Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhea?

Detailed characterization of the first high-level ceftriaxone resistant strain.

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Running title: N. gonorrhoeae - soon a superbug causing untreatable gonorrhea?
Recently, the first Neisseria gonorrhoeae strain (H041) that is highly resistant to the extended-spectrum cephalosporin (ESC) ceftriaxone, the last remaining option for empirical first-line treatment, was isolated. We performed a detailed characterization of H041, phenotypically and genetically, to confirm the finding, examine its antimicrobial resistance (AMR) and elucidate the resistance mechanisms. H041 was examined using seven species-confirmatory tests, antibiograms (30 antimicrobials), porB sequencing, N. gonorrhoeae multi-antigen sequence typing (NG-MAST), multilocus sequence typing (MLST) and sequencing of ESC-resistance determinants (penA, mtrR, penB, ponA and pilQ). Transformation, using appropriate recipient strains, was performed to confirm the ESC-resistance determinants. H041 was assigned serovar Bpyust, MLST ST7363 and the new NG-MAST ST4220. H041 proved highly resistant to ceftriaxone (2-4 µg/ml, which is 4-8-fold higher than any previously described isolate) and all other cephalosporins, as well as most other antimicrobials tested. A new penA mosaic allele caused the ceftriaxone resistance. In conclusion, N. gonorrhoeae has now shown its ability to develop also ceftriaxone resistance. Despite that the biological fitness of ceftriaxone resistance in N. gonorrhoeae remains unknown, N. gonorrhoeae may soon become a true superbug causing untreatable gonorrhea. A reduction in global gonorrhea burden by enhanced disease control activities combined with wider strategies for general AMR control and enhanced understanding of mechanisms of emergence and spread of AMR, which need to be monitored globally, and public health response plans for global (and national) perspectives are important. Ultimately, new drugs are necessary to develop for efficacious gonorrhea treatment.
Gonorrhea, caused by *Neisseria gonorrhoeae* (gonococcus), is the second most prevalent bacterial sexually transmitted infection globally. The disease is associated with high morbidity and socioeconomic consequences and remains a public health problem worldwide (29, 36, 47). In absence of a vaccine appropriate diagnostics and antimicrobial therapy are the key elements for reduction and control of gonorrhea, the further transmission of the infection as well as the development of associated severe complications and sequelae (34, 36).

The treatment options, however, have rapidly diminished because of emergence and worldwide spread of antimicrobial resistance (AMR) to all drugs previously used or considered as first-line, i.e. penicillins, early generation cephalosporins, tetracyclines, macrolides and fluoroquinolones. Furthermore, rapid emergence of resistance to spectinomycin was observed when it was widely used for treatment in the past (4), and this antimicrobial is not suitable for treatment of pharyngeal gonorrhea or currently available in many countries (3, 14, 36). Accordingly, spectinomycin is no promising candidate for first-line empirical treatment of gonorrhea. Worryingly, in recent years susceptibility to the currently recommended first-line antimicrobials the extended-spectrum cephalosporins (ESCs), i.e. ceftriaxone (injectable) and cefixime (oral), has also decreased globally (3, 14, 16, 36). Furthermore, since several years, cefixime treatment failures have been recognized in Japan (8, 36, 48), which excluded cefixime from their treatment guidelines already in 2006 (36). More recently failures have also been verified in Europe (41). However, despite that susceptibility to ceftriaxone (the last remaining option for empirical first-line treatment), is decreasing globally, *in vitro* and clinical (resulting in treatment failure of urogenital gonorrhea) resistance has been lacking (3, 14, 16, 36).

