Antimicrobial Agents and Chemotherapy

Antimicrobial Resistance and Molecular Typing of Neisseria gonorrhoeae Isolates in Kyoto and Osaka, Japan, 2010 to 2012: Intensified Surveillance after Identification of the First Strain (H041) with High-Level Ceftriaxone Resistance

Ken Shimuta, Magnus Unemo, Shu-ichi Nakayama, Tomoko Morita-Ishihara, Misato Dorin, Takuya Kawahata and Makoto Ohnishi

Antimicrob. Agents Chemother. **2013, 57(11):5225. DOI: 10.1128/AAC.01295-13.**
Published Ahead of Print 12 August 2013.

AAC
Journals.ASM.org

Antimicrobial Resistance and Molecular Typing of *Neisseria gonorrhoeae* Isolates in Kyoto and Osaka, Japan, 2010 to 2012: Intensified Surveillance after Identification of the First Strain (H041) with High-Level Ceftriaxone Resistance

Ken Shimuta,[a] Magnus Unemo,[b] Shu-ichi Nakayama,[a] Tomoko Morita-Ishihara,[a] Misato Dorin,[a] Takuya Kawahata,[c] Makoto Ohnishi,[a] on behalf of the Antibiotic-Resistant Gonorrhea Study Group

National Institute of Infectious Diseases, Tokyo, Japan[a]; WHO Collaborating Centre for Gonorrhoea and other STIs, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden[b]; Osaka Prefectural Institute of Public Health, Osaka, Japan[c]

In 2009, the first high-level ceftriaxone-resistant *Neisseria gonorrhoeae* strain (H041) was isolated in Kyoto, Japan. The present study describes an intensified surveillance (antimicrobial resistance and molecular typing) of *Neisseria gonorrhoeae* isolates in Kyoto and its neighboring prefecture Osaka, Japan, in 2010 to 2012, which was initiated after the identification of H041. From April 2010 to March 2012, 193 *N. gonorrhoeae* isolates were collected and the MICs (μg/ml) to six antimicrobials, including ceftriaxone, were determined. All isolates showed susceptibility to ceftriaxone and cefixime (MIC values, <0.5 μg/ml), and spectinomycin. The rates of resistance (intermediate susceptibility) to azithromycin, penicillin G, and ciprofloxacin were 3.6% (19.7%), 24.4% (71.0%), and 78.2% (0.5%), respectively. Multilocus sequence typing (MLST) showed that 40.9%, 19.2%, and 17.1% of isolates belonged to ST1901, ST7359, and ST7363, respectively. Furthermore, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) revealed that 12 (63%) of the 19 isolates with decreased susceptibility to ceftriaxone (MIC > 0.064 μg/ml) were of ST1407. NG-MAST ST1407 was also the most prevalent ST (16.1%; 31 of 193 isolates). In those NG-MAST ST1407 strains, several mosaic type *penA* alleles were found, including SF-A type (penicillin binding protein 2 allele XXXIV) and its derivatives. These were confirmed using transformation of the *penA* mosaic alleles as critical determinants for enhanced cefixime and ceftriaxone MICs. The intensified surveillance in Kyoto and Osaka, Japan, did not identify any dissemination of the high-level ceftriaxone-resistant *N. gonorrhoeae* strain H041, suggesting that H041 might have caused only a sporadic case and has not spread further.

Neisseria gonorrhoeae infections are major public health concerns worldwide. In 2008, the World Health Organization (WHO) estimated 106 million gonorrhea cases among adults globally, which places the infection as the most prevalent bacterial sexually transmitted infection (STI) (with a global incidence similar to that of genital chlamydial infections) (1). Resistance in *N. gonorrhoeae* to previously recommended first-line antimicrobials for treatment of gonorrhea is also prevalent worldwide. During the recent decade, the susceptibility to the extended-spectrum cephalosporins (ESCs) cefixime (oral) and ceftriaxone (parenteral), which currently are the first-line antimicrobials in most countries, has decreased rapidly worldwide (2–12). Clinical treatment failures with cefixime have been verified in many countries (13–19). In regard to ceftriaxone, which is the last remaining option for empirical first-line antimicrobial monotherapy in most countries, a few cases of failure in treating pharyngeal gonorrhea despite relatively low ceftriaxone MICs of the gonococcal strains have been confirmed in Australia (20), Sweden (21), and Slovenia (22). However, these cases likely reflected the fact that pharyngeal gonorrhea commonly is harder to treat than urogenital gonorrhea and not treatment failure due to the slightly increased ceftriaxone MICs of the gonococcal strains. Nevertheless, it is most perturbing that recently the first extensively drug-resistant (XDR) gonococcal strain H041 (23, 24) in Kyoto, Japan, followed by an XDR strain in France (14) and Spain (25), which all have been confirmed to have a high-level resistance to ceftriaxone, were described. If these strains start to spread globally, gonorrhea will become untreatable in certain circumstances and especially in settings where dual antimicrobial therapy is not feasible and/or affordable. In response to this developing situation, the WHO (26, 27), the European Centre for Disease Prevention and Control (ECDC), and the Centers for Disease Control and Prevention (CDC) in the United States have published global and region-specific response plans for their respective regions, i.e., the European Union/European Economic Area (EU/EEA) countries (28) and the United States (29). One main component of these action/response plans is to enhance the surveillance of antimicrobial resistance and treatment failures. Nevertheless, for future treatment of gonorrhea it is imperative to develop new treatment options.

In Japan, a sentinel surveillance system for sexually transmitted infections, including gonorrhea, was launched in 1999. This system includes approximately 1,000 sentinel sites that monthly report their gonorrhea cases. The number of reported gonorrhea cases peaked in 2002 (n = 21,921; 23.9 cases/site) but has since declined to 10,247 (10.6 cases/site) in 2011. Unfortunately, this

Received 18 June 2013 Returned for modification 13 July 2013
Accepted 3 August 2013
Published ahead of print 12 August 2013
Address correspondence to Makoto Ohnishi, ohnishi7@nih.go.jp.

doi:10.1128/AAC.01295-13

TABLE 1 Antibiotic susceptibility of *Neisseria gonorrhoeae* isolates from Kyoto and Osaka, Japan, 2010 to 2012 (n = 193)

Antimicrobial	Breakpoints[a] (μg/ml)	No. (%) of isolates showing:		
		Susceptibility	Intermediate susceptibility	Resistance
Ceftriaxone	S ≤ 0.25/R > 0.25	193 (100)		0
Cefixime	S ≤ 0.25/R > 0.25	193 (100)[b]		0
Penicillin G	S ≤ 0.06/R > 1	9 (4.7)	137 (71.0)	47 (24,4)
Ciprofloxacin	S ≤ 0.06/R > 0.5	41 (21.2)	1 (0.5)	151 (78.2)
Azithromycin	S ≤ 0.25/R > 0.5	148 (76.7)	38 (19.7)	7 (3.6)
Spectinomycin	S ≤ 64/R > 64	193 (100)		0

[a] Antibiotic susceptibility and resistance criteria according to the Clinical and Laboratory Institute (CLSI; www.clsi.org), with the exception of azithromycin (not stated by the CLSI), for which the breakpoints from the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) were applied. S, susceptible; R, resistant.

