

**Molecular characterization of *Neisseria gonorrhoeae* isolates collected through a national surveillance programme in Japan, 2013: evidence of the emergence of a ceftriaxone-resistant strain from a ceftriaxone-susceptible lineage**

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## Abstract

**Objectives:** To investigate the spread of ceftriaxone-resistant *Neisseria gonorrhoeae* lineages similar to strains H041 (2009) and FC428 (2015), we characterized 55 strains collected in 2013 from hospitals across Japan.

**Methods:** Susceptibility testing and whole-genome sequencing.

**Results:** Susceptibility rates were 58% for cefixime and 98% for ceftriaxone. The 55 strains were whole-genome sequenced and classified into nine MLST-STs. MLST-ST1901 was the most prevalent ( $n=19$ ) followed by MLST-ST7363 ( $n=12$ ) and MLST-ST7359 ( $n=11$ ). The most prevalent *penA* [encoding penicillin binding protein 2 (PBP2)] mosaic types, based on the *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) scheme, were 10.001 ( $n=20$ ) followed by 34.001 ( $n=13$ ). The H041 and FC428 strains were not detected; however, a single ceftriaxone-resistant strain (TUM15748) with a MIC of 0.5 mg/L ceftriaxone was identified. The TUM15748 strain belonged to MLST-ST7359 and *N. gonorrhoeae* multiantigen sequence typing-ST6771, and had a novel PBP2 (PBP2<sub>TUM15748</sub>, *penA* type 169.001). The amino acid sequence of PBP2<sub>TUM15748</sub> showed partial similarity to that of PBP2 from *N. gonorrhoeae* GU140106 and commensal *Neisseria perflava* and *Neisseria cinerea*. Natural transformation and recombination experiments using full-length TUM15748 *penA* showed that the ceftriaxone MICs of transformants increased 16-fold or more compared with the parental ceftriaxone-susceptible recipient strain (NG9807, belonging to MLST-ST7363). No ceftriaxone-resistant MLST-ST7359 strains have previously been reported.

**Conclusions:** We showed here that a ceftriaxone-susceptible lineage acquired a mutant PBP2 mosaic type, integrating partial PBP2 sequences from commensal *Neisseria* species, resulting in the emergence of ceftriaxone-resistant strains.

## Introduction

Resistance of *Neisseria gonorrhoeae* to extended-spectrum cephalosporins (ESCs) is a global public health concern.<sup>1,2</sup> Ceftriaxone is one of the last remaining recommended therapies for treatment of gonococcal infections. Thus, dissemination of ceftriaxone-resistant isolates may limit the availability of medical interventions.<sup>3–5</sup>

The main determinants of resistance to ESCs are mosaic *penA* alleles encoding penicillin-binding protein 2 (PBP2) variants with reduced affinity for ESCs.<sup>6</sup> *N. gonorrhoeae* can import extracellular DNA fragment by natural transformation and homologous recombination, thus acquiring antimicrobial resistance determinants from other *Neisseria* species.<sup>7</sup> Transformation of mosaic *penA* alleles can increase the MIC of ceftriaxone for recipient cells.<sup>8–10</sup> In Japan, the first high-level ceftriaxone-resistant gonococcal strain (H041) was isolated in Kyoto in 2009.<sup>8</sup> Subsequently, intensified surveillance conducted from 2010 to 2012 in the same district did not identify dissemination of the H041 strain.<sup>11</sup> Five years after the emergence of H041, two ceftriaxone-resistant strains belonging to different lineages (GU140106<sup>12</sup> and FC428<sup>13</sup>) were isolated in Japan (in 2014 and 2015, respectively). Their mosaic *penA* alleles were proposed to originate from commensal *Neisseria* species and parts of the *penA* sequences of GU140106 and FC428 were similar to *Neisseria cinerea penA*.<sup>13–16</sup>

Worldwide, the most prevalent MLST-STs of cefixime- and ceftriaxone-resistant gonococcal strains are MLST-ST7363, MLST-ST1901, and MLST-ST1903.<sup>13,17–20</sup> The ceftriaxone-resistant

H041,<sup>8</sup> A8806,<sup>21</sup> and GU140106<sup>12</sup> strains belong to MLST-ST7363, the F89 strain<sup>22</sup> belongs to MLST-ST1901, and the FC428 strain<sup>13</sup> belongs to MLST-ST1903. Most *N. gonorrhoeae* strains with mosaic *penA* alleles were classified as MLST-ST1901 and MLST-ST7363, and therefore close attention must be paid to trends in the ceftriaxone resistance of strains belonging to these two lineages.<sup>13,17</sup> However, the diversity of mosaic *penA* alleles is not always reflected in MLST results because *penA* and housekeeping genes can transform separately. A full explanation of the characteristics of resistant strains requires whole-genome sequences.

