MICROBIAL DRUG RESISTANCE
Volume 00, Number 00, 2016

DOI: 10.1089/mdr.2016.0086

Genetic Resistance Determinants for Cefixime and Molecular Analysis of Gonococci Isolated in Italy

Anna Carannante,[1] Paola Vacca,[1] Valeria Ghisetti,[2,*] Maria Agnese Latino,[3,*] Marco Cusini,[4,*] Alberto Matteelli,[5,*] Caterina Vocale,[6,*] Grazia Prignano,[7,*] Christian Leli,[8,*] Patrizia Ober,[9,*] Raffaele Antonetti,[10,*] Federica Poletti,[11,*] and Paola Stefanelli[1]

A strictly defined subset of gonococci ($n=65$) isolated in Italy from 2011 to 2014 was characterized by antimicrobial susceptibility for cefixime (CFM) and ceftriaxone (CRO) and by sequencing of resistance determinant genes (*penA*, *mtrR*, *porB1b*, *ponA*) for extended-spectrum cephalosporins and *Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST). The *penA* mosaic alleles XXXIV and XXXV were found in all resistant (R) and decreased susceptibility (DS) gonococci to CFM, except for one. They were associated with an adenine deletion in the *mtrR* promoter plus amino acid substitutions, H105Y or G45D, in the coding region and *ponA* L421P. The *penA* mosaic allele XXXIV, and one variant, was found exclusively among genogroup (G) 1407 and its closely related sequence types (STs), as in CFM-DS as well as in CFM-R isolates. Single or combined mutation patterns in *penA*, *mtrR*, *porB1b*, and *ponA* genes were associated with different CFM susceptibility patterns and NG-MAST STs. Genotyping and antimicrobial resistance (AMR) determinant analyses can be valuable to enhance the gonococcal AMR surveillance.

Introduction

The prevalence of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* continues to increase and multidrug-resistant isolates have been reported worldwide.[1–5] Among the extended-spectrum cephalosporins (ESCs), cefixime (CFM) and more rarely ceftriaxone (CRO) resistance is spreading in *N. gonorrhoeae* isolates.[1–5] Furthermore, treatment failures to ESCs contribute to consider gonorrhea as a potentially untreatable disease.[4,6] In the literature, ESCs resistance determinants in *N. gonorrhoeae* is mostly attributable to polymorphism in *penA* gene, encoding for penicillin-binding protein 2 (PBP2).[7–9] Three important mosaic PBP2 residues (I312M, V316T, G545S) and epistasis in mosaic structure variant in *penA* gene are mentioned as causes of decreased susceptibility (DS) and resistant (R) to CFM and CRO.[7,10–13] Furthermore, the *penA* mosaic allele XXXIV, for the first time described in San Francisco in 2008,[14] is now considered associated to CFM-R gonococci.[14–16] Moreover, overexpression of the MtrCDE efflux pump, due to mutations in the promoter and in the coding region of the *mtrR* gene, and the decreased membrane permeability, caused by *porB1b* gene mutations, may contribute to DS and resistance to ESCs.[9] Finally, the role of *ponA* gene (encoding penicillin-binding protein 1 [PBP1]) remains undefined. Molecular epidemiology typing by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) identified sequence type (ST) 1407, and its genogroup (G), G1407, as the main clonal group associated with DS or R gonococci to ESCs.[15,17,18] The combination of genetic resistant determinants with NG-MAST provides valuable data concerning the emergence and spread of resistant strain.

[1]Department of Infectious, Parasitic, and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy.
[2]Laboratory of Microbiology and Virology, Amedeo di Savoia Hospital, Turin, Italy.
[3]Department of "Medicina di Laboratorio," P. O. Sant'Anna, Città della Salute e della Scienza di Torino, Turin, Italy.
[4]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico Milano, Milan, Italy.
[5]Clinic of Infectious and Tropical Diseases, University of Brescia, Brescia, Italy.
[6]Unit of Clinical Microbiology, CRREM Laboratory, St. Orsola-Malpighi, University Hospital, Bologna, Italy.
[7]San Gallicano Dermatologic Institute, IRCSS, Rome, Italy.
[8]Microbiology Section, Department of Experimental Medicine, University of Perugia, Perugia, Italy.
[9]Microbiology and Virology Laboratory, "Santa Chiara Hospital, Trento," Trento, Italy.
[10]Department of Clinical Pathology, "Azienda Ospedaliero-Universitaria OORR," Foggia, Italy.
[11]Department of Infectious Diseases, "Ospedali Castelli ASL VCO," Verbania, Italy.
*These authors contributed equally to this work.

