CSIRO PUBLISHING
Sexual Health, 2019, **16**, 488–499
https://doi.org/10.1071/SH18227

Using the genetic characteristics of *Neisseria gonorrhoeae* strains with decreased susceptibility to cefixime to develop a molecular assay to predict cefixime susceptibility

Xiaomeng Deng[A,F], *Lao-Tzu Allan-Blitz*[B,C] *and Jeffrey D. Klausner*[A,D,E]

[A]David Geffen School of Medicine, University of California Los Angeles, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA.
[B]Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.
[C]Department of Medicine, Children's Hospital of Boston, 300 Longwood Avenue, Boston, MA 02115, USA.
[D]Division of Infectious Disease, Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Center for Health Sciences, 37–121, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA.
[E]Department of Epidemiology, Fielding School of Public Health, University of California Los Angeles, 650 Charles E. Young Drive S., Los Angeles, CA 90095, USA.
[F]Corresponding author. Email: dengxiaomeng0416@gmail.com

Abstract. ***Background*:** In the last two decades, gonococcal strains with decreased cefixime susceptibility and cases of clinical treatment failure have been reported worldwide. Gonococcal strains with a cefixime minimum inhibitory concentration (MIC) $\geq$0.12 μg mL^{-1} are significantly more likely to fail cefixime treatment than strains with an MIC <0.12 μg mL^{-1}. Various researchers have described the molecular characteristics of gonococcal strains with reduced cefixime susceptibility, and many have proposed critical molecular alterations that contribute to this decreased susceptibility. ***Methods*:** A systematic review of all published articles in PubMed through 1 November 2018 was conducted that report findings on the molecular characteristics and potential mechanisms of resistance for gonococcal strains with decreased cefixime susceptibility. The findings were summarised and suggestions were made for the development of a molecular-based cefixime susceptibility assay. ***Results*:** The penicillin-binding protein 2 (PBP2) encoded by the *penA* gene is the primary target of cefixime antimicrobial activity. Decreased cefixime susceptibility is conferred by altered *penA* genes with mosaic substitute sequences from other *Neisseria (N.)* species (identifiable by alterations at amino acid position 375–377) or by non-mosaic *penA* genes with at least one of the critical amino acid substitutions at positions 501, 542 and 551. Based on this review of 415 international cefixime decreased susceptible *N. gonorrhoeae* isolates, the estimated sensitivity for an assay detecting the aforementioned amino acid alterations would be 99.5% (413/415). ***Conclusions*:** Targeting mosaic *penA* and critical amino acid substitutions in non-mosaic *penA* are necessary and may be sufficient to produce a robust, universal molecular assay to predict cefixime susceptibility.

Additional keywords: antimicrobial resistance, antimicrobial stewardship.

Received 4 December 2018, accepted 1 April 2019, published online 24 June 2019

Introduction

Neisseria (*N.*) *gonorrhoeae*, is the world's second most prevalent sexually transmissible bacterial infection.[1] However, *N. gonorrhoeae* is a naturally competent organism that can acquire new genes from the microbial environment.[2] Those newly acquired genetic variations can render the organism less susceptible, or even resistant, to antimicrobial therapy.[2]

As the organism has developed resistance to multiple classes of antibiotics such as sulfas, penicillins, tetracyclines, fluoroquinolones and macrolides, the third-generation extended-spectrum cephalosporins, like cefixime, are among the few reliable efficacious treatment options left.[3] Cefixime is a highly useful antibiotic used for the treatment of gonorrhoea. *N. gonorrhoeae* remains susceptible to cefixime in most but not all countries.[4] Currently, the World Health Organization (WHO) recommends cefixime, in combination with azithromycin, as dual therapy for oropharyngeal, genital and anorectal gonococcal infection.[5] In settings where local

resistance data confirm cefixime susceptibility, the WHO recommends cefixime in a single dose for genital and anorectal gonococcal infection. In the USA, the Centers for Disease Control and Prevention also recommends cefixime, in combination with azithromycin, as an alternative regimen where ceftriaxone is not available.[6] In the UK, oral cefixime in combination with azithromycin is also recommended in penicillin-allergic patients for whom intramuscular injection is contraindicated or refused.[7] Cefixime has a serum half-life of 3–4 h in patients with normal renal function, high bioavailability after a single oral dose and is very well tolerated even in penicillin-allergic patients.[6] However, in the last two decades, various investigators have reported cases of *N. gonorrhoeae* infection with strains that have decreased susceptibility to cefixime.[8–30] Furthermore, in the past 10 years, cases of *N. gonorrhoeae* treatment failure in patients treated with cefixime have also been reported in Japan,[31] Norway,[32] UK,[33,34] South Africa,[28] France,[9] Australia[35] and Canada.[24] Various research teams and governmental institutions have expressed the need for more research on the mechanisms of cefixime resistance and the development of new tools to predict cefixime susceptibility.[36–38]

One target protein that is critical for the antimicrobial activity of many β-lactam antibiotics, including cefixime, is the penicillin-binding protein 2 (PBP2), which is a protein essential for the development of bacterial cell walls.[39] PBP2 is a transpeptidase encoded by the *penA* gene. Mosaicism of the *penA* gene in *N. gonorrhoeae* was first described by Ameyama *et al.*[40] who found that some *penA* nucleotide sequences of *N. gonorrhoeae* contained portions that highly resemble those of other non-pathogenic or commensal *Neisseria* species, such as *N. perflava*, *N. cinerea*, *N. flavescens* and *N. meningitidis*. *penA* mosaicism, along with other point mutations in *penA*, helps *N. gonorrhoeae* to develop resistance against extended-spectrum cephalosporins like cefixime.[14–27,41–43]

In this review, we describe molecular mechanisms of cefixime-decreased susceptibility in *N. gonorrhoeae*, summarise findings from published reports of various gene mutations contributing to that decreased susceptibility and make suggestions on how to develop a molecular-based cefixime susceptibility assay.

Methods

Definition of cefixime-decreased susceptibility

Historically, defining decreased susceptibility or resistance of *N. gonorrhoeae* to cefixime has been challenging due to the scarcity of treatment failure cases.[37] The most up-to-date recommendations of antimicrobial minimum inhibitory concentration (MIC) breakpoints by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) in the USA are as follows:

EUCAST: susceptible $\leq$0.125 μg mL^{-1}; resistant >0.125 μg mL^{-1}.[44]

CLSI: susceptible $\leq$ 0.25 μg mL^{-1}; intermediate: not applicable; resistant: not applicable.[45]

However, those recommendations might be too liberal when we compare those breakpoints to reported cases of clinical treatment failure. Allen *et al*. reported that the frequency of treatment failure with cefixime for gonococcal infections with an MIC $\geq$0.12 μg mL^{-1} was 25%, compared with 1.9% among those with an MIC <0.12 μg mL^{-1}.[24] For this review, we have described the molecular characteristics of all *N. gonorrhoeae* strains reported, to date, with a MIC $\geq$0.12 μg mL^{-1} as having a 'decreased susceptibility' to cefixime.