Genomic data on ESC-susceptible and -resistant *N. gonorrhoeae* clinical strains in Japan are limited.<sup>23</sup> Only a few reports have described the sequences of isolates from outpatient clinics in the area where H041 was detected (Kyoto and Osaka, Japan), from 2010 to 2012 and in 2015.<sup>11,13</sup>

In this study, we characterized 55 *N. gonorrhoeae* strains collected through national surveillance efforts conducted 4 years following the detection of the high-level ceftriaxone-resistant H041 and 2 years prior to detection of the ceftriaxone-resistant FC428 strain. We determined the draft whole-genome sequences of these strains and analysed the mechanisms underlying the development of ceftriaxone resistance by *in vitro* natural transformation of *penA* alleles.

## Materials and methods

### *Strains*

In 2013, 55 *N. gonorrhoeae* clinical isolates were identified at 25 medical facilities in 16 prefectures in Japan as part of a post-marketing survey of levofloxacin.<sup>24</sup> This study was conducted with approval of the Ethics Committee of the Faculty of Medicine, Toho University (no. A16039).

### ***Antimicrobial susceptibility testing***

MICs were determined by the agar dilution method according to the CLSI M07-ED11 guidelines.<sup>25</sup> The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints version 10.0 ([https://www.eucast.org/clinical\\_breakpoints/](https://www.eucast.org/clinical_breakpoints/)). Because the breakpoint for levofloxacin is indeterminate, that of ciprofloxacin was substituted. Strains were incubated on GC agar for 24 h at 35°C in the presence of 5% CO<sub>2</sub>. The MICs of the following six antimicrobial agents were measured: penicillin G (concentration range: 0.063–16 mg/L), cefixime (0.063–16 mg/L), ceftriaxone (0.063–4 mg/L), spectinomycin (0.063–64 mg/L), levofloxacin (0.063–64 mg/L) and azithromycin (0.063–64 mg/L). *N. gonorrhoeae* strains ATCC 49226, NG9807<sup>8</sup> and FC428<sup>13</sup> were used for antimicrobial susceptibility testing quality control.

### ***Molecular characterization by draft whole-genome sequencing***

Genomic DNA was obtained by phenol/chloroform extraction of bacteria and purified using a QIAamp PCR purification kit (Qiagen, Valencia, CA, USA). To determine draft whole-genome

sequences, DNA libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). Libraries were sequenced on a MiSeq system using a 600-cycle reagent kit (2×300 bp paired-end reads) and draft genome contigs were obtained by *de novo* assembly using SPAdes (v3.13.0). MLST and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) were performed using the pubMLST (<https://pubmlst.org/neisseria>)<sup>26</sup> and NG-MAST (<https://www.ng-mast.net>)<sup>27</sup> schemes, respectively. Genotyping of *penA* (encoding PBP2) was conducted using the *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) scheme (<https://ngstar.canada.ca>).<sup>28</sup> Multiple sequence alignment used CLASTALW and phylogenetic analyses used Maximum Likelihood were performed using MEGA X.<sup>29</sup> Core genome SNP-based phylogenetic analysis was performed using draft whole genome sequences. The MiSeq sequencing data were aligned to the genome sequence of the reference *N. gonorrhoeae* strain WHO Y (GenBank accession no. LT592161) using the Burrows-Wheeler Aligner (BWA) with ‘MEM’ option.<sup>30</sup> We constructed a core genome alignment using SAMtools (version 1.1) mpileup<sup>31</sup> and VarScan (version 2.3.7) mpileup2cns<sup>32</sup> and a maximum-likelihood phylogenetic tree using PhyML.<sup>33</sup> Using this as the starting tree, we inferred homologous recombination events that imported DNA fragments from outside the ST and constructed a clonal phylogeny with corrected branch lengths using ClonalFrameML.<sup>34</sup> A final phylogenetic tree was constructed using RAXML (version 8.2.12).<sup>35</sup>

### ***penA* natural transformation**

To assess whether the *penA* gene (encoding PBP2) of the ceftriaxone-resistant strain TUM15748 (*penA*<sub>TUM15748</sub>) contributed to the increased MIC of ceftriaxone, full-length *penA*<sub>TUM15748</sub> was amplified by PCR and a recipient strain, *N. gonorrhoeae* NG9807, was transformed with the *penA*<sub>TUM15748</sub> amplicon as previously described.<sup>36</sup> *N. gonorrhoeae* NG9807, which belongs to MLST-ST7363, NG-MAST-ST4093, had a ceftriaxone MIC of 0.016 mg/L.

