraW_F	GTGAGTGGAGCAGAAAG TTACCG	
mraW_S1	CCGTTACTGGTCATCG	Sequencing of the PCR product from penA_5′R and mraW_F
mraW_S2	TATCGGACCGGCAGTC	
mraW_S3	CCTCGTGCAAATCCTG	
mraW_S4	GGCGGTCAGAGAAGC	
penA_3′F	GCGGCAGCCTGAACATC TTGG	Amplification of the region downstream of *penA*[b]
dcaA_R	GGACACATCGGTAGCG GCTG	
murE_S1	TTCAAGATCGGAAA AACG	Sequencing of PCR product from penA_3′F and dcaA_R
murE_S2	TTGGCACAAAGCAAGG	
murE_S3	TGCGCGGTTTCTTCC	
murE_S4	TCGGACGGTTCAACG	
murE_S5	GCAGGCTTTGTTAACTC	
dcaA_S1	TCAATATCTTAACCG TATC	
dcaA_S2	GCGTATCGGGCAATGG	
dcaA_S3	CGGGAAGATTGCCGAC	
dcaA_S4	GGGGTATTTGCTGACG	
dcaA_S5	AGCTTGGCGAAGCAGG	
dcaA_S6	CGGTTTGATGCATGTCG	

[a] Amplification conditions were 96°C for 2 min and 30 cycles of 96°C for 10 s, 65°C for 10 s, and 72°C for 2 min.

[b] Amplification conditions were 96°C for 2 min and 30 cycles of 96°C for 10 s, 63°C for 10 s, and 72°C for 2 min.

counts of NG0202 and NG0003 were determined on GC agar plates containing either cefixime (0.031 μg/ml) or ciprofloxacin (2 μg/ml). The experiment was repeated three times. The transformation frequency was estimated on the basis of the number of viable recipient NG0202 colonies that grew on the Cef+Cip agar plates compared to the number of NG0202 colonies that grew on Cip agar plates. The MICs of clones (n = 12) resistant to both cefixime and ciprofloxacin were determined; and MLST typing, PFGE, and sequence analysis of the *penA*-flanking region of the clones were performed.

Nucleotide sequence accession numbers. The nucleotide sequences revealed in this study have been deposited in the DDBJ sequence library and assigned accession numbers AB511942 for *penA*-XXX, AB511943 for *penA*-XXXI, AB511944 for *penA*-XXXII, and AB511945 and AB511946 for the *penA*-flanking regions.

RESULTS

***penA* sequence variation.** To examine the possibility of the expansion of a single clone with reduced susceptibility to cefixime, we sequenced the *penA* alleles of the Cef^{Rs} isolates in our collection. Among 32 Cef^{Rs} isolates obtained from 1998 to 2005, five PBP 2 types were revealed, including three newly identified types. PBP 2-X was the predominant type (26/32, 81.3%), which is consistent with the findings presented in previous reports (10, 27). PBP XXVI, originally designated mosaic 4 (25), was also found. Newly identified types PBP 2-XXX and PBP 2-XXXI had replacements of Ala by Val at positions 502 and 533, respectively, compared with the sequence of PBP 2-X. PBP 2-XXXII was identical to PBP 2-X from positions 1 to 548, but its C-terminal portion was identical to that of the PBP 2-I allele from a strain that is susceptible to cefixime, strain LM306, suggesting the creation of a new mosaic structure.

Using phylogenetic analysis, we demonstrated that the amino acids sequences of the PBP 2 alleles among the Cef^{Rs} strains varied; however, the variation was restricted to a cluster, which was distinct from the other cluster formed by the PBP 2 types of Cef-susceptible isolates (Fig. 1), suggesting that *penA* of the Cef^{Rs} isolates evolved from a single origin through a point mutation or the replacement of a short segment, such as that in PBP 2-XXXII.

Multilocus sequence typing of Cef^{Rs} isolates. In order to examine whether the whole genomes of the Cef^{Rs} strains were clonal, we applied an MLST strategy. Thirty-two Cef^{Rs} isolates were divided into six different STs (Table 3), including three singleton STs. The predominant ST was newly assigned ST7363 (n = 23, 71.9%). ST1901 (n = 3) and ST1596 (n = 3) were the second most dominant STs among the Cef^{Rs} isolates.

ST7363 and ST1588 differed from ST1596 only in the *pdhC* locus and the *fumC* locus, respectively, suggesting that ST7363, ST1588, and ST1596 are closely related to each other (Table 3). The MLST sequence type might alter during passages *in*

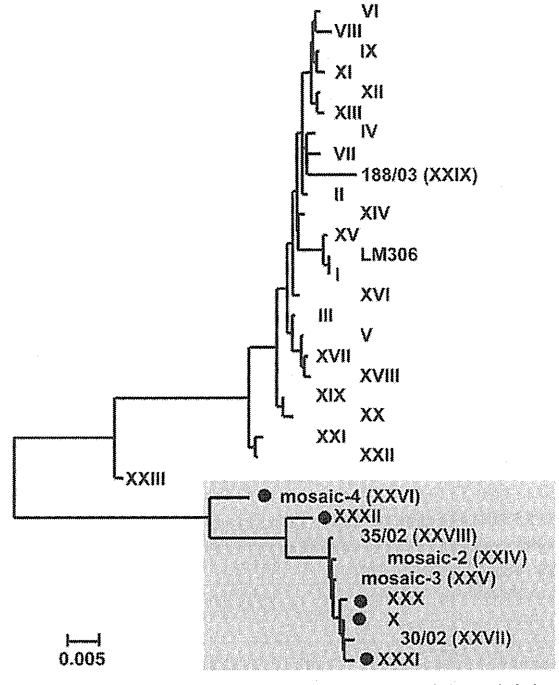

FIG. 1. Relationships of 33 PBP 2 types. A neighbor-joining tree was constructed from the PBP 2 amino acid sequences. The tree contains the LM306, PBP 2, and the PBP 2 types reported by Ito et al. (10), Whiley et al. (27), Takahata et al. (25), and Lindberg et al. (14) and in this study. The PBP 2 types that resulted in the reduced susceptibility of *N. gonorrhoeae* to cefixime are shaded in gray. Black dots indicate the PBP 2 types found in this study.

