fixation reaction；CF），中和反応（neutralization test；NT），EIA（enzyme immunoassay）による抗体測定である。

図6に一般的な液性免疫の推移を示す。通常CF，NTなどの抗体検査は急性期（発病後早期）と回復期（発病後14～21日）のペア血清の抗体価が4倍以上上昇した場合に有意と判断する。初感染の場合には一般に有意な上昇がみられ診断価値が高いが，再発型の場合には抗体価の変動を捉えることは難しい。CF法は感染後の上昇は他の検査法よりやや遅く，比較的短期間に抗体価が低下あるいは消失する傾向がある。NT法は感染後1週間ぐらいから上昇し，長期間持続し特異性は高いが他法に比べ結果が出るまでに時間がかかる。

NT法は1型および2型の抗体価を別々に測定することが可能なため，型特異的血清診断が一見可能のように思われるが，HSV-1とHSV-2は高い共通抗原を有するためにこの方法では現実的に型別は不可能であり，NT法の結果で患者に型別の説明をしてはならない。

2）EIA法

EIA法はIgGおよびIgM抗体の分画が可能であるのが特徴で，感度・特異度ともに優れている。図6のように初感染後7～10日でIgM抗体の上昇が認められる。EIA法ではある程度定量的なデータとして結果が得られるため，ペア血清でその推移を確認することで感染病態の推測が可能である。ただし再発型では，抗体価の変動は捉えることが難しいため血清診断は困難である。また，再発型性器ヘルペスでもIgMが検出されることが16.7%あることに注意する[1]。

3）HSV gG ELISA法

前述の抗体価検査ではHSVの型別は不可能である。GHにおいて1型と2型では特にその予後，再発の頻度についての差が大きいため型別が可能であれば診療上の利点は大きい。

型別の可能な血清抗体価検査としては，HSV gG ELISA（enzyme-linked immunosorbent assay）法がある。これはHSV-1型とHSV-2型で共通部分がきわめて少ない，エンベロープに存在する糖蛋白glycoprotein G（gG）を用いた識別方法である。

感染後上昇するまでが比較的遅く，40日以内の検出率は6～7割程度との報告はあるものの[2]，抗体陰性を確認した者が12週後以降陽性に転じた場合は新規のヘルペス感染を疑うことができる[3]。わが国でも検査会社に依頼可能

図6 ウイルス感染症に伴う一般的な液性免疫の推移

凡例：IgG，IgM，NT，CF
縦軸：抗体価
横軸：感染後経過日数（7日 14日 1カ月 3カ月 6～12カ月 2年）
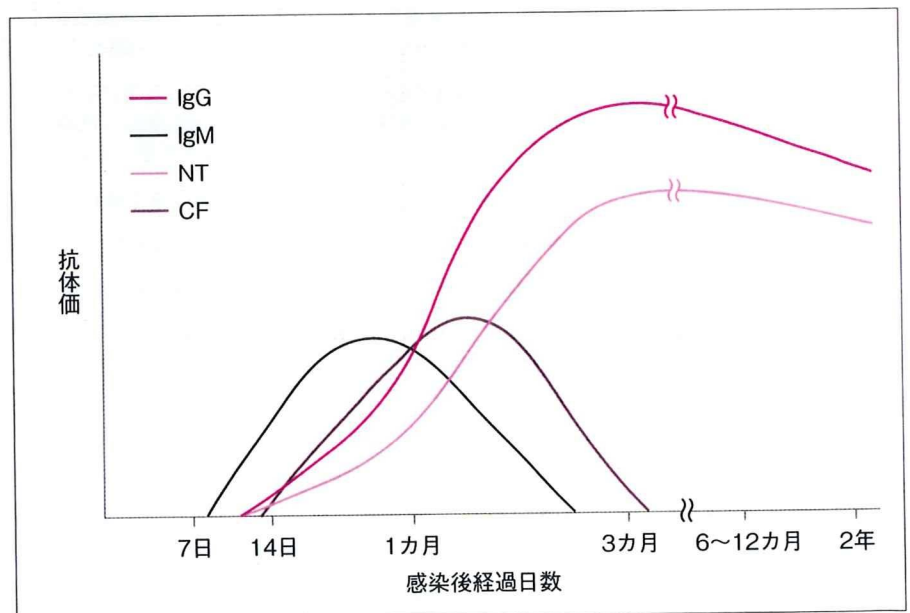
（文献6より引用）

であるが，保険適用とはなっていない。

◆核酸増幅法

核酸増幅法はウイルスのDNA（deoxyribonucleic acid）を検出する検査方法で，後述のウイルス分離培養に比べ感度や迅速性に優れる検査方法である。

水疱内容液，びらん面，腟分泌物などを検体として，HSVに特異的なプライマーを用いたPCR（polymerase chain reaction）法やreal-time PCR法，LAMP（loop-mediated isothermal amplification）法を施行することにより，HSV感染症であることが証明でき型別も可能である。

感度が高いゆえに，セックスパートナーなどからのコンタミネーションによる偽陽性に注意が必要である。検査会社に依頼可能であるが，保険適用外でありやや高価である。

◆ウイルス分離

Vero細胞などの培養細胞にウイルスを接種しその形態的変化である細胞変性効果（cytopathic effect；CPE）を観察し，抗原検査などから診断する方法である。水疱内容液，びらん面から1週間程度で分離でき確定診断することができる検査方法である。

ただし保険の適用外であり高価であるため，現状では使用する機会は限られてしまっている。乾燥，痂皮化あるいは時間の経過した皮疹からの検体，不適切に採取された検体や再発病変からの検体などでは感度が低い。

シェルバイアル法は感受性のある培養細胞にウイルスを接種した後24時間培養し，モノクローナル抗体を用いた蛍光抗体法で培養により発現したウイルス蛋白抗原を同定し陽性・陰性の判定を行うもので，分離培養に比べて短期間で結果が出る点で優れる。

◆病理組織学的検査

病理組織像では水疱形成における球状変性，網状変性，さらには好酸性に腫大した表皮細胞や多核巨細胞，核内封入体などの所見がみられる。組織内でのHSVの局在を検討する場合には，特異抗体による免疫染色や*in situ* hybridization法が用いられる。侵襲のある検査方法のため，HSV感染症を疑う場合に施行することはあまりない。

治療・カウンセリング

初感染時の治療

初感染では抗ウイルス薬の内服が勧められる。現在保険適用があるのはアシクロビルおよびバラシクロビルで，ファムシクロビルはわが国では現在保険適用外である。特に理由がなければ，保険適用があり1日2回の内服で治療の可能なバラシクロビルが現状ではわが国で推奨される薬剤であろう。バラシクロビル1回500mg1日2回投与を症状にあわせて1週間から10日間程度内服する。

再発例での治療

再発においても抗ウイルス薬の内服が勧められる。CDC（Centers for Disease Control and Prevention）のガイドラインでも指摘されているように，外用療法は臨床的な利点が小さく，特に理由がなければ推奨されない[4]。

再発した場合には，初感染と同様バラシクロビルの投与を推奨したい。わが国での保険適用はバラシクロビル1回500mg1日2回を5日間までの投与である。

再発の場合抗ウイルス薬の内服は前駆症状の出現している間かあるいは皮疹の出現後1日以内に開始するのが望ましい。あらかじめ抗ウイルス薬を処方しておき，患者が前駆症状の生じたあるいは皮疹が出現した直後，早期に抗ウイルス薬の内服を開始するpatient-initiated treatmentを考慮すべきであるが，現状では厳密には保険適用外の処方である。