Literature and sequence database review

One author (X. Deng) searched all articles published on PubMed from 1 January 1995 to 1 November 2018 under the search terms '*Neisseria gonorrhoeae*', 'cefixime' and 'molecular' and reviewed relevant articles cited as references. We identified a total of 74 articles from that search. We included articles that presented epidemiological or experimental evidence of certain molecular alterations contributing to cefixime-decreased susceptibility in *N. gonorrhoeae*; there was a total of 25 reports. All *N. gonorrhoeae* strains with a reported MIC $\geq$0.12 μg mL^{-1} and specific *penA* alterations associated with cefixime decreased susceptibility were included in Table 1, along with their specific MIC plus the time and location of collection.

The nomenclature of *penA* reflects the differences in amino acid sequence rather than nucleotide sequence. Historically, each new amino acid sequence gets a sequential whole number, and is classified into mosaic, semi-mosaic (alterations of either the first or second half of the *penA* gene only[27]), non-mosaic (point mutations only) and wild-type (*penA* peptide sequence identical to that of the *N. gonorrhoeae* reference strain, M32091[49,50]). Each different DNA sequence of an existing amino acid sequence gets a decimal number;[27,28] for example, the eighth DNA sequence reported for *penA* allele type 2 is assigned allele number *penA*2.008. Our review has exposed conflicting nomenclature for the same *penA* peptide sequence (Table 2); likely due to the lack of a single, centralised database requiring new submissions of sequences to be compared with existing entries and subsequently given appropriate designations. We also noticed a general lack of consensus in the standard style of nucleotide or amino acid sequence reporting. Many sequences reported were truncated, leading to incomplete information that hindered data interpretation. We highly recommend future researchers to submit complete gene sequencing data to GenBank (https://www.ncbi.nlm.nih.gov/genbank/), which is supported by the National Center for Biotechnology Information (NCBI) and *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR, https://ngstar.canada.ca/), which is supported by the Government of Canada, for easy reference. To prevent misclassification in this manuscript, we further verified *penA* sequences from each research article against the *penA* profiles in NG-STAR, a centralised, comprehensive and publicly accessible database for standardised characterisation of molecular alterations in *N. gonorrhoeae* worldwide.[27] One author (X. Deng) conducted all the multiple peptide sequence alignment using the Multiple Sequence Alignment tool by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Table 1. Summary of *penA* alterations in reported gonococcal strains with a cefixime minimum inhibitory concentration of ≥0.12 μg mL^{-1}[1A]
CFX, cefixime; WHO, World Health Organization

Reference	Country of collection	Year	Frequency	CFX MIC (μg mL^{-1})	*penA* type	*penA* mosaicism	Amino acid alteration at numbered position									
							A311	I312	V316	D345 insertion	T483	A501	N512	G542	G545	P551
14	Japan	2001	8/55[B]	0.125	1~9[C]	non-mosaic				x		V[D]		S[E]		
15	Argentina	2009–13	1/1987	0.125	5	non-mosaic				x				S		
8	Vietnam	2011	1/108	0.125	5+[F]	non-mosaic				x		V		S		
16	Canada	2001–10	1/155	0.5	5+	non-mosaic				x				S		
9	(WHO reference strain L)			0.25	7	non-mosaic				x		S		S		S
15	Argentina	2009–13	1/1987	0.125	9	non-mosaic				x						L
			1/1987	0.5	12	non-mosaic				x						S
16	Canada	2001–10	3/155	≥0.125	12+	non-mosaic				x						S
17	Spain	2013	13/329	≥0.125	12+[G]	non-mosaic				x		T				L
			2/329							x						
			2/329							x						L
			1/329							x				S		
			1/329							x		S		S		
18	China	2014–15	3/126	0.125	13	non-mosaic				x		V				S
15	Argentina	2009–13	1/1987	0.125	13	non-mosaic				x		V				S
10	Korea	2011–2013	6/210	0.12	13	non-mosaic				x		V				S
			13/210	0.25						x		V				S
			2/210	0.5						x		V				S
18	China	2014–15	1/126	0.25	13+	non-mosaic				x		V	Y			S
16	Canada	2001–10	1/155	0.125	13+	non-mosaic				x		V				S
8	Vietnam	2011	1/108	0.25	18	non-mosaic				x		T		S		
			2/108	0.125						x		T		S		
			1/108	0.125						x		T		S		
			2/108	0.125						x		T		S		
			1/108	0.125						x		T		S		
18	China	2014–15	1/126	0.125	21	non-mosaic				x		V				
19	Japan	2002	20/58	0.25	10	mosaic		M	T				Y		S	
			4/58	0.5				M	T				Y		S	
14	Japan	2001	37/55[B]	≥ 0.5	10	mosaic		M	T				Y		S	
			8/55[B]	0.25				M	T				Y		S	
			2/55[B]	0.125				M	T				Y		S	
18	China	2014–15	3/126	0.25	10[H]	mosaic		M	T				Y		S	
15	Argentina	2009–13	3/1987	0.5	10	mosaic		M	T				Y		S	
10	Korea	2011–2013	3/210	0.25	10	mosaic		M	T				Y		S	
			1/210	0.5				M	T				Y		S	
46	(Modified laboratory strain)			>0.25	10	mosaic		M	T				Y		S	
9	(WHO reference strain K)			0.5	10	mosaic		M	T				Y		S	
	(Modified laboratory strain)			0.5	10[I]	mosaic		M	T				Y		S	
16	Canada	2001–10	12/155	≥ 0.25	10	mosaic		M	T				Y		S	
20	Canada	2008	8/149	0.25	10[H]	mosaic		M	T				Y		S	
11	Japan	1998–2007	25/36[J]	0.25	10	mosaic		M	T				Y		S	
		2003–04	5/36[J]	0.5				M	T				Y		S	

