Clusters of circulating Neisseria gonorrhoeae strains and association with antimicrobial resistance in Shanghai

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Objectives: (i) To distinguish Neisseria gonorrhoeae isolates in Shanghai by porB typing; (ii) to ascertain the congruence of porB DNA sequence typing with cases linked epidemiologically; (iii) to determine the association of specific PorB mutations with antimicrobial resistance to penicillin or tetracycline.

Methods: porB DNA sequences of 174 N. gonorrhoeae isolates, collected from 143 male patients and 31 female sexual partners in Shanghai were determined. Phylogenetic analysis was used to determine sequence associations and concordance with epidemiologically linked cases. PorB protein sequences were compared with the wild-type sequence to identify mutations associated with antimicrobial resistance to penicillin and tetracycline.

Results: porB1a genotypes comprised 27.0% of the isolates and included 15 distinct DNA sequences, while 73.0% of the isolates carried porB1b genotypes with 63 distinct DNA sequences. porB DNA sequence typing was congruent with patient-reported sexual contacts. In addition, porB DNA sequence analysis revealed a number of strains with identical DNA sequences not identified through traditional epidemiological methods. The porB1b isolates had a significantly higher percentage of chromosomally mediated resistance to tetracycline and higher MIC₅₀ to penicillin and ciprofloxacin. G120K/A121D mutations were observed in 71.1% of PIB isolates and were associated with resistance to penicillin and/or tetracycline. The majority of the PIA isolates (82.1%) also carried G120D/A121G double mutations. The index of discrimination for porB DNA sequence analysis was 95%.

Conclusions: The porB1b genotype was found to be predominant in Shanghai. porB DNA sequence typing was sufficiently discriminatory for differentiating N. gonorrhoeae isolates and was congruent with epidemiological linkages. Novel porB sequences of N. gonorrhoeae and novel mutations of PorB proteins were identified.

Keywords: N. gonorrhoeae, molecular epidemiology, molecular typing, sexually transmitted diseases

Introduction

Neisseria gonorrhoeae is highly variable in its genotype and phenotype.¹–³ This genetic variability has been exploited to characterize gonococcal strains circulating in a given community, thereby identifying patterns of specific strain transmission and guiding control strategies.⁴–⁸ DNA sequence analysis of gonococcal porB genes can provide a high index of discrimination for isolates of N. gonorrhoeae⁷,⁹ and has been used to identify circulating strains/clusters,⁴,¹⁰–¹³ to track strain transmission in sexual contacts⁴,⁷,⁸,¹¹,¹⁴ and to investigate antibiotic resistance.¹⁵–¹⁷

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PorB proteins have been associated with cell viability, pathogenesis\(^1\)\(^{-}\)\(^8\) and antimicrobial susceptibility.\(^1\)\(^6\)\(^{-}\)\(^7\) The two \textit{porB} alleles, \textit{porB}1a and \textit{porB}1b, are mutually exclusive in \textit{N. gonorrhoeae} isolates, although some hybrid genes have been identified.\(^1\)\(^9\) The two alleles encode one of the two PorB proteins, either \textit{PIA} or \textit{PIB}.\(^2\)\(^3\) Gonococcal PorB is a transmembrane protein comprising eight highly variable loops separated by nine conserved regions.\(^5\)\(^-\)\(^7\) The loops, which are surface exposed, can elicit an immune reaction during infection\(^6\)\(^-\)\(^8\) and were targeted for differentiating isolates when serovar determination with monoclonal antibodies was widely available.\(^2\)\(^4\)\(^-\)\(^5\)\(^0\)

Gonorrhoea is a significant public health problem worldwide.\(^3\)\(^1\) The rate of sexually transmitted infections (STIs) in Shanghai, the trade capital of China and a city with a population of more than 17 million, has increased over the past two decades and is currently the highest in China; between 2001 and 2005 (STI Reports, Shanghai), gonorrhoea prevalence in Shanghai was 75–107 per 100 000 when compared with rates of 13–19 per 100 000 in China overall.\(^3\)\(^2\)\(^-\)\(^4\) The genotypic characterization and transmission patterns of \textit{N. gonorrhoeae} in Shanghai have not been studied previously. This report summarizes a pilot study undertaken to understand STI strain transmission in patients accessing the Shanghai Skin Disease and STD Hospital over a 7 month period in 2004–05. We determined the \textit{porB} DNA sequences of isolates, ascertained what strains comprised circulating clusters and associated \textit{porB} types with antimicrobial susceptibility. The congruence of \textit{porB} sequence typing and epidemiological sexual links was ascertained, and clusters identified by \textit{porB} sequence analysis were compared with those revealed by patients’ self-reported connections.