In Italy, the annual proportion of CFM-R gonococci ranged around 2% in the period, with a peak of 3.3% in 2012.[19] We previously reported the results of an in-depth analysis of susceptibility to CFM and CRO in gonococci circulating in Italy up to 2010, together with genetic resistance determinants and NG-MAST analyses.[20] Herein, we investigated the antimicrobial susceptibility and the molecular characteristics of a subsample of *N. gonorrhoeae* isolates collected in Italy from 2011 to 2014, which were classified as susceptible (S), DS, and R to CFM.

Materials and Methods

N. gonorrhoeae *isolates and microbiological methods*

From 2011 to 2014, laboratories from local hospitals confirmed 900 gonorrhea cases by culture. Isolates were sent to the Istituto Superiore di Sanità (ISS) for antimicrobial susceptibilities and molecular epidemiology investigation. After growth on Thayer Martin medium (Oxoid Ltd.) with 1% IsoVitaleX (Oxoid Ltd.) at 37°C in a 5% CO_2 atmosphere, the antimicrobial susceptibility tests were performed following the European Gonococcal Antimicrobial Surveillance Program (EURO-GASP) guidelines.[21] In particular, antimicrobial susceptibility to CFM and CRO was assessed by Etest (bioMérieux) and MIC TEST STRIP method (Liofilchem Diagnostici), carried out in agreement with the manufacturer's instructions. MIC values were interpreted referring to the EUCAST clinical breakpoint criteria (version 6.0, 2016).[22] The World Health Organization (WHO) *N. gonorrhoeae* G, K, M, O, and P control strains were used in each assay.[23] Ethical approval was not required as clinical isolates were collected, processed, and stored as part of routine clinical care by the hospitals participating in the study. Anonymous data were analyzed at ISS using EpiInfo software, version 3.3.2.

Molecular analyses of penA, mtrR, porB1b, *and* ponA *genes, and NG-MAST analysis*

For molecular investigation purposes a total of 65 *N. gonorrhoeae* isolates were selected on the basis of susceptibility patterns to CFM, which included all viable CFM-R in our collection ($n = 25$, MIC value >0.125 mg/L), 25 DS (MIC range >0.064–0.125 mg/L), and 15 S (MIC range 0.016–0.064 mg/L) isolates. Chromosomal DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

Sequencing of *penA*, *mtrR*, *porB1b*, *ponA,* and *porB*, and *tbpB* genes for NG-MAST analysis, was achieved using primers and amplification parameters, as described elsewhere.[20,24,25] The *porB* and *tbpB* alleles were assigned according to the NG-MAST website (www.ng-mast.net), following the interpretative procedures.[25] Closely related STs were clustered using published definitions.[17,18] In particular, all STs, which shared one allele and showed >99% similarity in the other allele (≤5 bp difference for *porB* and ≤4 bp for *tbpB*), were included in the same genogroup.[17] Multiple sequence and amino acid alignments were obtained using ChromasPro version 1.15 and Clustal Omega website (www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Clinical data of patients

Out of the 65 selected gonococci, 59 were collected from men (90.8%) and 52 (80%) from patients aged ≥25 years. Fifty-three of the patients were Italians (81.5%) and 33 were men who have sex with men (56%). A total of 57 patients reported urethral discharge (87.7%) and 8 anorectal infection (12.3%). Seven patients (10.8%) were HIV infected and 5 (8%) had coinfections with other sexually transmitted pathogens.

Antimicrobial susceptibility

Of the 65 isolates, 25 were R (MIC >0.125 mg/L), 25 were DS (MIC range >0.064–0.125 mg/L) and 15 were S (MIC range 0.016–0.064 mg/L) to CFM.