再発抑制療法は抗ウイルス薬を連日内服する治療法で，わが国でもバラシクロビルで保険適用がある。保険適用がある使用方法はバラシクロビル1日1回500mg連日内服するもので，抑制療法中に再発を繰り返す場合には，1回250mg1日2回または1日1回1,000mgの投与など用法用量の変更を考慮する。再発抑制療法は再発頻度の減少，再発時の症状の軽減化および無症候性排泄の抑制ひいてはセックスパートナーへの感染のリスク軽減に効果がある。特に無症候性排泄の抑制効果のある治療法は現状では他にない。ただし完全に無症候性排泄を抑制するものではないため，感染の予防のためにはコンドームの適正使用が望ましい[5]。

免疫不全の患者等で重症化した場合や髄膜炎や脳炎などの合併症がある場合等では，アシクロビルの点滴も考慮する。また抗ウイルス薬の投与時には，主に腎障害あるいは腎機能の低下のあ

る患者や高齢者で血中濃度が高度になって生じる，いわゆるアシクロビル脳症に注意が必要であり，投与量や投与間隔の変更を考慮する必要がある。

カウンセリング

GHの診断を受けた患者とそのパートナーにとって，カウンセリングは診療上不可欠である。不正確な情報を説明される，あるいは必要な説明をされずに無用な不安を抱える患者や感染のリスクを理解しない患者を生み出さないようにしたい。特にGHという病気の性質，再発の可能性，無症候性排泄とそれに伴う感染のリスク，感染の予防のために必要な措置，治療方法に関しては十分な説明が不可欠である。

病気について詳しく説明したパンフレットなどの使用は限られた診察時間内で情報を提供するうえで有用であろう。現在グラクソ・スミスクライン株式会社などより提供されているGHに関するパンフレットは内容が充実している。また同社の運営するウェブサイト「Herpes.jp」（http://herpes.jp/）は患者向けにGHに関するさらに正確で詳しい情報とエキスパートによるQ&Aなどのコーナーもあり，こちらも利用を勧めるのも有効であろう。

◎文献
1) 本田まりこ，小松崎　眞，峰咲幸哲ほか：尿中単純ヘルペスウイルス抗体検査の有用性. 臨皮 2000; 54: 71-5.
2) Ashley RL, Militoni J, Lee F, et al: Comparison of Western blot (immunoblot) and glycoprotein G-specific immunodot enzyme assay for detecting antibodies to herpes simplex virus types 1 and 2 in human sera. J Clin Microbiol 1988; 26: 662-7.
3) Gupta R, Warren T, Wald A: Genital herpes. Lancet 2007; 370: 2127-37.
4) Department of health and human services, Centers for Disease Control and Prevention: Sexually Transmitted Diseases Treatment Guidelines, 2006. Morbidity and Mortality Weekly Report, Vol.55/R-11: 16-20, 2006.
5) Wald A, Langenberg AG, Link K, et al: Effect of Condoms on Reducing the Transmission of Herpes Simplex Virus Type 2 from men to women. JAMA 2001; 285: 3100-6.
6) 尾上智彦，伊東秀記，松尾光馬ほか：性器ヘルペスの診断. Derma 2008; 147: 13-22.

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

Makoto Ohnishi,[1]* Yuko Watanabe,[2] Emi Ono,[3] Chieko Takahashi,[2] Hitomi Oya,[2] Toshiro Kuroki,[2] Ken Shimuta,[1] Norio Okazaki,[2] Shu-ichi Nakayama,[1] and Haruo Watanabe[1]

Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan[1]; Department of Microbiology, Kanagawa Prefectural Institute of Public Health, Kanagawa, Japan[2]; and Department of Moleculo-Genetic Science, Graduate School of Health Care Science, Tokyo Medical and Dental University, Tokyo, Japan[3]

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 $\geq$ 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

* Corresponding author. Mailing address: Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1163. E-mail: ohnishi7@nih.go.jp.

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*
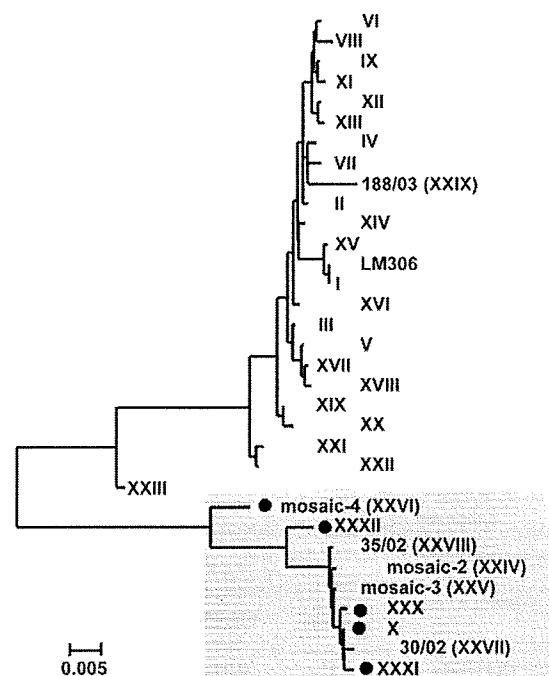

FIG. 1. Relationships of 33 PBP 2 types. A neighbor-joining tree was constructed from the PBP 2 amino acid sequences. The tree contains the LM306, PBP 2, and the PBP 2 types reported by Ito et al. (10), Whiley et al. (27), Takahata et al. (25), and Lindberg et al. (14) and in this study. The PBP 2 types 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 se-

lected 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.
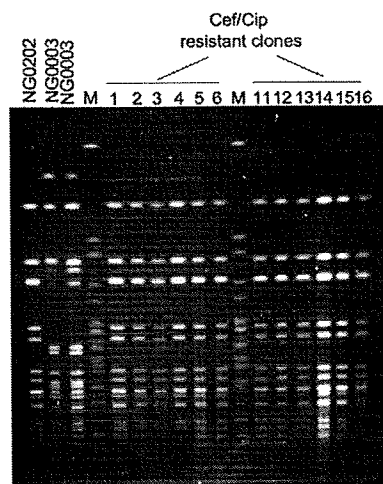[d] Containing 0.031 μg/ml of cefixime and 2 μg/ml of ciprofloxacin.

