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NG-STAR genotypes are associated with MDR in *Neisseria gonorrhoeae* isolates collected in 2017 in Shanghai

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Objectives: To determine the association of *Neisseria gonorrhoeae* antimicrobial resistance and genotypes using *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR).

Methods: We characterized 124 *N. gonorrhoeae* isolates for their antimicrobial susceptibility profiles and NG-STAR ST characteristics using the guidelines of CLSI and EUCAST. The NG-STAR STs of seven loci were analysed. *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) and MLST analysis was conducted in isolates with specific NG-STAR STs.

Results: NG-STAR differentiated 124 *N. gonorrhoeae* isolates into 84 STs, of which 66 STs were novel to the NG-STAR database. NG-STAR ST-199, ST-348, ST-428, ST-497 and ST-1138 were the predominant STs. Three *N. gonorrhoeae* isolates with ceftriaxone and cefixime MICs $\geq$1.0 mg/L were grouped as NG-STAR ST-233. NG-STAR ST-202 isolates (n=4) were associated with high azithromycin MICs and had an identical NG-MAST ST. The NG-STAR ST-348 group (n=5) comprised more isolates with reduced susceptibility to cefixime (n=4) than cefixime-susceptible isolates (n=1).

Conclusions: NG-STAR analysis differentiated *N. gonorrhoeae* isolates in settings with a high prevalence of antimicrobial resistance. Specific NG-STAR STs are associated with reduced susceptibility to ceftriaxone or cefixime and resistance to azithromycin in *N. gonorrhoeae*.

Introduction

Neisseria gonorrhoeae has developed antimicrobial resistance to most antimicrobials in the past few decades.[1–3] Molecular determinants of chromosomally mediated resistance have been identified.[4] Quinolone resistance of *N. gonorrhoeae* is associated with the quinolone resistance determinants in the *gyrA* and *parC* genes.[5] Penicillin resistance and reduced susceptibility to the third-generation cephalosporins (cefixime or ceftriaxone) have been associated with mutations in the *penA*, *porB*, *mtrR* and *ponA* genes.[4] Determinants of high-level tetracycline resistance include the presence of *tet*(M) or mutations in *rpsJ*.[6] Azithromycin resistance is associated with mutations in *mtrR* and 23S rRNA.[7]

Molecular typing methods have been used to differentiate genotypes of *N. gonorrhoeae* isolates and to study the association between *N. gonorrhoeae* genotypes and antimicrobial resistance phenotypes, such as *porB* sequence typing,[8] MLST,[8] *N. gonorrhoeae* multiantigen sequence typing (NG-MAST)[8,9] and *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR).[10] NG-STAR STs are determined using an allelic profile of the seven antimicrobial-resistance-associated loci: *penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC* and 23S rRNA.[10] NG-STAR analysis has been used in studies of *N. gonorrhoeae* isolates from Canada,[10] Australia[11,12] and Japan.[13] Specific NG-STAR STs are associated with unique antimicrobial resistance phenotypes. Reduced susceptibility to ceftriaxone or cefixime is associated with NG-STAR ST-90; resistance to azithromycin is associated with NG-STAR ST-58, ST-61 and ST-64.[10] This is the first study in China, to the best of our knowledge, that has employed the NG-STAR database to differentiate antimicrobial-resistant *N. gonorrhoeae* recovered from settings with a high prevalence of *N. gonorrhoeae* antimicrobial resistance.

Table 1. Association of NG-STAR STs with MICs for 124 *N. gonorrhoeae* isolates collected in 2017 in Shanghai