TABLE 3. MLST types and *penA* alleles of isolates with reduced susceptibility to cefixime

ST	No. of isolates	Allele at locus[a]:							No. of isolates with *penA* allele:			
		abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*	X	XXVI	XXX	XXXI	XXXII
7363	23	59	39	67	78	148	153	65	21	0	2	0	0
1596	3	59	39	67	78	148	**71**	65	3	0	0	0	0
1588	1	59	39	67	**158**	148	**71**	65	1	0	0	0	0
1590	1	**126**	39	67	78	**149**	153	65	0	0	0	1	0
7358	1	**109**	39	67	78	**149**	153	**133**	0	1	0	0	0
1901	3	**109**	39	**170**	**111**	148	153	65	1	0	0	0	2

[a] Boldface data indicate alleles different from that of ST7363.

vivo and *in vitro* due to a point mutation or an interstrain recombinational event. However, the other three STs, ST1901, ST1590, and ST7358, showed at least two differences from the other STs of the Cef^{Rs} isolates. It is unlikely that this was because of allele exchange in all these isolates, indicating that the concept of the expansion of a single clone of Cef^{Rs} could not completely explain the spread of Cef^{Rs}.

Correlation of *penA* allele type with MLST typing. If Cef^{Rs} isolates emerged as different STs through independently generated mosaic structures of the *penA* allele, we would expect isolates with unique *penA* alleles in each ST. As shown in Table 3, the *penA-X* of the dominant PBP, PBP 2-X, was widely distributed in four different STs, ST7363, ST1596, ST1588, and ST1901, while unique *penA* alleles of PBP 2-XXX and PBP 2-XXXII, which were found in more than two isolates, were detected only in ST7358 and ST1901, respectively. From the results, we speculate that one of the possible reasons for this is that in some Cef^{Rs} isolates the transfer of the *penA-X* allele occurs between different *N. gonorrhoeae* strains.

***In vitro* transfer of *penA* gene.** To explore the possibility that the *penA-X* allele spread between different *N. gonorrhoeae* strains, we tested whether *penA-X* could be transferred by the *in vitro* cocultivation of Cef^{Rs} isolate (NG0003, ST7363) and a cefixime-susceptible (Cef^{s}) strain (strain NG0202, ST1901). NG0003 is susceptible to ciprofloxacin, and NG0202 is resistant to ciprofloxacin.

When a portion (0.1 ml) of a 16-h static culture of strain NG0003 or strain NG0202 (0.71×10^8 and 1.01×10^8 CFU/ml, respectively) was plated on a Cef+Cip GC agar plate, no colonies appeared, indicating that no spontaneous antibiotic resistance mutations occurred (Table 4). When a mixture of NG0003 and NG0202 was plated after cocultivation for 16 h, we obtained colonies resistant to both drugs (4.33×10^3 CFU/ml) on Cef+Cip GC agar plates (Table 4). We randomly selected 12 colonies from these mutants. All clones were ST1901, and the PFGE profiles of all the resistant clones were identical to the PFGE profile of NG0202 (Fig. 2), suggesting that NG0202 received *penA-X* from NG0003 and became resistant to cefixime.

The transformation frequency was estimated to be 2.1×10^{-4} (Table 4). When DNase (200 μg/ml) was present in the cocultivated mixture, no colonies resistant to both drugs were obtained, suggesting that the transfer was dependent on naked DNA released from the donor strain in the broth.

Sequence comparison of *penA* alleles. To confirm the transfer of the *penA-X* allele, we determined the nucleotide sequence of the *penA* allele (1,752 bp) in the double-resistant clones derived from NG0202, which originally possessed a *penA-V* allele. Sequence diversity between the *penA-X* and the *penA-V* alleles was identified at a total of 221 polymorphic sites after nucleotide position 294 of the *penA* gene, and the overall sequence identity was 87.3%. Eight of 12 clones had the same *penA-X* allele as NG0003. The clones with the other *penA* alleles, clones Tf-3, Tf-13, Tf-14, and Tf-15, had alleles highly similar to the alleles in *penA-X* (99.6 to 99.9%), and all se-

TABLE 4. *In vitro* transfer of reduced susceptibility to cefixime

Strain[a]	No. of CFU on plates with:		
	Cefixime[b]	Ciprofloxacin[c]	Cefixime and ciprofloxacin[d]
NG0202 (ST1901)	<10	0.71×10^8	<10
NG0003 (ST7363)	1.01×10^8	<10	<10
NG0202 + NG0003	1.03×10^8	0.2×10^8	4.33×10^3

[a] Strain NG0202, strain NG0003, and a suspension of equal numbers of cells of both strains (OD_{600}, 0.02) were incubated for 16 h.
[b] Containing 0.031 μg/ml of cefixime.
[c] Containing 2 μg/ml of ciprofloxacin.
[d] Containing 0.031 μg/ml of cefixime and 2 μg/ml of ciprofloxacin.

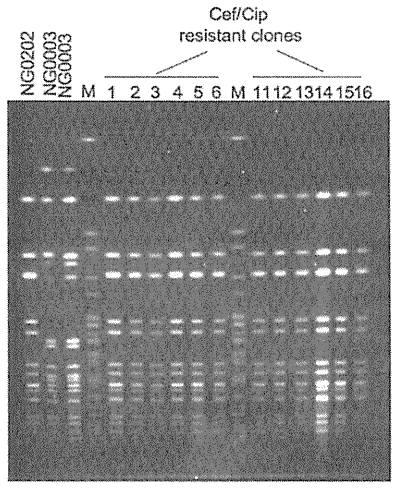

FIG. 2. PFGE patterns of clones obtained by *in vitro penA-X* transfer. NG0202 (Cef^{s} of ST1901) and NG0003 (Cef^{Rs} of ST7363) were cocultivated overnight, and then colonies that were resistant to both cefixime (Cef) and ciprofloxacin (Cip) were identified by using GC agar plates containing 0.031 μg/ml of cefixime and 2 μg/ml of ciprofloxacin. SpeI-digested genomic DNA from 12 of the clones obtained was analyzed by PFGE. Lanes M, size marker consisting of SpeI-digested *Salmonella enterica* serovar Braendecup strain H9812 genomic DNA.