**Materials and methods**

**Patient recruitment, \textit{N. gonorrhoeae} isolate collection and identification**

Male patients accessing the STD Clinic of the Shanghai Skin Disease and STD Hospital (the Shanghai Hospital), China, with gonorrhoea, were referred to trained interviewers by attending physicians. A consent form and an anonymous questionnaire were administered to each patient to gather demographic information, STI history and information related to sexual practices. Male patients with gonorrhoea (\(n = 143\)) were consecutively recruited as index patients between November 2004 and May 2005. Sexual partners were identified by index patient nomination and were invited into the study, of whom, 31 females were \textit{N. gonorrhoeae} positive. Subsequently, a total of 174 \textit{N. gonorrhoeae} isolates were included in this study comprising 143 isolates from male index patients and 31 isolates from their female sexual partners. Alternatively, these isolates comprised 113 isolates from individual male patients, 58 isolates from 29 sexual pairs and 3 isolates from 1 index patient and 2 female sexual partners, respectively. The primary isolation and identification of gonococcal isolates was performed at the Diagnostic Centre of the Shanghai Skin Disease and STD Hospital, as reported previously.\(^3\)\(^5\) After primary isolation, isolates were subcultured on GC agar base (Oxoid; GuangZhou LOSO Science Ltd) supplemented with 1% IsoVitalex (Oxoid) prior to antimicrobial susceptibility testing and chromosomal DNA extraction procedures. Isolates were stored at \(-80^\circ\)C in Brain Heart Infusion broth (Difco; distributed by Shanghai Chemical Reagent Co., China National Medicine Group, Shanghai, China) containing 20% glycerol. Ethical approval for this study was obtained from the Ottawa Hospital Research Ethics Board and the Ethics Committee of the Shanghai Municipal Bureau of Public Health.

**Determination of antimicrobial susceptibility**

The MICs for all isolates to penicillin, tetracycline, ciprofloxacin, spectinomycin and ceftriaxone were determined using an agar dilution method\(^6\) and results were reported previously.\(^2\)\(^3\) Beta-Lactamase production was determined for all isolates using a chromogenic cephalosporin test (Oxoid). Interpretative MIC criteria\(^1\)\(^6\) and antimicrobial resistance phenotypes included the following classifications: CIP\(^6\) (ciprofloxacin resistance MICs \(\geq 1\) mg/L), PPNG (beta-lactamase positive), TRNG (isolates having tetracycline MICs \(\geq 16\) mg/L), PP/TRNG (beta-lactamase positive and MICs to tetracycline \(\geq 16\) mg/L), CMPR (chromosomal penicillin resistance; non-PPNG isolates with penicillin MICs \(\geq 2\) mg/L), CMTR (chromosomal tetracycline resistance; non-TRNG isolates with tetracycline MICs \(\geq 8\) mg/L) and CMRNG (non-PPNG and non-TRNG isolates with penicillin MICs \(\geq 2\) mg/L and tetracycline MICs \(\geq 2\) to \(\leq 8\) mg/L).

**Preparation of chromosomal DNA**

\textit{N. gonorrhoeae} isolates were retrieved from storage and incubated for 18–24 h at 35°C in a humid environment supplied with 5% CO\(_2\). Bacterial suspensions were prepared in 0.9% saline to a turbidity equivalent to that of a 0.5 McFarland standard (Remel, Lenexa KS, USA; \(\sim 10^8\) cfu/mL). One millilitre of the cell suspension was used for chromosomal DNA extraction. Gonococcal DNA was extracted using the Genomic DNA Purification Kit following the manufacturer’s manual (Shanghai Promega Biological Products Ltd, Shanghai, China). DNA was eluted with 100 mL of ddH\(_2\)O. The quality of DNA was verified by agarose electrophoresis.