[19]	Japan	2002	4/58	0.12	10	mosaic		M	T			Y	S		
[47]	(Modified laboratory strains)			0.25	10+[K]	mosaic		M	T		S	Y	S		
				0.25				M	T		T	Y	S		
				0.25				M	T		V	Y	S		
				0.5				M	T		R	Y	S		
				0.5				M	T		P	Y	S		
[48]	(Modified laboratory strains)			0.125	10+[K]	mosaic		M	T			Y	S		
				>0.125			V	M	T			Y	S		
				0.25				M	P			Y	S		
				0.25			V	M	T	S		Y	S		
				>0.5			V	M	P			Y	S		
				>1.5			V	M	P	S		Y	S		
[11]	Japan	2003	1/36[J]	0.5	30	mosaic		M	T	V		Y	S		
		2003	1/36[J]	1				M	T	V		Y	S		
		2001	1/36[J]	0.25	31	mosaic		M	T			Y	S		
		2005	1/36[J]	0.25	32	mosaic		M	T			Y	S		
		2005	1/36[J]	0.5				M	T			Y	S		
[15]	Argentina	2009–13	15/1987	0.25	34	mosaic		M	T			Y	S		
			20/1987	0.125				M	T			Y	S		
[21]	Italy	2011–2014	45/50[B]	>0.125	34	mosaic		M	T			Y	S		
[17]	Spain	2013	28/329	≥0.125	34	mosaic		M	T			Y	S		
[12]	Japan	2011	Case report	0.125	34	mosaic		M	T			Y	S		
[13]	Ireland	2014–16	10/608	0.125	34	mosaic		M	T			Y	S		
[22]	Slovenia	2006–2012	28/194	0.125	34	mosaic		M	T			Y	S		
[23]	Switzerland	2010	1/34	0.19	34	mosaic		M	T			Y	S		
		2011	1/34	0.19				M	T			Y	S		
			1/34	0.125				M	T			Y	S		
			1/34	0.125				M	T			Y	S		
[46]	(Modified laboratory strain)			>0.125	34	mosaic		M	T			Y	S		
[28]	South Africa	2012	Case report	0.25	34	mosaic		M	T			Y	S		
[24]	Canada	2010–11	7/9[L]	0.12	34	mosaic		M	T			Y	S		
[20]	Canada	2008	1/149	0.125	34	mosaic		M	T			Y	S		
			1/149	0.25				M	T			Y	S		
[25]	USA	2008	Case report	0.25	34	mosaic		M	T			Y	S		
				0.125				M	T			Y	S		
[21]	Italy	2011–2014	1/50[B]	>0.125	34+	mosaic		M	T		V	Y	S		
[29]	Republic of Georgia	Did not report	Case report	0.5	34+	mosaic		M	T			Y	S		
[12]	Japan	2012	Case report	0.125	34+	mosaic		M	T		V	Y	S		
[46]	(Modified laboratory strain)			>0.35	34+	mosaic		M	T			Y	S	S	
				>0.5				M	T		S	Y	S		
[26]	Spain	Did not report	Case report	1.5	34+	mosaic		M	T		P	Y	S		

Table 1. (*continued*)

Reference	Country of collection	Year	Frequency	CFX MIC (μg mL^{-1})	*penA* type	*penA* mosaicism	Amino acid alteration at numbered position								
							A311	I312	V316	D345 insertion	T483	A501	N512	G542	G545	P551
[12]	Japan	2011	Case report	0.25	34+	mosaic		M	T				Y		S	S
				0.25				M	T				Y		S	S
				0.25				M	T				Y		S	S
		2012		0.25				M	T				Y		S	S
				0.25				M	T				Y		S	S
[20]	Canada	2008	2/149	0.25	35[M]	mosaic		M	T							
[30]	Japan	2009	Case report	4	37	mosaic	V	M	P		S		Y		S	
[48]	(Modified laboratory strain)			1.6	37[N]	mosaic	V	M	P		S		Y		S	
[30]	France	2010	Case report	2	42	mosaic		M	T			P	Y		S	
[9]	France	2010	Case report	4	42°	mosaic		M	T			P	Y		S	
[30]	Japan	2015	Case report	1	60	mosaic	V	M	T		S		Y		S	
	Japan	2014		1			V	M	T		S		Y		S	
	Denmark	2017		1			V	M	T		S		Y		S	
	Canada	2017		2			V	M	T		S		Y		S	
	Australia	2013		2	64	mosaic	V	M	T		S		Y		S	
[21]	Italy	2011–2014	4/50[B]	>0.125	not reported	mosaic	(Amino acid sequence not reported)									
[16]	Canada	2001–10	17/155	≥0.125	not reported	mosaic		M	T				Y		S	
			1/155	0.25				M	T							
[11]	Japan	2003	1/36[J]	0.5	not reported	mosaic		M	T				Y		S	

[A]An 'x' indicates the presence of the amino acid alteration indicated in the header row. Other one-letter abbreviations indicate the substitution into the corresponding amino acid.
[B]Among isolates with a cefixime MIC ≥ 0.125 μg mL^{-1}.
[C]In the manuscript, the authors reported the MIC values for a group of isolates with decreased susceptibility to cefixime with *penA* type 1~9.
[D]A501V mutation present only in *penA* 7 and 8; other *penA* types are wild-type at the 501 position.
[E]G542S mutation present only in *penA* 4, 5, 7, 8; other *penA* types are wild-type at the 542 position.
[F]In this entry and hereafter, '+' in the '*penA* type' column indicates that researchers reported the isolate having a *penA* sequence closely resembling the reported type.
[G]In the manuscript, the authors reported the *penA* type as closely resembling *penA* 36.
[H]In the manuscript, the authors reported the *penA* type as *penA* 35.
[I]In the manuscript, the authors reported the *penA* type as *penA* 28.
[J]Among isolates with a cefixime MIC ≥0.25 μg mL^{-1}.
[K]In the manuscript, the authors reported the *penA* type as closely resembling *penA* 35.
[L]Among isolates that failed clinical treatment.
[M]In the manuscript, the authors reported the *penA* type as *penA* 38.
[N]In the manuscript, the authors reported the *penA* type as *penA* 41.
[O]In the manuscript, the authors reported the *penA* type as *penA* 51.

Table 2. *penA* gene types with conflicting nomenclature
NG- STAR, *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance

Reference strain	*penA* type by NG-STAR	*penA* type reported in literature
35/02	10	mosaic-1 by Takahata *et al.*[19] 28 by Unemo *et al.*[9] 35 by Allen *et al.*[20] Tomberg *et al.*[47,48,51] Jiang *et al.*[18]
F98	42	51 by Unemo *et al.*[9] 42 by Lahra *et al.*[30]
H041	37	50 by Unemo *et al.*[9] 41 by Tomberg *et al.*[48] 37 by Lahra *et al.*[30]
FA6140	12	36 by Allen *et al.*[20] Serra-Pladevall *et al.*[17]
/	35	38 by Allen *et al.*[20] Martin *et al.*[16]

Estimation of assay sensitivity

We proposed a parsimonious group of *penA* amino acid locations to predict decreased susceptibility to cefixime in *N. gonorrhoeae* strains. We estimated the sensitivity of a hypothetical assay for predicting decreased susceptibility using those locations by calculating the number of isolates with genotypic mutations in those locations divided by the number of phenotypically decreased susceptible isolates using the data summarised in Table 1.

Results

Overview of the molecular mechanisms of cefixime-decreased susceptibility

Table 3 shows a list of *penA* types from the NG-STAR database and the presence or absence of amino acid alterations associated with cefixime-decreased susceptibility. Decreased susceptibility to cefixime has been associated with many genetic alterations in the *penA* gene. There is strong laboratory and epidemiological research supporting mosaicism[14,40] and other point mutations[19,47,48,51] of the *penA* gene as mechanisms for cefixime-decreased susceptibility by means of PBP2 target alteration. Evidence supporting the importance of alterations in other genes are not as compelling. However, there is evidence that isolates with identical *penA* alleles could have different MIC levels (susceptible with an MIC <0.125 μg mL^{-1} vs highly resistant with an MIC ≥ 0.5 μg mL^{-1}), indicating the involvement of other genes in decreasing susceptibility to cefixime.[27] Several candidate genes contributing to cefixime decreased susceptibility include *mtrR*, the transcriptional regulator for the *mtrCDE* efflux pump system;[52] *ponA*, encoding for penicillin-binding protein 1 (PBP1);[53] *pilQ*, encoding for the type IV pilus secretin;[54] and *penB* (alias: *porB1b*), encoding for a major outer membrane protein, *porB*, a porin.[55]

penA – penA mosaicism

Mosaicism in *penA* was found to be the primary determinant of cefixime-decreased susceptibility by many studies.[14–27,41–43] From an aggregate of 415 *N. gonorrhoeae* isolates with a MIC ≥0.12 μg mL^{-1} from 25 reports representing 22 countries, 83.1% (345 out of 415) were found to have mosaic *penA* genes (Table 1). Among all the different mosaic patterns, *penA*10[10,56,57] and *penA*34[15,17,21–23,27,42,56,58] were the most frequently reported mosaic *penA* patterns of all isolates with mosaic *penA* gene mutations among *N. gonorrhoeae* strains with reduced cefixime susceptibility [accounting for 39.1% (135 out of 345) and 49.9% (172 out of 345) respectively]. While *penA*34 was found worldwide, *penA*10 was mostly found in Asia, and was also associated with resistance to, and treatment failure by, another third-generation cephalosporin, ceftibuten.[59]