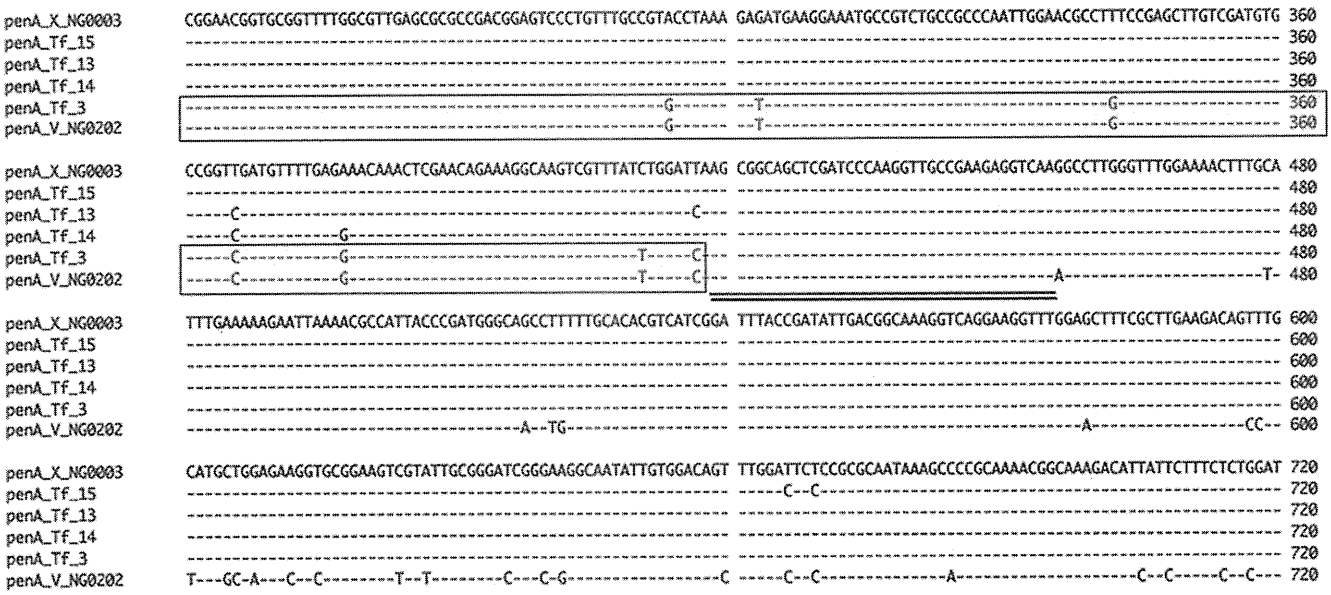

FIG. 3. Comparison of sequences from nucleotides 241 to 720 among *penA-X*, *penA-V*, and minor variants of the transformants. Dashes indicate the same nucleotide as *penA-X* of strain NG0003 (shown on the first line). Shaded boxes in *penA* Tf-3 and NG0202 (positions 241 to 417) indicate regions where the sequences between them are identical. The underlined region indicates the possible junction site of Tf-3.

quence divergences were found between nucleotide positions 294 and 669 of *penA* (Fig. 3). We concluded that all clones analyzed acquired *penA-X* or its derivatives and that these were responsible for the reduced susceptibility to cefixime in the clones. We also showed that *penA-X* allelic diversity was generated in the Tf-3, Tf-13, Tf-14, and Tf-15 clones.

Junction site of recombination of *penA-X*. The 5′ portion of *penA* (positions 1 to 417) in clone Tf-3 was identical to that of *penA-V* in strain NG0202, while the 3′ portion after nucleotide position 456 was identical to that of *penA*-X (Fig. 3), implying that a recombination junction site was located between positions 417 and 456 in *penA* of Tf-3. To determine the junction sites of the other clones, we first sequenced the *penA*-flanking regions (6,299 bp) in strains NG0003 and NG0202 (Fig. 4A). The overall nucleotide sequence identity of the region between NG0003 and NG0202 was 95.7%, significantly less than the identity of the concatenated seven loci of ST7363 and ST1901 determined by MLST analysis (3 bp different in 3,284 bp; 99.9%). As shown in Fig. 4A, the sequence divergence accumulated in the *penA* locus and also in the 5′ part of *murE*. Only three polymorphic sites were identified in the *dcaA* gene (1,647 bp), at about nucleotide position 5500, outside the highly variable region (Fig. 4A and 4B).

As shown in Fig. 4A and B, since the upstream region (positions 1 to 1590) was highly conserved and there were no polymorphic sites between strains NG0003 and NG0202, we could not determine the left junction site, other than that of Tf-3. As for the right junction site, we detected a possible junction site within the highly variable region in the *penA*-flanking region of Tf-15 (Fig. 4B). Although we could not determine the right junction site for *penA* recombination other than that in Tf-15, our analysis of the other 11 clones showed that the nucleotide sequence of *dcaA* was identical to that of NG0003, indicating that *penA-X* was replaced along with *murE* and *dcaA*.

Sequencing analysis of *murE-dcaA* region of ST1901 clinical isolates with PBP 2-X allele. To investigate the horizontal transfer of *penA*, we analyzed a *murE-dcaA* region of additional an ST1901 Cef^S isolate (n = 1) and ST1901 Cef^{Rs} isolates (n = 5) (Table 1). As shown in Fig. 4C, the *penA-murE-dcaA* region of the ST1901 Cef^S clinical isolate (NG0402) was identical to that of NG0202. The sequence of the *penA-murE-dcaA* region of the ST1901 Cef^{Rs} strains NG0002, NGON03-079, NGON03-092, and NGON03-115 was identical to that of NG0003 and most of the clones (type I) obtained in the *in vitro* experiment. NGON07-002 had a *murE* sequence identical to that of NG0003, but the polymorphism sites in *dcaA* of NGON07-002 were the same as those of NG0202, implying that the recombination junction of NGON07-002 was within the region from positions 4100 to 5500. The results suggested that similar DNA transfer and recombination events involving *penA-X* might occur *in vivo*.

DISCUSSION

Reduced susceptibility to cefixime has been associated with the mosaic-type *penA-X* allele encoding PBP 2-X or its derivatives with minor differences (10, 25, 27). However, the genetic relatedness between Cef^{Rs} *N. gonorrhoeae* isolates has not been completely elucidated. In the study described here, we applied MLST analysis to reveal the clonality of the Cef^{Rs} *N. gonorrhoeae* isolates in our collection and showed that Cef^{Rs} *N. gonorrhoeae* isolates belong to six different MLST types. One possible explanation for the wide distribution of Cef^{Rs} *N. gonorrhoeae* is the introduction of *penA* from other species to these STs by interspecies recombination (3, 10). We found that the minor types of PBP 2, PBP 2-XXX and PBP 2-XXXII, were seen only in ST7363 and ST1901 strains, respectively. Although we should analyze more Cef^{Rs} isolates, this may imply that the independent