All the 65 gonococci were fully susceptible to CRO. In particular, for 25 CFM-R gonococci, the ceftriaxone MIC range was 0.016–0.094 mg/L, for the 25 CFM-DS isolates the range was 0.016–0.064 mg/L, and for the 15 CFM-S gonococci it was 0.002–0.032 mg/L. Only one CFM-R isolate (MIC = 0.25 mg/L), collected in 2014, showed a DS to CRO with a MIC value of 0.094 mg/L.

penA, mtrR, porB1b, *and* ponA *mutation patterns*

The *penA* sequences were compared with those of *N. gonorrhoeae* strain LM306 (GenBank Accession No. M3209) and NG-3 (GenBank Accession No. AB071984). The *penA* mosaic allele was identified in 80% (52/65) of the total analyzed. As shown in Table 1, out of 25 CFM-R gonococci, 21 (84%) showed the *penA* profiles belonging to XXXIV family (XXXIV and XXXIV plus the A501V amino acid substitution).[16] Moreover, 4 (16%) isolates showed the *penA* mosaic allele XXXV (Table 1). The *penA* mosaic allele XXXIV was also found in all CFM-DS gonococci.

The 15 CFM-S gonococci, except for 2 with *penA* allele XXXIV and XXXVI, displayed the *penA* nonmosaic alleles, containing an aspartic acid insertion after position 345 (termed as D345a); 11 showed the *penA* allele IV or its variants (73.3%). As shown in Table 1, *penA* allele IV with two additional amino acid substitutions A501T and P551L, detected in this study (Accession No. KP677512), was found in six CFM-S gonococci (MIC range 0.016–0.023 mg/L); *penA* allele IV with an amino acid substitution G542S was found in one isolate; *penA* allele IV in four isolates and *penA* allele XIX in two isolates with MIC values of 0.023 and 0.064 mg/L, respectively. Finally, *penA* mosaic allele XXXIV[26] and XXXVI[27] were detected in two CFM-S isolates.

As shown in Table 1, all CFM-R and DS isolates harbored the single deletion of adenine (A) in the *mtrR* promoter region, wherever 10/15 (66.7%) of the CFM-S isolates showed the same deletion. A total of five mutation patterns were observed in *mtrR* gene. In particular, the amino acid substitution H105Y was predominant among CFM-R isolates (21/25, 84%). The amino acid substitution G45D in the DNA-binding motif of MtrR was found in four CFM-R gonococci (16%), whereas among CFM-DS was present exclusively the amino acid substitution H105Y (25/25, 100%). Among CFM-S isolates, 6 (40%) harbored the mutation pattern D79N/T86A/H105Y, 3 (20%) the amino acid substitution A39T, 3 (20%) the mutation pattern A39T/R44H, 2 (13.3%) the amino

TABLE 1. MUTATION PATTERNS OF *penA, mtrR, ponA, AND porB1*B GENES, ANTIMICROBIAL SUSCEPTIBILITY CATEGORIES TO CEFIXIME AND *NEISSERIA GONORRHOEAE* MULTIANTIGEN SEQUENCE TYPING RESULTS IN 65 GONOCOCCI