The I312M, V316T, N512Y and G545S amino acid substitutions are frequently seen in mosaic *penA* patterns. Researchers have found that amino acid substitutions, I312M,[19,25] V316T/P[19,25,48] and G545[19,25,60,61] are associated with reduced cefixime susceptibility. However, a gene transformation study by Tomberg *et al.*[51] in 2010 showed that when introduced into a wild-type *penA*, I312M, V316T and G545S together only minimally elevated the cefixime MIC. The reversion of the triple mutation in a cefixime-resistant strain 35/02 (with *penA*10) back into wild-type returned its MIC to that similar to the wild-type *penA* MIC, indicating that those three mutations are important to cefixime resistance only in the context of other mutations found in mosaic *penA*10 alleles.

In the same study by Tomberg *et al.*,[51] chimeric *penA* genes were created by replacing sequential portions of *penA*10 with the corresponding regions of a wild-type *penA* gene. Reversion of amino acid regions 309–353 (containing I312M and V316T and 13 other substitutions), 489–528 (containing N512Y and two other mutations) and 528–581 (containing G545S and nine other substitutions), showed significant decrease in MIC. Among the three, the reversion of region 528–581 decreased the MIC to such a significant degree that the chimera could not be selected despite multiple attempts, indicating mutations in this region may be critical factors in influencing cefixime susceptibility. When the G545S substitution was re-introduced to the 528–581-wild-type *penA*10, the resulting strain's cefixime MIC only rose to 0.05 μg mL^{-1}, suggesting that other mutations in the 528–581 region were necessary to significantly elevate the cefixime MIC to >0.125 μg mL^{-1}.

The reversion of region 489–528 (containing N512Y and two other mutations) decreased the MIC from ~0.125 to ~0.05 μg mL^{-1}. Y512N reversion alone presented a decreased MIC from ~0.125 to ~0.06 μg mL^{-1}. Accounting for most of the effect on cefixime resistance by mutations in the 489–528 region, N512Y showed major importance in conferring cefixime resistance in the context of other mosaic changes.

In summary, I312M, V316T, N512Y and G545S were found to be important to cefixime-decreased susceptibility or resistance only in the context of other mutations found in mosaic *penA* alterations. All of them were associated with, but none was necessary or sufficient for, cefixime-decreased susceptibility or resistance.

penA – non-mosaic penA with point amino acid alterations – A501 + G542 + P551

Although the *penA*34 mosaicism was present in 98% of all isolates with a MIC ≥0.25 μg mL^{-1} in the study by

Table 3. *penA* types: mosaicism and amino acid alterations associated with cefixime-decreased susceptible *Neisseria gonorrhoeae* infections
The one-letter abbreviation of amino acid indicates the substitution of the amino acid described in the header row. The lack of a one-letter abbreviation indicates that the position contains the wild-type amino acid. The x indicates the insertion of an aspartate at amino acid 345 position.

penA type	Mosaicism	Amino acid alteration at number position									
		A311	I312	V316	D345 insertion	T483	A501	N512	G542	G545	P551
0 (Wild-type)	Non-mosaic										
1	Non-mosaic				x						
2	Non-mosaic				x						
3	Non-mosaic				x						
4	Non-mosaic				x				S		
5	Non-mosaic				x				S		
7	Non-mosaic				x		S		S		
9	Non-mosaic				x						L
10	Mosaic		M	T				Y		S	
11	Non-mosaic				x		V				L
12	Non-mosaic				x						S
13	Non-mosaic				x		V				S
14	Non-mosaic				x						
15	Non-mosaic										
17	Non-mosaic				x		V		S		
18	Non-mosaic				x		T		S		
19	Non-mosaic				x						
21	Non-mosaic				x		V				
22	Non-mosaic				x						
26	Mosaic		M	T				Y		S	
27	Mosaic		M	T				Y		S	
30	Mosaic		M	T		V		Y		S	
31	Mosaic		M	T				Y		S	
32	Mosaic		M	T				Y		S	
34	Mosaic		M	T				Y		S	
35	Mosaic		M	T							
37	Mosaic	V	M	P		S		Y		S	
38	Mosaic										
39	Semi-mosaic				x						
40	Non-mosaic				x						
41	Non-mosaic				x				S		
42	Mosaic		M	T			P	Y		S	
43	Non-mosaic				x		V				
44	Non-mosaic				x		T				L
45	Non-mosaic										
46	Non-mosaic				x						
47	Semi-mosaic		M	T							
48	Non-mosaic				x						
49	Non-mosaic				x		T				
50	Non-mosaic				x						A
51	Mosaic		M	T				Y		S	
52	Mosaic		M	T				Y		S	
53	Mosaic			T				Y		S	A
54	Non-mosaic				x		V				A
55	Mosaic		M	T				Y		S	
56	Non-mosaic				x		V				G
57	Non-mosaic				x		V				A
58	Mosaic							Y		S	A
59	Mosaic					S		Y		S	
60	Mosaic	V	M	T		S		Y		S	
61	Non-mosaic				x						L
62	Mosaic							Y			
63	Mosaic		M	T							
64	Mosaic	V	M	T		S		Y		S	