NG-STAR ST[a]	No. of isolates	Ceftriaxone MIC range (mg/L)	Cefixime MIC range (mg/L)	Azithromycin MIC range (mg/L)	Ciprofloxacin MIC range (mg/L)	Penicillin MIC range (mg/L)	Tetracycline MIC range (mg/L)
38	3	0.008–0.06	0.015–0.125	0.03–0.25	2.0–16.0	0.25–16.0	0.5–2.0
199	7	0.03–0.06	0.03–0.06	0.06–0.5	4.0–16.0	1.0–16.0	0.5–32.0
202	4	0.015–0.06	0.03–0.06	8.0	8.0	2.0–16.0	32.0
233	3	≥1.0	≥1.0	0.125–0.25	16.0	2.0–4.0	1.0–2.0
346	2	0.06	0.06–0.125	0.25	16.0	2.0–16.0	2.0–4.0
348	5	0.03–0.125	0.015–1.0	0.06–0.25	16.0	1.0–4.0	1.0–2.0
428	5	0.03–0.25	0.06–0.25	0.06–0.5	8.0–16.0	1.0–16.0	1.0–16.0
497	5	0.03–0.25	0.06–0.25	0.125–1	8.0–16.0	1.0–32.0	1.0–4.0
501	2	0.06–0.125	0.06–0.125	0.06–0.25	16.0	2.0–4.0	1.0
1138	5	0.015–0.06	0.03–0.25	0.25–2	16.0	4.0–16.0	2.0–4.0
1144	2	0.03	0.06	0.125–0.25	8.0–16.0	16.0	32.0
1146	3	0.06	0.06–0.125	0.125–0.25	2.0–8.0	2.0–16.0	1.0
1150	2	0.03	0.06–0.125	0.125	8.0	1.0–16.0	16.0
1151	2	0.03–0.06	0.06–0.125	0.25	8.0–16.0	2.0	2.0–16.0
1155	2	0.03–0.06	0.06–0.125	0.25–0.5	4.0–8.0	1.0–2.0	16.0
1187	3	0.06–0.125	0.125	0.125–0.5	16.0	4.0–16.0	2.0–4.0
1190	2	0.06	0.06	0.25	16.0	16.0	1.0
Other STs (67)[b]	1/ST	0.008–0.25	0.008–1.0	0.003–16.0	0.004–16.0	0.5–16.0	0.125–32.0
Total no. of STs = 84	total = 124	0.008 to ≥1.0	0.008 to ≥1.0	0.003–16.0	0.004–16.0	0.25–16.0	0.125–32.0

[a]Eight STs in bold are novel to the NG-STAR database.
[b]STs with single isolates are STs 51, 90, 138, 493, 496, 506, 515, 909, 1109, **1136**, **1137**, **1140–1143**, **1145**, **1147–1149**, **1152–1154**, **1156–1186**, **1188**, **1189**, **1191–1198**, **1217**, **1436**, **1463** and **1464**, among which the 58 STs in bold are new to the NG-STAR database.

Materials and methods

N. gonorrhoeae isolates were collected from male patients at Shanghai Skin Disease Hospital from January to December 2017 through the national Gonococcal Antimicrobial Susceptibility Surveillance Program. The first 10 *N. gonorrhoeae* isolates of each month were used in this study (n=120). Additionally, a ciprofloxacin-susceptible *N. gonorrhoeae* isolate and three *N. gonorrhoeae* isolates with ceftriaxone or cefixime MICs ≥1.0 mg/L, collected in 2017, were also included in this study. These 124 *N. gonorrhoeae* isolates were isolated and identified as previously described.[5] *N. gonorrhoeae* isolates were cultivated on Thayer–Martin (T–M) medium supplemented with 1% IsoVitaleX and identified using the oxidase test, Gram staining and glucose utilization tests. MICs for six antimicrobials were determined using the agar dilution method according to CLSI.[14] *N. gonorrhoeae* ATCC 49226 was used as a reference strain. The criteria for resistance phenotypes were as follows: MICs ≥2 mg/L for penicillin or tetracycline; MICs ≥1 mg/L for ciprofloxacin;[14] MICs ≥1 mg/L for azithromycin; and MICs ≥0.25 mg/L for ceftriaxone or cefixime, using the breakpoints described previously.[15]

For genotyping, specific primers were used to amplify and to sequence various loci (Table S1, available as Supplementary data at *JAC* Online). Seven NG-STAR loci (*penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC* and 23S rRNA) were PCR-amplified as previously described.[5] DNA sequencing was performed at Sangon Biotech Co. DNA sequences were analysed and edited using Geneious and Vector NTI.