**Amplification and DNA sequence analysis of \textit{porB}, \textit{piIA} and \textit{abcZ}**

\textit{porB} was amplified by PCR using \textit{porB}-F and \textit{porB}-R primers (purchased from Invitrogen Canada, Burlington, Ontario, Canada) as described previously (Table 1).\(^1\)\(^1\) The PCR mixture (50 \(\mu\)L) contained 5 \(\mu\)L of genomic DNA, 2.5 U of \textit{Taq} DNA polymerase (Amersham Bioscience), 1 \(\times\) PCR buffer with 1.5 mM MgCl\(_2\), 0.25 mM dNTPs, 0.5 \(\mu\)M of each primer. Amplification (Perkin Elmer 9600 Thermo Cycler, Wellesley, MA, USA) was performed as follows: an initial denaturing step at 94°C for 4 min, followed by 30 sequential cycles of 94°C for 1 min, 50°C for 45 s and 72°C for 1 min, and a final extension phase at 72°C for 10 min. Following the purification of PCR products (PCR Purification Kit, Qiagen, Mississauga, Ontario, Canada), DNA sequences of both strands were determined using the Applied Biosystems 3730x1 DNA Analyzer (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada) with primers \textit{porB}-F or \textit{porB}-R, respectively (Table 1). The \textit{porB} DNA sequences determined in our study comprise seven polymorphic areas for encoding the surface-exposed loops (I–VII) and six conserved inter spacer regions (II–VII), which accounts for 85% of the entire \textit{porB} gene.\(^2\)\(^1\)\(^3\)\(^7\) The sequences of these regions were determined and used for analysis because they can be ascertained in a single DNA sequencing reaction of upper- and lower-strand of DNA and because the discriminatory power is high enough for sufficient molecular epidemiology studies.\(^3\)\(^8\)\(^-\)\(^4\)\(^0\)
An initial experiment was conducted to determine the discriminatory power of \textit{porB}, \textit{pilA} or \textit{abcZ} DNA sequence analysis either alone or in combination.\textsuperscript{8} Thus, 33 clinical isolates were randomly selected from a clinical collection in Shanghai in 2004 prior to this study. The identification of \textit{N. gonorrhoeae} for these isolates was confirmed by the oxidase test,Gram’s stain and glucose utilization tests, as reported previously.\textsuperscript{35} \textit{PorB} (primers indicated above), \textit{pilA} (primers \textit{pilA-F/pilA-R}) and \textit{abcZ} (primers \textit{abcZ-F/abcZ-R}) were PCR-amplified (Table 1).\textsuperscript{8} PCR parameters were those described above except for an extension phase of 2 min for \textit{abcZ}. Each strand of the amplicons was sequenced with the primers listed in Table 1.

### Verification and editing of DNA sequences

DNA sequences were verified by the alignment of the complementary strands using Multalin version 5.4.1 (http://prodes.toulouse.inra.fr, date last accessed 20 September 2006)\textsuperscript{44} and occasionally by manual examination.\textsuperscript{5,9} Sequences were aligned using ClustalW\textsuperscript{42} and edited using Jalview Alignment Editor (http://www.ebi.ac.uk/clustalw, date last accessed 28 September 2006).\textsuperscript{41} \textit{PorB} sequences (e.g. \textit{porB}1a and \textit{porB}1b) from \textit{N. gonorrhoeae} MS11 (\textit{porB}1a, GenBank #J03029) or FA1090 (\textit{porB}1b, GenBank #J03017) were used as prototypes. \textit{PorB} genotypes were assigned to \textit{porB}1a or \textit{porB}1b based on the presence or absence of two nucleotide sequences in the loop V coding region, as described previously.\textsuperscript{6,9,21} \textit{PorB}1a was characterized by the absence of ~50 nucleotides (nt) corresponding to the positions nt681–705 and nt718–736 of \textit{porB}1b. DNA sequences of \textit{pilA} (GenBank #AF520319) and \textit{abcZ} (GenBank #AF520352) were previously described by Viscidi and Demma.\textsuperscript{8}

### Discriminatory powers

Simpson’s index of diversity (ID) was used to determine the abilities of either single gene sequence analysis (\textit{porB}, \textit{pilA} or \textit{abcZ}) or multilocus sequencing typing (MLST) methods to discriminate between isolates.\textsuperscript{38,39} For MLST, each unique sequence of \textit{porB}, \textit{pilA} and \textit{abcZ} was assigned a distinct allele number. Each isolate was given a 3-integer allelic profile and each unique profile was given a number representing a sequence type.