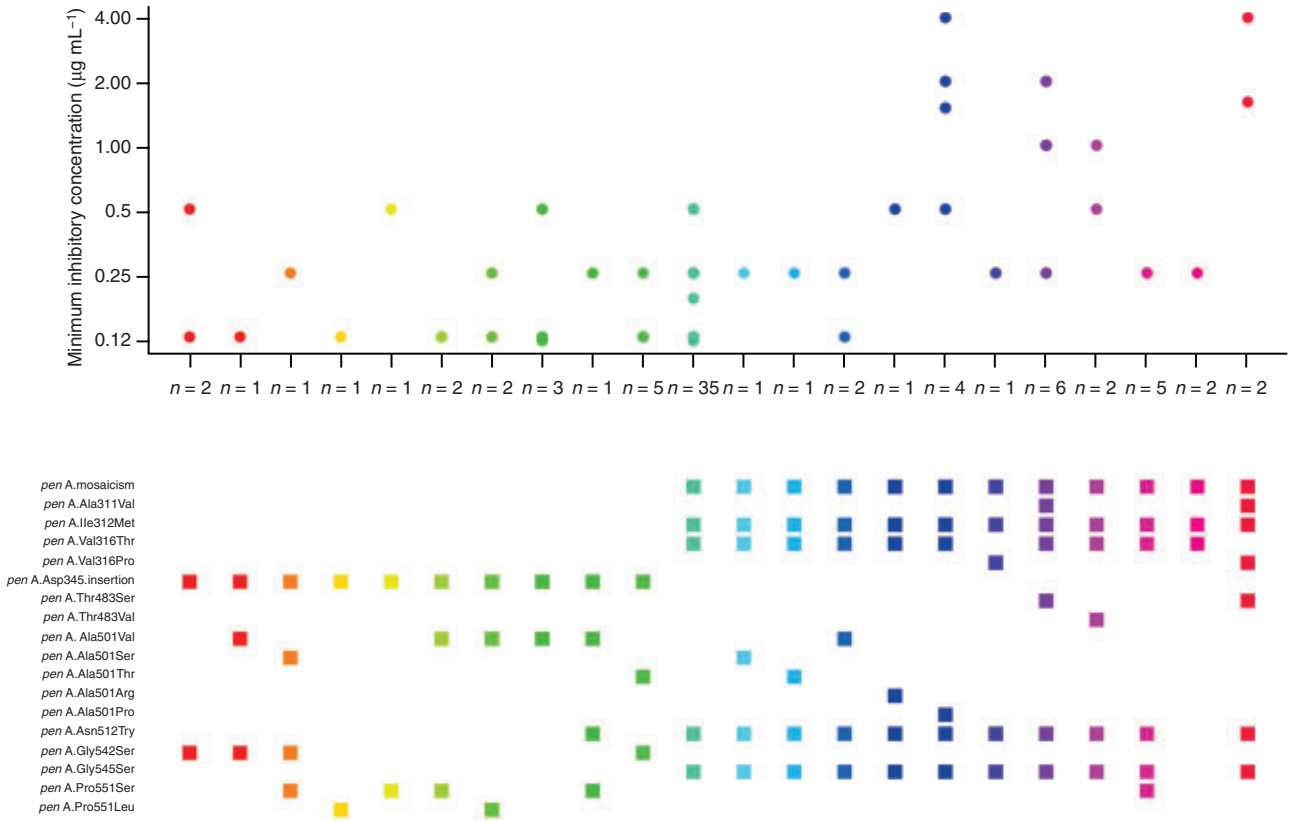

Fig. 1. Summary of cefixime minimum inhibitory concentration (MIC) of *Neisseria gonorrhoeae* strains by different combinations of cefixime-decreased susceptibility-related *penA* amino acid alterations. Minimum inhibitory concentrations are on the *y*-axis. Combinations of *penA* amino acid alterations associated with cefixime-decreased susceptibility are on the *x*-axis; different colours indicate different mutation combinations.

Grad *et al*. involving more than 1100 *N. gonorrhoeae* isolates collected in the USA, the percentage lowered to 91% when a lower and more clinically relevant MIC breakpoint (≥0.125 μg mL^{-1}) was used.[27] That indicates the role of other non-*penA*34 mutations, most notably, other amino acid substitutions in the *penA* gene. In the last decade, more than a dozen cefixime-resistant *N. gonorrhoeae* strains reported in Asia[8,10,14,18] and Europe[17] were found to have non-mosaic *penA* alleles (Table 1). Among reports from Asia, over 20% of *N. gonorrhoeae* strains with decreased susceptibility to cefixime had non-mosaic *penA* mutations. Gene transformation experiments have identified multiple, important amino acid substitutions in non-mosaic *penA* that significantly decrease cefixime susceptibility.[47]

Three amino acid substitutions, A501S/V/T/P, G542S and P551S/L/P, have been associated with cefixime-decreased susceptibility independent from *penA* mosaicism.[16,21] The study by Tomberg *et al*. demonstrated that an independent A501 substitution potentially decreased cefixime susceptibility by increasing the rigidity of the PBP2 active site.[47]

In Table 1, we have summarised the cefixime-decreased susceptible related *penA* amino acid alterations in all *N. gonorrhoeae* strains with a cefixime MIC ≥0.12 μg mL^{-1}. One important finding is that 68 out of 70 (97.1%) non-mosaic *penA* strains with reduced susceptibility to cefixime have a point mutation in at least one of the three codons, A501, G542 and P551. Additionally, decreased susceptible *N. gonorrhoeae* strains with non-mosaic *penA* have mostly been reported in Asia and Europe.

In Figure 1, we have summarised the MIC levels of all *N. gonorrhoeae* strains with cefixime MIC ≥ 0.12 μg mL^{-1} by different combinations of decreased susceptible-related *penA* amino acid alterations (n = 240). Strains lacking a specific cefixime MIC value or *penA* alteration records (n = 175) were excluded.

mtrR

mtrR is a repressor gene that regulates the expression of the *mtrCDE* efflux pump system, an important mechanism in transporting antimicrobial agents out of the bacterial cell.[52] Changes in the promoter or coding sequence of the *mtrR* gene can potentially decrease antimicrobial susceptibility by increased efflux.[62] There are conflicting reports on the importance of the *mtrR* gene in cefixime-decreased susceptibility. Mutations frequently found in strains with cefixime-decreased susceptibility include a –35A deletion in the promoter region,[13,22] plus A39T and G45D[22] in the coding region. We did not find gene transformation studies that looked into *mtrR* alterations' contribution to cefixime resistance independent from *penA* changes. Nonetheless, other studies report that mutations in the *mtrR* gene have little or no association with cefixime susceptibility.[58,62]

penB (*porB1b*)

penB, also known as *porB1b*, encodes for an outer membrane porin and is thought to increase penicillin resistance by changing the bacterial membrane permeability to certain antibiotics when *penA* and *mtrR* mutations are also present, although its role in cefixime resistance is unclear.[58,63] Alterations found in strains with cefixime-decreased susceptibility include G120K and A121N/D substitutions.[42] While the study by Grad *et al*. in the USA suggested no correlation between cefixime-decreased susceptibility and G120K or G120D/A121D, the lack of G120K *and* A121N mutations strongly predicted susceptibility.[27]

Two other mutations in the *penB* gene commonly found in cefixime-decreased susceptible strains are G101K/D and A102D/N/S. Combinations of mutations at those two sites were found in all 48 strains with a cefixime MIC >0.125 μg mL^{-1} out of the total 329 *N. gonorrhoeae* strains in the Serra-Pladevall *et al*. study[17,22] and among all 127 strains with a cefixime MIC ≥0.125 μg mL^{-1} out of the total 194 *N. gonorrhoeae* strains in the study by Jeverica *et al*.[22]

We have found no evidence that alterations in the *penB* gene alone can confer decreased susceptibility to cefixime.

ponA

ponA encodes for penicillin-binding protein 1 (PBP1), an additional cell wall protein important in β-lactam antibiotic antimicrobial activity. Alterations in the *ponA* gene were associated with penicillin resistance by PBP1 target mutation, although its role in cefixime-decreased susceptibility is unclear.[19,37,58] One mutation, L421P,[17,42,64] was frequently found in cefixime-decreased susceptible strains, but a gene transformation study showed that a *ponA* L421P substitution does not contribute additional decreased susceptibility to cefixime without a mosaic *penA* gene.[62]

pilQ

pilQ encodes for a type IV pili secretin.[54] Mutations in the *pilQ* gene are thought to increase penicillin resistance by changing the bacterial membrane permeability when *penA*, *mtrR* or *penB* mutations are also present, although its role in cefixime resistance is also unclear.[58,63]

While the study by Whiley *et al*.[63] concluded that changes in the *pilQ* gene are unlikely associated with cefixime-decreased susceptibility, the study by Grad *et al*.[27] found that a 176–183 deletion (vs full length), N341S, D526N/G or N648S each strongly predicted *N. gonorrhoeae* susceptibility to cefixime. Notably, among those four mutations, N648S was the only mutation that was found to be relatively common (in 23.7% of all isolates compared with ≤10% for any of the other three).