Recently the first high-level ceftriaxone-resistant gonococcal strain (H041) was isolated from the pharynx of a female commercial sex worker in Kyoto, Japan (22). H041 displayed a minimum inhibitory concentration (MIC) of ceftriaxone of 2 µg/ml. This is a very high level
of resistance and, previously, only one isolate having an MIC $>0.25 \ \mu g/ml$ (MIC=$0.5 \ \mu g/ml$ (33)) has been reported worldwide. Unfortunately, it was not possible to definitively verify that H041 caused a treatment failure because a post-treatment isolate was not available; however, treatment failure seems likely (see the present Discussion section). Furthermore, H041 belonged to multilocus sequence typing (MLST) ST7363 and was closely related to the successful gonococcal cefixime-resistant sub-clones of ST7363 (22), which are prevalent in Japan (23) and now also transmitted in Europe. Accordingly, H041 may be a sub-clone of the MLST ST7363 cefixime-resistant strains that has acquired additional resistance determinant(s), resulting in high-level ceftriaxone resistance. Historically, gonococcal AMR has mostly emerged in the World Health Organization (WHO) Western Pacific Region (WPR) and, in particular, in Japan. This resistance has rapidly spread via sex tourists, long distance truck drivers and forced migration in the WHO WPR, to the Pacific Rim countries, including the USA, to South-East and Central Asia, Europe and globally (36). The spread of ceftriaxone-resistant gonococcal strains worldwide will likely follow the same pattern. Consequently, it is crucial to examine the first high-level ceftriaxone-resistant gonococcal strain worldwide that recently was isolated in Japan (H041, (22)) in detail, including elucidation of the mechanisms causing the ceftriaxone resistance.

The most common mechanism in gonococci for decreased ESC susceptibility is alteration of the penA gene, i.e. acquisition of a penA mosaic allele or alterations of amino acid A501 in the encoded penicillin-binding protein 2 (PBP2) (1, 3, 10, 13-17, 20, 22, 23, 26, 27, 32, 33, 36, 39, 41, 42, 45, 51). Mutations in the promoter and/or coding sequence of the repressor gene mtrR cause an over-expression of the MtrCDE efflux pump system, which further decreases ESC susceptibility (3, 10, 11, 15-17, 30, 36, 39, 44, 49, 51). Furthermore, specific porB1b mutations that alter amino acid G101 and A102 in the PorB1b porin (the penB resistance determinant) result in additionally decreased ESC susceptibility (3, 10, 15-17, 24,
Nevertheless, based on the relatively few studies of gonococcal isolates with decreased ESC susceptibility polymorphisms in *ponA* (encoding PBP1) and *pilQ* (encoding the pore-forming secretin PilQ protein in the type IV pili), which both can be involved in high-level penicillin resistance, do not seem to substantially enhance the MICs of ESCs (10, 28, 46, 50, 51). At least one unknown resistance determinant exists (10, 17, 39, 51).

The aims of this study were to perform a detailed characterization, phenotypically and genetically, of the first identified high-level ceftriaxone-resistant *N. gonorrhoeae* strain (H041) worldwide in order to confirm this finding, to thoroughly examine its antimicrobial resistance and to elucidate the ESC resistance mechanisms.

**MATERIAL AND METHODS**

**Neisseria gonorrhoeae** strains. The high-level ceftriaxone-resistant strain H041 (22) and gonococcal strains (n=9) selected for transformation assays to verify the resistance mechanisms, i.e. five clinical strains and four of the eight 2008 WHO *N. gonorrhoeae* reference strains (42), were included. For gonococcal species-verification of H041, morphology on selective culture medium, catalase and oxidase tests, microscopy after Gram-staining and seven species-confirmatory tests [sugar utilization, ID-test HN-20 Rapid System (Nissui, Tokyo, Japan), PhadeBact GC Monoclonal Test (Bactus AB, Solna, Sweden), PhadeBact GC Monoclonal Serovar Test (Bactus AB, Solna, Sweden), MicroTrak *N. gonorrhoeae* culture confirmation test (Trinity Biotech, Wicklow, Ireland), *porA* pseudogene PCR (12) and dual-target PCR (*porA* and *opa*) (9)] were used. All strains were grown on GC culture media as previously described (43).