### Phylogenetic analysis

Edited DNA sequences were separated into two data sets (e.g. \textit{porB}1a and \textit{porB}1b). Phylogenetic analyses were conducted with PAUP version 4.0b10.\textsuperscript{44} Alignments were not stripped of gaps before phylogenetic analysis.\textsuperscript{24} Maximum parsimony trees were found with 1000 heuristic search including parsimony-informative characters in stepwise (random) addition, and tree bisection and reconstruction as branch swapping algorithm. MAXTREES were set to 5000, branches of zero length were collapsed and all parsimonious trees were saved. Branch support for all parsimony analyses was estimated by performing 1000 bootstrap replicates\textsuperscript{45} with a heuristic search consisting of 10 random-addition replicates for each bootstrap replicate. Trees were constructed using Treeview.\textsuperscript{46}

To determine clusters of \textit{N. gonorrhoeae} isolates based on \textit{porB} sequences, an average distance tree (using BLOSUM62) for the \textit{porB}1a or \textit{porB}1b data set, respectively, was constructed using ClustalW.\textsuperscript{42} The program allows for selection of continuous cut-off values of alignment distances. Upon selection of a cut-off value, we defined two clusters for \textit{porB}1a isolates and three clusters for \textit{porB}1b isolates.

### Analysis of \textit{porB} types/mutations and antimicrobial susceptibility

In order to avoid analysis bias due to clonal effects, only gonococcal isolates from the male index patients (n = 143) were used in this analysis. They comprised the majority of the isolates (90%, 143/159) tested, as reported previously.\textsuperscript{35} To determine the co-relationship between amino acid mutations of \textit{PorB} proteins and the antimicrobial susceptibilities of relevant isolates, each \textit{porB} sequence from 143 male index patients was translated into an amino acid sequence using Proteomics and Sequence Tools of ExPasy Proteomics Server (http://ca.expasy.org, date last accessed 20 October 2006). Alignments of the deduced protein sequences were carried out using ClustalW. \textit{PorB} sequences from \textit{N. gonorrhoeae} MS11 (\textit{porB}1a, GenBank #J03029) or FA1090 (\textit{porB}1b, GenBank #J03017) were used as prototypes. Chi-squared tests were performed on the tetracycline antimicrobial susceptibility profiles for \textit{porB}1a and \textit{porB}1b isolates to determine the significance of the percentage differences among data sets. Statistical tests for percentages of isolates resistant to penicillin, ciprofloxacin, spectinomycin or ceftriaxone are not indicated since most of the isolates were resistant to penicillin and ciprofloxacin, and were susceptible to spectinomycin and ceftriaxone.

### Gonococcal \textit{porB} sequence deposit

\textit{PorB} DNA sequences identified in this study were blasted against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST, date last accessed 3 April 2007) and were deposited in GenBank with deposit numbers of EF540591–EF540669.
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Results

Discrimination of N. gonorrhoeae isolates by porB, pilA and abcZ DNA sequence analysis

The ID values for porB, pilA and abcZ DNA sequence typing or MLST by combining these three genes were determined. In 33 isolates randomly selected from a clinical collection in Shanghai in 2004, the ID values were 95%, 74% and 68% for single locus typing of porB, pilA or abcZ, respectively, and 99% for the combination of the three loci (data not shown). Thus, we determined that porB DNA sequence typing alone would be sufficiently discriminatory to use in subsequent analyses.

Identification of porB clusters of N. gonorrhoeae

The 174 N. gonorrhoeae isolates tested included 47 isolates (27.0%) typed as porB1a and 127 isolates (73.0%) typed as porB1b [Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. The porB1a isolates comprised two distinct clusters, porB1a-1 and porB1a-2 (Figure 1), with a total of 15 different DNA sequence groups. Cluster porB1a-1 included 37 isolates displaying nine DNA sequence groups. The majority (29/37) of porB1a-1 isolates comprised two different sequence groups with either 14 isolates (all having sequence #166) or 15 isolates (all having sequence #26), respectively; the former contained two epidemiologically linked pairs and an epidemiologically linked triplet, and the latter had two epidemiologically linked pairs. One porB1a-1 sequence (#63F) comprised two isolates from a sexual pair. Each of the other six sequences comprised single isolates (Table S1). Cluster porB1a-2 contained 10 isolates having a total of six unique DNA sequences; one porB1a-2 sequence group comprised five isolates (representative sequence #115), which included one sexual pair. Each of the other five sequences comprised a single isolate (Table S1).