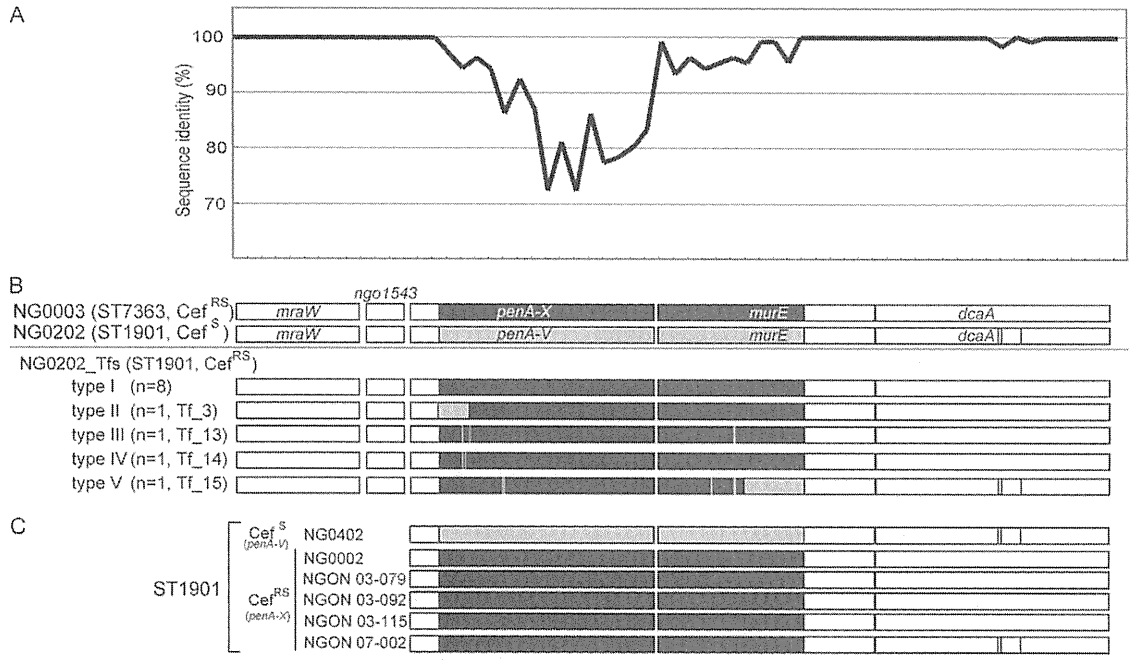

FIG. 4. Sequence diversity in *penA*-flanking regions (6,299 bp) among strain NG0003, strain NG0202, and the transformants generated by *in vitro* cocultivation. (A) Sequence identity of each 100 bp between NG0003 and NG0202. (B) Boxes indicate the five open reading frames in this region, *mraW*, *NGO1543*, *penA*, *murE*, and *dcaA*. Gray boxes, the highly variable region; dark gray boxes, sequences that are identical to the sequence of the *penA-X*-flanking region of NG0003; bright gray boxes, sequences identical to the sequence of the *penA-V*-flanking region of NG0202; fine vertical lines (white and black), polymorphic sites that match the nucleotide bases of NG0202. (C) Sequence diversity in the *penA-murE-dcaA* regions of additional clinical isolates of ST1901.

introduction of a DNA segment from a putative common ancestor has occurred, as proposed previously (24).

However, the putative original *penA-X* allele was the predominant allele among Cef^{Rs} strains in this study as well as in other studies (10, 27). All nine types of Cef^{Rs}-associated PBP 2 seem to be derived from the putative original *penA-X* (Fig. 1). Therefore, another possible explanation for the wide distribution of Cef^{Rs} is that a putative original Cef^{Rs} clone may emerge in a given lineage and clonally expand worldwide (14, 27). This is also suggested by our finding that ST7363 with the *penA-X* allele is predominant. During the spread of the Cef^{Rs} clone, mutations may be introduced, resulting in the emergence of new variants of *penA-X*. Another possibility is that the observed predominance may reflect fitness. If the *penA-X* allele has an advantage in cell growth over other alleles, the result is the elimination of the other alleles, although there is no evidence for such a difference.

The horizontal transfer of the *penA-X* allele shown in the present study can explain the clonality of Cef^{Rs}-associated PBP 2 even in isolates of different STs. We demonstrated the *in vitro* transfer of the *penA-X* allele from Cef^{Rs} ST7363 to Cef^{S} ST1901. Our sequence analysis of the *penA*-flanking region in the clones that acquired *penA-X* (8 of 12) showed that *penA* and the downstream open reading frames for *murE* and *dcaA* were replaced. Furthermore, the sequences from the Cef^{Rs} clinical isolates of ST1901 were also identical to those of the clones generated *in vitro*, supporting the possibility of the *in vivo* spread of the *penA*-flanking DNA segment. To our knowledge, this is the first case that suggests the interstrain transfer of a chromosomally encoded antibiotic resistance-conferring gene in *N. gonorrhoeae* by natural transformation in nature.

In addition to the horizontal transfer of *penA*, we observed the generation of *penA* allele diversity by the introduction of point mutations and the formation of a mosaic structure between a donor and a recipient *in vitro*. The *penA* alleles of one-third (4 of 12) of the transformants analyzed had minor differences from those of both the donor and the recipient. This is inconsistent with an observation mentioned by Spratt et al. (24). They could not detect any sequence variation during experimental transformation by using a PCR-amplified *N. meningitidis penA* gene. This discrepancy may be due to differences in the experimental procedures used, for example, a coculture assay versus transformation by use of a PCR product. However, we should examine more details about the natural transformation system, including the repair process, in *N. gonorrhoeae*. Nonetheless, the dynamic change observed in the allele during transformation may explain the diversity of the *penA* allele-derived Cef^{Rs} clinical isolate. Determination of the mutation rate for the *penA* allele during *in vitro* passages and analysis of more Cef^{Rs} isolates from various geographical areas will help improve our understanding of the diversity of the *penA* allele.

N. gonorrhoeae is a highly recombinogenic pathogen. DNA transformation contributes to the interspecies acquisition of chromosomally encoded antibiotic resistance (10, 23). DNA uptake in *Neisseria* is directly affected by piliation of the cells and the 10-bp-specific DNA uptake sequence (1, 9). After the DNA is internalized, it can be efficiently recombined with a homologous sequence on the recipient chromosome. As the efficiency of homologous recombination is correlated with se-

quence homology, intraspecies genetic exchange may be more efficient than interspecies exchange (8). If so, once *N. gonorrhoeae* acquires a genetic element from another bacterium that provides an advantage for *N. gonorrhoeae* survival *in vivo*, the acquired element would easily be spread among *N. gonorrhoeae* strains under selective pressure.