penA	mtrR *promoter*	mtrR	ponA	porB1b	*CFM*			*Sequence type* (tbpB; porB)	*Total*
					S	*DS*	*R*		
XXXIV	A del	H105Y	L421P	G120K/A121N	—	13	13	1407 (110; 908)	26
XXXIV	A del	H105Y	L421P	G120K/A121N	1	—	1	2212 (110; 1388)	2
XXXIV	A del	H105Y	L421P	G120K/A121N	—	—	1	5622 (110; 3411)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	—	1	3149 (110; 1903)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	—	1	4843 (1021; 908)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	8095 (110; 4826)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	2	—	4706 (110; 2851)	2
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	4936 (110; 2992)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	8826 (110; 5213)	1
XXXIV A501V	A del	H105Y	L421P	G120K/A121N	—	—	1	9829 (110; 5796)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	—	1	3499 (110; 2115)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	1	8096 (110; 4827)	2
XXXIV	A del	H105Y	L421P	wt	—	—	1	8343 (110; 266)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	10566[a] (1021; 6174)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	2	—	5339 (1128; 1388)	2
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	6210 (1262;1914)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	5843 (4; 1388)	1
XXXIV	A del	H105Y	L421P	G120K/A121N	—	1	—	11665 (6; 2078)	1
XXXV	A del	G45D	L421P	G120K/A121D	—	—	3	312 (113; 206)	3
XXXV	A del	G45D	L421P	wt	—	—	1	11712[a] (113; 6815)	1
IV A501T/P551L	A del	D79N/T86A/H105Y	L421P	G120K/A121D	2	—	—	6360 (563; 3957)	2
IV A501T/P551L	A del	D79N/T86A/H105Y	L421P	G120K/A121D	3	—	—	10128 (563; 5942)	3
IV A501T/P551L	A del	D79N/T86A/H105Y	L421P	G120K/A121D	1	—	—	11713[a] (563; 6876)	1
IV G542S	A del	A39T	L421P	G120K/A121D	1	—	—	10564 (6; 6172)	1
IV	wt	A39T/R44H	wt	wt	1	—	—	7576 (29; 1388)	1
IV	wt	A39T/R44H	wt	wt	1	—	—	5194 (29; 3149)	1
IV	wt	A39T/R44H	wt	A121S	1	—	—	7445 (24; 28)	1
IV	A del	G45D	wt	G120K/A121D	1	—	—	2997 (21; 866)	1
XIX	wt	A39T	L421P	G120K/A121G	1	—	—	10194 (1809; 997)	1
XIX	wt	A39T	L421P	G120K/A121G	1	—	—	6961 (137; 4157)	1
XXXVI	A del	H105Y	L421P	G120K/A121D	1	—	—	4980 (4; 3019)	1
Total					15	25	25		65

Light gray signifies Genogroup (G) 1407 and dark gray signifies Genogroup (G) 2400.
[a]New sequence types.
A del, adenine deletion in the *mtrR* promoter; CFM, cefixime; DS, decreased susceptibility; R, resistant; S, susceptible; wt, wild-type.

acid substitution H105Y and, finally, 1 isolate (6.7%) showed the G45D amino acid substitution in the DNA-binding motif of MtrR (Table 1).

The L421P substitution in the PBP1, encoded by the *ponA* gene, was detected in all isolates belonging to CFM-R and DS categories (25/25, 100%) and in 11/15 (73.3%) isolates belonging to CFM-S (Table 1).

PorB1b showed four mutation patterns: the G120K/A121N predominant in the CFM-R gonococci (20/25, 80%) and in one CFM-S isolate (1/15, 6.7%); three CFM-R and nine CFM-S isolates showed the G120K/A121D (12% and 60%, respectively) (Table 1). All the CFM-DS gonococci showed the G120K/A121N (25/25, 100%). The G120K/A121G pattern was found in two CFM-S gonococci (13.3%) and the A121S in one CFM-S isolate (6.7%) (Table 1). Moreover, the G101K and A102G amino acid substitutions were identified in all examined gonococci (data not shown). The combination of mutation patterns *penA* mosaic allele XXXIV, *mtrR* promoter A deletion, H105Y in the MtrR, the L421P in PBP1, and G120K/A121N in PorB1b, was mainly associated with CFM-R as well as DS isolates. Moreover, the combination of *penA* mosaic allele XXXV together with A deletion in *mtrR* promoter, G45D in the DNA-binding motif of MtrR, the L421P in PBP1 and G120K/A121D in PorB1b, was associated exclusively with CFM-R isolates.

N. gonorrhoeae *multiantigen sequence typing*

To examine whether there was a clonal expansion of a specific isolate among those analyzed, all selected gonococci were typed by NG-MAST. As shown in Table 1, among the 65 examined gonococci, 2 main genogroups have been identified: G1407 and G2400. Out of 37 belonging to G1407, 13 CFM-R and 13 CFM-DS had the ST1407 (*tbpB* 110, *porB* 908).