[b] All isolates were categorized as susceptible to cefixime according to the CLSI breakpoints; however, 48 (24.9%) of the isolates were resistant to cefixime according to the European EUCAST breakpoints (MIC > 0.12 μg/ml).

sentinel surveillance system does not include any requirements to perform antimicrobial susceptibility testing on the identified gonococcal isolates. Nevertheless, after the identification of the first high-level ceftriaxone-resistant XDR strain H041 (23, 24) in Kyoto, Japan, surveillance of antimicrobial resistance in *N. gonorrhoeae* was initiated in April 2010 in Kyoto and in April 2011 in the neighboring prefecture Osaka.

The present study describes the antimicrobial resistance and molecular typing of *Neisseria gonorrhoeae* isolates in Kyoto and Osaka, Japan, in 2010 to 2012, a study initiated after the identification of the first high-level ceftriaxone-resistant XDR *N. gonorrhoeae* strain H041 in Kyoto (23, 24).

MATERIALS AND METHODS

Study settings and *N. gonorrhoeae* isolates. Five outpatient clinics, three in Kyoto and two in Osaka, Japan, provided mainly consecutive urethral discharge samples to the National Institute of Infectious Diseases (NIID), Tokyo, for isolation of *N. gonorrhoeae*. The specimens were transported using Seed swab γ no. 2 (Eiken Chemical, Tokyo, Japan) to NIID, where the samples were inoculated on selective BBL Modified Thayer Martin (MTM II) agar plates (Becton, Dickinson and Company, Sparks, MD). After 18 to 24 h of incubation, suspected colonies on the MTM II agar plates were verified as *N. gonorrhoeae* by identification of Gram-negative diplococci in microscopy, by the rapid oxidase reaction, and with the biochemical test ID Test HN-20 Rapid (Nissui Pharmaceutical, Tokyo, Japan). If culture was negative after 18 to 24 h of incubation, the agar plates were incubated for an additional 18 to 24 h before being classified as negative for *N. gonorrhoeae*. From April 2010 to March 2012, in total 154 males and 39 females were culture positive for *N. gonorrhoeae*, and accordingly, 193 *N. gonorrhoeae* isolates (one per patient) were obtained (53 from Kyoto and 140 from Osaka).

Antimicrobial susceptibility testing. The MICs (μg/ml) of ceftriaxone, cefixime, penicillin G, ciprofloxacin, azithromycin, and spectinomycin were determined using the Etest method (AB bioMérieux, Solna, Sweden) according to the manufacturer's instructions. MIC breakpoints used for determination of susceptibility, intermediate susceptibility, and resistance (Table 1) were in accordance with the Clinical and Laboratory Standards Institute (CLSI; www.clsi.org), with the exception of azithromycin, for which CLSI does not state any breakpoints. For azithromycin, the breakpoints stated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) were used. The 2008 WHO *N. gonorrhoeae* reference strains (30) were used for quality control in all antimicrobial susceptibility testing.

DNA extraction. The bacterial isolates were suspended in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and boiled for 10 min. After centrifugation to remove the cell debris, the supernatant was promptly used as the template DNA for the PCRs.

Molecular epidemiological characterization. Molecular epidemiological characterization by means of multilocus sequence typing (MLST) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) was performed as described previously (31, 32). The diversity index for MLST and NG-MAST was calculated as described earlier (33). Neighbor-joining trees based on partial *porB* gene sequences (490 bp) were generated by using MEGA 4. Similarity of alleles was evaluated by individual pairwise alignment against the representative alleles in each clade (*porB4*, *porB1059*, *porB206*, *porB1785*, *porB908*, and *porB2569*) to determine the numbers of base pair differences. Alleles of *porB* showing ≥99% similarity (<5-bp difference) were grouped.

***penA* sequencing.** The *penA* gene was PCR amplified and sequenced by using the previously described primers penA_F and penA_R (34). Briefly, 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. The PCR products were subsequently purified with an ExoSAP IT kit (GE Healthcare Limited, Buckinghamshire, United Kingdom). Both DNA strands of the PCR products were sequenced with an ABI BigDye Terminator cycle sequencing kit (version 3.1) on an ABI 3130 xl sequencer, in accordance with the instructions from the manufacturer (Applied Biosystems, Foster City, CA).

Transformation assays. To assess the capacity of a unique *penA* allele to result in increased MICs of cefixime and ceftriaxone, the full-length *penA* allele was PCR amplified and transformed into a recipient strain as previously described (34). The recipient gonococcal strain NG9807 was of MLST ST7363 and NG-MAST ST4093 and had a ceftriaxone as well as cefixime MIC of 0.016 μg/ml (23). Briefly, the recipient was suspended in GC broth containing 1.5% (wt/vol) proteose peptone 3 (Becton, Dickinson and Company, Sparks, MD), 0.4% (wt/vol) K_2HPO_4, 0.1% (wt/vol) KH_2PO_4, 0.5% (wt/vol) NaCl, and 1% (vol/vol) isovitalex (1×10^8 cells/100 μl) and incubated with 0.2 μg of PCR product of the specific *penA* allele (after purification using the High Pure PCR Product Purification kit [Roche Diagnostics GmbH, Mannheim, Germany]) for 4 h. Aliquots of 10 μl and 100 μl were inoculated on GC agar containing cefixime (0.064 μg/ml). After incubation for 18 to 24 h, the obtained colonies were subcultured on an antimicrobial-free GC agar plate for single-clone isolation. For confirmation, the full-length *penA* allele was sequenced in all transformants.