NG-STAR analysis was conducted using the NG-STAR database (https://ngstar.canada.ca).[10] NG-MAST was performed using the *porB* and *tbpB* loci (Table S1)[9] and NG-MAST STs were assigned using the NG-MAST database (http://www.ng-mast.net). MLST analysis was performed using seven loci (*abcZ*, *adk*, *aroE*, *fumC*, *phdC*, *gdh* and *pgm*) (Table S1)[8] and MLST STs were assigned using the MLST database (https://pubmlst.org/neisseria).

Phylogenetic analysis based on seven NG-STAR loci was performed using Molecular Evolutionary Genetics Analysis (MEGA) and neighbour-joining trees and bootstrapping were analysed (https://www.megasoftware.net). Simpson's diversity index was used to evaluate the discriminatory levels of NG-STAR.[16] Statistical analysis was performed using Fisher's exact test (SPSS Statistics version 22.0). A P value of <0.05 was considered statistically significant. DNA sequences were submitted to GenBank of the NCBI. The GenBank accession numbers are listed in Table S2.

Results

A high level of antimicrobial resistance was observed among the 124 *N. gonorrhoeae* isolates (Table S3). The proportions of *N. gonorrhoeae* isolates with resistance to penicillin, tetracycline, ciprofloxacin and azithromycin were 81.5% (n=101), 59.7% (n=74), 99.2% (n=123) and 8.1% (n=10), respectively. The proportions of *N. gonorrhoeae* isolates with resistance to ceftriaxone or cefixime (MICs ≥0.25 mg/L) were 6.5% (n=8) and 16.9% (n=21), respectively.

NG-STAR analysis differentiated the 124 *N. gonorrhoeae* isolates into 84 STs. There were 66 new NG-STAR STs, comprising 79 *N. gonorrhoeae* isolates (Table 1). ST-199 (n=7) was the predominant ST. The next most common STs were ST-348, ST-428, ST-497 and ST-1138; each of these STs was represented by five *N. gonorrhoeae* isolates. Four isolates were ST-202. Four NG-STAR STs were each associated with three isolates. Seven NG-STAR STs were each represented by two isolates. Sixty-seven NG-STAR STs were each associated with a single isolate. Three *N. gonorrhoeae* isolates with ceftriaxone or cefixime MICs ≥1.0 mg/L exhibited NG-STAR ST-233, having PenA type 60. Four *N. gonorrhoeae* isolates with NG-STAR ST-202 showed an identical NG-MAST ST (ST1866) and two MLST STs (ST10899 and ST12039). Five *N. gonorrhoeae*