All gonococci belonging to G1407 showed the *penA* mosaic allele XXXIV, and its variant,[27] together with the A deletion in *mtrR* promoter, the substitution H105Y in MtrR, the G120K/A121N in PorB1b, and the L421P in PBP1. A

total of 10 gonococci (3 CFM-R and 7 CFM-DS) with different STs, not included in G1407, but with *penA, mtrR*, *porB1b*, and *ponA* genes identical to those found among isolates associated with G1407, have been identified. In particular, four isolates were associated with ST3499, ST8096, and ST8343, showing the *tbpB* 110. Moreover, one CFM-DS isolate was associated with ST10566, a new ST with *tbpB* 1021, 1 bp variant of *tbpB* 110, and *porB* 6174, 1 bp variant of *porB* 908. Two isolates with ST5339, characterized by *tbpB* 1128, 1 bp variant of *tbpB* 110, and by *porB* 1388, 1 bp variant of *porB* 908, were also detected (Table 1). Three CFM-DS isolates associated with ST6210, ST5843, and ST11665, with 1 or 2 bp of difference with *porB* 908, were recovered.

Finally, four CFM-R isolates were associated with ST312 and ST11712, both of them harboring the same *tbpB* 113 allele. The ST11712, with a new *porB* and *tbpB* allele combination, was associated with *penA* mosaic allele XXXV, the A deletion in *mtrR* promoter, G45D in the DNA-binding motif of MtrR, and the L421P in *ponA* gene.

Concerning the 15 CFM-S gonococci, 6 belonged to G2400 comprising ST6360, ST10128, and ST1173 (a new ST due to a new *porB* 6876 allele); all of them shared the same *tbpB* allele 563. These CFM-S isolates showed the *penA* nonmosaic allele IV variant with two additional amino acid substitutions A501T and P551L (Accession No. KP677512), the substitutions D79N/T86A/H105Y in the MtrR, the L421P in *ponA* gene, and the substitutions G120K/A121D in PorB1b. Finally, the remaining CFM-S isolates were associated with ST10564, ST7576, ST5194, ST7445, ST2997, ST10194, ST6961, and ST4980 as singletones and with *penA* nonmosaic alleles, except for one, and the amino acid substitutions A39T and R44H in the MtrR.

Discussion

Increased resistance to ESCs has been described among gonococci in recent years and associated with cases of treatment failure.[2–6,28] DS or resistance to ESCs is more likely related to *penA* gene mosaicism.[13,15] In fact, several studies have reported that the presence of *penA* mosaic allele XXXIV is associated to cefixime-resistant or decreased susceptible gonococci (MIC ranging from 0.032 to 0.25 mg/L) indicating that this mosaicism might play a role in the entire mechanism of susceptibility to ECSs.[14–17,26,27]

Our findings suggest that all CFM-R and DS gonococci, except for one, harbored the *penA* mosaic alleles XXXIV and XXXV. In particular, those belonging to the genogroup G1407, and its closely related STs, contained *penA* mosaic allele XXXIV combined with the A deletion in the *mtrR* promoter and the H105Y amino acid substitution in MtrR protein, together with L421P amino acid substitution in PBP1, as previously described.[4,16,29] Furthermore, the international epidemic clone G1407 emerged as the predominant among the cefixime-resistant gonococci of recent isolation in the country[19,20] suggesting a high rate of successful transmission.

Specific mutation patterns were found exclusively among CFM-S gonococci, as, for example, the *penA* nonmosaic allele (*i.e.*, IV and its variants), the A39T, R44H, D79N, T86A amino acid substitutions in MtrR and the G120K/A121G and A121S substitutions in PorB1b. Isolates containing *penA* nonmosaic allele, that is, IV, were most likely to be associated to cefixime-susceptible isolates and assigned to G2400 clonal group. Although the limited number of isolates analyzed it is possible to hypothesize that the synergistic mechanism of multiple mutations might contribute to the cefixime-resistant pattern. Taken together, our findings are in agreement to Thakur *et al.*,[30] Shimuta *et al.*,[12] and Unemo *et al.*[3] In particular, *penA* mosaicism, the A deletion in the *mtrR* promoter, and H105Y change in MtrR protein were associated with isolates with higher MIC values for cefixime; moreover, *penA* nonmosaic allele variants containing A501, G542, or P551 changes were found in isolates with low MIC values. However, as already reported, possibly unknown antimicrobial-resistant determinants could not to be ruled out to play a role in the development and in the spreading of a specific resistant isolate. In the near future, strengthening the surveillance of gonococcal antimicrobial susceptibility by Next Generation Sequencing (NGS) may certainly detect the molecular relationships and AMR determinants among isolates; NGS together with epidemiologic contact-tracing data will permit to monitor more efficiently the spread of AMR gonococci.