### ***Complete whole-genome sequencing***

To identify any unintended mutations and recombination events in the transformant strain chromosomes, we determined the complete whole-genome sequences of the donor, recipient, and transformant strains. High molecular weight DNA was extracted using NucleoBond AXG 20 (TaKaRa, Shiga, Japan) and prepared for MinION (Oxford Nanopore Technologies, Oxford, UK) sequencing. DNA libraries were prepared using the Rapid Barcoding Kit (Oxford Nanopore Technologies). Pooled libraries were sequenced using a Nanopore flow cell R9.4. Hybrid *de novo* assemblies of Illumina and MinION reads were performed with Unicycler (v0.4.8-beta).<sup>37</sup> Additional nucleotide error correction was performed using pilon (v1.22) three times.<sup>38</sup> Gene annotation and analysis of genome structure was performed using DFAST (v1.1.15) (<https://dfast.nig.ac.jp/>) and MAUVE (v2.1.1),<sup>39</sup> respectively.

### ***Nucleotide sequence accession numbers***

The sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) under BioProject number PRJDB9563. The draft genome sequences of 55 *N. gonorrhoeae* strains were deposited in the DDBJ and under GenBank accession numbers SAMD00217291 to SAMD00217345. The accession numbers of the complete genome sequences determined in this study were SAMD00220608 for TUM15748, SAMD00220610 for TUM16691 (NG9807), and SAMD00257605 for TUM19855 (NG9807/PBP2<sub>TUM15748</sub>). Datasets S1 and S2, available as Supplementary data at *JAC* Online, show the sequencing data statistics.

## **Results**

### ***Antimicrobial susceptibility***

The results of the antimicrobial susceptibility testing of the 55 *N. gonorrhoeae* isolates are summarized in Table 1 and Figure 1. Of the 55 isolates, 42% were susceptible to cefixime and 98% were susceptible to ceftriaxone. The susceptibility rates to benzylpenicillin, azithromycin and levofloxacin were 2%, 95% and 25%, respectively. All 55 strains were susceptible to spectinomycin. Only one strain, *N. gonorrhoeae* TUM15748, was resistant to ceftriaxone (MIC: 0.5 mg/L). This strain had low susceptibility to benzylpenicillin (MIC: 0.5 mg/L) but was susceptible to azithromycin, spectinomycin and levofloxacin (Table 1).

### ***Genotyping and phylogenetic analysis using MLST, NG-MAST and core genome SNPs***

A total of 55 draft whole-genome sequences were obtained at an average depth of 51.0-fold (SD 18.8-fold) (see Dataset S1). Assembled genomes had an average of 218.0 (SD 119.0) contigs and an average N50 value of 24683.0 (SD 7095.0) bp. Genotyping of the 55 *N. gonorrhoeae* strains by MLST and NG-MAST is summarized in Table S1. The 55 strains were classified into nine MLST-STs. MLST-ST1901 was the most prevalent ( $n=19$ , 35%), followed by MLST-ST7363 ( $n=12$ , 22%) and MLST-ST7359 ( $n=11$ , 20%). The 55 strains were classified into 38 NG-MAST-STs. NG-MAST-ST6543, NG-MAST-ST6771 and NG-MAST-ST6798 were the most prevalent ( $n=4$ , 7% each), followed by NG-MAST-ST1407 ( $n=3$ , 5%). Each NG-MAST-ST could be assigned to a specific MLST-ST, except for NG-MAST-ST4018, which was associated with both MLST-ST1594 and MLST-ST15378. MLST-ST15378, NG-MAST-ST19619, NG-MAST-ST19620, NG-MAST-ST19621, NG-MAST-ST19622, NG-MAST-ST19623, and NG-MAST-ST19706 have not been reported previously. Of the 55 *N. gonorrhoeae* strains, three shared the same STs as the ceftriaxone-resistant strain F89 (also called strain WHO Y) (MLST-ST1901 and NG-MAST-ST1407)<sup>22</sup> and four shared the same STs with the ceftriaxone-resistant strain GU140106 (MLST-ST7363 and NG-MAST-ST6543).<sup>12</sup>