Nucleotide sequence accession numbers. The nucleotide sequences of *penA* determined in this study have been deposited in the DDBJ sequence library and assigned the accession numbers AB819103 to AB819115.

RESULTS

Antimicrobial susceptibility testing. The results of the antimicrobial susceptibility testing of the *N. gonorrhoeae* isolates (n = 193) are summarized in Table 1. All 193 examined isolates were susceptible to ceftriaxone, cefixime, and spectinomycin. The rates of resistance (intermediate susceptibility) to azithromycin, peni-

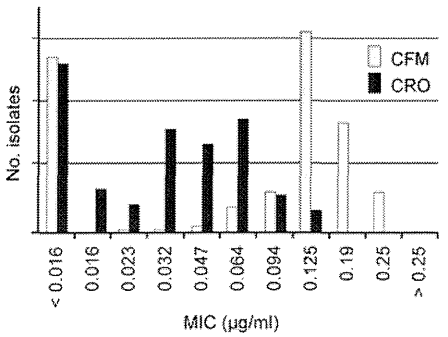

FIG 1 Cefixime (CFM) and ceftriaxone (CRO) MIC distribution of *Neisseria gonorrhoeae* isolated in Kyoto and Osaka, Japan, in 2010 to 2012 (n = 193).

cillin G, and ciprofloxacin were 3.6% (19.7%), 24.4% (71.0%), and 78.2% (0.5%), respectively. However, although there were no isolates resistant to cefixime and ceftriaxone according to the CLSI breakpoints (MIC, >0.25 μg/ml), 48 (24.9%) isolates were resistant to cefixime according to the European breakpoints (EUCAST; MIC, >0.125 μg/ml) and the MIC_{90} value for ceftriaxone was 0.094 μg/ml (Fig. 1).

Multilocus sequence typing investigation and relationship between MLST and cephalosporin resistance. In Japan, the initial emerged *N. gonorrhoeae* strains with intermediate susceptibility and resistance to cefixime possessed a *penA* mosaic allele X, which encodes a penicillin binding protein 2 (PBP 2) X. These strains belonged mainly to two major MLST sequence types (ST), particularly ST7363 but also ST1901 (34). These STs and their *penA* alleles have subsequently further evolved. The first strain with high-level resistance to ceftriaxone identified in Kyoto, Japan (H041; 23, 24), was of ST7363. The additional high-level ceftriaxone-resistant strains isolated in France (14) and Spain (25) were of ST1901. In the present study, a total of 29 MLST STs were found among the 193 isolates from Kyoto and Osaka. ST1901 was the most common MLST ST (n = 79, 40.9%), followed by ST7359 (n = 37, 19.2%), ST7363 (n = 33, 17.1%), and ST7819 (n = 17, 8.8%) (Table 2). The diversity index for MLST of the 193 isolates was 0.76.

Among those four most prevalent MLST STs, isolates of ST1901, ST7363, and ST7819 showed relatively high MIC values for cefixime with similar MIC distribution patterns (Fig. 2). In regard to ceftriaxone, isolates of ST1901 showed slightly higher MICs than did the ST7363 and ST7819 isolates. In contrast, all ST7359 isolates were highly susceptible to both cefixime and ceftriaxone (Fig. 2).

***N. gonorrhoeae* multiantigen sequence typing analysis and relationship between NG-MAST and MLST.** NG-MAST has a substantially higher discriminatory power than MLST, and NG-MAST has been applied for molecular epidemiologic investigations for gonococci isolated worldwide (10, 35). In recent years, NG-MAST ST1407 has been a prevalent ST in many countries, and this ST has also accounted for a substantial proportion of the decreased susceptibility and resistance to ESCs in those countries (10). Furthermore, the high-level ceftriaxone-resistant XDR isolates cultured in France (14) and in Spain (25), which might represent one and the same strain, were of NG-MAST ST1407. In the present study, a total of 95 NG-MAST STs were identified among the 193 isolates from Kyoto and Osaka (Table 2). ST1407 (n = 31, 16.1% of all isolates) was the most prevalent NG-MAST ST, followed by ST4186 (n = 10, 5.2%), ST3505 (n = 8, 4.1%), and ST6780 (n = 7, 3.6%) (Table 2). The diversity index for NG-MAST of the 193 isolates was 0.96.

N. gonorrhoeae isolates assigned to the four most common MLST STs, ST1901, ST7359, ST7363, and ST7819, were subdivided into 30 (intra-MLST ST diversity index, 0.83), 18 (diversity index, 0.89), 23 (diversity index, 0.94), and 7 (diversity index, 0.71) NG-MAST STs, respectively. These results clearly illustrate the substantially higher discriminatory power of NG-MAST compared to MLST. However, the number of different *tbpB* alleles within these MLST STs was relatively small, that is, MLST ST1901, ST7363, ST7359, and ST7819 possessed only six, two (*tbpB10* and *tbpB3*), one (*tbpB241*), and one (*tbpB27*) different *tbpB* alleles, respectively. Accordingly, the high discriminatory power of NG-MAST was highly dependent on the diversified *porB* gene (see below), and the different level of genetic heterogeneity within the main MLST STs might reflect how long these STs have been spreading and thus evolving. As aforementioned, ST1407 (n = 31) was the most prevalent NG-MAST ST, and all except one (belonging to MLST ST10241) of these isolates belonged to MLST ST1901 (30/79 [38.0%] of all ST1901 isolates). The MIC_{50}s (ranges) of cefixime and ceftriaxone for the NG-MAST ST1407 isolates were 0.19 μg/ml (0.064 to 0.25 μg/ml) and 0.064 μg/ml (0.016 to 0.125 μg/ml), respectively. Furthermore, 12 (63%) of the 19 isolates with decreased susceptibility to ceftriaxone (MIC, >0.064 μg/ml) were of ST1407.

***porB* and *penA* sequencing.** To further examine the genetic relationships of the 193 *N. gonorrhoeae* isolates from Kyoto and Osaka, a phylogenetic analysis of the *porB* sequences (490 bp) used in the NG-MAST was performed.