**Antimicrobial susceptibility testing.** MIC determination of ceftriaxone was performed using the Etest method (AB bioMérieux, Solna, Sweden) according to the manufacturer’s instructions. The ceftriaxone MIC of H041 was also confirmed using the agar dilution method.
according to the Clinical Laboratory and Standards Institute (CLSI) (7). Finally, H041 was examined for its MICs of 29 additional antimicrobials (using the Etest method) and tested with the calibrated dichotomous sensitivity (CDS) disc diffusion method (35, 37), which is used in resistance surveillance of *N. gonorrhoeae* in many countries in WHO WPR (seven antimicrobials) (Table 1). β-lactamase production was tested using nitrocefin discs. The 2008 WHO *N. gonorrhoeae* reference strains (42) were used for quality controls of all antimicrobial susceptibility testing.

**Genetic characterization.** DNA was isolated in the NorDiag Bullet instrument (NorDiag ASA Company, Oslo, Norway) using the BUGS’n BEADS™ STI-fast kit (NorDiag ASA Company, Oslo, Norway) according to the manufacturer’s instructions. For molecular epidemiological examination, strains were genotyped by means of MLST (23), *porB* gene sequencing and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) as described previously (40). PCR and sequencing of resistance determinants, i.e. *penA*, *mtrR*, *porB1b*, *ponA* and *pilQ* genes, were performed as described elsewhere (17, 42, 46).

**Sequence alignments and phylogenetic analysis.** Multiple-sequence alignments (nucleotide and amino acid sequences) were performed using the BioEdit Sequence Alignment Editor software (version 7.0.9.0). For examination of the evolutionary relationships of H041 with other *penA* mosaic strains displaying decreased ESC susceptibility and circulating worldwide, a phylogenetic analysis using the full-length *porB* sequences in H041 and previously reported *penA* mosaic strains (10) was performed with TREECON (version 1.3b) as previously described (40).

**Transformation assays.** To confirm that the unique *penA* allele in H041 (*penA-H041*) caused the high-level resistance to ceftriaxone the full-length *penA-H041* was PCR-amplified and transformed into nine recipient strains as previously described (23). These nine recipient strains displayed different molecular epidemiological sequence types, ceftriaxone MICs and
composition of ESC resistance mechanisms such as \textit{penA} alleles, \textit{mtrR} promoter and \textit{penB} sequences (Table 2). Briefly, the recipients were suspended in GC broth (1×10^8 cells/100 µl) and incubated with 0.2 µg of \textit{penA-H041} PCR product (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 with a four-fold higher ceftriaxone concentration than the MIC of the respective recipient. After incubation, obtained colonies were subcultured on an antimicrobial-free GC agar plate for single clone isolation. For confirmation, the transformation assay was performed three times for each recipient.

**RESULTS**

**Phenotypic characterization of the high-level ceftriaxone-resistant \textit{N. gonorrhoeae} strain H041.** All conventional bacteriological diagnostic tests and the seven species confirmatory assays verified that H041 was a gonococcal strain, which was also assigned serovar Bpyust.

The results of the antimicrobial susceptibility testing using the Etest method (30 antimicrobials) and CDS disc diffusion method (seven antimicrobials) are summarized in Table 1. Briefly, H041 was resistant to various antimicrobials, including all β-lactam antimicrobials (with possible exceptions of carbapenems [at least ertapenem and meropenem] and piperacillin/tazobactam for which no breakpoints are available), fluoroquinolones, macrolides, tetracycline, trimetoprim/sulphamethoxazole, chloramphenicol and nitrofurantoin. The MICs of all the cephalosporins, including the recommended first-line ESCs, were very high (e.g., 2-4 µg/ml of ceftriaxone and 8 µg/ml of cefixime). H041 did not produce any β-lactamase. The strain, however, was susceptible to spectinomycin and rifampicin. Furthermore, the MICs of aminoglycosides and tigecycline were also relatively low (no
breakpoints are available for these antimicrobials). The CDS disc diffusion method also identified H041 as resistant to ceftriaxone, cefpodoxime, penicillin G, tetracycline and ciprofloxacin but susceptible to spectinomycin (Table 1).

Genetic characterization and elucidation of the mechanisms causing the high-level ceftriaxone resistance in *N. gonorrhoeae* strain H041. The molecular epidemiological characterization assigned H041 as MLST ST7363 and the not previously described NG-MAST ST4220 (www.ng-mast.net). A phylogenetic analysis using *porB* sequences showed that H041 is closely related to other *penA* mosaic strains, with decreased ESC susceptibility, that have been shown to circulate worldwide (Fig. 1).