### ***Core genome SNP-based phylogeny, antimicrobial susceptibility, and determinants of antimicrobial resistance***

The relationships between a core genome SNP-based phylogeny, antimicrobial susceptibility, and antimicrobial resistance determinants are shown in Figure 1. Of the 55 strains, 53 could be divided into three clades (A, B, and C). Clades A, B, and C could be subdivided into two (A-1 and A-2), three (B-1, B-2, and B-3), and two (C-1 and C-2) subclades, respectively. The two remaining isolates (TUM15789 and TUM15795) were not closely related to other strains and were independent of each other. Three MLST-STs (MLST-ST7359, MLST-ST1594, and MLST-ST15378) belonged to clade A. Except for TUM15748, subclade A-2 consisted of ESC-susceptible *N. gonorrhoeae* strains. The cefixime- and ceftriaxone-resistant strain TUM15748 harbored a novel *penA* type 169 (mosaic) (Figure 1, Figure 2, and Table S1). Subclade B-1 consisted of MLST-ST1901 and MLST-ST7360. Subclades B-2 and B-3 were mixed, containing MLST-ST1901 and MLST-ST1579. Subclade B-2 harboured strains of *penA* types 34.001 (mosaic) and 72.001 (mosaic). Except for TUM15759, subclade B-3 harboured strains of *penA* type 10.001 (mosaic). All clade B strains harboured mutations in GyrA (S91F and D95G) and ParC (S87R) and were resistant to levofloxacin. Clade C consisted of MLST-ST7363. Subclade C-1 harboured strains of *penA* types 10.001 (mosaic) and 168.001 (mosaic). Subclade C-2 harboured strains of *penA* type 150.001 (semi-mosaic). Subclade C-1 strains showed reduced susceptibility to cefixime. Of the three subclade C-2 *penA* type 150.001 strains, only one showed reduced susceptibility to cefixime. In clade C, only one *penA* type 10.001 strain and one *penA* type 168.001 strain showed reduced susceptibility to ceftriaxone. All clade C strains harbouring mutations in GyrA (S91F and D95N) and ParC (S87R and S88P) were resistant to levofloxacin. All

subclade A-2 strains harboured the MtrR A39T mutation and nearly all clade B strains harboured the –35A deletion. Almost all clade B and all subclade C-1 strains harbored the PorB G120K and A121D mutations. No penicillinase genes or 23S rRNA gene mutations were detected in any strains.

### ***Characteristics of the PBP2<sub>TUM15748</sub> amino acid sequence***

Phylogenetic analysis was performed to investigate the relevance of the novel PBP2 sequence of TUM15748 (PBP2<sub>TUM15748</sub>) through comparison with previously reported PBP2 sequences (Figure 2). The amino acid sequence of PBP2<sub>TUM15748</sub> was most similar to that of PBP2<sub>GU140106</sub> (94.7% identity). PBP2<sub>TUM15748</sub> bore two conserved amino acid substitutions, A311V and T483S, which contribute to reduced susceptibility to ESCs.<sup>8,40</sup> Regional similarity of PBP2 amino acid sequences across *Neisseria* species is shown in Figure 3. Based on regional amino acid sequence similarity, PBP2 could be divided into seven regions. PBP2<sub>TUM15748</sub> was highly similar (>95% identity) to the region-1, -2, -5, and -7 sequences of PBP2<sub>GU140106</sub>. PBP2<sub>TUM15748</sub> shared high similarity in region-1, -2, -3, and -5 with *N. perflava* PBP2<sub>NSU-per\_52</sub> and region-2, -3, -5, and -7 of *N. cinerea* PBP<sub>SI57-1</sub>. Interestingly, the sequences of these four PBP2s were highly similar in region-5, and except for PBP2<sub>NSU-per\_52</sub>, shared a conserved A311V amino acid substitution.