The porB1b isolates were divided into three clusters porB1b-1, porB1b-2 and porB1b-3 comprising 63 individual DNA sequences (Figure 2). Cluster porB1b-1 contained 72 isolates with 27 distinct porB1b sequence groups: one sequence contained a large group of 33 isolates (representative sequence #104, Figure 2, black arrow) comprising 12 isolates from six sexual pairs and 21 individual isolates. Seven sequences each comprised two isolates from epidemiologically linked pairs; six sequences each contained two isolates with no confirmed sexual linkages (Table S1). Each of the other 13 DNA sequence groups of the porB1b-1 cluster comprised an individual isolate (Table S1). Cluster porB1b-2 had 15 unique DNA sequence groups comprising a total of 23 isolates; one group (representative sequence #29) comprised five isolates including one pair; another sequence group (representative sequence #118) had four isolates including one sexual pair; and another sequence group was from a single sexual pair. Each of the other 12 sequence groups contained a single isolate (Table S1). Cluster porB1b-3 had 21 sequence groups comprising 32 isolates; one sequence group (representative sequence #16) comprised five isolates with no sexual linkages; each of the other seven sequence groups contained two isolates and five of them were sexual pairs. Each of the other 13 sequence groups in the porB1b-3 cluster contained a single isolate (Table S1).

Congruence of porB sequence typing and epidemiological linkages

The porB sequences of N. gonorrhoeae isolates (n = 61) from 29 epidemiologically identified pairs as well as an additional male patient having two female partners were analysed for congruence (Figure 3a). Fifteen of these isolates exhibited porB1a genotypes which were distributed into four sequences: one sequence comprised seven isolates including two pairs (56/56F and 61/61F) and the epidemiologically linked triplet (75/75F1/75F2); a second sequence comprised two pairs (26/26F and 162/162F); each of the other porB1a sequences comprised one pair (63/63F and 164/164F, respectively). Forty-six isolates comprising 23 pairs were typed as porB1b representing 20 DNA sequences (Figure 3a): one sequence comprised 12 isolates from six pairs (34/34F, 64/64F, 89/89F, 101/101F, 134/134F and 135/135F); each of other 15 sequences comprised one pair (Figure 3a). Two epidemiologically linked pairs (41/41F and 141/141F) exhibited different porB1b sequences (Figure 3a, asterisks).

In order to better illustrate the correlation between porB sequence and epidemiologically linked isolates, DNA sequence and epidemiological data were plotted (Figure 3b and c). All linked porB1a isolates exhibited identical porB sequences (Figure 3b); seven isolates comprising two sexual pairs and the
linked trio had an identical sequence, and two pairs shared a porB1a sequence (Figure 3b, open dashed rectangles). Of 23 sexually linked porB1b pairs, 21 pairs had identical sequences (Figure 3c), while 2 pairs showed different porB sequences between isolates (Figure 3c, dashed circle); 12 isolates from six sexual pairs shared an identical porB1b sequence (Figure 3c, open dashed rectangles).

**Antimicrobial susceptibility and porB types**

The antimicrobial susceptibilities of 143 clinical gonococcal isolates (porB1a, n = 39; porB1b, n = 104) from male patients have been reported previously. Most isolates were resistant to penicillin (porB1a 89.8% and porB1b 95.3%), ciprofloxacin (porB1a 91.5% and porB1b 99.2%) and tetracycline (porB1a 38.4% and porB1b 62.5%). More than 95% of the isolates were susceptible to spectinomycin, and all isolates were susceptible to ceftriaxone, although decreasing levels of susceptibility were noted (Figure 4). The percentage of tetracycline-resistant isolates was significantly higher in porB1b isolates than in porB1a isolates (P < 0.025). Nevertheless, porB1b isolates had higher MIC50s to penicillin (≥64.0 mg/L) or ciprofloxacin (8.0 mg/L) than porB1a isolates (8.0 mg/L for penicillin and 3.0 mg/L for ciprofloxacin). Plasmid-mediated resistance to penicillin and tetracycline accounted for 59.0% (porB1a) and 57.7% (porB1b) of the isolates, and there was no significant difference between the two genotypes. The percentage of porB1a or porB1b isolates with chromosomally mediated resistance to penicillin and tetracycline was 48.7% and 65.4%, respectively, and the latter (porB1b) was significantly higher (Table 2). The percentages of CMPR, CMTR or CMRNG for porB1b isolates were 21.2%, 24.0% and 20.2%, respectively, whereas those of porB1a isolates...
were 38.5%, 5.1% and 5.1%, respectively (data not shown). A few isolates had MICs to penicillin at intermediate levels, but there was no significant difference of percentages between \( \text{porB}_{1a} \) (10.3%, \( n = 4 \)) and \( \text{porB}_{1b} \) (4.8%, \( n = 5 \)) phenotypes. The percentages of tetracycline susceptible isolates (S) and isolates with intermediate levels of MICs (I) were significantly higher in \( \text{porB}_{1a} \) (61.5%) than \( \text{porB}_{1b} \) (37.5%) isolates (Table 2). There was no difference in the MIC 50s to spectinomycin (16.0 mg/L) or ceftriaxone (0.03 mg/L) between \( \text{porB}_{1a} \) and \( \text{porB}_{1b} \) isolates (Figure 4). The MIC 50's to ciprofloxacin for \( \text{porB}_{1b} \) isolates (32.0 mg/L) was significantly higher than that for \( \text{porB}_{1a} \) isolates (8.0 mg/L), while the MIC 50's to penicillin, tetracycline, spectinomycin and ceftriaxone were not significantly different between \( \text{porB}_{1a} \) and \( \text{porB}_{1b} \) isolates (data not shown).