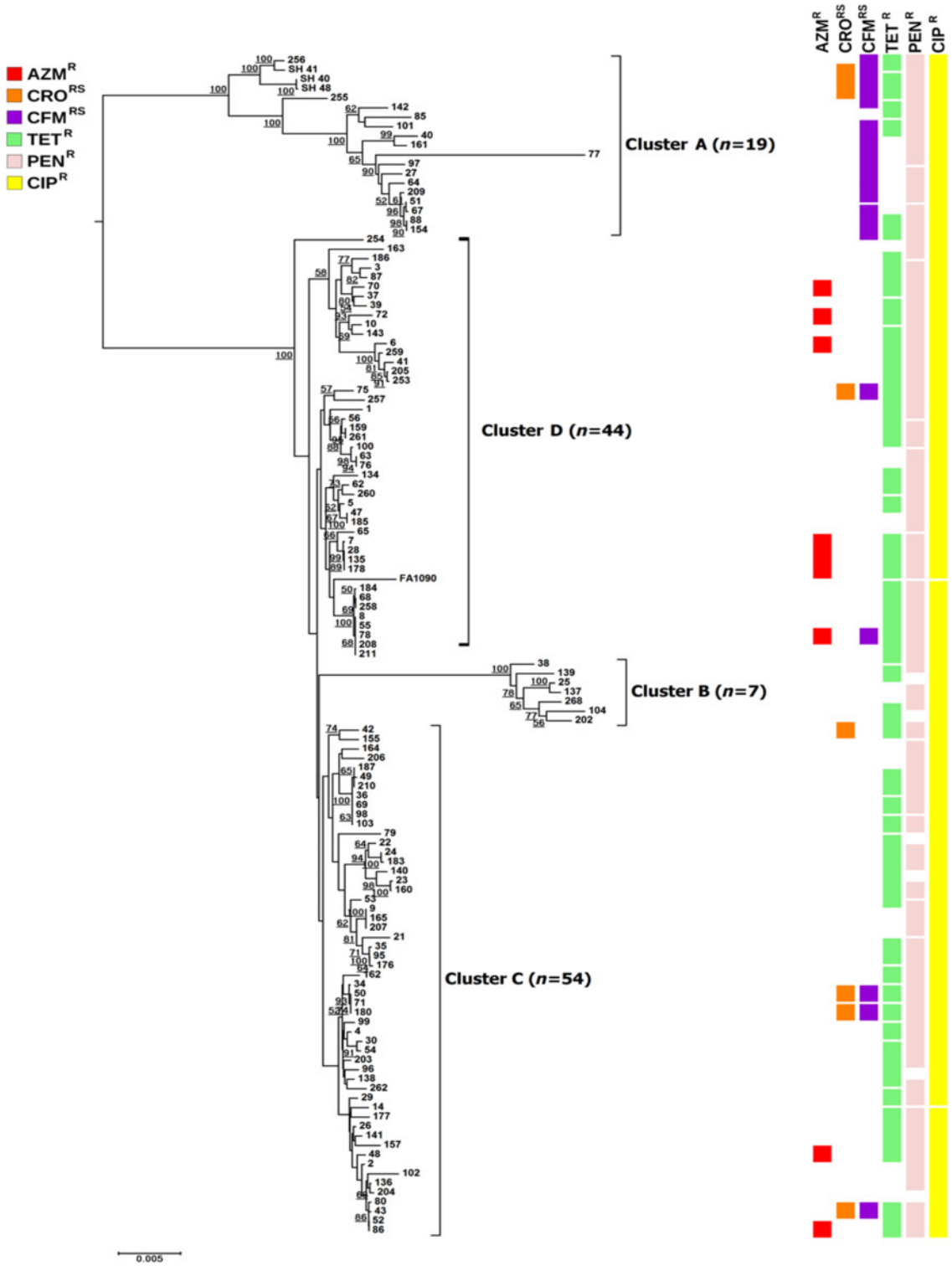

Figure 1. Phylogenetic analysis based on the seven loci for NG-STAR. The phylogenetic tree was constructed using MEGA 6 software. Horizontal lines are proportional to phylogenetic distance. Resistance phenotypes are shown as coloured squares for each isolate and each square represents one isolate. Numbers on the tips are *N. gonorrhoeae* isolate numbers. Bootstrap values of ≥50 are shown at the nodes of the tree branches. Four clusters (clusters A–D) are defined arbitrarily. AZM^{R}, azithromycin resistance; CRO^{RS}, reduced susceptibility to ceftriaxone; CFM^{RS}, reduced susceptibility to cefixime; TET^{R}, tetracycline resistance; PEN^{R}, penicillin resistance; CIP^{R}, ciprofloxacin resistance.

isolates with NG-STAR ST-348 had four different NG-MAST STs and two MLST STs (ST7363 and ST14283). The discrimination index of NG-STAR was 0.989.

Phylogenetic analysis based on the seven NG-STAR loci differentiated the *N. gonorrhoeae* isolates into four clusters (Figure 1). Cluster A had 15.3% (19/124) of the isolates, including 37.5% (3/8) of the isolates with reduced susceptibility to ceftriaxone and 76.2% (16/21) of the isolates with reduced susceptibility to cefixime. The three isolates with ceftriaxone and cefixime MICs ≥1.0 mg/L were present in cluster A. Cluster B contained seven isolates, which had ciprofloxacin resistance phenotypes (resistance to both ciprofloxacin and penicillin, or resistance to both ciprofloxacin and tetracycline). The *N. gonorrhoeae* isolates in clusters C (n=54) and D (n=44) had diverse resistance phenotypes. *N. gonorrhoeae* isolates with azithromycin resistance were scattered across the tree.