### ***PBP2<sub>TUM15748</sub> raised the ESC MICs of susceptible strains***

Ceftriaxone-susceptible *N. gonorrhoeae* NG9807 was transformed with *penA*<sub>TUM15748</sub>. The ceftriaxone and cefixime MICs of transformant NG9807/PBP2<sub>TUM15748</sub> increased 16-fold or more compared with the recipient strain (Table 2). By contrast, the MIC of benzylpenicillin was unaffected. The complete whole-genome sequence of the donor, recipient, and transformant strains showed that the recipient full-length *penA* was completely homologous with *penA*<sub>TUM15748</sub>. No unexpected mutations or recombination events were detected in transformant chromosomes.

## Discussion

Following the identification of the high-level ceftriaxone-resistant *N. gonorrhoeae* H041 strain in 2009, the spread of this strain has been carefully monitored through surveillance efforts in Japan.<sup>11,41–43</sup> Fortunately, the H041 strain has not been detected, and we also did not observe dissemination of H041 in this study. The ceftriaxone-resistant FC428 strain has likewise not been detected. The rate of azithromycin susceptibility in the present study (95%) was higher than that reported in a previous study (76.7%) undertaken in Japan from 2010 to 2012 ( $n=193$ ).<sup>11</sup> Almost all *N. gonorrhoeae* strains with reduced susceptibility to ESCs identified to date have belonged to MLST-ST1901, MLST-ST1903, or MLST-ST7363.<sup>8,13,22,43,44</sup> In this study, we found that of 32 gonococcal strains with reduced susceptibility or resistance to cefixime, 31 belonged to clade B and subclade C-1 according to core genome SNP-based phylogenetic analysis. Almost all strains belonging to clade B and subclade C-1 were MLST-ST1901, MLST-ST7363, and MLST-ST1579. The mutations G120K and A121D in PorB have been reported to produce slight reductions in

susceptibility to penicillin, cefixime, and ceftriaxone,<sup>45,46</sup> and therefore these mutations contribute to the rising MIC baselines of penicillin, cefixime, and ceftriaxone in most strains of clade B and subclade C-1 (Figure 1). Our data showed that the MLST-ST1579 lineage may be developing reduced susceptibility to cefixime in Japan; only one strain of ceftriaxone-resistant *N. gonorrhoeae* of MLST-ST1579 has been previously reported.<sup>47</sup> Assessment of quinolone- and macrolide-resistance determinants, as well as PBP2 sequences, using draft whole-genome sequences is important to understand the molecular epidemiology of antimicrobial resistance in *N. gonorrhoeae*.

In this study, we found that the ESC-susceptible lineage MLST-ST7359 included only one ceftriaxone-resistant strain, TUM15748. According to previous studies in Japan, all MLST-ST7359 strains were susceptible to both cefixime and ceftriaxone.<sup>11,43</sup> TUM15748 was inferred to have developed ceftriaxone resistance by acquiring a novel type 169.001 *penA* (mosaic) encoding a PBP2 variant with decreased affinity for ceftriaxone. PBP2<sub>TUM15748</sub> shared regional amino acid similarity with PBP2<sub>SI57-1</sub> and PBP2<sub>NSU-per\_52</sub> from commensal *N. cinerea* and *N. perflava*, respectively (Figure 3). Our data support the possibility that the previously reported partial sequence of *N. cinerea* PBP2 is a component of PBP2 variants with decreased ceftriaxone affinity in *N. gonorrhoeae*.<sup>16</sup> There has been no report of strains with genetic similarity to TUM15748 since TUM15748 was isolated. Mutations in *penA* are predicted to have a negative impact on gonococcal fitness,<sup>8,48</sup> so it is possible that PBP2<sub>TUM15748</sub> imposes fitness costs on *N. gonorrhoeae*, limiting its spread.

The number of samples analysed in our study was quite small compared with the estimated number of gonorrhoea cases in 2013 in Japan (25 606). Therefore, low sample size is one possible reason for our inability to detect strain H041 in this study. However, we detected the ceftriaxone-resistant *N.*

*gonorrhoeae* strain TUM15748 harbouring a novel *penA* allele. This finding suggests that novel *penA* alleles associated with reduced susceptibility to ESCs may have a prevalence of around 2%.

Appropriate use of ESCs is required to avoid selecting for ESC-resistant *N. gonorrhoeae* strains emerging at low frequency.