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.
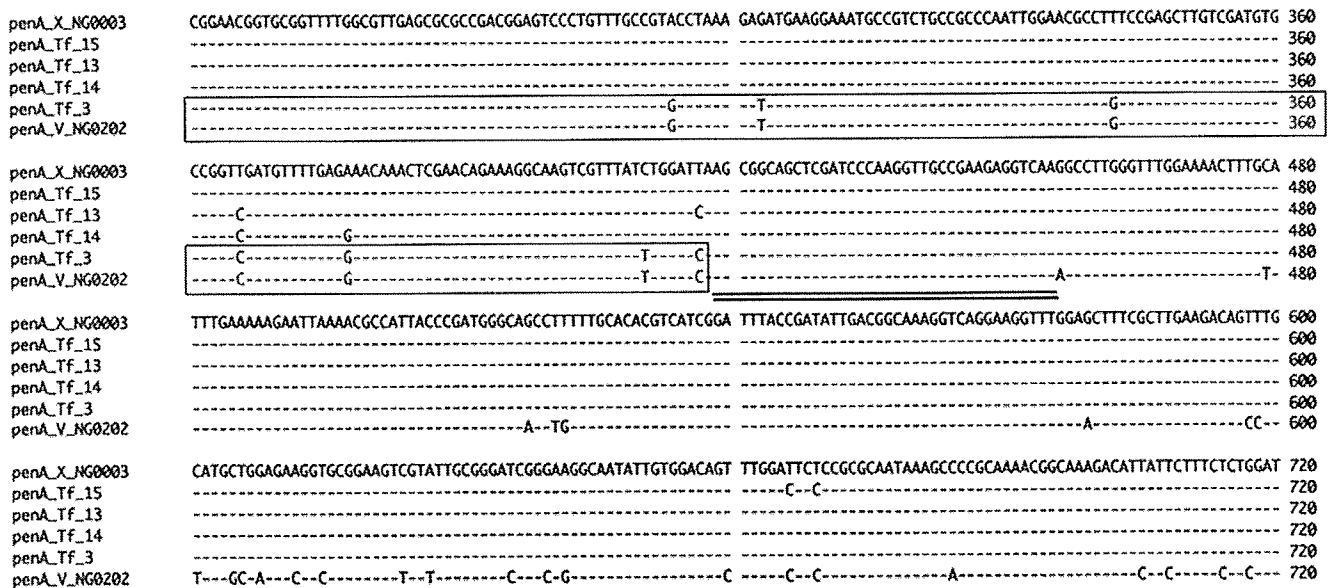
FIG. 3. Comparison of sequences from nucleotides 241 to 720 among *penA-X*, *penA-V*, and minor variants of the transformants. Dashes indicate the same nucleotide as *penA-X* of strain NG0003 (shown on the first line). Shaded boxes in *penA* Tf-3 and NG0202 (positions 241 to 417) indicate regions where 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
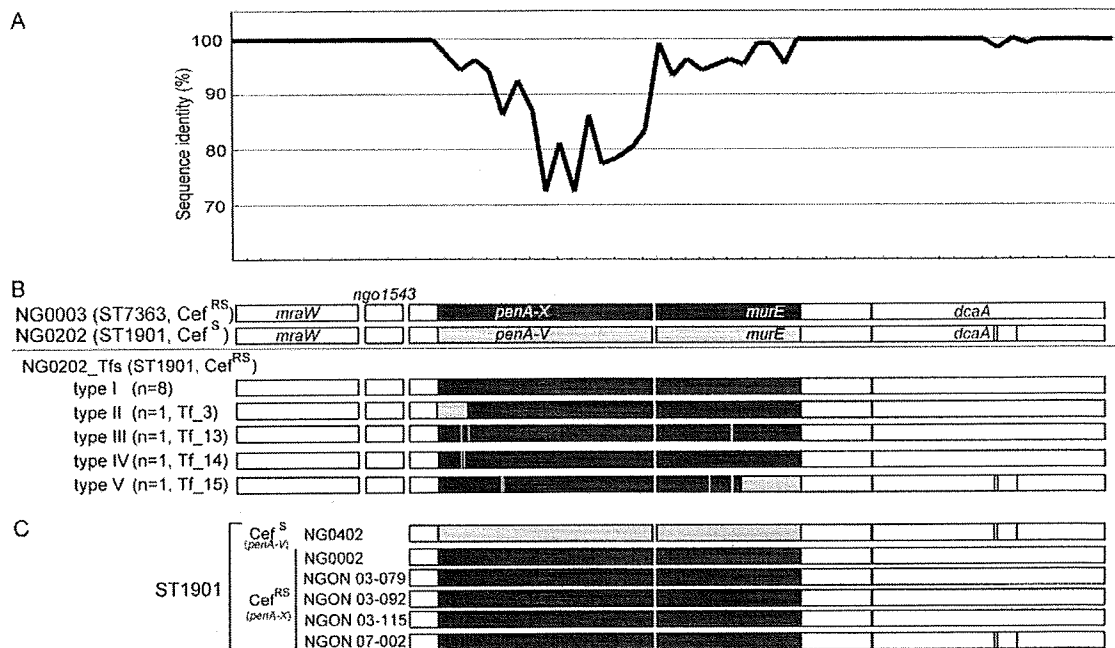
FIG. 4. Sequence diversity in *penA*-flanking regions (6,299 bp) among strain NG0003, strain NG0202, and the transformants generated by *in vitro* cocultivation. (A) Sequence identity of each 100 bp between NG0003 and NG0202. (B) Boxes indicate the five open reading frames in this region, *mraW*, *NGO1543*, *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.