Discussion

This is the first study, to the best of our knowledge, to investigate the association of NG-STAR STs and antimicrobial resistance in *N. gonorrhoeae* clinical isolates from settings in which MDR is prevalent. We found that *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone or cefixime could be grouped into a distinguishable cluster. Three isolates with ceftriaxone or cefixime MICs ≥1.0 mg/L exhibited a single NG-STAR type (ST-233) and appeared in the same cluster. Our results suggest that NG-STAR STs are associated with antimicrobial resistance phenotypes and can be potentially used in determining clonal expansion of antimicrobial resistance in settings in which MDR is prevalent.

In our study, the majority of NG-STAR STs were novel to the NG-STAR database (66/84), similar to that observed in a previous report.[11] The most prevalent NG-STAR STs in our study were ST-199, ST-348, ST-428, ST-497 and ST-1138; whereas NG-STAR ST-90, ST-42, ST-91, ST-64 and ST-139 were most common in Canada[10] and the frequencies of NG-STAR ST-755 and ST-NV9 were high in Australia.[11] This regional distribution of NG-STAR STs suggests a correlation with geographic locations.

As previously reported,[10,12] specific NG-STAR STs tended to be associated with characteristic antimicrobial resistance phenotypes such as ST-233 isolates with high ceftriaxone and cefixime MICs (≥1.0 mg/L). The first isolate reported as NG-STAR ST-233 was from Japan in 2015 (FC428).[12] NG-STAR ST-233 was also reported in 2017 from Australia,[12] Canada[12] and France,[17] followed by a report from the UK in 2018.[18] The NG-STAR ST-233 isolates reported in all of these countries were associated with travel to Southeast Asia. The NG-STAR ST-233 *N. gonorrhoeae* isolates would, to the best of our knowledge, be the first report in China. However, further investigation of the social or epidemiological connections of the patients is warranted. In-depth genomic analysis would provide detailed information on the association of NG-STAR ST-233 and clone FC428. The NG-STAR STs associated with reduced susceptibility to ceftriaxone (ST-91 or ST-97) reported by another group[10] were not observed in this study, which might be due to the criteria for reduced susceptibility to ceftriaxone used in the Canadian report (≥0.06 mg/L).[10]

The percentage of isolates identified as NG-STAR ST-202 was significantly higher among azithromycin-resistant *N. gonorrhoeae* isolates than azithromycin-susceptible isolates (P<0.001), indicating that ST-202 is associated with resistance to azithromycin, which is consistent with another report.[10] *N. gonorrhoeae* isolates identified as NG-STAR ST-202 (n=4) exhibited an identical NG-MAST ST (ST1866) and two MLST STs (ST10899 and ST12039). It has been reported that NG-MAST ST1866 and MLST ST10899/ST12039 were the predominant STs among high-level azithromycin-resistant *N. gonorrhoeae* isolates.[19] The percentage of isolates identified as NG-STAR ST-348 was significantly higher among *N. gonorrhoeae* isolates with reduced susceptibility to cefixime (4/21) than that for isolates susceptible to cefixime (1/103) (P<0.05), indicating that NG-STAR ST-348 could be associated with reduced susceptibility to cefixime. However, the five *N. gonorrhoeae* isolates with NG-STAR ST-348 had various cefixime MICs and had four different NG-MAST STs and two MLST STs (Table 1).

This study only investigated a low number of isolates (<2%) from all of the 5711 *N. gonorrhoeae* cases reported in Shanghai in 2017 (data not shown). However, it is representative of the institution where the isolates were collected. A study with a larger sample size is required to extrapolate a broader strain distribution.

In conclusion, we preliminarily characterized NG-STAR STs in *N. gonorrhoeae* in Shanghai. The majority of NG-STAR STs were novel to the database. The most prevalent NG-STAR STs differed from those in the reports from Canada and Australia. NG-STAR ST-233 is associated with high ceftriaxone or cefixime MICs (≥1.0 mg/L). NG-STAR ST-348 is associated with reduced susceptibility to cefixime. NG-STAR ST-202 is associated with azithromycin resistance in *N. gonorrhoeae*.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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