TABLE 2 MLST and NG-MAST sequence types of *N. gonorrhoeae* isolates from Kyoto and Osaka, Japan, 2010 to 2012

ST	No. (%) of isolates
MLST ST	
1901	79 (40.9)
7359	37 (19.2)
7363	33 (17.1)
7819	17 (8.8)
7358	3 (1.6)
1579	2 (1.0)
1584	2 (1.0)
7371	2 (1.0)
7827	2 (1.0)
10241	2 (1.0)
Others (19 STs)	24 (12.4)
NG-MAST ST	
1407	31 (16.1)
4186	10 (5.2)
3505	8 (4.1)
6780	7 (3.6)
2958	6 (3.1)
6771	6 (3.1)
4019	5 (2.6)
6767	5 (2.6)
3431	4 (2.1)
4015	4 (2.1)
7381	4 (2.1)
6765	4 (2.1)
6754	3 (2.1)
6769	3 (2.1)
Others (81 STs)	93 (48.2)

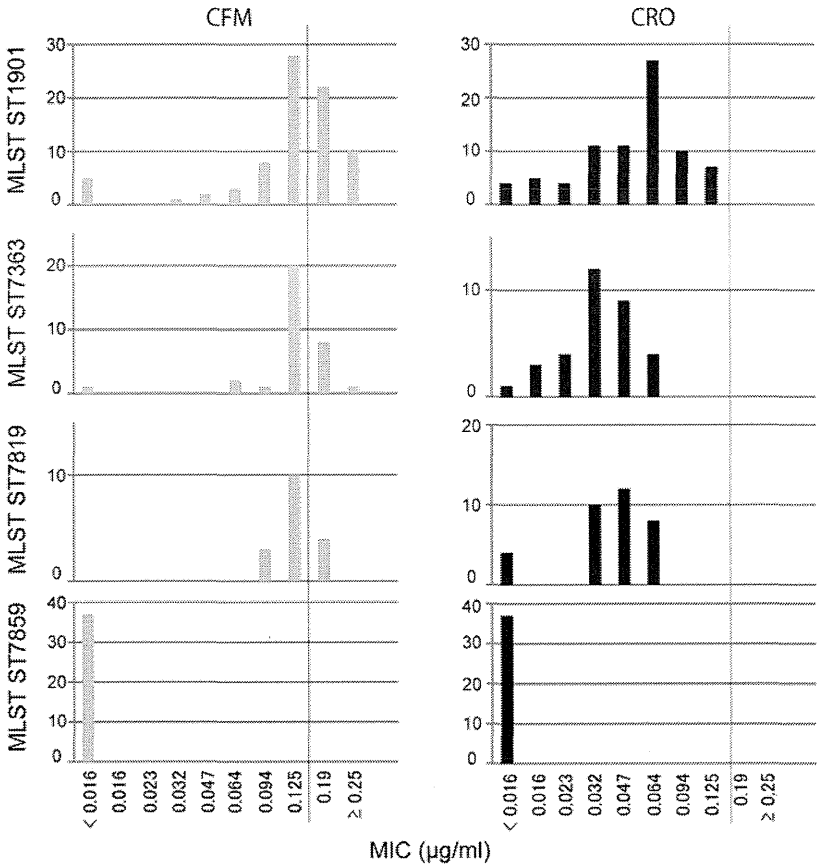

FIG 2 MIC of cefixime (CFM) and ceftriaxone (CRO) in *Neisseria gonorrhoeae* isolates from Kyoto and Osaka, Japan, in 2010 to 2012 belonging to the four major multilocus sequence typing (MLST) sequence types (STs), that is, ST1901 (n = 79), ST7363 (n = 33), ST7819 (n = 17), and ST7359 (n = 37).

The phylogenetic analysis identified six clades, in which common representative alleles for each clade were *porB4*, *porB1059*, *porB206*, *porB1785*, *porB908* (the *porB* allele in NG-MAST ST1407), and *porB2569*, respectively (Fig. 3A). The MIC distributions for cefixime and ceftriaxone varied substantially for those different *porB* clades. Interestingly, the isolates in the *porB908* clade (including all 31 NG-MAST ST1407 isolates but also 31 additional isolates of other STs [n = 15]) had clearly the highest MICs of cefixime and particularly ceftriaxone; in fact, this clade contained all isolates showing an MIC of 0.125 μg/ml for ceftriaxone (Fig. 3D). NG-MAST ST1407 isolates have been previously described to possess a *penA* mosaic allele XXXIV encoding a PBP 2 mosaic XXXIV, which results in elevated MICs of ESCs (10, 14). Accordingly, in the present study the *penA* gene in all isolates from the *porB908* clade (n = 62) and, for comparison, isolates from the *porB1059* clade (n = 17) and the *porB1785* clade (n = 25), which also had substantially elevated MICs of cefixime particularly (Fig. 3B and C), were sequenced. In total, 11 *penA* alleles, including nine mosaic alleles, were revealed among those 104 isolates (Fig. 4). Of the nine *penA* mosaic alleles, five encoded PBP 2 mosaic X or closely related sequence variants (found in 50 of the isolates), which were distributed in all three examined *porB* clades. In contrast, all isolates possessing a *penA* mosaic allele XXXIV or closely related sequence variants (in 51 isolates) were in the *porB908* clade, with the exception of one isolate from the *porB1785* clade (Fig. 4), indicating that this *penA* mosaic allele might have emerged in the *porB908* clade (including the 31 NG-MAST ST1407 isolates). Nevertheless, only a few (n = 5) of the isolates in the *porB908* clade possessed the authentic *penA* mosaic allele XXXIV, and most contained PBP 2 mosaic XXXIV with an additional P551S substitution (n = 44) (Fig. 4). Furthermore, all isolates showing an MIC of 0.125 μg/ml of ceftriaxone possessed this PBP 2 mosaic XXXIV with an additional P551S alteration, with the exception of one isolate with PBP 2 mosaic XXXIV containing an additional A501V substitution. As a matter of great concern, these results indicate that the authentic *penA* mosaic XXXIV allele is evolving, resulting in further enhanced MICs of ESCs.