Discussion

Prediction of cefixime susceptibility using molecular markers

Antimicrobial stewardship is important in the control of antimicrobial resistance in infectious diseases.[65] Antimicrobial use results in selective pressure, favouring the development of resistant organisms.[65] Therefore, the use of molecular assays to predict antimicrobial susceptibility at the time of treatment allows for targeted therapy, enabling the use of antimicrobials shown to be highly effective and rapidly bactericidal, as well as the use of older medications previously deemed non-effective because of prevalent resistance.[66,67] Such molecular assays to predict susceptibility could be a valuable complement to antimicrobial stewardship and novel drug development in slowing the emergence of antimicrobial resistance.[38]

Previously, we developed a polymerase chain reaction-based assay using high resolution melt analysis to predict *N. gonorrhoeae* susceptibility to ceftriaxone and cefixime by targeting the *penA*34 type.[68] While that assay showed greater than 98% sensitivity in predicting cefixime-decreased susceptibility in North America, where the *penA*34 is the most common *penA* type,[27,50] the assay is limited by its inability to identify cefixime-decreased susceptibility as a result of *N. gonorrhoeae* strains with non-mosaic *penA* or other non-34 mosaic *penA* patterns. As *N. gonorrhoeae* strains with various non-34 *penA* types account for many of the cefixime-resistant strains reported in Asia and Europe, it is likely only a matter of time for such strains to emerge in other parts of the world, including in the USA.

Additional amino acid alterations should be considered when developing a molecular assay to predict cefixime susceptibility, especially when a lower and more clinically relevant MIC breakpoint (<0.12 μg mL^{-1}) is used.

For the *penA* gene, either of the following patterns of alterations could lead to elevated cefixime MIC levels: (1) mosaic *penA* types with characteristic polymorphisms I312M, V316T, N512Y, G545S; or (2) non-mosaic *penA* types with mutations at any combinations of the three amino acid positions: A501, G542 or P551. A multiple peptide sequence alignment showed that a wild-type sequence of amino acid region 375–377 can reliably distinguish 32 out of 33 types of wild-type and non-mosaic *penA* types (with the only exception being the non-mosaic *penA*49 type possessing a A377V substitution) from all 21 reported mosaic *penA* types (data not shown). Other researchers have targeted other regions or used the characteristic amino acid polymorphisms in mosaic *penA* types such as I312M, V316T and G545S.[19,69]

Non-mosaic *penA* types with critical point mutations can be identified by the absence of changes at amino acid region 375–377, and the presence of one of the three critical point mutations A501, G542 or P551. A molecular assay that detects any *penA* alterations at amino acid positions 375–377, 501, 542 or 551 would be the most parsimonious assay to predict decreased susceptibility to cefixime. That modified assay would be more effective in predicting cefixime susceptibility than the assay we previously developed,[68] with greater sensitivity for diverse international isolates. Based on the molecular characteristics of all reported strains with decreased susceptibility to cefixime as recorded in Table 1, the estimated sensitivity for the proposed assay would be 99.5% (413 out of 415).

Other genes, such as *mtrR*, *ponA*, *penB* and *pilQ*, have a less clear role in cefixime resistance and seem to further increase cefixime MIC values only when *penA* alterations are present. Although certain alterations in these genes were found to be highly associated with full susceptibility as mentioned above, detecting these changes appears to be unnecessary as *N. gonorrhoeae* will constantly undergo

selection by antimicrobial agents and gradually gain mutations that eventually lead to cefixime resistance.

Conclusion

Because of the continued challenge in treating antimicrobial-resistant *N. gonorrhoeae* infections, there have been many reports published regarding the genetic alterations associated with decreased susceptibility to third-generation cephalosporins. Researchers have provided epidemiological and molecular evidence that alterations in the *penA* gene are the primary determinants of cefixime-decreased susceptibility, while other genes in the presence of an altered *penA* gene further contribute in reducing cefixime susceptibility but are neither necessary nor sufficient in independently conferring decreased susceptibility. Based on those data, we proposed the optimal targets for novel molecular assays aiming to predict *N. gonorrhoeae* susceptibility to cefixime.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Funding was provided by the National Institute of Allergy and Infectious Diseases, grant R21 AI117256, to Jeffrey D. Klausner. Jeffrey D. Klausner has received advisory fees or research support from Cepheid, SpeedX Diagnostics, Shield Diagnostics, Click Diagnostics and Hologic Inc. The results presented are subject of a pending U.S. patent application.

References

1 Unemo M, Shafer WM. Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. *Ann N Y Acad Sci* 2011; 1230: E19–28. doi:10.1111/j.1749-6632.2011.06215.x

2 Tapsall JW, Ndowa F, Lewis DA, Unemo M. Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. *Expert Rev Anti Infect Ther* 2009; 7(7): 821–34. doi:10.1586/eri.09.63

3 Tanvir SB, Qasim SSB, Shariq A, Najeeb S, Shah AH. Systematic review and meta-analysis on efficacy of cefixime for treating gonococcal infections. *Int J Health Sci (Qassim)* 2018; 12(5): 90–100.

4 Unemo M, Lahra MM, Cole M, Galarza P, Ndowa F, Martin I, Dillon JR, Ramon-Pardo P, Bolan G, Wi T. The WHO Global Gonococcal Antimicrobial Surveillance Programme (67 surveyed countries in 2015–2016) – an observational study emphasising essential global actions. *Sex Health* 2019. In press.

5 World Health Organization. WHO guidelines for the treatment of *Neisseria gonorrhoeae*. Geneva: World Health Organization; 2016.

6 Workowski KA, Bolan GA. Sexually transmitted diseases treatment guidelines, 2015. *MMWR Recomm Rep* 2015; 64(RR-03): 1–137.

7 Fifer H, Saunders J, Soni S, Sadiq ST, FitzGerald M. British Association for Sexual Health and HIV national guideline for the management of infection with *Neisseria gonorrhoeae*. Macclesfield: BASHH; 2019. Available online at: https://pcwhf.co.uk/wp-content/uploads/2019/03/gc-2019.pdf [verified 1 June 2019].