ACKNOWLEDGMENTS

REFERENCES

1. **Aas, F. E., M. Wolfgang, S. Frye, S. Dunham, C. Lovold, and M. Koomey.** 2002. Competence for natural transformation in *Neisseria gonorrhoeae*: components of DNA binding and uptake linked to type IV pilus expression. Mol. Microbiol. **46:**749–760.
2. **Akasaka, S., T. Muratani, Y. Yamada, H. Inatomi, K. Takahashi, and T. Matsumoto.** 2001. Emergence of cephem- and aztreonam-high-resistant *Neisseria gonorrhoeae* that dose not produce beta-lactamase. J. Infect. Chemother. **7:**49–50.
3. **Ameyama, S., S. Onodera, M. Takahata, S. Minami, N. Maki, K. Endo, H. Goto, H. Suzuki, and Y. Oishi.** 2002. Mosaic-like structure of penicillin-binding protein 2 gene (*penA*) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. Antimicrob. Agents Chemother. **46:**3744–3749.
4. **Bennett, J., K. A. Jolley, P. F. Sparling, N. J. Saunders, C. A. Hart, I. M. Feavers, and M. C. J. Maiden.** 2007. Species status of *Neisseria gonorrhoeae*: evolutionary and epidemiological inferences from multilocus sequence typing. BMC Biol. **5:**35.
5. **Chung, G. T., J. S. Yoo, H. B. Oh, Y. S. Lee, S. H. Cha, S. J. Kim, and C. K. Yoo.** 2008. The complete genome sequence of *Neisseria gonorrhoeae* NCCP11945. J. Bacteriol. **190:**6035–6036.
6. **Enright, M. C., and B. G. Spratt.** 1999. Multilocus sequence typing. Trends Microbiol. **7:**482–487.
7. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
8. **Fraser, C., W. P. Hanage, and B. G. Spratt.** 2007. Recombination and the nature of bacterial speciation. Science **315:**476–480.
9. **Goodman, S. D., and J. J. Scocca.** 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. U. S. A. **85:**6982–6986.
10. **Ito, M., T. Deguchi, K. Mizutani, M. Yasuda, S. Yokoi, S. Ito, Y. Takahashi, S. Ishihara, Y. Kawamura, and T. Ezaki.** 2005. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harbouring mosaic-like structure of penicillin-binding protein 2 in central Japan. Antimicrob. Agents Chemother. **49:**137–143.
11. **Janda, W. M., and C. A. Gaydos.** 2007. *Neisseria*, p. 601–620. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of clinical microbiology, 9th ed. ASM Press, Washington, DC.
12. **Jolley, K. A.** 2001. Multi-locus sequence typing, p. 173–186. *In* A. J. Pollard and M. C. Maiden (ed.) Meningococcal diseases: methods and protocols. Humana Press, Totowa, NJ.
13. **Jolley, K. A., M. S. Chan, and M. C. Maiden.** 2004. mlstdbNet—distributed multi-locus sequence typing (MLST) database. BMC Bioinform. **5:**86.
14. **Lindberg, R., H. Fredlund, R. Nicholas, and M. Unemo.** 2007. *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA*, *mtrR*, *porB1b*, and *ponA*. Antimicrob. Agents Chemother. **51:**2117–2122.
15. **Lynn, F., M. M. Hobbs, J. M. Zenilman, F. M. T. F. Behets, K. Van Damme, A. Rasamindrakotroka, and M. C. Bash.** 2005. Genetic typing of the porin of *Neisseria gonorrhoeae* from clinical noncultured samples for strain characterization and identification of mixed gonococcal infections. J. Clin. Microbiol. **43:**368–375.
16. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U. S. A. **95:**3140–3145.
17. **Martin, I. M. C., and C. A. Ison.** 2003. Detection of mixed infection of *Neisseria gonorrhoeae*. Sex. Transm. Infect. **79:**56–58.
18. **Muratani, T., S. Akasaka, T. Kobayashi, Y. Yamada, H. Inatomi, K. Takahashi, and T. Matsumoto.** 2001. Outbreak of cefzopran (penicillin, oral cephems, and aztreonam)-resistant *Neisseria gonorrhoeae* in Japan. Antimicrob. Agents Chemother. **45:**3603–3606.
19. **National Committee for Clinical Laboratory Standards.** 1999. Performance standards for antimicrobial susceptibility testing, 9th informational supplement M7-A4 (M100-S9). National Committee for Clinical Laboratory Standards, Wayne, PA.
20. **Ochiai, S., H. Ishiko, M. Yasuda, and T. Deguchi.** 2008. Rapid detection of the mosaic structure of the *Neisseria gonorrhoeae penA* gene, which is associated with decreased susceptibilities to oral cephalosporins. J. Clin. Microbiol. **46:**1804–1810.
21. **O'Rourke, M., and B. G. Spratt.** 1994. Further evidence for the non-clonal population structure of *Neisseria gonorrhoeae*: extensive genetic diversity within isolates of the same electrophoretic type. Microbiology **140:**1285–1290.
22. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
23. **Spratt, B. G.** 1988. Hybrid penicillin-binding proteins in penicillin-resistant gonococci. Nature **332:**173–176.
24. **Spratt, B. G., L. D. Bowler, Q.-Y. Zhang, J. Zhou, and J. M. Smith.** 1992. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. J. Mol. Evol. **34:**115–125.

25. Takahata, S., N. Senju, Y. Osaki, T. Yoshida, and T. Iida. 2006. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **50:**3638–3645.

26. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.

27. Whiley, D. M., E. A. Limnios, S. Ray, T. P. Sloots, and J. W. Tapsall. 2007. Diversity of *penA* alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob. Agents Chemother. **51:**3111–3116.

28. Yamai, S., Y. Obara, T. Nikkawa, Y. Shimoda, and Y. Miyamoto. 1979. Preservation of *Neisseria gonorrhoeae* by the gelatin-disc method. Br. J. Vener. Dis. **55:**90–93.

Review Article

Human papillomavirus vaccines: current issues & future

Kei Kawana, Toshiharu Yasugi & Yuji Taketani

Department of Obstetrics & Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Cervical cancer is the leading cause of cancer mortality among women in worldwide. Some 99 per cent of cervical cancer cases are linked to genital infection with human papillomaviruses (HPVs) comprised of approximately 15 oncogenic genital HPV types. Most HPV infections resolve spontaneously. But, the remainder persist and may then progress to cervical cancer in some women. In high-resource countries, the best way to prevent cervical cancer is to implement organised gynaecological screening programs with appropriate treatment of the detected pre-cancerous lesions. However, in developing countries, this method is not practicable because of cost and complexity of proper screening. Vaccines against HPV infections hold promise to reduce incidence of cervical cancer cost-effectively. Two Prophylactic HPV vaccines have been thus far developed: Gardasil®, a quadrivalent vaccine targeting HPV-6, -11, -16 and -18) and Cervarix®, a bivalent vaccine which targets HPV-16 and -18. Both vaccines contain L1 virus-like particles (VLPs) derived from HPV-16 and -18 which are most frequently associated with cervical cancer. The L1-VLP vaccines are HPV type-specific and therefore can effectively prevent infection of a HPV type in question alone. Therefore, the L1-VLP vaccines are hoped to be multivalent for 15 oncogenic HPV types, which comes at a price. Otherwise, costly cytologic screening for cervical cancer is still necessary. The current HPV vaccines thus may not be ultimate strategy and study on new HPV vaccines is needed. Broad-spectrum prophylactic vaccines against all oncogenic HPV types and therapeutic vaccines for clearance of HPV-related cervical lesion are being developed.

Epidemiology of HPV infection

At present, there are about 100 identified genotypes (types) of human papillomavirus (HPV), of which about 40 are genital HPV types that invade the genital organs such as the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are classified into high-risk types commonly associated with cervical cancer and low-risk types known causative pathogens of condyloma acuminatum. This classification varies among researchers, but, in general, types 16/18/31 /33/35/39/45/51/52/56/58/66/68 are classified as high-risk and 6/11/40/42/43/44/54/61/72 as low-risk types[1]. Interestingly, the HPV type distribution varies depending on the discrete stage of cervical neoplasia (Fig. 1).

The HPV-DNA detection rate in the genital organs of healthy adult females varies between advanced and developing countries, being approximately 20-40 per cent collectively[2,3]. In Japan, the HPV-positive rate in pregnant women aged 20-29 yr has been reported to be 20-30 per cent similar to, or higher than in the same age group in the US[4]. The World Health Organization
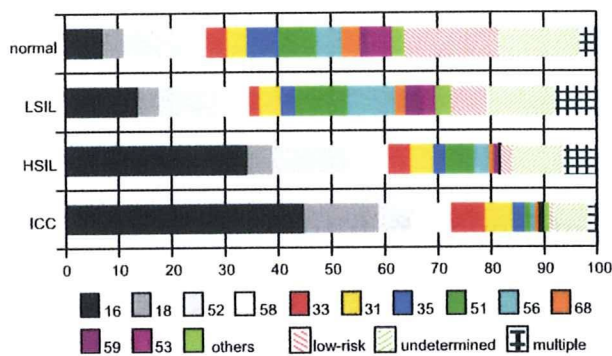
341

Fig. 1. HPV type distribution in cervical neoplasia in Japan[18]. HPV16 and 18 are the most common types in invasive cervical cancer (ICC) while more than 40 per cent of the invasive cancer is associated with the other types in Japan. HPV52 is the most common type in female with normal cytology in Japan[18].

(WHO) estimated an annual increase of 3 hundred million in the number of HPV carriers in the world[5,6]. Overall HPV prevalence in 157,879 women with normal cervical cytology was estimated to be 10.4 per cent[6]. In the US, epidemiological data show HPV infection at least once in life in 3 out of every 4 females[3]. Thus, HPV infection is a common disease affecting any female but not an event those in particular populations. High sexual activity has been reported to increase the risk of HPV infection[7]; in some women.