Transformation of specific *penA* mosaic alleles. To assess the capacity of specific *penA* mosaic alleles to result in increased MICs of ceftriaxone and cefixime, the full-length *penA* mosaic alleles X, XXXIV, XXXIV-P551S, and XXXIV-A501V were separately transformed into the ESC-susceptible strain NG9807 (ceftriaxone MIC, 0.016 μg/ml; cefixime MIC, 0.016 μg/ml). As shown in Fig. 5, the transformants with *penA* XXXIV variants (P551S and A501V) showed ceftriaxone MICs of 0.125 μg/ml (and cefixime MICs of 0.25 μg/ml and 0.5 μg/ml, respectively), which were clearly higher than those of transformants with *penA* XXXIV (ceftriaxone MIC, 0.064 μg/ml; cefixime MIC, 0.125 μg/ml). The authentic *penA* mosaic allele X transformant showed a ceftriaxone MIC of 0.125 μg/ml and a cefixime MIC of 0.25 μg/ml. Accord-

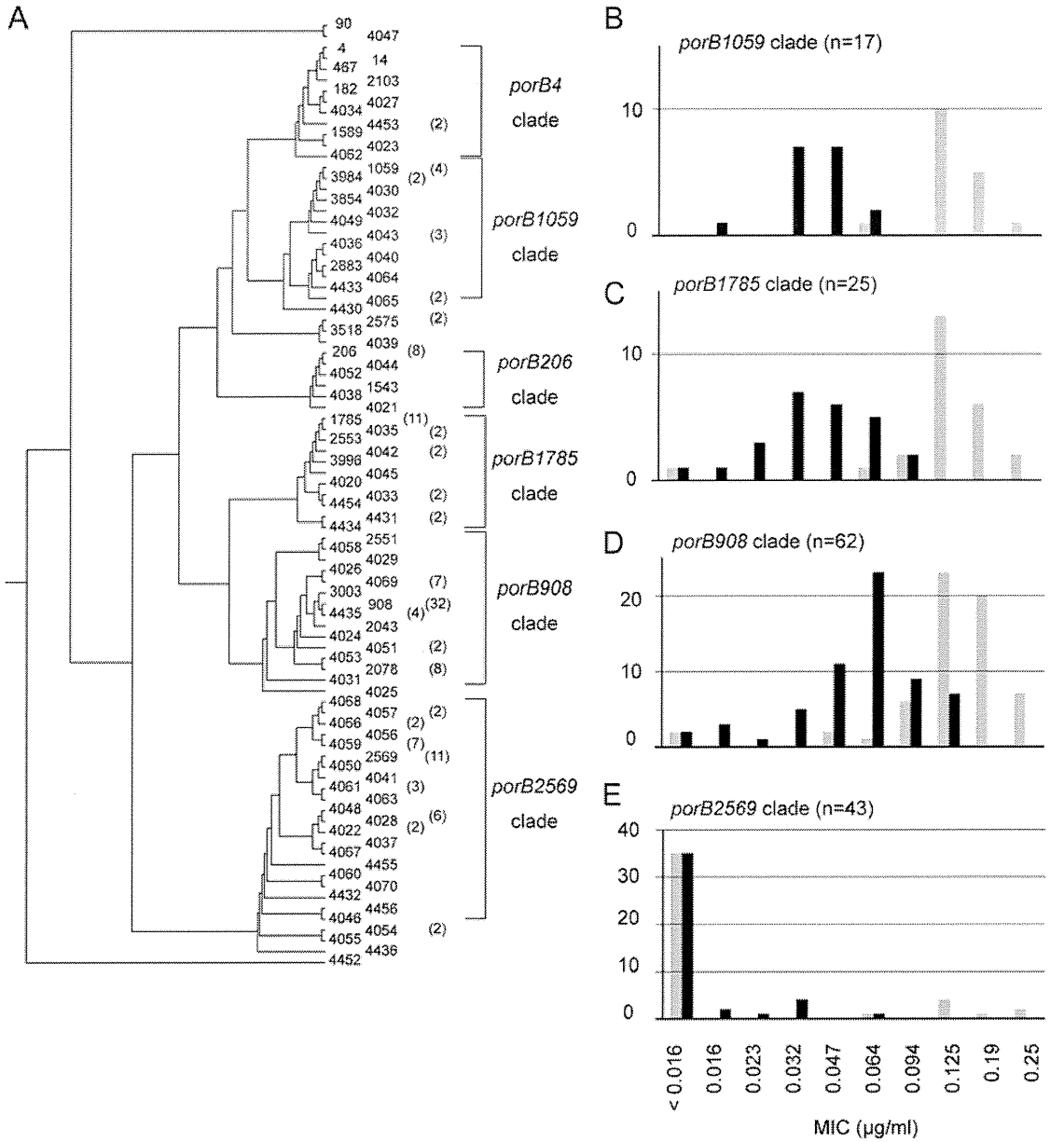

FIG 3 Sequence comparison of *porB* alleles (490 bp) from *N. gonorrhoeae* isolates (n = 193) from Kyoto and Osaka, Japan, and cefixime and ceftriaxone MIC profile of each *porB* group. (A) An internal region of the *porB* genes including loops 3, 4, and 5 (490 bp), which is used in NG-MAST, was utilized to create a phylogenetic tree with the MEGA 4 program. The numbers indicate *porB* allele numbers assigned in the NG-MAST database. The number of isolates with the same *porB* allele is indicated in parentheses. (B to E) Similar *porB* alleles are grouped as *porB1059*, *porB1785*, *porB908*, and *porB2569* clades, and the MICs to cefixime (gray bars) and ceftriaxone (black bars) are shown in panels B to E, respectively.

ingly, in regard to ceftriaxone, *penA* alleles XXXIV-P551S and XXXIV-A501V showed impact on MIC identical to that of the *penA* X allele, and the MIC was raised twice as much as that seen with the *penA* XXXIV allele. Interestingly, the *penA* allele XXXIV-A501V could increase the MIC of cefixime up to 0.5 μg/ml, which is above the resistance breakpoint (Fig. 5).

DISCUSSION

In 2009, the first high-level ceftriaxone-resistant *N. gonorrhoeae* strain (H041) was isolated in Kyoto, Japan (23, 24). H041 was also the first extensively drug-resistant (6) *N. gonorrhoeae* strain, displaying resistance to most available antimicrobials (23). The present study describes an intensified surveillance (antimicrobial resistance and molecular typing) of *N. gonorrhoeae* isolates in Kyoto and its neighboring prefecture Osaka, Japan, in 2010 to 2012, which was initiated after the identification of H041 (23, 24). All *N. gonorrhoeae* isolates (n = 193), collected at five clinics, were susceptible to ceftriaxone and cefixime (MIC values, <0.5 μg/ml) and to spectinomycin. The rates of resistance (intermediate susceptibility) to azithromycin, penicillin G, and ciprofloxacin were