Acknowledgments

Neisseria gonorrhoeae Italian Study Group—S. Del Re: Microbiology and Virology Laboratory, Infectious Diseases, Amedeo di Savoia Hospital, Turin, Italy; I. Dal Conte: STI Clinic, Department of Infectious Diseases, Amedeo di Savoia Hospital, Turin, Italy; A. Di Carlo, A. Cristaudo, M. Giuliani, A. Latini, A. De Santis: San Gallicano Dermatologic Institute, IRCSS, Rome, Italy; L. Scioccati, S. Ramoni, L. Corti: Foundation IRCCS Ca' Granda ''Ospedale Maggiore Policlinico Milano,'' Milan, Italy; M.A. De Francesco: ''Dipartimento di Medicina Molecolare e Traslazionale, Sezione di Microbiologia,'' University of Brescia, Italy; M.P. Landini: Unit of Clinical Microbiology, University Hospital S.Orsola-Malpighi, Bologna, Italy; A. D'Antuono: Unit of Clinical Microbiology, CRREM Laboratory, St. Orsola-Malpighi, University Hospital, Bologna, Italy; A. Di Taranto, R. De Nittis: Department of Clinical Pathology, ''Azienda Ospedaliero-Universitaria OORR,'' Foggia, Italy; M. Gaino, R. Predazzer: Microbiology and Virology Laboratory, Santa Chiara Hospital, Trento; F. Urbani: U.O. Dermatology, Santa Chiara Hospital, Trento; V. Petix: Institute for Maternal and Child Health—IRCCS ''Burlo Garofolo,'' Trieste; S. Del Monte: STI Clinic of San Lazzaro Dermatological Hospital, A.O. Città della Salute e della Scienza di Torino; A.M. Barbui, S. Brossa: Microbiology and Virology Laboratory, Molinette Hospital, Turin, Italy; A. Mencacci: Microbiology Section, Department of Experimental Medicine, University of Perugia; E. Gaido: Department of ''Medicina di Laboratorio,'' P.O. Sant'Anna, Città della Salute e della Scienza di Torino, Turin, Italy; C.L. Bonanno, M.C. Cava: Microbiology and Virology Laboratory, Sandro Pertini Hospital, Rome, Italy; M. Busetti: Microbiology Unit, University Hospital ASUITs, Trieste, Italy. This work was supported by the Italian Ministry of Health grant CCM 2014, ''Sorveglianza di laboratorio della farmaco resistenza di *Neisseria gonorrhoeae* come malattia emergente.''

Disclosure Statement

No competing financial interests exist.