In this study, we did not detect the high-level ceftriaxone-resistant H041 strain and the ceftriaxone-resistant FC428 strain in 2013 in Japan. One *N. gonorrhoeae* strain in the ESC-susceptible lineage MLST-ST7359 acquired resistance to ceftriaxone mediated by a novel *penA* allele.

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## Transparency declarations

None to declare.

## *Author contributions*

M.H. and K.A. were co-investigators and responsible for the data analysis. Y.I. was responsible for the organization and coordination of the study. K.S. and M.O. provided research strains and guided some experiments. K.T. was the primary investigator. All authors contributed to the writing of the final manuscript and met the ICMJE authorship criteria.

## Supplementary data

Table S1 and Datasets S1 and S2 are available as Supplementary data at *JAC* Online.

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**Table 1.** Antimicrobial susceptibility of 55 *Neisseria gonorrhoeae* strains identified in a national hospital surveillance study in Japan, 2013

Antimicrobial	Breakpoints <sup>a</sup> (mg/L)	No. (%) of isolates <sup>d</sup>			MIC <sub>50</sub> <sup>e</sup>	MIC <sub>90</sub> <sup>f</sup>
		S	I	R		
Benzylpenicillin	S ≤0.06/R >1	1 (2)	31 (56)	23 (42)	1	4
Cefixime	S ≤0.125/R >0.125	23 (42)	0 (0)	32 (58)	0.25	0.5
Ceftriaxone	S ≤0.125/R >0.125	54 (98)	0 (0)	1 (2)	0.06	0.125
Azithromycin	ECOFF 1 mg/L <sup>b</sup>	52 (95)	0 (0)	3 (5)	0.25	0.5
Spectinomycin	S ≤64/R >64	55 (100)	0 (0)	0 (0)	16	16
Levofloxacin	S ≤0.03/R >0.06 <sup>c</sup>	14 (25)	0 (0)	41 (75)	8	16

<sup>a</sup>European Committee on Antimicrobial Susceptibility Testing breakpoints version 10.0 are shown.

<sup>b</sup>The clinical breakpoint for azithromycin monotherapy is undefined. With the aim of detecting acquired resistance mechanisms, the ECOFF (epidemiological cut-off value) is 1 mg/L. Susceptible strains have MICs below the ECOFF and resistant strains have MICs equal to or greater than the ECOFF.

<sup>c</sup>The breakpoint of ciprofloxacin was applied to levofloxacin.

<sup>d</sup>S, susceptible; I, intermediate; R, resistant.

<sup>e</sup>MIC<sub>50</sub>, MIC of an antimicrobial inhibiting 50% of isolates.

<sup>f</sup>MIC<sub>90</sub>, MIC of an antimicrobial inhibiting 90% of isolates.

**Table 2.** Phylogenetic and genetic characteristics of *N. gonorrhoeae* strains used for the transformation assay

Characteristic	Donor	Recipient	Transformant
Strain name	TUM15748 (this study)	NG9807 (9)	NG9807/PBP2 <sub>TUM15748</sub>
MLST-ST	7359	7363	7363
NG-MAST-ST	6771	4093	4093
<i>penA</i> type	169.001 <sup>a</sup>	2.001	169.001
MIC (mg/L)			
Benzylpenicillin	0.5	2	2
Cefixime	2	<0.06	4
Ceftriaxone	0.5	<0.06	0.5

Abbreviations: NG-MAST-ST, *Neisseria gonorrhoeae* multiantigen sequence typing-defined sequence type; PBP2, penicillin binding protein 2.

<sup>a</sup>New *penA* type identified in this study.

## Figure legends

**Figure 1.** Phylogenetic relationships, antimicrobial susceptibility, and antimicrobial resistance determinants of *N. gonorrhoeae* strains analysed in this study. Phylogenetic analysis was performed using core-genome single-nucleotide polymorphisms and strain STs were characterized by MLST. Genotyping was conducted using the *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) scheme. The MICs of penicillin G (PCG), cefixime (CFIX), ceftriaxone (CTRX), levofloxacin (LVFX), azithromycin (AZM) and spectinomycin (SPCT) were determined. S, susceptible; I, intermediate; R, resistant. The scale distance corresponds to the number of substitutions per site. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