The sequencing of ESC resistance determinants showed that H041 possessed a unique *penA* mosaic allele (*penA-H041*) and the previously described *mtrR, penB* and *ponA1* (L421P) resistance determinants. No new *pilQ* mutations were found. Thus, the only new resistance determinant, which consequently was suspected to cause the high ESC MICs, was *penA-H041* (GenBank accession number AB546858). *penA-H041* was highly similar (i.e. 97.6% nucleotide identity and only 12 PBP2 amino acid differences that clustered in two regions) to the previously described *penA* mosaic allele X that has been correlated to cefixime treatment failures in Japan. Of these 12 amino acids, five were unique compared with any gonococcal PBP2 sequence previously described, but one of these (I486) has been found in *N. meningitidis* and *N. flavescens* (Fig. 2). Accordingly, *penA-H041* contained only four PBP2 amino acid residues that have not been previously reported in any Neisseria species, i.e. compared with *penA* mosaic X, these consisted of A311V, T316P, A328T and T484S (Fig. 2).

Transformation assays confirming that the unique *penA-H041* caused the high-level resistance to ceftriaxone and other extended-spectrum cephalosporins. Based on their different genotypes, ceftriaxone MICs and composition of ESC resistance determinants, nine strains were selected as recipients of *penA-H041* in transformation assays (Table 2).
By transforming penA-H041, the ceftriaxone MICs of the recipients increased to 0.125-8 µg/ml, i.e. by 16- to 500-fold. Accordingly, the ceftriaxone MICs of all recipients, with the exception of NG9901 (0.125 µg/ml), increased above the resistance breakpoint (>0.25 µg/ml; (7)) independent of other resistance determinants. Remarkably, WHO F, which has wild type alleles of all ESC and penicillin resistance determinants, displayed a ceftriaxone MIC of 0.5 µg/ml after transformation (500-fold MIC increase) (Fig. 3).

All single clone transformants (derived from all recipient strains) showed identical mtrR, penB and ponA sequences as in the recipients. All the single clone transformants did also contain the penA-H041 allele. In most transformants, the transformed penA-H041 sequence was identical to the sequence in H041. However, in a few transformants, such as those derived from the WHO F and WHO M strains, some point mutations differed from the penA-H041 sequence. These were considered to represent spontaneous mutations, mutations in junctions for recombination and/or belonging to the penA allele of the recipient. The majority of these mutations were nonsynonymous and none was located in any segment of the mosaic penA allele affecting the ceftriaxone MICs. Consequently, the transformation experiments confirmed that penA-H041 was the sole cause of the high-level ceftriaxone resistance.

**DISCUSSION**

The present study describes the detailed phenotypic and genetic confirmation and characterization, including elucidation of the resistance mechanisms, of the first identified *N. gonorrhoeae* strain (H041) displaying high-level resistance to ceftriaxone worldwide. H041 was isolated from a female commercial sex worker in Japan (22), and the ceftriaxone MIC of H041 was four- to eight-fold higher than previously observed. Ceftriaxone is also the last remaining option for empirical first-line treatment of gonorrhea. Accordingly, *N. gonorrhoeae*
has now shown its ability to develop resistance to also ceftriaxone and, despite that the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, the gonococcus may become a true superbug that initiates a future era of untreatable gonorrhea.