References

1. World Health Organisation (WHO). 2012. Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. Available at http://whqlibdoc.who.int/publications/2012/9789241503501_eng.pdf
2. Cole, M.J., G. Spiteri, S.A. Chisholm, S. Hoffmann, C.A. Ison, M. Unemo, and M. Van de Laar. 2014. Emerging cephalosporin and multidrug-resistant gonorrhea in Europe. Euro. Surveil. 19:pii: 20955.
3. Unemo, M., and W.M. Shafer. 2015. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st Century: past, evolution, and future. Clin. Microbiol. Rev. 27: 587–613.
4. Unemo, M., D. Golparian, R. Nicholas, M. Ohnishi, A. Gallay, and P. Sednaoui. 2012. High-level cefixime and ceftriaxone resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. Antimicrob. Agents Chemother. 56:1273–1280.
5. Cole, J.M., G. Spiteri, S. Jacobsson, R. Pitt, V. Grigorjev, M. Unemo; Euro-GASP Network. 2015. Is the tide turning again for cephalosporin resistance in *Neisseria gonorrhoeae* in Europe? Results from the 2013 European surveillance. BMC Infect. Dis. 15:321–328.
6. Ohnishi, M., D. Golparian, K. Shimuta, T. Saika, S. Hoshina, K. Iwasaku, S. Nakayama, J. Kitawaki, and M. Unemo. 2011. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob. Agents Chemother. 55:3538–3545.
7. Ito, M., T. Deguchi, K.S. Mizutani, M. Yasuda, S. Yokoi, S. Ito, Y. Takahashi, S. Ishihara, Y. Kawamura, and T. Ezaki. 2005. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. Antimicrob. Agents Chemother. 49:137–143.
8. Whiley, D.M., E.A. Limnios, S. Ray, T.P. Sloots, and J.W. Tapsall. 2007. Diversity of *penA* alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob. Agents Chemother. 51:3111–3116.
9. Ochiai, S., H. Ishiko, M. Yasuda, and T. Deguchi. 2008. Rapid detection of the mosaic structure of the *Neisseria gonorrhoeae penA* gene, which is associated with susceptibilities to oral cephalosporins. J. Clin. Microbiol. 46: 1804–1810.
10. Takahata, S., N. Senju, Y. Osaki, T. Yoshida, and T. Ida. 2006. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. 50:3638–3645.
11. Tomberg, J., M. Unemo, C. Davies, and R.A. Nicholas. 2010. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae*: role of epistatic mutations. Biochemistry 49: 8062–8070.
12. Shimuta, K., Y. Watanabe, S. Nakayama, T. Morita-Ishihara, T. Kuroki, M. Unemo, and M. Ohnishi. 2015. Emergence and evolution of internationally disseminated cephalosporin-resistant *Neisseria gonorrhoeae* clones from 1995 to 2005 in Japan. BMC Infect. Dis. 15:378–388.
13. Lee, H., M. Unemo, H.J. Kim, Y. Seo, K. Lee, and Y. Chong. 2015. Emergence of decreased susceptibility and resistance to extended-spectrum cephalosporins in *Neisseria gonorrhoeae* in Korea. J. Antimicrob. Chemother. 70:2536–2542.
14. Pandori, M., P.M. Barry, A. Wu, A. Ren, W.L. Whittington, S. Liska, and J.D. Klausner. 2009. Mosaic penicillin-binding protein 2 in *Neisseria gonorrhoeae* isolates collected in 2008 in San Francisco, California. Antimicrob. Agents Chemother. 53:4032–4044.
15. Jeverica, S., D. Golparian, M. Matičič, M. Potočnik, B. Mlakar, and M. Unemo. 2014. Phenotypic and molecular characterization of *Neisseria gonorrhoeae* isolates from Slovenia, 2006–2012: rise and fall of the multi-drug-resistant NG-MAST genogroup 1407 clone? J. Antimicrob. Chemother. 69:1517–1525.
16. Shimuta, K., M. Unemo, S. Nakayama, T. Morita-Ishihara, M. Dorin, T. Kawahata, Ohnishi M; Antibiotic-Resistant Gonorrhea Study Group. 2013. Antimicrobial resistance and molecular typing of *Neisseria gonorrhoeae* isolates in Kyoto and Osaka, Japan, 2010 to 2012: intensified surveillance after identification of the first strain (H041) with high-level ceftriaxone resistance. Antimicrob. Agents Chemother. 57:5225–5232.
17. European Centre for Disease Prevention and Control. 2012. Molecular typing of *Neisseria gonorrhoeae*. Results from a pilot study 2010–2011. Available at www.ecdc.europa.eu/en/publications/Publications/201211109-Molecular-typing-gonorrhea.pdf
18. Chisholm, S.A., M. Unemo, N. Quaye, E. Johansson, M.J. Cole, C.A. Ison, and M.J. Van de Laar. 2013. Molecular epidemiological typing within the European Gonococcal Antimicrobial Resistance Surveillance Programme reveals predominance of a multidrug-resistant clone. Euro. Surveil. 18:20358–20368.
19. Carannante, A., G. Renna, I. Dal Conte, V. Ghisetti, A. Matteelli, G. Prignano, G. Impara, M. Cusini, A. D'Antuono, C. Vocale, R. Antonetti, M. Gaino, M. Busetti, M.A. Latino, A. Mencacci, C. Bonanno, M.C. Cava, C. Giraldi, and P. Stefanelli. 2014. Changing antimicrobial resistance profiles among *Neisseria gonorrhoeae* isolates in Italy, 2003 to 2012. Antimicrob. Agents Chemother. 58:5871–5876.
20. Carannante, A., G. Prignano, M. Cusini, A. Matteelli, I. Dal Conte, V. Ghisetti, A. D'Antuono, F. Cavrini, R. Antonetti, and P. Stefanelli. 2012. Cefixime and ceftriaxone susceptibility of *Neisseria gonorrhoeae* in Italy from 2006 to 2010. Clin. Microbiol. Infect. 18:558–564.
21. Spiteri, G., M. Cole, M. Unemo, S. Hoffmann, C. Ison, and M. van de Laar. 2013. The European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP)—a sentinel approach in the European Union (EU)/European Economic Area (EEA). Sex Transm. Infect. 89:16–18.
22. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2016. Breakpoints tables for interpretation of MICs and zone diameters, Version 6.0. Available at www.eucast.org
23. Unemo, M., O. Fasth, H. Fredlund, A. Limnios, and J. Tapsall. 2009. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. J. Antimicrob. Chemother. 63:1142–1151.
24. Lee, S.G., H. Lee, S.H. Jeong, D. Yong, G.T. Chung, Y.S. Lee, Y. Chong, and K. Lee. 2010. Various *penA* mutations together with *mtrR*, *porB* and *ponA* mutations in *Neisseria*