Risk factors for the progression of cervical neoplastic diseases

The incidence of cervical epithelial dysplasia (corresponding to squamous intraepithelial lesion: SIL) is about 1 per 10 females with HPV infection[8]. The incidence of high-SIL (corresponding to cervical intraepithelial neoplasia 2 and 3: CIN2 and CIN3, respectively) is about 3 per 10 females with low-SIL, and that of CIN3 is about 1-2 per 10 females with low-SIL[9]. Since therapeutic interventions are performed for CIN3, the actual incidence of cervical cancer is about 1 per 600 females with HPV infection. Without treatment, the incidence of the progression of CIN3 to cervical cancer is about 30 per cent[10]. Therefore, the incidence of the spontaneous development of cervical cancer is about 1 per 200-300 females with HPV infection.

Factors associated with progression to cervical cancer in females with HPV infection have been extensively studied[1]. Many prospective studies have identified persistent HPV infection as the most important risk factor, and also showed that the persistent infection tends to occur in high-risk type HPV. Persistent HPV infection generally involves

persistent virus proliferation, as verified by the detection of virus DNA from cervical exfoliated cells. Chronic virus proliferation induces the active proliferation/ differentiation of infected epithelial cells, and some infected cells incidentally immortalize, which is the first step of carcinogenesis[1].

On the other hand, transient infection involves short-term virus proliferation followed by long-term latent presence of low copies of the viral genome in the basal cells of the genital epithelium. A fate of HPV infection leading to transient, but not persistent, is determined by cellular immunocompetence against HPV. It is unlikely that transient infection progresses to cervical cancer[1].

Prophylactic vaccines

Development of the current L1-VLP vaccines

HPV is the causative virus (requirement) for genital cancers with cervical cancer being most prevalent. Thus, theoretically, if HPV infection could be completely eradicated, most of genital cancers could be prevented. The study of HPV vaccines began about 10 years ago. In 2002, Koutsky *et al* were the first to show the clinical prophylactic effects of an HPV vaccine[11]. Merck in the US and Glaxo Smith Kline (GSK) in Europe launched full-scale development of prophylactic vaccines against HPV, and their vaccines were approved and commercially available a few years ago. The vaccine antigens of the two companies are virus-like particles (VLP) produced using HPV type16 L1 protein overexpressed in yeasts or insect cells. These particles externally have a 3-dimensional structure similar to that of virus particles, but have no contents, and, therefore, are not infective. The vaccine reported by Koutsky *et al*[11] also uses HPV16L1-VLP as an antigen.

However, the main problem of the L1-VLP vaccine is its negligible prophylactic effects on other HPV types[12]. Therefore, GSK and Merck developed cocktail vaccines composed of L1-VLPs corresponding to HPV types as targets. The vaccine developed by Merck is a tetravalent vaccine against types 6, 11, 16, and 18 (Gardasil®)[13] and that developed by GSK was a bivalent vaccine against types 16 and 18 (Cervarix®)[15]. A follow-up after inoculation with the quadrivalent vaccine showed the prevention of persistent infection with all 4 HPV types in 96 per cent[14]. Though the antibody titers have been maintained for 4-5 years[13-15], whether the antibody titers can be maintained for longer periods is unknown.

Clinical trials led by the two companies are ongoing in Japan and elsewhere.

Issues regarding the currently prevailing L1-VLP vaccines

The current HPV vaccines developed by GSK and Merck are used for uninfected females to prevent HPV infection/spread. For mass prophylactic vaccination in uninfected females, vaccination should be performed at the age of about 10 years before sexual activity begins. A recent phase III clinical study (FUTURE 1 & 2) in which females aged about 20 years were randomly inoculated with Gardasil® revealed prophylactic effects on the development of CIN2-3 associated with HPV types 16 and 18 in more than 98 per cent of females who completed the vaccination protocol[16,17]. However, prophylactic effects were observed in only 13-22 per cent of females inoculated just once or twice or by intention-to-treat analysis including prophylactic effects on other HPV types[16,17].

At present, antibody titers induced by L1-VLP vaccines are confirmed to be maintained for only 5 yr. There is no guarantee that the prophylactic effects of the vaccine inoculated at the age of 10 yr will be maintained, beyond the sexual activity period. Even if the prophylactic effects of the current HPV vaccines continue for life, only cases of cervical cancer due to HPV types 16 and 18, which constitute less than 60 per cent of all invasive cervical cancer cases in Japan[18], can be prevented (Fig. 1). Indeed, the HPV type distribution in cervical cancer varies depending on regions in the world[19]. HPV16 and 18-associated cervical cancer is more than 70 per cent in North America, Europe and Australia, about 65 per cent in Africa, about 60 per cent in South and Central America, and less than 60 per cent in Asia including Japan[18,19]. Therefore, females who undergo vaccination and receive the current vaccine may have a risk for the development of cervical cancer and thereby need not undergo cervical cancer screening. Providing such information to females undergoing this vaccination is the most important for the introduction of the current HPV vaccines. A single dose of the present HPV vaccines costs about 100 USD. There is need for reduction of this high cost. In addition, the L1-VLP vaccines are highly protective against infection corresponding to the papillomavirus type used to derive the immunogen, but are ineffective against all but the most closely related HPV types. Therefore, the L1-VLP vaccines should be ultimately multivalent for

15 oncogenic HPV types. This makes the prophylactic vaccine more expensive than the current vaccines.

In some countries and states, the current HPV vaccines are distributed for free, or inoculation is covered by pubic expenses[20]. However, considering the progression of HPV infection to cervical cancer in only 1 per 300 females, vaccines effecting the prevention of only limited types, the relatively widespread cancer screening, and the high cost of such vaccines, it mandatory mass preventive inoculation with the current HPV vaccines is of value in developed country such as Japan may not be feasible. In addition, the current HPV vaccines targeting only HPV types 16 and 18 do not enable the omission of cancer screening, and vaccination at public expenses has no advantage in terms of medical economics. In Japan, voluntary inoculation during the sexual activity period should be performed first at the expense of each woman. The mass prevention employing the current HPV vaccines is a matter of debate.

Second generation HPV prophylactic vaccines

The main problem regarding the current L1-VLP vaccines is the induction of type-specific immunity. To overcome this, broad-spectrum vaccines that are also effective for the prevention of high-risk type HPV infection are under development. L2 as the other structural proteins of virus particles contains many conserved regions among all HPV types (Fig. 2). We
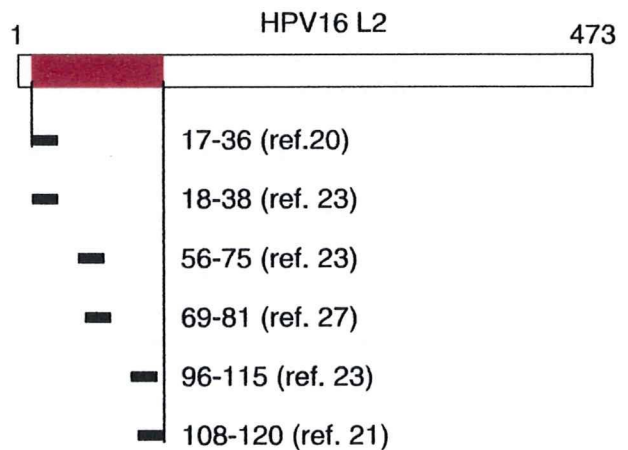