Although a post-treatment isolate was unavailable (only one specimen positive with SDA (ProbeTec ET, Becton-Dickinson) sampled two weeks after treatment) to definitively verify treatment failure using 1 g ceftriaxone intravenously (22), it seems likely that this was the first gonorrhea clinical failure, caused by high resistance in the bacteria solely, using ceftriaxone based on the post-treatment positive SDA sample (all residual gonococcal DNA is expected to be eliminated before two weeks post-treatment (2)), the very high ceftriaxone MIC and all available data regarding pharmacodynamic parameters for ESCs. Thus, according to Monte Carlo simulations, ceftriaxone 1 g intravenously that was used for treatment (in full concordance with treatment recommendations for urogenital and pharyngeal gonorrhea in the Japanese treatment guidelines) results in median times of free ceftriaxone above the MIC ($f_{T>MIC}$) of only 6.0 h (0-20.3 h) and 0 h (0-5.6 h) for the detected MICs of 2 µg/ml (agar dilution method) and 4 µg/ml (Etest method), respectively (5). Accordingly, using ceftriaxone 1 g for treatment the very ceftriaxone MIC of H041 will make the strain escape eradication in most (if not all) patients. Furthermore, this was a case of pharyngeal gonorrhea that is substantially harder to treat compared with urogenital gonorrhea (3, 36) and most likely the infection resolved spontaneously within three months. Nevertheless, despite that a clinical history was recorded, re-reinfection can not be completely excluded, especially as the patient was a commercial sex worker.

The resistance determinants causing the high ESC MICs in H041 were also elucidated. Accordingly, the unique penA-H041 mosaic allele was responsible: by transforming penA-H041 into recipients with different ESC MICs and resistance mechanisms, their ceftriaxone MICs increased to 0.125-8 µg/ml, i.e. by 16-fold to 500-fold. Nevertheless, additional
resistance determinants, especially \textit{mtrR} and \textit{penB} (and “Factor X”, i.e. the still unidentified determinant), were needed to reach the same level of ceftriaxone MIC as H041, a synergy that was previously reported (17, 36, 51). Factor X was not transformable using the H041 genome (data not shown), which was also previously described (17, 51). \textit{penA-H041} was highly similar to the previously described \textit{penA} mosaic allele X (causing ceftriaxone MICs of only 0.064-0.125, Table 2), which has been correlated to cefixime treatment failures in Japan, i.e. only 12 PBP2 amino acid differences clustering in two regions. Of these 12 amino acids, only four have not been previously reported in any Neisseria species, i.e. compared with \textit{penA} mosaic X, these consisted of A311V, T316P, A328T (in Region A) and T484S (in Region B) (Fig. 2). It was also confirmed that transformation of only the \textit{penA-H041} Region A into the recipients caused mainly as high ceftriaxone MIC as transforming the full-length \textit{penA-H041} (data not shown). Although further confirmatory studies are needed, it is highly probable that A311V and T316S are the alterations causing the high ceftriaxone resistance, i.e. due to the proximity to the β-lactam active site in PBP2. Despite this fact, the MICs of some β-lactam antimicrobials, such as penicillins (especially piperacillin/tazobactam) and carbapenems (particularly ertapenem and meropenem), were surprisingly low. \textit{penA-H041} could also easily be transformed to other gonococcal strains in co-cultivation experiments (data not shown) performed as previously described (23), which shows that this ceftriaxone resistance can rapidly spread within the \textit{N. gonorrhoeae} population.

H041 seems to represent a sub-clone of the MLST ST7363 cefixime-resistant \textit{N. gonorrhoeae} previously described circulating in Japan (22, 23). This clone has caused treatment failures using oral ESCs and successfully spread worldwide, and now seems to have further evolved and developed resistance to ceftriaxone as well. The fear is that this ceftriaxone resistant sub-clone will now spread in Japan, to WHO WPR countries, Pacific Rim countries and globally, which has been the scenario for emergence and worldwide spread.
of most gonococcal AMR. Based on previous experiences (e.g., for fluoroquinolones), AMR can be widely disseminated internationally only one to two decades after the first emergence of AMR in WHO WPR (34, 36). The finding of this single high-level ceftriaxone-resistant gonococcal strain is important, especially because it was identified in a female commercial sex worker that belongs to a high-risk frequently transmitting population, no national gonococcal antimicrobial resistance surveillance programs (including no sentinel sites for identification of gonorrhea treatment failures) are active in Japan. Accordingly, the strain should have excellent opportunities for a rapid spread. An enhanced, but still limited, gonococcal antimicrobial resistance surveillance in Kyoto, Japan was initiated after the finding of H041, however, any secondary spread of H041 (or additional treatment failures) has yet not been identified. Despite the suboptimal Japanese surveillance systems, this fact may indicate that H041 has a lower biological fitness that results in limited further spread. Accordingly, the biological fitness of H041, compared to its “wild type” lacking penA-H041 that causes the ceftriaxone resistance, would be valuable to examine in a well-designed study, i.e. investigating quantitatively the fitness in vitro (different culture media; solid and liquid based) and also in an appropriate animal model, i.e. in vivo.