**Figure 2.** Phylogenetic analysis of PBP2 amino acid sequences determined this study and from previously reported *Neisseria gonorrhoeae* strains and commensal *Neisseria* spp.<sup>49</sup>. This phylogenetic tree was constructed using maximum likelihood methods in MEGA X. Sequence accession numbers were as follows: B904096.1, *Neisseria perflava* NSU-per\_52 PBP2; LC410041, *Neisseria polysaccharea* SI28-1 PBP2; LC410042, *Neisseria lactamica* SI60-1 PBP2; LC410043, *Neisseria cinerea* SI57-1 PBP2; LC410044, *Neisseria subflava* SI94-3 PBP2, AB904142, *N. subflava* SH43-1 PBP2; AB904147, *Neisseria* sp. SH43-3 PBP2; AB904125, *N. lactamica* NLA-1 PBP2; AB904126, *N. perflava* NSU-per\_57 PBP2; and LC316656, *N. cinerea* AM1601 PBP2. PBP2<sub>TUM15748</sub> is highlighted in grey. Commensal *Neisseria* spp. are shown in bold text. The scale distance corresponds to the number of substitutions per site.

**Figure 3.** Regional amino acid sequence similarity of PBP2. PBP2<sub>TUM15748</sub> amino acid sequence similarity with PBP2<sub>GU140106</sub>, PBP2<sub>NSU-per\_52</sub>, and PBP2<sub>SI57-1</sub> was calculated by splitting PBP2 into 50 amino acid segments and sliding the 50-residue window by one amino acid at a time. PBP2-encoding *penA* sequences were downloaded from NG-STAR (<https://ngstar.canada.ca/alleles/penA>). *Neisseria*

*perflava* NSU-per\_52 and *Neisseria cinerea* SI57-1 PBP2 amino acid sequences were downloaded from GenBank (accession nos. BAO96787.1 and LC410043, respectively). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Figure 1.

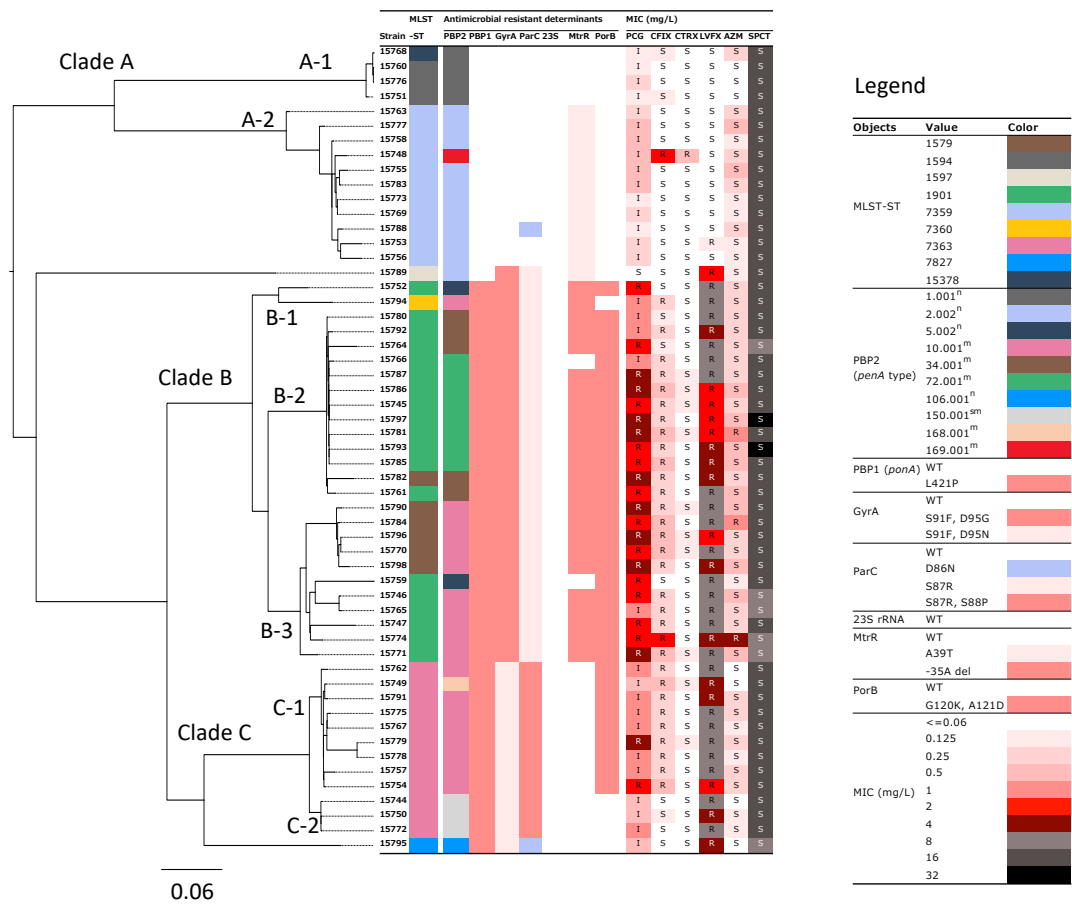


Figure 2.