8 Olsen B, Pham TL, Golparian D, Johansson E, Tran HK, Unemo M. Antimicrobial susceptibility and genetic characteristics of *Neisseria gonorrhoeae* isolates from Vietnam, 2011. *BMC Infect Dis* 2013; 13: 40. doi:10.1186/1471-2334-13-40

9 Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. 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* 2012; 56(3): 1273–80. doi:10.1128/AAC.05760-11

10 Lee H, Unemo M, Kim HJ, Seo Y, Lee K, Chong Y. Emergence of decreased susceptibility and resistance to extended-spectrum cephalosporins in *Neisseria gonorrhoeae* in Korea. *J Antimicrob Chemother* 2015; 70(9): 2536–42. doi:10.1093/jac/dkv146

11 Ohnishi M, Watanabe Y, Ono E, Takahashi C, Oya H, Kuroki T, Shimuta K, Okazaki N, Nakayama S, Watanabe H. Spread of a chromosomal cefixime-resistant penA gene among different *Neisseria gonorrhoeae* lineages. *Antimicrob Agents Chemother* 2010; 54(3): 1060–7. doi:10.1128/AAC.01010-09

12 Morita-Ishihara T, Unemo M, Furubayashi K, Kawahata T, Shimuta K, Nakayama S, Ohnishi M. Treatment failure with 2 g of azithromycin (extended-release formulation) in gonorrhoea in Japan caused by the international multidrug-resistant ST1407 strain of *Neisseria gonorrhoeae*. *J Antimicrob Chemother* 2014; 69(8): 2086–90. doi:10.1093/jac/dku118

13 Ryan L, Golparian D, Fennelly N, Rose L, Walsh P, Lawlor B, Mac Aogain M, Unemo M, Crowley B. Antimicrobial resistance and molecular epidemiology using whole-genome sequencing of *Neisseria gonorrhoeae* in Ireland, 2014–2016: focus on extended-spectrum cephalosporins and azithromycin. *Eur J Clin Microbiol Infect Dis* 2018; 37(9): 1661–72. doi:10.1007/s10096-018-3296-5

14 Ito M, Deguchi T, Mizutani KS, Yasuda M, Yokoi S, Ito S, Takahashi Y, Ishihara S, Kawamura Y, Ezaki T. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. *Antimicrob Agents Chemother* 2005; 49(1): 137–43. doi:10.1128/AAC.49.1.137-143.2005

15 Gianecini R, Romero MLM, Oviedo C, Vacchino M, Galarza P. Emergence and spread of *Neisseria gonorrhoeae* isolates with decreased susceptibility to extended-spectrum cephalosporins in Argentina, 2009 to 2013. *Sex Transm Dis* 2017; 44(6): 351–5. doi:10.1097/OLQ.0000000000000603

16 Martin I, Sawatzky P, Allen V, Hoang L, Lefebvre B, Mina N, Wong T, Gilmour M. Emergence and characterization of *Neisseria gonorrhoeae* isolates with decreased susceptibilities to ceftriaxone and cefixime in Canada: 2001–2010. *Sex Transm Dis* 2012; 39(4): 316–23. doi:10.1097/OLQ.0b013e3182401b69

17 Serra-Pladevall J, Barbera MJ, Rodriguez S, Bartolome-Comas R, Roig G, Juve R, Andreu A. *Neisseria gonorrhoeae* antimicrobial susceptibility in Barcelona: penA, ponA, mtrR, and porB mutations and NG-MAST sequence types associated with decreased susceptibility to cephalosporins. *Eur J Clin Microbiol Infect Dis* 2016; 35(9): 1549–1556. doi:10.1007/s10096-016-2696-7

18 Jiang FX, Lan Q, Le WJ, Su XH. Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolates from Hefei (2014–2015): genetic characteristics of antimicrobial resistance. *BMC Infect Dis* 2017; 17: 366. doi:10.1186/s12879-017-2472-z

19 Takahata S, Senju N, Osaki Y, Yoshida T, Ida T. Amino acid substitutions in mosaic penicillin-binding protein 2 associated with reduced susceptibility to cefixime in clinical isolates of *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2006; 50(11): 3638–45. doi:10.1128/AAC.00626-06

20 Allen VG, Farrell DJ, Rebbapragada A, Tan J, Tijet N, Perusini SJ, Towns L, Lo S, Low DE, Melano RG. Molecular analysis of antimicrobial resistance mechanisms in *Neisseria gonorrhoeae* isolates from Ontario, Canada. *Antimicrob Agents Chemother* 2011; 55(2): 703–12. doi:10.1128/AAC.00788-10

21 Carannante A, Vacca P, Ghisetti V, Latino MA, Cusini M, Matteelli A, Vocale C, Prignano G, Leli C, Ober P, Antonetti R, Poletti F,

Stefanelli P. Genetic resistance determinants for cefixime and molecular analysis of gonococci isolated in Italy. *Microb Drug Resist* 2017; 23(2): 247–52. doi:10.1089/mdr.2016.0086

22 Jeverica S, Golparian D, Maticic M, Potocnik M, Mlakar B, Unemo M. Phenotypic and molecular characterization of *Neisseria gonorrhoeae* isolates from Slovenia, 2006–12: rise and fall of the multidrug-resistant NG-MAST genogroup 1407 clone? *J Antimicrob Chemother* 2014; 69(6): 1517–25. doi:10.1093/jac/dku026

23 Endimiani A, Guilarte YN, Tinguely R, Hirzberger L, Selvini S, Lupo A, Hauser C, Furrer H. Characterization of *Neisseria gonorrhoeae* isolates detected in Switzerland (1998–2012): emergence of multidrug-resistant clones less susceptible to cephalosporins. *BMC Infect Dis* 2014; 14: 106. doi:10.1186/1471-2334-14-106

24 Allen VG, Mitterni L, Seah C, Rebbapragada A, Martin IE, Lee C, Siebert H, Towns L, Melano RG, Low DE. *Neisseria gonorrhoeae* treatment failure and susceptibility to cefixime in Toronto, Canada. *JAMA* 2013; 309(2): 163–70. doi:10.1001/jama.2012.176575

25 Pandori M, Barry PM, Wu A, Ren A, Whittington WL, Liska S, Klausner JD. Mosaic penicillin-binding protein 2 in *Neisseria gonorrhoeae* isolates collected in 2008 in San Francisco, California. *Antimicrob Agents Chemother* 2009; 53(9): 4032–4. doi:10.1128/AAC.00406-09

26 Cámara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, Ardanuy C. Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. *J Antimicrob Chemother* 2012; 67(8): 1858–60. doi:10.1093/jac/dks162

27 Grad YH, Harris SR, Kirkcaldy RD, Green AG, Marks DS, Bentley SD, Tress D, Lipsitch M. Genomic epidemiology of gonococcal resistance to extended-spectrum cephalosporins, macrolides, and fluoroquinolones in the United States, 2000–2013. *J Infect Dis* 2016; 214(10): 1579–87. doi:10.1093/infdis/jiw420