Nevertheless, N. gonorrhoeae has now shown its ability to develop resistance to also ceftriaxone and, despite that the biological fitness of H041 remains unknown, a serious public health problem in which case gonorrhea may become untreatable in certain circumstances seems to be approaching. To at least detain spread of ESC (cefixime and ceftriaxone) resistance, timely and decisively public health multidisciplinary and multicomponent actions are essential not only in Japan but also globally. A recent expert review described WHO initiatives and approaches to AMR containment and how to meet public health challenges of untreatable gonorrhea (36). Nevertheless, to succeed with any AMR containment enhanced gonorrhea control activities are needed to reduce the burden of infection (36). Furthermore, it
is crucial to explore options, in industrialized settings as well as in less-resourced settings, for future treatment of ESC-resistant gonorrhea. This include exploration of optimized dose regimens of presently used antimicrobials, new antimicrobials (or “rediscovery” of old such as gentamicin, ertapenem and perhaps piperacillin/tazobactam in emergent situations of ESC-resistant *N. gonorrhea*) or other substances, and combination therapy (5, 6, 18, 19, 21, 31, 36, M. Unemo and J. Tapsall, unpublished data).

In conclusion, the first high-level ceftriaxone-resistant *N. gonorrhea* strain has now been characterized in detail, including an elucidation of the resistance mechanisms. Accordingly, *N. gonorrhea* has now shown its ability to develop also ceftriaxone resistance and, despite that the biological fitness of ceftriaxone resistance in *N. gonorrhea* remains unknown, the gonococcus may soon become a true superbug that initiates a future era of untreatable gonorrhea. To at least detain spread of ESC (cefixime and ceftriaxone) resistance, a reduction in global gonorrhea burden by enhanced disease prevention and control activities combined with the implementation of much wider strategies for general AMR control and a better understanding of mechanisms of emergence and spread of AMR, which need to be monitored globally, and public health response plans (including sustainable clinical, microbiological and epidemiological components) for global and national perspectives, are crucial. However, any such plan alone will most probably not be able to prevent the emergence, establishment and spread of ceftriaxone resistance, nevertheless, these plans will be valuable to detain a global spread of ESC-resistant (cefixime and ceftriaxone). Ultimately, a major focus important for public health globally is to timely develop new effective drugs (for single or combined use) for the treatment of gonorrhea.
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501


503  Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime


505
TABLE 1. Minimum inhibitory concentration (MIC) using the Etest method and zone sizes with the calibrated dichotomous sensitivity (CDS) disc diffusion method of *Neisseria gonorrhoeae* H041 to various antimicrobials

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Class</th>
<th>MIC Etest&lt;sup&gt;a&lt;/sup&gt; (S, I, or R)&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>CDS (mm)&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
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<tr>
<td>Penicillin G</td>
<td>β-lactams, penicillins</td>
<td>4 (R)&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
<td>Ampicillin</td>
<td></td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Mecillinam</td>
<td></td>
<td>&gt;256</td>
<td>ND</td>
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<tr>
<td>Piperacillin/Tazobactam</td>
<td></td>
<td>0.25</td>
<td>ND</td>
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<td>β-lactam, monobactam</td>
<td>128</td>
<td>ND</td>
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<tr>
<td>Cefuroxime</td>
<td>β-lactams, 2nd generation cephalosporins</td>
<td>16 (R)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
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<td>16 (R)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>16 (R)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
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<td>8 (R)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>ND</td>
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<tr>
<td>Cefixime</td>
<td></td>
<td>8 (8)&lt;sup&gt;+&lt;/sup&gt; (R)&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
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</tr>
<tr>
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<td></td>
<td>16 (R)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Ciprofloxacin</td>
<td>Fluoroquinolones</td>
<td>&gt;32 (R)&lt;sup&gt;b,c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Spectinomycin</td>
<td>Aminocyclitol</td>
<td>16 (S)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Tetracycline</td>
<td>4 (R)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>Glycylcycline</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Trimetoprim/Sulphamethoxazole</td>
<td>Folic acid antagonists</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td></td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Rifampicin</td>
<td></td>
<td>-</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Antimicrobial susceptibility testing was performed using the Etest method (AB bioMérieux, Solna, Sweden) on all antimicrobials according to the instructions from the manufacturer (results in µg/ml were rounded up to 23
whole MIC dilutions). Furthermore, agar dilution was additionally performed for ceftriaxone and cefixime (in parentheses) according to the method described by the Clinical Laboratory and Standards Institute (CLSI) (7).