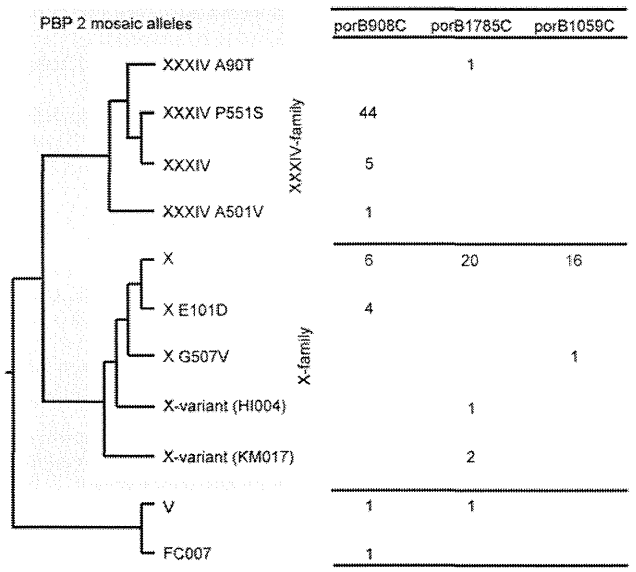

FIG 4 Phylogenetic tree of the amino acid sequences of penicillin binding protein 2 encoded by different *penA* mosaic alleles. The *penA* genes from a total of 104 *N. gonorrhoeae* isolates possessing alleles belonging to the *porB908*, *porB1785* and *porB1059* clades (C) were sequenced, and a phylogenetic tree based on the PBP 2 amino acid sequences was constructed by the MEGA 4 program. Two major *penA* mosaic allele groups of PBP 2 were revealed, the PBP 2 X family and the PBP 2 XXXIV family. Strains HI004 and KM017 had 3 and 11, respectively, amino acid substitutions in their PBP 2 X group alleles.

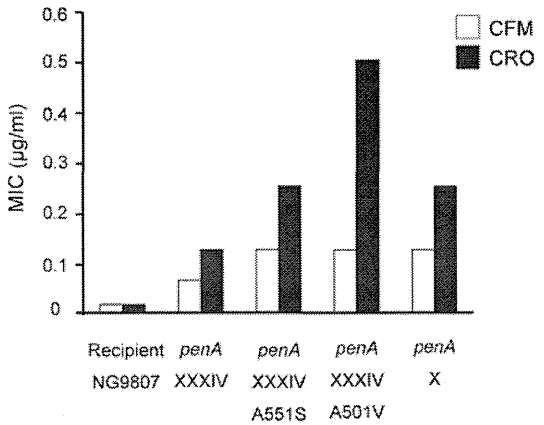

FIG 5 Capacity for increased MIC conferred by the mosaic PBP 2 alleles harbored by *penA* X and *penA* XXXIV, including amino acid substituted mutants identified in the present study. Each allele was introduced into the cefixime- and ceftriaxone-susceptible strain NG9807, and MIC values of each transformant are shown.

3.6% (19.7%), 24.4% (71.0%), and 78.2% (0.5%), respectively (Table 1). These data are basically consistent with a previous recent report from Japan, except for the azithromycin resistance (12). In a previous publication (12), only 0.4% (1 of 242 isolates) was reported to be resistant to azithromycin. Accordingly, the high-level ceftriaxone-resistant *N. gonorrhoeae* strain H041 might have caused only a sporadic event, and no further dissemination in the Kyoto or Osaka area could be found. The reasons for this are unknown; however, the high-level ceftriaxone resistance (i.e., the remodeled PBP 2) might affect the biological fitness of H041. Studies investigating the biological fitness of H041 in competitive experiments *in vitro* and *in vivo* (in a mouse model) are in progress. In the present study, no other ceftriaxone-resistant strain was identified either. However, only five clinics were included in the present surveillance and, due to this lack of complete surveillance, it cannot be entirely excluded that some ceftriaxone-resistant gonococcal strain was spreading in the region. The antimicrobial resistance surveillance in this region has continued, further strengthened in the area, and should now be expanded nationally.

All clinics included in this surveillance adhered to the treatment guideline from the Japanese Society for STI, which recommends using 1 g ceftriaxone intravenously or 2 g spectinomycin intramuscularly. However, in some cases also azithromycin (2 g orally) can be used. During the study period, no cases of suspected treatment failure using 1 g ceftriaxone were identified, which further supports the lack of ceftriaxone resistance in the region.

Recently, the antimicrobial resistance surveillance for *N. gonorrhoeae* has been enhanced worldwide (26–28), and some of those surveillance systems have also performed molecular typing of the *N. gonorrhoeae* isolates (36). As to molecular typing for *N. gonorrhoeae*, MLST (31) and particularly NG-MAST (32) have been most widely applied worldwide (35). MLST, which identifies allelic variations in seven housekeeping genes, describes the phylogenetic relationship between isolates. However, since the housekeeping genes are relatively conserved, the discrimination of MLST is rather low and the method is more suitable for long-term epidemiology, macroepidemiology (e.g., on a global scale), and evolutionary studies (35). In contrast, NG-MAST is more applicable to short-term epidemiology and microepidemiology because it is based on sequence variations of genes encoding two bacterial cell surface proteins, that is, PorB and TbpB (35). These antigens are exposed to the human immune response, and this selective pressure results in antigenic changes giving the *N. gonorrhoeae* strain an opportunity to escape from the human immune system. Although NG-MAST has a very high ability to discriminate *N. gonorrhoeae* isolates, several publications have reported a high prevalence of the NG-MAST ST1407, which appears to be disseminated basically worldwide and account for a high proportion of the decreased susceptibility and increased resistance to ESCs and multidrug resistance (10, 14, 18, 36). As found in the present study, the NG-MAST ST1407 (30 of those 31 isolates belonged to MLST ST1901) was also the most prevalent ST in Osaka and Kyoto, Japan, and as in other countries the ST1407 isolates had decreased susceptibility to ESCs and were resistant to fluoroquinolones (data not shown).