HPV16 L2

1 — 473

17-36 (ref.20)
18-38 (ref. 23)
56-75 (ref. 23)
69-81 (ref. 27)
96-115 (ref. 23)
108-120 (ref. 21)

Fig. 2. Broad-spectrum neutralization epitopes of HPV16 L2 capsid protein. Many studies reveals linear epitope cross-neutralizing infection with many HPV types in L2 capsid protein. Each epitope includes amino acid conserved regions between genital HPV types and has potential of neutralization of HPV infection. These are candidates for type-common prophylactic vaccines to HPV[21,23,26,27].

and Kondo *et al* have sought a way to develop novel vaccines using partial regions of L2 containing type-common neutralization epitope[21,22]. Recently, Kondo *et al* identified a vaccine candidate for the prevention of all types by developing newly type-common neutralization epitopes of L2 and optimizing the regions[23]. Roden *et al.* also studied the type-common neutralization epitope of L2[24-26]. They devised strategies to use the entire L2 for vaccines, and their joint study with Christensen *et al*[20]. confirmed its suppressive effects on infection with a broad spectrum of HPV types in animal experiments[25]. Furthermore, they discovered a new region (17-36 amino acid of HPV16 L2) of L2 which contains broad-spectrum neutralization epitopes[26]. It is certain that L2 will be a vaccine antigen candidate for common-type vaccines for the prevention of HPV infection.

The problem of L2 is its lower antigenicity than that of L1-VLP[22]. To apply L2 to humans, there are various problems such as the incidence of non-responders to the vaccine and the necessity for adjuvants. Several groups have recently revealed that chimeric VLP in which the cross-neutralization epitope of L2 inserted induce cross-neutralizing antibodies more effectively[27,28]. If high-risk type HPV infection can be suppressed using L2, the benefits of mass prevention by prophylactic HPV vaccine should be increased.

Other vaccine strategies for cervical cancer

Vaccine and cancer prevention strategies for cervical cancer depend on the medical/economic situations of each country. In low-resource settings, prophylactic vaccines against HPV infections have clearly the potential to reduce incidence of cervical cancer cost-effectively. By contrast, in developed countries, where precursor lesions of cervical cancer can be detected early based on well-established cancer screening program, the following diverse vaccine strategies warrant consideration (Fig. 3): (*i*) vaccines for the prevention of infection in uninfected females, (*ii*) vaccines for the reduction of viral load at the cervical mucosa in females with low-SIL and prevention its progression, (*iii*) vaccines for treatment in females with high-SIL, and (*iv*) immunotherapy for cervical cancer. The current HPV vaccines are those for the prevention of infection described in (*i*). On the other hand, (*iii*) and (*iv*) are considered to be therapeutic vaccines used for females with disease, and many clinical studies on such vaccines have been performed worldwide[29]. However, none of the vaccines exhibited statistically significant clinical effects with
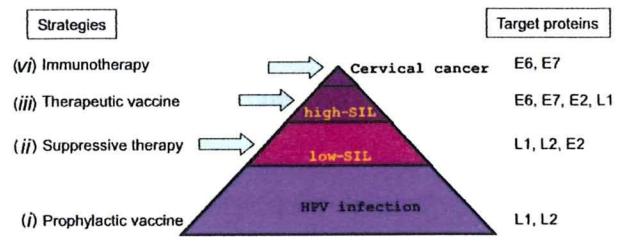

Fig. 3. Possible strategies utilizing immunological responses to HPV proteins for HPV-associated lesion and the target viral proteins for each strategy. (*i*) vaccines for the prevention of infection in uninfected females, (*ii*) vaccines for the reduction of viral load at the cervical mucosa in females with low-SIL to prevent from progression, (*iii*) vaccines for treatment in females with high-SIL, and (*iv*) immunotherapy for cervical cancer.

adequate cellular immunological responses induced by the vaccines. Since prophylactic vaccines such as the current HPV vaccines are preceding, the development of the latter seems to be delayed at present.

Possible suppressive therapy for cervical neoplasia

Long-term effects of the current HPV vaccines on HPV infection are still unclear. Clinical studies were already initiated by inoculating females aged about 20 yr with this HPV vaccine irrespective of the presence/absence of HPV infection. A recent study revealed that the current HPV vaccines tended to protect women who had already oncogenic HPV infection as well as cytological abnormalities from progression to high-grade CIN at 15 months follow-up[30]. We reported that HPV16-associated CIN1-2 tends to regress at 24 months follow-up in patients positive for serum high-titer neutralizing antibodies to HPV16[31]. Both evidences were not based on long-term follow-up. The current HPV vaccines are known to have a marked ability to induce neutralizing antibodies. Given these considerations, current HPV vaccines are likely to eliminate persistent HPV infection and subsequent malignant transformation. This raises the expectation that the vaccines can work so as to suppress HPV infection as described in 2). The results of further clinical studies are awaited.

Therapeutic vaccines

Because of limitations of the current HPV vaccines as mentioned above, necessity of therapeutic vaccines for the treatment of HPV-associated lesions is still in demand even after the prophylactic vaccine program are implemented in the world[29]. Development of the HPV therapeutic vaccines has been performed for the

Table. Clinical trials of therapeutic vaccine for HPV-associated cervical lesion

Trial phase	Target proteins	Vaccine vectors	Inoculation	Target HPVs
Ph-I/II[34]	L1, E7	Chimera-VLP	sc	16
Ph-II[33]	E7	Hsp (SGN-00101)	sc	16
Ph-II[35]	E6, E7	Vaccinia virus (TA-HPV)	sc	16, 18
Ph-II[36]	L2, E6, E7	Fusion protein L2E6E7 (TA-CIN)	im	16, 18
Ph-II[37]	BPV E2	Vaccinia virus (MVA-E2)	intrauteral	all
Ph-III[38]	E6, E7	Plasmid vaccine (ZYC101a)	im	16, 18

sc, subcutaneous injection; im, intramuscular injection; BPV, bovine papillomavirus

last two decades. The following vaccines have been well evaluated in clinical studies (Table).

1. SGN-00101 (sc) is a fusion protein consisting of heat shock protein (Hsp) of *Mycobacterium bovis* and HPV type 16 E7. The Ph-II study looking at effect of SGN-00101 in cases with CIN3 revealed histological CR in 13 (22.5%) of 58 cases, although immunological responses was not determined[32]. Another Ph-II study in cases with CIN showed 7 (35%) of 20 patients. In 5 of the 7 cases, the induction of CTL against HPV16E7 in peripheral monocytes was shown[33].

2. L1VLP-E7 (sc) is a vaccine using chimera particles composed of HPV type 16 L1-VLP and E7. In the Ph-I/II study in CIN2-3 cases, histological PR was shown in 39 per cent of vaccine recipients compared with 25 per cent of placebo recipients although there was no significant difference[34]. The clinical efficacy was coupled with cellular immune responses in some cases.

3. TA-HPV (im) is a recombinant vaccinia virus expressing HPV16/18 E6 and E7. The Ph-II study of TA-HPV in VIN cases revealed PR was shown in 8 of 13 cases and reduction of viral load was also shown in 6 of 8 lesion responders. The responders showed increase of lesion-infiltrating CD4 and 8-positive cells[35].