Where available, interpretative criteria (S, Susceptible; I, Intermediate susceptible; R, resistant) from the Clinical and Laboratory Standards Institute (CLSI) (7) were used.

Where CLSI breakpoints are not available, interpretative criteria (S, I, R) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used. Available online at: http://eucast.org/ (Accessed: April 30, 2011).

Calibrated dichotomous sensitivity (CDS) disc diffusion method (35, 37) is used for antimicrobial resistance testing in many countries in the World Health Organization (WHO) Western Pacific Region.
TABLE 2. *Neisseria gonorrhoeae* strains containing different ceftriaxone MICs and divergent genetic ceftriaxone resistance mechanisms and used as recipients in transformation of the full-length penA gene from H041.

<table>
<thead>
<tr>
<th>Strains</th>
<th>MLST</th>
<th>NG-MAST</th>
<th>Ceftriaxone MIC (µg/ml)</th>
<th>penA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>mtrR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>penB&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ponA&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG9901</td>
<td>ST7363</td>
<td>ST240</td>
<td>&lt;0.002&lt;sup&gt;e&lt;/sup&gt;</td>
<td>penA XXXVI (Mosaic)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>WHO F</td>
<td>NEW</td>
<td>ST3303</td>
<td>&lt;0.002&lt;sup&gt;e&lt;/sup&gt;</td>
<td>penA XV (WT)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>NG9903</td>
<td>ST7359</td>
<td>ST4058</td>
<td>0.004</td>
<td>penA II (A345a)</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>NG9807</td>
<td>ST7363</td>
<td>ST4093</td>
<td>0.016</td>
<td>penA II (A345a)</td>
<td>A-del Yes</td>
<td>L421P</td>
<td></td>
</tr>
<tr>
<td>WHO M</td>
<td>ST7367</td>
<td>ST3304</td>
<td>0.016</td>
<td>penA II (A345a)</td>
<td>A-del Yes</td>
<td>L421P</td>
<td></td>
</tr>
<tr>
<td>WHO K</td>
<td>ST7362</td>
<td>ST1424</td>
<td>0.064</td>
<td>penA X (Mosaic)</td>
<td>A-del Yes</td>
<td>L421P</td>
<td></td>
</tr>
<tr>
<td>NG0003</td>
<td>ST7363</td>
<td>ST4068</td>
<td>0.125</td>
<td>penA X (Mosaic)</td>
<td>A-del Yes</td>
<td>L421P</td>
<td></td>
</tr>
<tr>
<td>35/02</td>
<td>ST7363</td>
<td>ST326</td>
<td>0.125</td>
<td>penA XXVIII (Mosaic)</td>
<td>A-del Yes</td>
<td>L421P</td>
<td></td>
</tr>
<tr>
<td>WHO L</td>
<td>ST1590</td>
<td>ST1422</td>
<td>0.125</td>
<td>penA VII (A501V)</td>
<td>A-del Yes</td>
<td>L421P</td>
<td></td>
</tr>
</tbody>
</table>

MLST, Multilocus sequencing typing (23); NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing (40); MIC, minimum inhibitory concentration (µg/ml); Etest results rounded up to whole MIC steps; WT, wild type.