Thus, the discriminatory ability of NG-MAST is substantially higher than MLST (35; this study); however, the majority of this discrimination is due to the high variation of the *porB* allele and far fewer *tbpB* alleles have been described. The *porB908* allele (present in the NG-MAST ST1407) and its closely related alleles (*porB908* group) were widely distributed in the isolates examined in the present study; these isolates also in general had higher MICs of ESCs. The other major *porB* groups were the *porB1059* group, the *porB1785* group, and the *porB2569* group (Fig. 3). The *porB2569* group isolates (mostly belonging to MLST ST7359) were mainly fully susceptible to ceftriaxone and cefixime, although some isolates showed higher MICs, that is, MICs of 0.016 to 0.064 μg/ml of ceftriaxone and MICs of 0.064 to 0.25 μg/ml of cefixime. Because *porB* alleles might be horizontally transferred among *N. gonor-*

rhoeae strains, it is difficult to strictly link *porB* alleles and accordingly NG-MAST STs to antimicrobial susceptibility phenotypes. Interestingly, seven of the eight isolates in the *porB2569* group that showed higher MICs of cefixime belonged to MLST ST7363 and MLST ST1901, which are the two major STs in which cefixime resistance has emerged. This might suggest that these MLST ST7363 and MLST ST1901 isolates have horizontally acquired their *porB2569* group allele from an authentic MLST ST7359 strain, which is the predominant MLST type among isolates within the *porB2569* group.

The first described isolate of NG-MAST ST1407 from San Francisco, CA, displayed a decreased susceptibility to cefixime, and this isolate had a mosaic type PBP 2 encoded by the *penA* mosaic allele SF-A, subsequently named *penA* allele XXXIV (23). The high-level ceftriaxone-resistant NG-MAST ST1407 isolates identified in France (14) and Spain (25) both had an identical mutated *penA* XXXIV allele (*penA* allele CI [14, 23, 25]) that encodes a PBP 2 XXXIV sequence with leucine replaced by proline at amino acid position 501 (L501P) (14). Accordingly, these high-level resistant strains have presumably evolved by a single nucleotide polymorphism in the *penA* XXXIV allele resulting in an A501P alteration in PBP 2 in an NG-MAST ST1407 (MLST 1901) strain. In the present study, although many NG-MAST ST1407 *N. gonorrhoeae* isolates with *penA* XXXIV allele or derivates of this allele were identified, no *penA* allele CI (14, 23, 25) was found. However, some other single-amino-acid-substituted alleles of *penA* XXXIV, i.e., *penA* XXXIV with P551S or A501V, were identified. The P551S and A501V amino acid substitutions have previously been associated with decreased susceptibility to ceftriaxone (10, 37, 38). In the present study, it was shown by transformation experiments that these substitutions in a *penA* XXXIV allele result in a 2- to 4-fold increase in the MICs of cefixime and ceftriaxone.

In conclusion, our intensified surveillance in 2010 to 2012 in the Kyoto and Osaka prefectures, Japan, did not identify any dissemination of the high-level ceftriaxone-resistant *N. gonorrhoeae* strain H041, suggesting that H041 caused only a sporadic case and has not further spread. Furthermore, no other ceftriaxone-resistant strain was identified. The antimicrobial resistance surveillance in this region has continued and should now be expanded nationally.

However, it is cause for great concern that *N. gonorrhoeae* strains with the *penA* XXXIV allele and its derivatives are also shown to be spreading in Japan, as in many other countries (10, 14). The *penA* mosaic XXXIV allele appears also to be evolving, resulting in further-enhanced MICs of ESCs, and it might be only a matter of time before an additional high-level ceftriaxone-resistant strain emerges. If this strain is not detected in a timely fashion and has a retained biological fitness, it will spread internationally and result in a large public health problem. Consequently, it is crucial not only to enhance the phenotypic antimicrobial susceptibility surveillance of *N. gonorrhoeae* but also to perform molecular typing and detection to monitor the spread and evolution of successful *N. gonorrhoeae* clones with decreased susceptibility and resistance to ceftriaxone worldwide.

ACKNOWLEDGMENTS

We thank Makiko Itoh, Haruka Tsuruzono, Tomoko Shimamura, and Kazuma Suzuki for technical assistance.

The laboratory work was performed at the National Institute of Infectious Diseases.

The present work was supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan (H24-Shinko-Ippan-001).

Members of the Antibiotic-Resistant Gonorrhea Study Group include Mitsufumi Fujiwara, Kei-ichi Furubayashi, Shuichi Hida, Yasuhiro Ishikawa, Mikio Itoh, Hiroshi Kameoka, Tomohiro Ueda, and Ryouji Yasumoto.

REFERENCES

1. **World Health Organization.** 2008. Global incidence and prevalence of selected curable sexually transmitted infections—2008. World Health Organization, Geneva, Switzerland. http://www.who.int/reproductivehealth/publications/rtis/2008_STI_estimates.pdf. Accessed 6 June 2013.
2. **Barry PM, Klausner JD.** 2009. The use of cephalosporins for gonorrhea: the impending problem of resistance. Expert Opin. Pharmacother. **10:** 555–577.
3. **Stoltey JE, Barry PM.** 2012. The use of cephalosporins for gonorrhea: an update on the rising problem of resistance. Expert Opin. Pharmacother. **13:**1411–1420.
4. **Deguchi T, Nakane K, Yasuda M, Maeda S.** 2010. Emergence and spread of drug resistant *Neisseria gonorrhoeae*. J. Urol. **184:**851–858.
5. **Deguchi T, Yasuda M, Yokoi S, Ishida K, Ito M, Ishihara S, Minamidate K, Harada Y, Tei K, Kojima K, Tamaki M, Maeda S.** 2003. Treatment of uncomplicated gonococcal urethritis by double-dosing of 200 mg cefixime at a 6-h interval. J. Infect. Chemother. **9:**35–39.
6. **Tapsall JW, Ndowa F, Lewis DA, Unemo M.** 2009. Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. Expert Rev. Anti Infect. Ther. **7:**821–834.
7. **Lewis DA.** 2010. The gonococcus fights back: is this time a knock out? Sex. Transm. Infect. **86:**415–421.
8. **Kirkcaldy RD, Ballard RC, Dowell D.** 2011. Gonococcal resistance: are cephalosporins next? Curr. Infect. Dis. Rep. **13:**196–204.
9. **Cole MJ, Unemo M, Hoffmann S, Chisholm SA, Ison CA, van de Laar MJ.** 2011. The European gonococcal antimicrobial surveillance programme, 2009. Euro Surveill. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19995.
10. **Unemo M, Nicholas RA.** 2012. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. Future Microbiol. **7:**1401–1422.
11. **Van de Laar M, Spiteri G.** 2012. Increasing trends of gonorrhoea and syphilis and the threat of drug-resistant gonorrhoea in Europe. Euro Surveill. **17**(29):pii=20225. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20225.
12. **Tanaka M, Koga Y, Nakayama H, Kanayama A, Kobayashi I, Saika T, Iyoda T.** 2011. Antibiotic-resistant phenotypes and genotypes of *Neisseria gonorrhoeae isolates* in Japan: identification of strain clusters with multidrug-resistant phenotypes. Sex. Transm. Dis. **38:**871–875.
13. **Lewis DA, Sriruttan C, Muller EE, Golparian D, Gumede L, Fick D, de Wet J, Maseko V, Coetzee J, Unemo M.** 2013. Phenotypic and genetic characterization of the first two cases of extended-spectrum-cephalosporin-resistant *Neisseria gonorrhoeae* infection in South Africa and association with cefixime treatment failure. J. Antimicrob. Chemother. **68:**1267–1270.
14. **Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P.** 2012. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. Antimicrob. Agents Chemother. **56:**1273–1280.
15. **Unemo M, Golparian D, Syversen G, Vestrheim DF, Moi H.** 2010. Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. Euro Surveill. **15**(47):pii=19721. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19721.
16. **Ison CA, Hussey J, Sankar KN, Evans J, Alexander S.** 2011. Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. Euro Surveill. **16**(14):pii=19833. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19833.
17. **Unemo M, Golparian D, Stary A, Eigentler A.** 2011. First *Neisseria gonorrhoeae* strain with resistance to cefixime causing gonorrhoea treatment failure in Austria. Euro Surveill. **16**(43):pii=19998. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19998.
18. **Allen VG, Mitterni L, Seah C, Rebbapragada A, Martin IE, Lee C,**