MLST is used for phylogenetic analysis for many other bacteria because the nucleotide sequence variation of housekeeping genes is likely to accumulate slowly and to be selectively neutral (4, 6, 16). However, the phylogeny of highly recombinogenic bacteria such as *Neisseria* species are difficult to study due to the exchange of DNA segments by natural transformation, resulting in the formation of nonclonal populations (21). Therefore, Cef^{Rs} isolates also might exchange the allele(s) utilized in MLST analysis by a recombinational event. As the allele profiles of ST7363, ST1588, and ST1596 were very similar to each other, these STs might be expected to be genetically related (the ST1596 complex). If we can assume that the housekeeping genes are exchangeable between strains, Cef^{Rs} isolates belonging to ST1596 complex might emerge by allele exchange, despite *penA* allele transfer. Other than the ST1596 complex, ST1901, which is one of the STs found in Cef^{Rs} isolates with the *penA-X* allele, has three loci, *abcZ*, *fumC*, and *aroE*, different from those in ST7363 (Table 3). These loci are scattered on the *N. gonorrhoeae* chromosome (5). Because even the loci closest to each other, *abcZ* and *fumC*, are 140 kb apart on the *N. gonorrhoeae* chromosome (5), evolution from ST7363 to ST1901 (or the other direction) would require three independent genetic events. However, we cannot suggest that this scenario is completely exclusive, since *N. gonorrhoeae* has a high likelihood of acquiring DNA from other cells.

As *N. gonorrhoeae* is an obligate human pathogen, there is neither transmission to other animals nor an environmental reservoir. Genetic exchange between two different strains must take place when one strain meets another strain within an individual host. Recently, two independent groups showed evidence for *N. gonorrhoeae* mixed infections (15, 17). The spread of an antibiotic resistance gene demonstrated in this study could also occur during a mixed infection, probably in highly sexually active persons. It should be noted that the frequency of *penA* allele transfer was relatively high (approximately 2 cells per 10^4 recipients). As expected previously and also as demonstrated in this study, the high natural competence of *N. gonorrhoeae* plays an important role in the transfer of a mosaic *penA* allele among different types of *N. gonorrhoeae* strains. As a result, the prevalence of the allele would be increasing in the population, although it remains unclear whether the other determinants are spread like the *penA* allele. If it is assumed that the spread occurs frequently, we need to reinforce surveillance for asymptomatic mixed gonococcal infections to prevent the spread of resistance-conferring genes.

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Identification of TEM-135 β-Lactamase in Penicillinase-Producing *Neisseria gonorrhoeae* Strains in Japan▽

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Ten penicillinase-producing *Neisseria gonorrhoeae* (PPNG) strains isolated from 2000 to 2008 were characterized by multilocus sequence typing, multiantigen sequence typing, and plasmid typing. Sequence analysis showed that 8 strains contained a TEM-1 β-lactamase gene. However, two other genetically distinct PPNG strains, isolated in 2004 and 2008, each contained a TEM-135 β-lactamase on different plasmids, a Toronto/Rio type R plasmid and an Asia type R plasmid, suggesting independent origins of these PPNG strains.

Antibiotic-resistant *Neisseria gonorrhoeae* is a major public health concern (15). An essential element in gonococcal-infection control is the availability of effective antimicrobial therapy. However, *N. gonorrhoeae* has developed resistance to multiple classes of antimicrobials. In Japan, the prevalence of fluoroquinolone-resistant *N. gonorrhoeae* strains is over 80% (12), and *N. gonorrhoeae* strains with reduced sensitivity and with resistance to cefixime (CFM) have emerged and spread nationwide (5, 7). In contrast to the high prevalence of *N. gonorrhoeae* strains with chromosomal β-lactam resistance genes, the prevalence of penicillinase-producing *N. gonorrhoeae* (PPNG) strains with a β-lactamase gene carried on a plasmid is relatively low in Japan. However, the prevalence of PPNG strains in other countries in Asia is high (16). To study the epidemiology of *N. gonorrhoeae*, nucleotide sequence-based typing methods, like multilocus sequence typing (MLST) and multiantigen sequence typing (MAST), are useful tools, since the analyses yield highly reproducible and easy-to-compare data from different laboratories.

Among the 719 *N. gonorrhoeae* strains isolated from January 2000 to December 2008 in the Nakano Sogo Hospital in Japan, 10 strains (1.4%) were found to be penicillinase-producing *N. gonorrhoeae* (PPNG) by the nitrocefin test (data not shown). The MICs of penicillin (PEN), cefixime (CFM), and ceftriaxone (CRO) were determined by the agar dilution method (6), suggesting that the strains were highly resistant to penicillin but not to cephalosporins (Table 1). This low prevalence was consistent with other reports (14, 16).

MLST and MAST (3, 4) were used to characterize these PPNG strains. As shown in Table 1, both MLST and MAST divided the 10 PPNG strains into 7 types, with 4 (NGON 00-002, NGON 00-027, NGON 04-025, and NGON 08-003) of the PPNG strains having unique sequence types (ST) by both MLST and MAST. However, three pairs of strains (NGON 05-042 and NGON 06-041, NGON 08-041 and NGON08-046, and NGON 08-043 and NGON 08-044) had identical sequence types by MLST and by MAST (Table 1). Although we have no information linking the patients from whom each pair of strains was isolated, transmission of PPNG strains might be considered in these cases.

Plasmids of the PPNG strains carrying the β-lactamase gene (*bla*) have been typed based on plasmid size, since deletion mutants have been reported previously (9). To investigate plasmid diversity in the PPNG strains in this study, plasmid DNAs were purified using QIAprep Spin miniprep kits (Qiagen). To estimate β-lactamase plasmid size, we amplified the complete DNA of each plasmid by long PCR using LA *Taq* polymerase (TaKaRa) and primers bla-IR, 5′-TCGTGGTGTCACGCTC GTCG, and bla-IF, 5′-CTGCAGCAATGGCAACAACGTTG, which anneal to nucleotides 7426 to 7404 and 1 to 23, respectively, of the 7,426-bp pJD4 plasmid (Fig. 1A) (9). The PCR products were incubated for 2 min at 96°C followed by 30 cycles of 10 s at 96°C, 10 s at 63°C, and 8 min at 72°C. As shown in Fig. 1B, analysis of the amplified plasmid DNAs in a 1% agarose gel showed three plasmid sizes: 5.2, 5.6, and 7.4 kb. By use of a multiplex PCR method for plasmid typing (10), the 5.2-, 5.6-, and 7.4-kb plasmids were identified as Toronto/Rio, Africa, and Asia type R plasmids, respectively (Fig. 1A and C).