**Discussion**

\( \text{porB} \) sequence analysis has been a useful method for studying the molecular epidemiology of \( N. \) gonorrhoeae isolates including identifying circulating clusters, \(^{4,10-13} \) tracking strain transmission networks \(^{8,9,11,14,15,47} \) and investigating mechanisms of antibiotic resistance. \(^{15,16} \) This method has a high discriminatory index similar to NG-MAST. \(^{13} \) Furthermore, sequence analysis of six variable regions (VRs) of \( \text{porB} \) has the same discriminatory

**Figure 3.** Congruence of \( \text{porB} \) types and sexual contacts. (a) Average distance tree of 61 \( \text{porB} \) sequences from 30 sexual partnerships. Branch lengths shown are proportional to the amount of genetic change. The identity of each sequence is shown at the tips of the branches; for example, #63 represents sequence number 63 from a male patient and 63F for the sequence from the female partner of #63. Stars indicate that sequences of the two epidemiologically linked pairs were different. Vertical lines indicate isolates that carry an identical sequence. (b and c) Congruence of \( \text{porB} \) sequence analysis and epidemiological data. The diagrams are drawn based on the phylogenetic tree in (a). Open rectangles, isolates from male index patients; filled circles, isolates from females; solid line, isolates with an identical \( \text{porB} \) sequence. Epidemiological linkages depicted by open circles. Open dashed rectangles represent isolates from several pairs sharing an identical \( \text{porB} \) sequence. Dashed circles are isolates from sexual pairs having different \( \text{porB} \) sequences (c). The black arrow represents the trios.

**Figure 4.** Antimicrobial susceptibility and \( \text{porB} \) types of 143 \( N. \) gonorrhoeae isolates from male patients. Bars indicate percentages of isolates that were classified as susceptible (white), intermediate (hatched) and resistant (black). PEN, penicillin; TET, tetracycline; CIP, ciprofloxacin; SPT, spectinomycin; CRO, ceftriaxone. Stars indicate statistically significant difference of TET resistance between the two \( \text{porB} \) types.

Mutations at residues G120 and A121 of gonococcal PorB proteins (i.e. PIB and PIA) are associated with decreased susceptibility to antibiotics. \(^{16,17} \) Of 104 PIB isolates in Shanghai (Table 3), 98.1% (102/104) carried mutations at residue G120: 84.6% (88/104) with a G120K mutation, 11.5% (12/104) with a G120D mutation, 1% (1/104) with a G120R and 1% (1/104) with a G120N mutation. The majority of the PIB isolates (87.5%, 91/104) had mutations at residue A121: A121D, A121G, A121N or A121H mutations accounted for 73.1% (76/104), 6.7% (7/104), 6.7% (7/104) or 1% (1/104) of the sequences, respectively. Double mutations of PIB G120/A121 were observed in 86.5% (90/104) of the isolates, including G120K/A121D (71.1%, 74/104), G120K/A121G (6.7%, 7/104), G120K/A121N (5.7%, 6/104), G120K/A121H (1%, 1/104), G120R/A121D (1%, 1/104) and G120N/A121D (1%, 1/104). A single G120 mutation (G120D) was noted in 11.5% (12/104) of the sequences, while a single A121 mutation (A121D) was observed in 1% (1/104). Two PIB isolates (2/104) did not carry any mutations at these two residues (Table 3). The mutations G120R or A121N have not been reported previously. Of 39 PIA isolates, the G120 mutation, G120D, was observed in 82.1% (32/39) of the sequences, while the rest (7/39) did not have a G120 mutation. All PIA isolates had a mutation at residue A121 (A121G). Double mutations of G120D/A121G were observed in 82.1% of PIA isolates, while 17.9% of PIA isolates had a single A121G mutation (Table 3).
power as sequencing the entire gene.\textsuperscript{6,22,48,49} This study aimed to distinguish circulating strain types of \textit{N. gonorrhoeae} in Shanghai and to determine the discriminatory powers of various gene sequence analysis methods. We demonstrated that \textit{porB} sequence typing alone has ID of 95%, very similar to multiple locus analysis of \textit{porB} (ID \textgreek{g}= 99%).