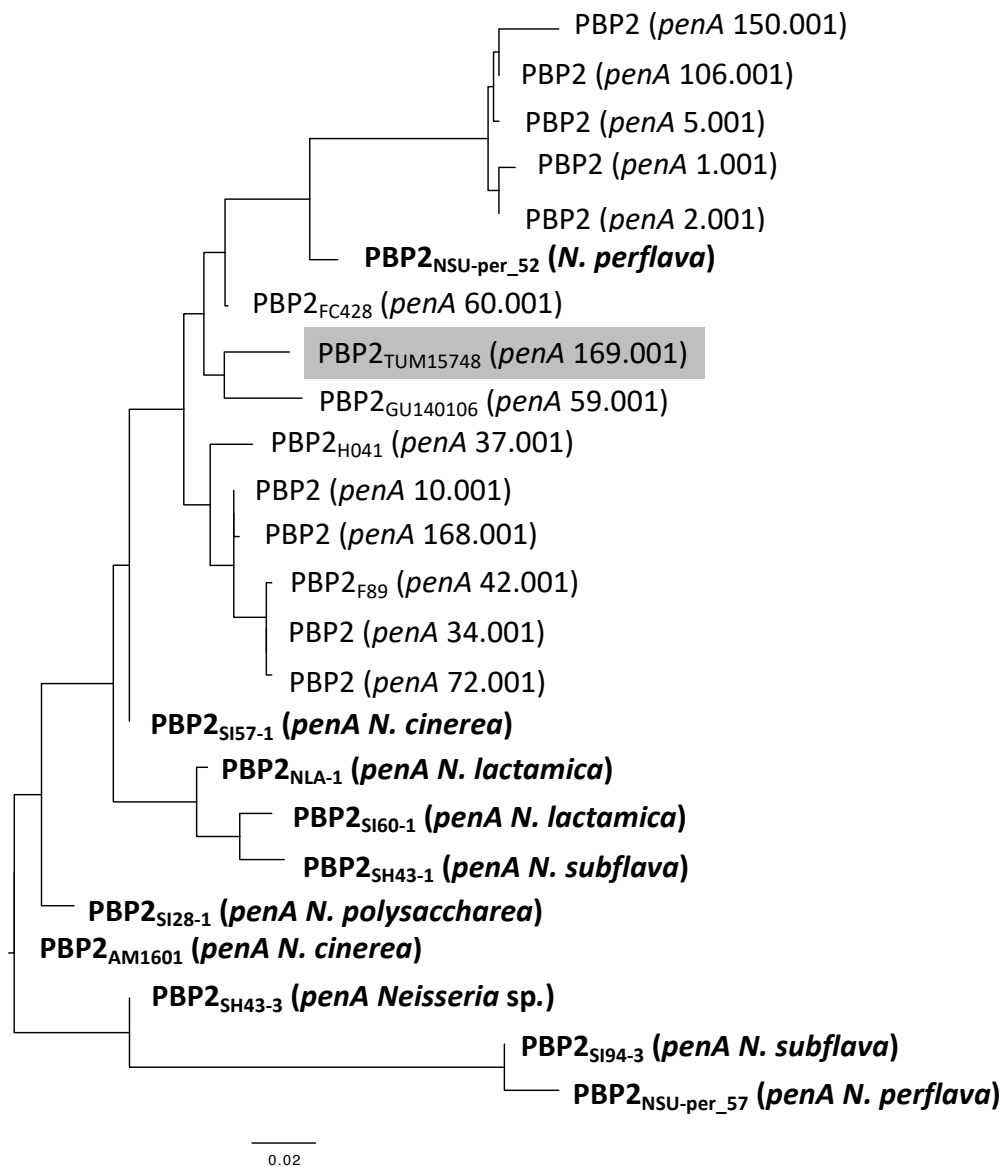


Figure 3.