4. TA-CIN (im) is a fusion protein consisting of E6, E7 and L2 of HPV types 16 and 18. The Ph-II study in VIN cases revealed that CR or PR was shown in only 6 of 29 cases. CTL against E6/E7 was induced in 4 of 29 cases[36]. The correlation between clinical efficacy and cellular immune responses to the vaccine are unclear.

5. MVA-E2 (TGA4001) (intrauteral) is also a recombinant vaccinia virus expressing bovine papilloma virus (BPV) E2. The Ph-II study in cases with CIN2-3 confirmed antibody responses in serum, CTL induction in peripheral blood, and the regression of CIN in some cases (19/34 cases). There was no significant clinical efficacy[37].

6. ZYC-101a (im) is a DNA vaccine synthesized from some proteins containing CTL epitopes against E6 and E7 of HPV types 16 and 18. The Ph-III test was performed in subjects with CIN2-3. CR or PR was observed in 41 per cent in the vaccination group and 27 per cent in the placebo group, with no significant difference. When the cases were limited to those aged ≤ 25 yr, the percentage showing CR or PR was significantly higher in the vaccination (72%) than in the placebo (23%) group. However, no correlation between CTL induction against E6/E7 and clinical effects was shown[38].

Thus, there are no therapeutic HPV vaccines so far with apparent clinical efficacy based on enhanced cellular immune responses induced by vaccines. The current therapeutic vaccines elicit systemic cellular immunity by intramuscular or subcutaneous injection and thereby the clinical trials have shown cellular immune responses to the vaccines in peripheral monocyte, but not mucosal immunity at cervical mucosa.

We consider that CTL induction in the cervical mucosa is indispensable for treating cervical mucosal lesions such as CIN. In addition, vaccination is an effective method in the induction of mucosal immunity. Therefore, we have attempted induction of mucosal T cell responses by stimulating intestinal mucosal immunity through mucosal administration, particularly oral administration. Bermudez-Humaran *et al*[39]. produced gene-recombinant type lactic acid-expressing HPV16E7 and IL-12 from live lactobacillus, and evaluated the induction of CTL activity following its nasal or oral administration as a live vaccine in an experiment using mice, and also its preventive and

reductive effects in a tumor challenge test . They also found more marked mucosal induction after nasal than oral administration and a more effective induction of immunity using *Lactobacillus plantarum* than *Lactococcus lactis*[40]. No information on clinical studies of this vaccine is available. We have worked with a lactobacillus HPV vaccine using the *Lactobaccilus casei* strain showing of inflammatory immune responses. We noted marked induction of mucosal T cells possessing CTL activity to HPV E7 at intestinal mucosa after its oral administration of *Lactobaccilus casei* expressing HPV16 E7 to mice (Kawana *et al*, unpublished data). Further studies are necessary to get a detailed picture of this approach.

Summary

The usefulness of the current HPV vaccines cannot be underestimated. These vaccines are a valuable step toward the control of cervical cancer. The mass prevention strategy by use of the current HPV vaccine is ongoing in many countries. However, a conclusion cannot be drawn until the results of large-scale clinical studies in progress and long-term follow-up data are available. In addition, the development of the next generation HPV vaccines is also essential.

References

1. zur Hausen H. Papillomavirus and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002; *2* : 342-50.

2. Dunne EF, Unger ER, Sternberg M, McQuillan G, Swan DC, Patel SS, *et al*. Prevalence of HPV infection among females in the United States. *JAMA* 2007; *297* : 813-9.

3. Bosch FX, de Sanjose S. Human papillomavirus and cervical cancer – burden and assessment of causality. *J Natl Cancer Inst Monogr* 2003; *31* : 3-13.

4. Masumoto N, Fujii T, Ishikawa M, Mukai M, Ono A, Iwata T, *et al*. Dominant human papillomavirus 16 infection in cervical neoplasia in young Japanese women; study of 881 outpatients. *Gynecol Oncol* 2004; *94*: 509-14.

5. The current status of development of prophylactic vaccines against human papillomavirus infection. Report of a technical meeting. WHO, Geneva; February 16-18, 1999.

6. Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med* 1998; *338* : 423-8.

7. de Sanjosé S, Diaz M, Castellsagué X, Clifford G, Bruni L, Muñoz N, *et al*. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. *Lancet Infect Dis* 2007; *7* : 453-9.

8. Koutsky L. Epidemiology of genital human papillomavirus infection. *Am J Med* 1997; *102* : 3-8.

9. Trottier H, Franco EL. The epidemiology of genital human papillomavirus infection. *Vaccine* 2006; *24S1*: S1/4-S1/15.

10. Holowaty P, Miller AB, Rohan T, To T. Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999; *91* : 252-8.

11. Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, *et al*. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med* 2002; *347* : 1645-51.

12. Ochi H, Kondo K, Matsumoto K, Oki A, Yasugi T, Furuta R, *et al*. Neutralizing antibodies against human papillomavirus types 16, 18, 31, 52, and 58 in serum samples from women in Japan with low-grade cervical intraepithelial neoplasia. *Clin Vaccine Immunol* 2008; *15* : 1536-40.

13. Villa LL, Ault KA, Giuliano AR, Costa RL, Petta CA, Andrade RP, *et al*. Immunologic responses following administration of a vaccine targeting human papillomavirus Types 6, 11, 16, and 18. *Vaccine* 2006; *24* : 5571-83.

14. Villa LL, Costa RL, Petta CA, Andrade RP, Paavonen J, Iversen OE, *et al*. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *Br J Cancer* 2006; *95* : 1459-66.

15. Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, *et al*. Sustained efficacy up to 4·5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* 2006; *367* : 1247-55.

16. Garland SM, Hernandez-Avila M, Wheeler CM, Perez G, Harper DM, Leodolter S, *et al*. FUTURE I investigators, Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. *N Engl J Med* 2007; *356* : 1928-43.

17. FUTURE II study group, Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. *N Engl J Med* 2007; *356* : 1915-27.

18. Miura S, Matsumoto K, Oki A, Satoh T, Tsunoda H, Yasugi T, *et al*. Do we need a different strategy for HPV screening and vaccination in East Asia? *Int J Cancer* 2006; *119* : 2713-15.

19. Clifford GM, Smith JS, Plummer M, Muñoz N, Franceschi S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer* 2003; *88* : 63-73.

20. Wright TC Jr. Current status of HPV vaccination recommendation. *HPV Today* 2008; *14* : 8-9.

21. Kawana K, Yoshikawa H, Taketani Y, Yoshiike K, Kanda T. Neutralizing epitope of L2 minor capsid protein common to human papillomavirus type 16 and 6. *J Virol* 1999; *73* : 6188-90.

22. Kawana K, Yasugi T, Kanda T, Kino N, Oda K, Okada S, *et al*. Safety and immunogenicity of a peptide containing the cross-neutralization epitope of HPV16 L2 administered nasally in healthy volunteers. *Vaccine* 2003; *21* : 4256-60.

23. Kondo K, Ishii Y, Oshi H, Matsumoto T, Yoshikawa H, Kanda T. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. *Virology* 2007; *358* : 266-72.

24. Roden RB, Yutzy WH 4th, Fallon R, Inglis S, Lowy DR, Schiller JT. Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. *Virology* 2000; *270* : 254-7.