gonorrhoeae isolates with reduced susceptibility to cefixime or ceftriaxone. J. Antimicrob. Chemother. 65:669–675.
25. Martin, I.M., C.A. Ison, D.M. Aanensen, K.A. Fenton, and B.G. Spratt. 2004. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J. Infect. Dis. 189:1497–1505.
26. Demczuk, W., T. Lynch, I. Martin, G. Van Domselaar, M. Graham, A. Bharat, V. Allen, L. Hoang, B. Lefebvre, G. Tyrrell, G. Horsman, D. Haldane, R. Garceau, J. Wylie, T. Wong, and M.R. Mulvey. 2015. Whole-genome phylogenomic heterogeneity of *Neisseria gonorrhoeae* isolates with decreased cephalosporin susceptibility collected in Canada between 1989 and 2013. J. Clin. Microbiol. 53:191–200.
27. Allen, V.G., D.J. Farrell, A. Rebbapragada, J. Tan, N. Tijet, S.J. Perusini, L. Towns, S. Lo, D.E. Low, and R.G. Melano. 2011. Molecular analysis of antimicrobial resistance mechanism in *Neisseria gonorrhoeae* isolates from Ontario, Canada. Antimicrob. Agents Chemother. 55:703–712.
28. Unemo, M. 2015. Current and future antimicrobial treatment of gonorrhoea—the rapidly evolving *Neisseria gonorrhoeae* continues to challenge. BMC Infect. Dis. 15: 364–378.
29. Gose, S., D. Nguyen, D. Lowenberg, M. Samuel, H. Bauer, and M. Pandori. 2013. *Neisseria gonorrhoeae* and extended-spectrum cephalosporins in California: surveillance and molecular detection of mosaic *penA*. BMC Infect. Dis. 13:570–578.
30. Thakur, S.D., S. Starnino, G.B. Horsman, P.N. Levett, and J.R. Dillon. 2014. Unique combined *penA/mtrR/porB* mutations and NG-MAST strain types associated with ceftriaxone and cefixime MIC increases in a 'susceptible' *Neisseria gonorrhoeae* population. J. Antimicrob. Chemother. 69:1510–1516.

Address correspondence to:
Paola Stefanelli, PhD
Department of Infectious, Parasitic and Immune-Mediated Diseases
Istituto Superiore di Sanità
Viale Regina Elena 299
Rome 00161
Italy

E-mail: paola.stefanelli@iss.it