Siebert H, Towns L, Melano RG, Low DE. 2013. *Neisseria gonorrhoeae* treatment failure and susceptibility to cefixime in Toronto, Canada. JAMA **309**:163–170.
19. **Yokoi S, Deguchi T, Ozawa T, Yasuda M, Ito S, Kubota Y, Tamaki M, Maeda S.** 2007. Threat to cefixime treatment for gonorrhea. Emerg. Infect. Dis. **13**:1275–1277.
20. **Tapsall J, Read P, Carmody C, Bourne C, Ray S, Limnios A, Sloots T, Whiley D.** 2009. Two cases of failed ceftriaxone treatment in pharyngeal gonorrhoea verified by molecular microbiological methods. J. Med. Microbiol. **58**:683–687.
21. **Unemo M, Golparian D, Hestner A.** 2011. Ceftriaxone treatment failure of pharyngeal gonorrhoea verified by international recommendations, Sweden, July 2010. Euro Surveill. 16(6):pii=19792. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19792.
22. **Unemo M, Golparian D, Potočnik M, Jeverica S.** 2012. Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia, September 2011. Euro Surveill. **17**(25): pii=20200. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20200.
23. **Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, Nakayama S, Kitawaki J, Unemo M.** 2011. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob. Agents Chemother. **55**:3538–3545.
24. **Ohnishi M, Saika T, Hoshina S, Iwasaku K, Nakayama S, Watanabe H, Kitawaki J.** 2011. Ceftriaxone-resistant *Neisseria gonorrhoeae*, Japan. Emerg. Infect. Dis. **17**:148–149.
25. **Camara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, Ardanuy C.** 2012. Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. J. Antimicrob. Chemother. **67**:1858–1860.
26. **World Health Organization (WHO), Department of Reproductive Health and Research.** 2012. Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*, p 1–36. WHO, Geneva, Switzerland. http://www.who.int/reproductivehealth/publications/rtis/9789241503501. Accessed 6 June 2013.
27. **Ndowa F, Lusti-Narasimhan M, Unemo M.** 2012. The serious threat of multidrug-resistant and untreatable gonorrhoea: the pressing need for global action to control the spread of antimicrobial resistance, and mitigate the impact on sexual and reproductive health. Sex. Transm. Infect. **88**:317–318.
28. **European Centre for Disease Prevention and Control.** 2012. Response plan to control and manage the threat of multidrug-resistant gonorrhoea in Europe, p 1–23. ECDC, Stockholm, Sweden. http://www.ecdc.europa.eu/en/publications/Publications/1206-ECDC-MDR-gonorrhoea-response-plan.pdf. Accessed 6 June 2013.
29. **Centers for Disease Control and Prevention.** 2012. Cephalosporin-resistant *Neisseria gonorrhoeae* public health response plan, p 1–43. http://www.cdc.gov/std/gonorrhea/default.htm. Accessed 6 June 2013.
30. **Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J.** 2009. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. J. Antimicrob. Chemother. **63**:1142–1151.
31. **Jolley KA.** 2001. Multi-locus sequence typing. Methods Mol. Med. **67**: 173–186.
32. **Martin IMC, Ison CA, Aanensen DM, Fenton KA, Spratt BG.** 2004. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J. Infect. Dis. **189**:1497–1505.
33. **Hunter PR, Gaston MA.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26**:2465–2466.
34. **Ohnishi M, Watanabe Y, Ono E, Takahashi C, Oya H, Kuroki T, Shimuta K, Okazaki N, Nakayama S, Watanabe H.** 2010. Spread of a chromosomal cefixime-resistant *penA* gene among different *Neisseria gonorrhoeae* lineages. Antimicrob. Agents Chemother. **54**:1060–1067.
35. **Unemo M, Dillon JA.** 2011. Review and international recommendation of methods for typing *Neisseria gonorrhoeae* isolates and their implications for improved knowledge of gonococcal epidemiology, treatment, and biology. Clin. Microbiol. Rev. **24**:447–458.
36. **Chisholm SA, Unemo M, Quaye N, Johansson E, Cole MJ, Ison CA, Van de Laar MJW.** 2013. Molecular epidemiological typing within the European Gonococcal Antimicrobial Resistance Surveillance Programme reveals predominance of a multidrug-resistant clone. Euro Surveill. **18**(3): pii=20358. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20358.
37. **Whiley DM, Goire N, Lambert SB, Ray S, Limnios EA, Nissen MD, Sloots TP, Tapsall JW.** 2010. Reduced susceptibility to ceftriaxone in *Neisseria gonorrhoeae* is associated with mutations G542S, P551S and P551L in the gonococcal penicillin-binding protein 2. J. Antimicrob. Chemother. **65**:1615–1618.
38. **Whiley DM, Limnios EA, Ray S, Sloots TP, Tapsall JW.** 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.