25. Gambhira R, Jagu S, Karanam B, Gravitt PE, Culp TD, Christensen ND, *et al*. Protection of rabbits against challenge

with rabbit papillomaviruses by immunization with the N terminus of human papillomavirus type 16 minor capsid antigen L2. *J Virol* 2007; *81* : 11585-92.

26. Alphs HH, Gambhira R, Karanam B, Roberts JN, Jagu S, Schiller JT, *et al*. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. *Pro Natl Acad Sci USA* 2008; *105* : 5850-5.

27. Slupetzky K, Gambhira R, Culp TD, Shafti-keramat S, Schellenbacher C, Christensen ND, *et al*. A papillomavirus-like particle (VLP, vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. *Vaccine* 2007; *25* : 2001-10.

28. Kondo K, Ochi H, Matsumoto T, Yoshikawa H, Kanda T. Modification of human papillomavirus-like particle vaccine by insertion of the cross-reactive L2-epitopes. *J Med Virol* 2008; *80* : 841-6.

29. Kanodia S, Da Silva DM, Kast WM. Recent advances in strategies for immunotherapy of human papillomavirus-induced lesions. *Int J Cancer* 2008; *122* : 247-59.

30. Paavonen J, Jenkins D, Bosch FX, Naud P, Salmerón J, Wheeler CM, *et al*. Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. *Lancet* 2007; *369* : 2161-70.

31. Kawana K, Yasugi T, Kanda T, Kawana Y, Hirai Y, Yoshikawa H, *et al*. Neutralizing antibodies against oncogenic human papillomavirus (HPV) as a possible determinant of the fate of low-grade cervical intraepithelial neoplasia. *Biochem Biophys Res Commun* 2002; *296* : 102-5.

32. Einstein MH, Kadish AS, Burk RD, Kim MY, Wadler S, Streicher H, *et al*. Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. *Gynecol Oncol* 2007; *106* : 453-60.

33. Roman LD, Wilczynski S, Muderspach LI, Burnett AF, O'Meara A, Brinkman JA, *et al*. A phase II study of Hsp-7

(SGN-00101) in women with high-grade cervical intraepithelial neoplasia. *Gynecol Oncol* 2007; *106* : 558-66.

34. Kaufmann AM, Nieland JD, Jochmus I, Baur S, Friese K, Gabelsberger J, *et al*. Vaccination trial with HPV16 L1E7 chimeric virus-like particles in women suffering from high grade cervical intraepithelial neoplasia (CIN 2/3). *Int J Cancer* 2007; *121* : 2794-800.

35. Davidson EJ, Boswell CM, Sehr P, Pawlita M, Tomlinson AE, McVey RJ, *et al*. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. *Cancer Res* 2003; *63* : 6032-41.

36. Fiander AN, Tristram AJ, Davidson EJ, Tomlinson AE, Man S, Baldwin PJ, *et al*. Prime-boost vaccination strategy in women with high-grade, noncervical anogenital intraepithelial neoplasia: clinical results from a multicenter phase II trial. *Int J Gynecol Cancer* 2006; *16* : 1075-81.

37. García-Hernández E, González-Sánchez JL, Andrade-Manzano A, Contreras ML, Padilla S, Guzmán CC, *et al*. Regression of papilloma high-grade lesions (CIN 2 and CIN 3) is stimulated by therapeutic vaccination with MVA E2 recombinant vaccine. *Cancer Gene Ther* 2006; *13* : 592-7.

38. Garcia F, Petry KU, Muderspach L, Gold MA, Braly P, Crum CP, *et al*. ZYC101a for treatment of high-grade cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet Gynecol* 2004; *103* : 317-26.

39. Bermúdez-Humarán LG, Cortes-Perez NG, Lefèvre F, Guimarães V, Rabot S, Alcocer-Gonzalez JM, *et al*. A novel mucosal vaccine based on live Lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. *J Immunol* 2005; *175* : 7297-302.

40. Cortes-Perez NG, Lefèvre F, Corthier G, Adel-Patient K, Langella P, Bermúdez-Humarán LG. Influence of the route of immunization and the nature of the bacterial vector on immunogenicity of mucosal vaccines based on lactic acid bacteria. *Vaccine* 2007; *25* : 6581-8.

性感染症クリニック女性受診者における Real-time PCR を用いた
Neisseria gonorrhoeae および *Chlamydia trachomatis* の検出性の検討

余田敬子、尾上泰彦、海野　壮

性感染症クリニック女性受診者における Real-time PCR を用いた *Neisseria gonorrhoeae* および *Chlamydia trachomatis* の検出性の検討

Performance of Real-time PCR for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in oropharyngeal specimens and endocervical swabs of women visiting an STI clinic

余田敬子[1]　　尾上泰彦[2]　　海野　壮[3]
Keiko YODA　Yasuhiko ONOYE　Tsuyoshi UNNO

淋菌（GC）およびクラミジア（CT）検査として開発中の COBAS TaqMan CT/NG（CTM）の検出性を、性感染症クリニック女性受診者 116 人で検討した。咽頭スワブとうがい液、子宮頸管スワブを採取し、咽頭の GC は CTM とプローブテック（SDA）、咽頭の CT、性器の GC および CT は CTM、SDA、アンプリコア（PCR）によって検出した。うがい液からの検出は CTM のみ行った。それぞれの陽性者数は、咽頭 GC は CTM うがい 14 人・スワブ 11 人、SDA 15 人、咽頭 CT は CTM うがい 7 人・スワブ 4 人、SDA 8 人、PCR 6 人、性器 GC は CTM 9 人、SDA 9 人、PCR 6 人、性器 CT は CTM 27 人、SDA 26 人、PCR 30 人であった。咽頭の GC および CT の CTM 検査では、陽性者数はうがい液がスワブを上回る結果であった。今回の検討で、うがい液、咽頭スワブ、子宮頸管スワブのいずれの検体においても CTM は GC および CT 検査として有用と考えられた。

COBAS TaqMan CT/NG (CTM) is a nucleic acid amplification test (NAAT) under development for detection of *Neisseria gonorrhoeae* (gonococcus : GC) and *Chlamydia trachomats* (CT). To evaluate the performance of CTM compared to the two existing NAATs, Becton Dickinson strand displacement amplification (SDA) and Roche PCR, three specimens, throat washing, throat swab and endocervical swab, were obtained from 116 women visiting an STI clinic. Expecting PCR was not adapted for the detection of GC in throat swabs, CTM, SDA and PCR were performed for the detection of GC and CT with throat swabs and endocervical swabs. Throat washings were assayed only by CTM. A positive result of GC or CT was defined as a positive result by two NAATs. According to these definitions, the sensitivities and specificities of throat washings of CTM, and throat swab of CTM and SDA of GC were 100, 78.6, and 100% and 96.1, 100 and 99.0%, respectively ; the sensitivities and specificities of throat washings of CTM, and throat swab of CTM, SDA and PCR of CT were 100, 57.1, 100 and 85.7% and 96.3, 100, 99.1 and 100%, respectively. The sensitivities and specificities of endocervical swabs of CTM, SDA and PCR of GC were 100, 100 and 66.7% and 100, 100 and 97.2%, respectively ; the sensitivities and specificities of endocervical swabs of CTM, SDA and PCR of CT were 100, 100 and 96.2% and 98.9, 100 and 94.4%, respectively. The performance of CTM with throat washings and endocervical swabs proved to be equivalent to SDA and to be superior to PCR.

127