Although the molecular sizes of *N. gonorrhoeae* R plasmids are diverse, plasmids carrying β-lactamases are genetically related and carry a TEM-1 type *bla* gene, $bla_{\text{TEM-1}}$ (12). To confirm the conservation of $bla_{\text{TEM-1}}$, the *bla* genes of the 10 PPNG isolates were analyzed by DNA sequencing (8). The primers used for amplification and sequencing were bla-F, 5′-CGCTCATGAGACAATAACCCTGG, and bla-R, 5′-GGGTCTGACGCTCAGTGGAACG. The PCR products were incubated for 2 min at 96°C followed by 30 cycles of 10 s at 96°C, 10 s at 60°C, and 1 min at 72°C. Nucleotide sequencing was carried out as described previously (8). As shown in Table 1, two distinct bla_{TEM} alleles were found: 8 PPNG strains contained $bla_{\text{TEM-1}}$, and the other 2 strains (NGON 04-025 and NGON 08-003) contained $bla_{\text{TEM-135}}$, a TEM allele originally identified in *Salmonella enterica* serovar Typhimurium (11). These alleles, $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$, had one base difference, which resulted in a single amino acid

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TABLE 1. Penicillinase-producing *Neisseria gonorrhoeae* strains isolated in Tokyo from 2000 to 2008

Strain	Time of isolation	Sex of patient[a]	Age (yr) of patient	Specimen type[b]	MLST type	MAST type	Plasmid type	*bla* type	MIC (μg/ml)		
									PEN	CFM	CRO
NGON 00-002	January 2000	M	26	UD	ST-1590	ST-270	Africa	TEM-1	16	0.032	0.016
NGON 00-027	June 2000	M	42	U	ST-1921	ST-1817	Asia	TEM-1	>64	0.008	0.008
NGON 04-025	April 2004	M	27	U	ST-1597	ST-1549	Toronto/Rio	TEM-135	>64	0.004	≤0.004
NGON 05-042	August 2005	F	30	VD	ST-1588	ST-4012	Africa	TEM-1	64	0.25	0.064
NGON 06-041	October 2006	M	56	U	ST-1588	ST-4012	Africa	TEM-1	32	0.25	0.032
NGON 08-003	January 2008	F	31	VD	ST-7823	ST-4013	Asia	TEM-135	>64	0.032	0.032
NGON 08-041	September 2008	M	52	U	ST-1584	ST-1478	Africa	TEM-1	64	0.008	0.004
NGON 08-043	September 2008	M	31	U	ST-7823	ST-1288	Asia	TEM-1	>64	0.064	0.064
NGON 08-044	September 2008	F	25	VD	ST-7823	ST-1288	Asia	TEM-1	>64	0.064	0.064
NGON 08-046	October 2008	F	59	VD	ST-1584	ST-1478	Africa	TEM-1	64	0.25	0.032

[a] M, male; F, female.
[b] U, urine; VD, vaginal discharge; UD, urethral discharge.

substitution, M182T (residue numbering follows that of Ambler et al. [1]).

Interestingly, the two PPNG strains with $bla_{TEM-135}$ were genetically different: the sequence types of strain NGON 04-025 were MLST ST-1597 and MAST ST-1549, and those of strain NGON 08-003 were MLST ST-7823 and MAST ST-4013 (Table 1). The plasmids carried by strains NGON 04-025 and NGON 08-003 were also distinct: the plasmid for the former was a Toronto/Rio type, and that for the latter was an Asia type. Taken together, these findings suggest that $bla_{TEM-135}$ may have been introduced independently into these two *N. gonorrhoeae* strains or may have emerged by a point mutation in each. Recently, Srifeungfung et al. (13) reported that a PPNG strain isolated in Thailand contained a $bla_{TEM-135}$ allele. PPNG strains containing $bla_{TEM-135}$ might be widespread in Asian countries, although further study is needed to determine the prevalence.

The TEM type β-lactamase genes, which are widely distributed in Gram-negative bacteria, are diverse in sequence and in substrate spectrum. Some types of TEM β-lactamases can hydrolyze extended-spectrum cephalosporins with an oxyimino side chain, including ceftriaxone, which is still an effective antibiotic for *N. gonorrhoeae*. The diverse substrate spectra of TEM β-lactamases are due to mutations in the bla_{TEM} gene that alter the amino acid configuration around the β-lactamase active site. Since bacteria with $bla_{TEM-135}$ have a restricted β-lactamase substrate spectrum, as reported in a previous study (10) and also in this study (Table 1), the selective pressure for emergence of *N. gonorrhoeae* $bla_{TEM-135}$ is not known. It is noteworthy that there are other TEM β-lactamases with extended substrate spectra that may have arisen as a single point mutation in bla_{TEM-1} or $bla_{TEM-135}$, e.g., bla_{TEM-29} and bla_{TEM-20} (2). Since point mutations in bla_{TEM-1} and $bla_{TEM-135}$ could lead to emergence of *N. gonorrhoeae* β-lactamases with extended substrate spectra, the antibiotic resistance profiles of PPNG strains should be monitored, especially in areas of high PPNG prevalence.

Nucleotide sequence accession number. The sequence data for the $bla_{TEM-135}$ gene have been assigned DDBJ accession number AB551787.