We identified 78 \textit{porB} sequences (15 \textit{porB}1a and 63 \textit{porB}1b) from 174 clinical isolates in Shanghai. The majority of these sequences (75/78) had not been previously reported to the GenBank database except for two \textit{porB}1a sequences and one \textit{porB}1b sequence. The \textit{porB}1a sequence (GenBank accession \#540667) from our study was identical to a \textit{porB}1a sequence (GenBank accession \#AF09018)\textsuperscript{37} reported from Nairobi, Kenya. Another of our \textit{porB}1a sequences (GenBank accession EF540668) was identical to a sequence previously reported (GenBank accession \#AF304403)\textsuperscript{7} for an isolate from Baltimore, MD, USA. One of our \textit{porB}1b sequences (GenBank accession \#EF540666) was identical to a previously reported \textit{porB}1b sequence (GenBank accession \#AF304396)\textsuperscript{7} from Baltimore, MD, USA.

Our results demonstrate that the \textit{porB}1b genotype of \textit{N. gonorrhoeae} is predominant in Shanghai. The geographical distribution of \textit{N. gonorrhoeae} isolates with \textit{porB}1a and \textit{porB}1b genotypes has been studied in several countries/regions. For example, the \textit{porB}1b type is more prevalent than the \textit{porB}1a type in \textit{N. gonorrhoeae} isolates in Germany,\textsuperscript{29} Russia,\textsuperscript{50} Sweden\textsuperscript{51} and the USA.\textsuperscript{48} Prior to DNA sequence analysis to establish the prevalence of \textit{porB} types, serological analysis was used to indicate temporal and geographic differences between \textit{porB}1a and \textit{porB}1b isolates.\textsuperscript{48} However, these studies used highly selected isolates and may not be fully representative of the broader \textit{N. gonorrhoeae} population. The reason for type differences in different regions is not well understood, although host physiological factors, behavioural characteristics and control programs such as antibiotic use may play roles in evolutionary selection.\textsuperscript{53,54}

Our results confirm that \textit{porB} sequence analysis was congruent with identified sexual contacts, confirming epidemiological information. However, a small number of patient self-identified sexual contacts exhibited different \textit{porB} sequences, suggesting that the corresponding male and/or female patients may have had multiple sexual partners infected with different \textit{N. gonorrhoeae} strains or that the patients may have had a mixed strain infection; mixed strain infections are common in sexually active groups.\textsuperscript{12,55} To detect multiple strains from cultures, \textit{porB} sequence typing of multiple individual colonies from the primary isolation plate is required. Alternatively, \textit{opa}-typing of multiple individual colonies\textsuperscript{25} or \textit{porB} VR typing from clinical non-cultured samples\textsuperscript{12,49} can be used; however, these methods were not included in the present study.

As reported in previous studies,\textsuperscript{4,7} several clusters with an identical \textit{porB} sequence but not associated through epidemiological connections were present in Shanghai. For example, three groups in the \textit{porB}1a cluster were identified comprising 14, 15 and 5 isolates, respectively. In \textit{porB}1b isolates, there were four groups each comprising 33, 4, 5 and 4 isolates, respectively. These groups were not identified through epidemiological methods. Furthermore, seven groups with two isolates each were classified by \textit{porB} sequence analysis while there were no identified epidemiological connections between the two isolates (Figure 2 and Table S1). Because discussions about sex are not well accepted in China, the patient self-reported sexual linkages are likely underestimated and thus sexual networks created from this information cannot be readily observed. This may result in discordance between \textit{porB} typing and sexual linkage data. It

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**Table 2. Antimicrobial resistant profiles of \textit{porB}1a and \textit{porB}1b \textit{N. gonorrhoeae} isolates**