<sup>a</sup> penA mosaic allele encodes a mosaic penicillin binding protein 2 (PBP2) that causes a decreased susceptibility to extended-spectrum cephalosporins (ESCs). Mosaic X has been found in cefixime resistant *N. gonorrhoeae* isolates in Japan (17, 36, 51).

<sup>b</sup> A-del: characteristic single nucleotide (A) deletion in the inverted repeat of the promoter region of mtrR that causes an overexpression of the MtrCDE efflux pump resulting in a further decreased susceptibility to ESCs (17, 36, 51).

<sup>c</sup> penB: alterations of amino acids 120 and 121 in the porin PorB that cause a decreased intake of ESCs and, accordingly, a further decreased susceptibility to ESCs (17, 36, 51).

<sup>d</sup> ponA1: alteration of amino acids 421 in PBP1 that causes a decreased susceptibility to penicillins (17, 28, 51).

<sup>e</sup> MICs of <0.002 µg/ml were calculated as 0.001 µg/ml in the MIC ratios in Fig. 3.

<sup>f</sup> penA mosaic allele that has not been previously described; GenBank accession number AB608050.
FIG. 1. Phylogenetic tree describing the evolutionary relationships of full-length porB gene sequences of the high-level ceftriaxone-resistant Neisseria gonorrhoeae strain H041 compared with previously published N. gonorrhoeae penA mosaic isolates (10). The 2008 WHO K reference strain (42), containing a penA mosaic allele X and cultured in Japan in 2001, was used to root the tree. The N. gonorrhoeae multiantigen sequence typing (NG-MAST) sequence type (ST) and number of isolates are indicated.

FIG. 2. A schematic figure describing all reported penicillin binding-protein 2 (PBP2) amino acid sequences in Neisseria gonorrhoeae, which are aligned to the wild type PBP2 sequence M32091. All amino acid alterations in the different PBP2 sequences are illustrated with a single capital letter. The amino acids differing from PBP2 mosaic X (n=12) are indicated (#). The four amino acid residues in the highly ceftriaxone-resistant N. gonorrhoeae strain H041 not previously observed in any Neisseria species, which explained the ceftriaxone resistance, are illustrated by shaded bold letters.

FIG. 3. Transformation of the full-length penA allele (penA-H041) from the high-level ceftriaxone-resistant Neisseria gonorrhoeae strain H041 (Donor) into N. gonorrhoeae strains (Recipients) with different ceftriaxone MICs and genetic resistance determinants affecting the susceptibility to ceftriaxone. The ceftriaxone MICs using the Etest method (as mean results of three repeated experiments) of the donor strain, recipient strains (R), transformants (T) and the MIC ratio (T/R) are illustrated.
**FIG. 2**

<table>
<thead>
<tr>
<th>Region A</th>
<th>Region B</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image_url" alt="Table Image" /></td>
<td><img src="image_url" alt="Table Image" /></td>
</tr>
</tbody>
</table>

*Description of the table:*

- The table compares two regions, A and B, with amino acid sequences aligned vertically.
- Each row represents a different strain or sample.
- The sequences are aligned based on homology or similarity.

*Notes and footnotes:*

- *Footnote 1:* This is a note explaining the significance of the table.
- *Footnote 2:* This is another note providing additional context.

*Other information:*

- The table is referenced by FIG. 2 in the text.
- The table contains a total of 12 columns and 20 rows.
- The table is used to compare and analyze the genetic sequences of two different regions.

---

*Additional notes or comments:* This figure provides a detailed view of the genetic comparison, allowing for a more nuanced understanding of the evolutionary relationships between the two regions.
FIG. 3

<table>
<thead>
<tr>
<th>Recipient strains</th>
<th>MIC ratio (T/R):</th>
<th>MIC ratio (T/R):</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG9903</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>WHO F</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>NG9807</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>WHO M</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>WHO K</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>NG0003</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>35/02</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>WHO L</td>
<td>125</td>
<td>500</td>
</tr>
</tbody>
</table>

Breakpoint for ceftriaxone resistance (7)

Transformant (T) □
Recipient (R) □

Ceftriaxone MIC (mg/L)