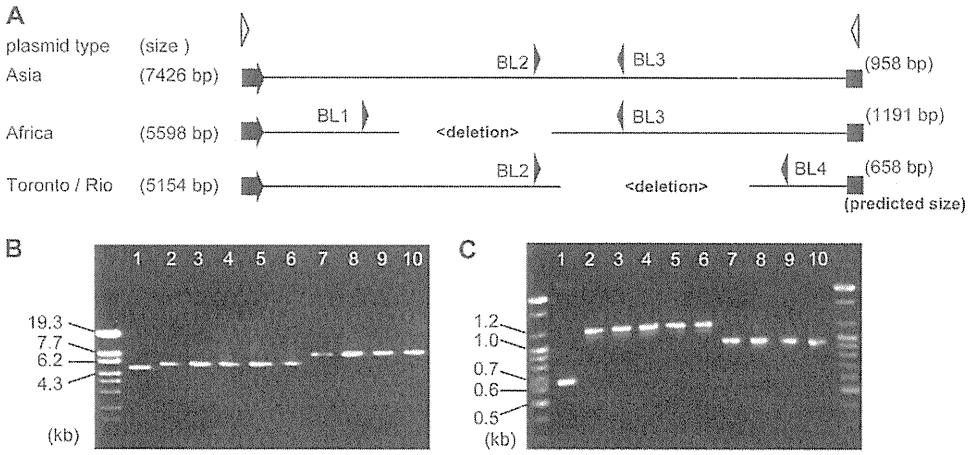

FIG. 1. Typing of plasmids carrying β-lactamases from *Neisseria gonorrhoeae* strains. (A) Schematics of Asia, Africa, and Toronto/Rio type plasmids. Each β-lactamase gene is shown by an arrowhead. The annealing sites of the primers used in this study for plasmid size determination (white arrowheads) and for plasmid type determination (black arrowheads) are shown. (B) Products of whole-plasmid PCR amplification, separated on a 1% agarose gel. (C) Products of multiplex PCR, separated on a 2% agarose gel. The size marker lanes contain StyI-digested lambda DNA (Toyobo) (B) or a 100-bp DNA ladder (Bioneer) (C). Lane 1, NGON 04-025; lane 2, NGON 00-002; lane 3, NGON 05-042; lane 4, NGON 06-041; lane 5, NGON 08-041; lane 6, NGON 08-046; lane 7, NGON 00-027; lane 8, NGON 08-003; lane 9, NGON 08-043; lane 10, NGON 08-044.

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Ceftriaxone-Resistant *Neisseria gonorrhoeae*, Japan

To the Editor: Spread of multidrug-resistant *Neisseria gonorrhoeae* is a major public health concern. Effective antimicrobial therapy is a key element in gonorrhea control. However, *N. gonorrhoeae* has developed resistance to multiple classes of antimicrobial drugs, including β-lactams, tetracyclines, and fluoroquinolones (*1–3*). Even an extended-spectrum oral cephalosporin-resistant, cefixime-resistant *N. gonorrhoeae* has emerged, and cefixime has now been withdrawn from use in Japan. Best practice treatment is limited to injectable extended-spectrum cephalosporins, such as ceftriaxone and spectinomycin. The emergence of ceftriaxone-resistant *N. gonorrhoeae* threatens effective disease control.

We identified a novel ceftriaxone-resistant *N. gonorrhoeae* isolated from a 31-year-old female commercial sex worker; MIC of ceftriaxone for this isolate was high (2 μg/mL). The woman visited a clinic in Kyoto for a routine examination for sexually transmitted infections in January 2009. Although she had no obvious symptoms or signs, a throat sample collected on her first visit yielded a positive result for *N. gonorrhoeae* by the strand displacement amplification test (ProbeTec ET, Becton Dickinson, Franklin Lakes, NJ, USA), but a vaginal sample taken at the same time was negative. After 2 weeks, another throat sample was positive for *N. gonorrhoeae* when cultured on Thayer-Martin medium, and the patient subsequently received 1 g ceftriaxone intravenously. Her pharyngeal sample was also *N. gonorrhoeae* positive by strand displacement amplification test on the third visit 2 weeks later, and further ceftriaxone treatment was prescribed. However, a culture for test of cure was not conducted because reinfection was considered. A negative result was finally obtained in April 2009.

The culture showed positive reactions in oxidase and catalase tests. Gram staining showed gram-negative diplococci. The ID-test HN-20 Rapid system (Nissui, Tokyo, Japan) classified the bacterium as *N. gonorrhoeae*. Susceptibility was determined by the agar dilution method (*4*). For this strain, named H041, MIC of ceftriaxone was high (2 μg/mL), and the strain was highly resistant to penicillin G (4 μg/mL), cefixime (8 μg/mL), and levofloxacin (32 μg/mL). However, it demonstrated susceptibility to spectinomycin (16 μg/mL) and reduced susceptibility to azithromycin (0.5 μg/mL).

To characterize the ceftriaxone-resistant *N. gonorrhoeae* H041, multilocus sequence typing characterized the strain as ST7363 (*5*), which is the predominant sequence type (ST) among cefixime-resistant clones (*6*). *N. gonorrhoea* multiantigen sequence typing (NG-MAST) was also performed (*7*). The NG-MAST strategy uses 2 genes, *por* and *tbpB*, for porin and a transferrin-binding protein, respectively. NG-MAST indicated that the strain H041 was ST4220 and contained the *por2594* allele and the *tbpB10* allele. NG-MAST 4220 is a novel ST. However, the *tbpB10* allele is the most frequently observed allele (76.5%) among multilocus sequence typing-ST7363 *N. gonorrhoeae* strains (n = 81) (M. Ohnishi, unpub. data).

Molecular typing suggested that the novel ceftriaxone-resistant *N. gonorrhoeae*, H041, is closely related to the ST7363 cefixime-resistant *N. gonorrhoeae*. Therefore, we compared *Spe*I-digested genomic DNA banding patterns of strain H041 with those of other *N. gonorrhoeae* strains by using pulsed-field gel electrophoresis as described (*8*). Four ST7363 strains, including *N. gonorrhoeae* H041, and 4 ST1901 strains (another major ST among cefixime-resistant *N. gonorrhoeae* strains) (*6*) were analyzed. The banding pattern of *Spe*I digested H041 genomic DNA was similar to that of other ST7363 strains and indistinguishable from that of cefixime-resistant but ceftriaxone-susceptible NG0207 (Figure).

We describe the emergence of ceftriaxone-resistant *N. gonorrhoeae*, isolated from a pharyngeal specimen from a female commercial sex worker. At 2 μg/mL, the MIC was 4-fold higher than that of the previously reported ceftriaxone nonsusceptible strain (*9*). Our susceptibility testing suggests that only azithromycin and spectinomycin are effective drugs for treating this strain. In this case, eradication was successful, although *N. gonorrhoeae* colonization of the pharynx may just be tempory because

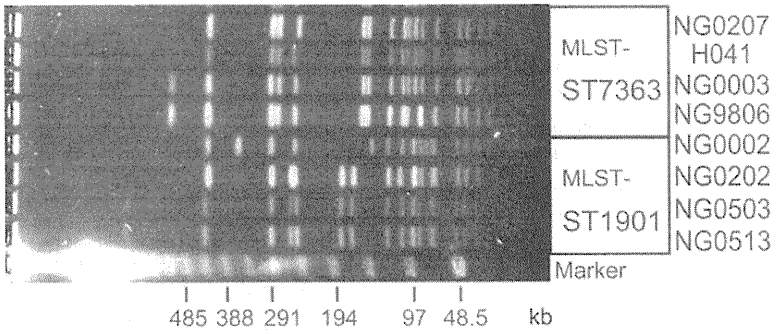

Figure. Pulsed-field gel electrophoresis patterns of ceftriaxone-resistant *Neisseria gonorrhoeae* strain H041 and other multilocus sequence typing (MLST) ST7363 and ST1901 strains. *Spe*I-digested genomic DNA from ceftriaxone-resistant *N. gonorrhoeae* H041, 3 of the MLST ST7363 strains and 4 of the MLST ST1901 strains were analyzed by pulsed-field gel electrophoresis. A lambda ladder standard (Bio-Rad, Hercules, CA, USA) was used as a molecular size marker.

the pharynx is not an ideal site for *N. gonorrhoeae* growth. From the routine examinations of commercial sex workers during January–March 2009, 40 *N. gonorrhoeae* were isolated in the clinic, but no other ceftriaxone-resistant strains were isolated. There is no evidence of dissemination of this strain in Kyoto.