<table>
<thead>
<tr>
<th>Antimicrobial resistant profiles</th>
<th>\textit{porB}1a, \textit{n}=39</th>
<th>\textit{porB}1b, \textit{n}=104</th>
<th>\textit{P value}\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid-mediated resistance (PPNG, PP/TRNG and TRNG)</td>
<td>23 (59.0%)\textsuperscript{b}</td>
<td>60 (57.7%)\textsuperscript{c}</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Chromosomally mediated resistance (CMPR, CMTR and CMRNG)</td>
<td>19 (48.7%)\textsuperscript{b}</td>
<td>68 (65.4%)\textsuperscript{c}</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Penicillin I/S\textsuperscript{d}</td>
<td>4 (10.3%)\textsuperscript{b}</td>
<td>5 (4.8%)\textsuperscript{c}</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Tetracycline I/S\textsuperscript{d}</td>
<td>24 (61.5%)\textsuperscript{b}</td>
<td>39 (37.5%)\textsuperscript{c}</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\text{PPNG, penicillinase-producing (PP) \textit{N. gonorrhoeae}, TRNG, plasmid-mediated tetracycline resistant \textit{N. gonorrhoeae}; CMPR, chromosomally mediated penicillin resistance; CMTR, chromosomally mediated tetracycline resistant; CMRNG, chromosomally mediated resistant \textit{N. gonorrhoeae}.}

\textsuperscript{a}P values were determined by \textit{\textgreek{chi}2} tests.

\textsuperscript{b}Numbers in parentheses are percentages of total number of \textit{porB}1a isolates.

\textsuperscript{c}Numbers in parentheses are percentages of total number of \textit{porB}1b isolates.

\textsuperscript{d}Susceptible isolates (S) or isolates with intermediate levels of MIC (I).
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should also be noted that isolates with identical porB sequences could possibly be further differentiated using different typing methods, such as opa-typing and MLST methods. Therefore, in-depth analysis of demographic, social and geographical data may reveal better concordance and, in combination with genotypic data, will better define specific transmission clusters.

China has experienced an increasing burden of antimicrobial resistance of N. gonorrhoeae isolates. This has provoked the ongoing review and modification of guidelines so that recommended antibiotics for the treatment of gonorrhoea are effective. The current recommended treatment of choice for uncomplicated gonococcal infections in Shanghai, China, includes ceftriaxone or spectinomycin, whereas penicillin, tetracycline and quinolones are no longer recommended. It would be useful to compare porB gene sequences with previously reported serovars so that comparison can be made for temporal trends and continuity of typing data.

Specific mutations in porB1b (spontaneously or experimentally) have been associated with decreased susceptibility to hydrophilic antibiotics such as β-lactams and tetracyclines. Mutations causing this decreased susceptibility include amino acid replacements of the hydrophobic amino acids glycine G120 and alanine A121 by lysine (K) and aspartate (D), respectively, i.e. G120K or A121D. Between 40% and 47% of porB1b isolates have been reported to have these double mutations.

In our study, the majority of porB1b isolates (71.1%) exhibited G120K/A121D double mutations, remarkably higher than previously reported, suggesting that these mutations may play a significant role in the high percentages of penicillin and tetracycline resistance in N. gonorrhoeae isolates from Shanghai. We discovered that porB1b G120 or A121 can be replaced by amino acids other than lysine and aspartate such as arginine (R), histidine (H) or asparagine (N). The novel replacements at these two residues include G120R, G120N, A121R, A121N or A121H, accounting for 14.5% of the porB1b isolates. It is presently unknown whether these mutations confer gonococcal antibiotic resistance as well and this hypothesis should be confirmed experimentally. The porB1a isolates exhibited different mutations at G120 and A121 residues than porB1b. The majority of our porB1a isolates (82.1%) had G120D/A121G double mutations, similar to porB1a isolates from Baltimore, MD, USA, which had G120D/A121D double mutations. However, A121 was replaced by a hydrophilic amino acid (G) in our isolates, while in Baltimore isolates it was replaced by a hydrophilic amino acid (D). The impact on antimicrobial susceptibility of these differences needs to be further investigated as there was no susceptibility data shown for the Baltimore strains.

In summary, this study illustrates the porB-typing based molecular epidemiology of N. gonorrhoeae from Shanghai. porB sequence analysis is a useful tool to track strain transmission and to provide additional information on sexual networks to the client’s self-reporting methods. porB sequence analysis can provide more information on sexual networks when compared with conventionally obtained epidemiological data, revealing hidden epidemiological linkages. The G120K/A121D mutations in porB1b were common in N. gonorrhoeae isolates from Shanghai and were associated with chromosomally mediated penicillin and tetracycline resistance. Novel replacements at the residues G120 and A121 were identified, and the association with antimicrobial resistance in N. gonorrhoeae warrants further investigation.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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