Three independent molecular subtyping methods indicated that the ceftriaxone-resistant H041 strain was *N. gonorrhoeae*, and it might originate from an ST7363 cefixime-resistant *N. gonorrhoeae* clone. There are several possible mechanisms for the acquisition of resistance, including formation of a new mosaic type *penA* allele as *penA-X* cefixime resistance and acquisition of an extended-spectrum β-lactamase gene. The H041 strain did not produce β-lactamase in a nitrocephin test. Further molecular analysis is needed to elucidate the precise mechanism of the ceftriaxone resistance of the H041 strain.

The emergence of ceftriaxone-resistant *N. gonorrhoeae* raises concerns for controlling gonorrhea because ceftriaxone is widely recommended and the first-line treatment for gonorrhea around the world. *N. gonorrhoeae* has a potential to gain an extraordinarily high MIC to ceftriaxone. Surveillance for ceftriaxone-resistant *N. gonorrhoeae* should be strengthened.

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Role of National Travel Health Network and Centre Website during Pandemic (H1N1) 2009

To the Editor: The National Travel Health Network and Centre (NaTHNaC) was created in 2002 by the Department of Health in England to provide authoritative guidance in travel medicine. The open-access NaTHNaC website (www.nathnac.org) is a key mode of communication, with both health professionals' and travelers' areas. Website country information pages (CIP) provide specific guidance for travel to each country of the world, and an outbreak surveillance database (OSD) detailing global outbreaks of disease is updated daily.

In late April 2009, influenza A virus (H1N1) of swine origin was identified in 2 children from California, USA (*1*). These cases were traced to travel to Mexico, and a widespread outbreak of influenza A (H1N1) in Mexico subsequently was recognized. On June 11, 2009, the World Health

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Is *Neisseria gonorrhoeae* Initiating a Future Era of Untreatable Gonorrhea?: Detailed Characterization of the First Strain with High-Level Resistance to Ceftriaxone▽†

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Recently, the first *Neisseria gonorrhoeae* strain (H041) that is highly resistant to the extended-spectrum cephalosporin (ESC) ceftriaxone, the last remaining option for empirical first-line treatment, was isolated. We performed a detailed characterization of H041, phenotypically and genetically, to confirm the finding, examine its antimicrobial resistance (AMR), and elucidate the resistance mechanisms. H041 was examined using seven species-confirmatory tests, antibiograms (30 antimicrobials), *porB* sequencing, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), multilocus sequence typing (MLST), and sequencing of ESC resistance determinants (*penA*, *mtrR*, *penB*, *ponA*, and *pilQ*). Transformation, using appropriate recipient strains, was performed to confirm the ESC resistance determinants. H041 was assigned to serovar Bpyust, MLST sequence type (ST) ST7363, and the new NG-MAST ST4220. H041 proved highly resistant to ceftriaxone (2 to 4 μg/ml, which is 4- to 8-fold higher than any previously described isolate) and all other cephalosporins, as well as most other antimicrobials tested. A new *penA* mosaic allele caused the ceftriaxone resistance. In conclusion, *N. gonorrhoeae* has now shown its ability to also develop ceftriaxone resistance. Although the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, *N. gonorrhoeae* may soon become a true superbug, causing untreatable gonorrhea. A reduction in the global gonorrhea burden by enhanced disease control activities, combined with wider strategies for general AMR control and enhanced understanding of the mechanisms of emergence and spread of AMR, which need to be monitored globally, and public health response plans for global (and national) perspectives are important. Ultimately, the development of new drugs for efficacious gonorrhea treatment is necessary.

Gonorrhea, caused by *Neisseria gonorrhoeae* (gonococcus), is the second-most-prevalent bacterial sexually transmitted infection globally. The disease is associated with high morbidity and socioeconomic consequences and remains a public health problem worldwide (36, 46; G. Schmid, presented at WHO/CDC symposium: Congenital syphilis and the 2005 WHO estimates of STI incidence and prevalence: using the second to help eliminate the first, 18th International Society for Sexually Transmitted Disease Research conference [ISSTDR], 28 June to 1 July 2009, London, United Kingdom). In the absence of a vaccine, appropriate diagnostics and antimicrobial therapy are the key elements for reduction and control of gonorrhea and the development of associated severe complications and sequelae, as well as further transmission of the infection (34, 36).

The treatment options, however, have diminished rapidly because of the emergence and worldwide spread of antimicrobial resistance (AMR) to all drugs previously used or considered first line, i.e., penicillins, narrow-spectrum cephalosporins, tetracyclines, macrolides, and fluoroquinolones. Furthermore, rapid emergence of resistance to spectinomycin was observed when it was widely used for treatment in the past (4), and this antimicrobial is not suitable for treatment of pharyngeal gonorrhea, nor is it currently available in many countries (3, 15, 36). Accordingly, spectinomycin is not a promising candidate for first-line empirical treatment of gonorrhea. Worryingly, in recent years, susceptibility to the currently recommended first-line antimicrobials, the extended-spectrum cephalosporins (ESCs), i.e., ceftriaxone (injectable) and cefixime (oral), has also decreased globally (3, 15, 17, 36). Furthermore, for several years, cefixime treatment failures have been recognized in Japan (9, 36, 47), where cefixime was already excluded from treatment guidelines in 2006 (36). More recently, failures have also been verified in Europe (40). However, despite the fact that susceptibility to ceftriaxone (the last remaining option for empirical first-line treatment) is decreasing globally, *in vitro* and clinical (resulting in treatment failure of urogenital gonorrhea) resistance has been lacking (3, 15, 17, 36).

Recently, the first high-level ceftriaxone-resistant gonococcal strain (H041) was isolated from the pharynx of a female commercial sex worker in Kyoto, Japan (23). H041 displayed a MIC of ceftriaxone of 2 μg/ml. This is a very high level of resistance and, previously, only one isolate having an MIC of >0.25 μg/ml (MIC = 0.5 μg/ml) (33) has been reported world-

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