28 Lewis DA, Sriruttan C, Muller EE, Golparian D, Gumede L, Fick D, de Wet J, Maseko V, Coetzee J, Unemo M. Phenotypic and genetic characterization of the first two cases of extended-spectrum-cephalosporin-resistant *Neisseria gonorrhoeae* infection in South Africa and association with cefixime treatment failure. *J Antimicrob Chemother* 2013; 68(6): 1267–70. doi:10.1093/jac/dkt034

29 Washington MA, Jerse AE, Rahman N, Pilligua-Lucas M, Garges EC, Latif NH, Akhvlediani T. First description of a cefixime- and ciprofloxacin-resistant *Neisseria gonorrhoeae* isolate with mutations in key antimicrobial susceptibility-determining genes from the country of Georgia. *New Microbes New Infect* 2018; 24: 47–51. doi:10.1016/j.nmni.2018.04.004

30 Lahra MM, Martin I, Demczuk W, Jennison AV, Lee KI, Nakayama SI, Lefebvre B, Longtin J, Ward A, Mulvey MR, Wi T, Ohnishi M, Whiley D. Cooperative recognition of internationally disseminated ceftriaxone-resistant *Neisseria gonorrhoeae* strain. *Emerg Infect Dis* 2018; 24(4): 735–43. doi:10.3201/eid2404.171873

31 Yokoi S, Deguchi T, Ozawa T, Yasuda M, Ito S, Kubota Y, Tamaki M, Maeda S. Threat to cefixime treatment for gonorrhea. *Emerg Infect Dis* 2007; 13(8): 1275–7.

32 Unemo M, Golparian D, Syversen G, Vestrheim DF, Moi H. Two cases of verified clinical failures using internationally recommended first-line cefixime for gonorrhoea treatment, Norway, 2010. *Euro Surveill* 2010; 15(47): 19721.

33 Ison CA, Hussey J, Sankar KN, Evans J, Alexander S. Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. *Euro Surveill* 2011; 16(14): 19833.

34 Forsyth S, Penney P, Rooney G. Cefixime-resistant *Neisseria gonorrhoeae* in the UK: a time to reflect on practice and recommendations. *Int J STD AIDS* 2011; 22(5): 296–7. doi:10.1258/ijsa.2009.009191

35 Unemo M, Golparian D, Stary A, Eigentler A. First *Neisseria gonorrhoeae* strain with resistance to cefixime causing gonorrhoea treatment failure in Austria, 2011. *Euro Surveill* 2011; 16(43): 19998. doi:10.2807/ese.16.43.19998-en

36 Cristillo AD, Bristow CC, Torrone E, Dillon JA, Kirkcaldy RD, Dong H, Grad YH, Nicholas RA, Rice PA, Lawrence K, Oldach D, Shafer WM, Zhou P, Wi TE, Morris SR, Klausner JD. Antimicrobial resistance in *Neisseria gonorrhoeae*: proceedings of the STAR Sexually Transmitted Infection-Clinical Trial Group Programmatic Meeting. *Sex Transm Dis* 2019; 46(3): e18–25. doi:10.1097/OLQ.0000000000000929

37 Barry PM, Klausner JD. The use of cephalosporins for gonorrhea: the impending problem of resistance. *Expert Opin Pharmacother* 2009; 10(4): 555–77. doi:10.1517/14656560902731993

38 Blank S, Daskalakis DC. *Neisseria gonorrhoeae* — rising infection rates, dwindling treatment options. *N Engl J Med* 2018; 379(19): 1795–7. doi:10.1056/NEJMp1812269

39 Dougherty TJ, Koller AE, Tomasz A. Penicillin-binding proteins of penicillin-susceptible and intrinsically resistant *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 1980; 18(5): 730–7. doi:10.1128/AAC.18.5.730

40 Ameyama S, Onodera S, Takahata M, Minami S, Maki N, Endo K, Goto H, Suzuki H, Oishi Y. Mosaic-like structure of penicillin-binding protein 2 gene (penA) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. *Antimicrob Agents Chemother* 2002; 46(12): 3744–9. doi:10.1128/AAC.46.12.3744-3749.2002

41 Gose S, Nguyen D, Lowenberg D, Samuel M, Bauer H, Pandori M. *Neisseria gonorrhoeae* and extended-spectrum cephalosporins in California: surveillance and molecular detection of mosaic penA. *BMC Infect Dis* 2013; 13: 570. doi:10.1186/1471-2334-13-570

42 Chen CC, Yen MY, Wong WW, Li LH, Huang YL, Chen KW, Li SY. Tracing subsequent dissemination of a cluster of gonococcal infections caused by an ST1407-related clone harbouring mosaic penA alleles in Taiwan. *J Antimicrob Chemother* 2013; 68(7): 1567–71. doi:10.1093/jac/dkt059

43 Ochiai S, Ishiko H, Yasuda M, Deguchi T. Rapid detection of the mosaic structure of the *Neisseria gonorrhoeae* penA Gene, which is associated with decreased susceptibilities to oral cephalosporins. *J Clin Microbiol* 2008; 46(5): 1804–10. doi:10.1128/JCM.01800-07

44 European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters, version 9.0. Basel: EUCAST; 2019. Available online at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf [verified 1 June 2019].

45 Clinical and Laboratory Standards Institute. M100 Performance Standards for Antimicrobial Susceptibility Testing, 28th edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

46 Shimuta K, Unemo M, Nakayama S, Morita-Ishihara T, Dorin M, Kawahata T, Ohnishi M. 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* 2013; 57(11): 5225–32. doi:10.1128/AAC.01295-13

47 Tomberg J, Fedarovich A, Vincent LR, Jerse AE, Unemo M, Davies C, Nicholas RA. Alanine 501 mutations in penicillin-binding protein 2 from *Neisseria gonorrhoeae*: structure, mechanism, and effects on cephalosporin resistance and biological fitness. *Biochemistry* 2017; 56(8): 1140–50. doi:10.1021/acs.biochem.6b01030

48 Tomberg J, Unemo M, Ohnishi M, Davies C, Nicholas RA. Identification of amino acids conferring high-level resistance to expanded-spectrum cephalosporins in the penA gene from

Neisseria gonorrhoeae strain H041. *Antimicrob Agents Chemother* 2013; 57(7): 3029–36. doi:10.1128/AAC.00093-13

49 Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, Nakayama S, Kitawaki J, Unemo M. 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* 2011; 55(7): 3538–45. doi:10.1128/AAC.00325-11

50 Demczuk W, Sidhu S, Unemo M, Whiley DM, Allen VG, Dillon JR, Cole M, Seah C, Trembizki E, Trees DL, Kersh EN, Abrams AJ, de Vries HJC, van Dam AP, Medina I, Bharat A, Mulvey MR, Van Domselaar G, Martin I. *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance, a novel antimicrobial resistance multilocus typing scheme for tracking global dissemination of *N. gonorrhoeae* strains. *J Clin Microbiol* 2017; 55(5): 1454–68. doi:10.1128/JCM.00100-17

51 Tomberg J, Unemo M, Davies C, Nicholas RA. 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* 2010; 49(37): 8062–70. doi:10.1021/bi101167x

52 Pan W, Spratt BG. Regulation of the permeability of the gonococcal cell envelope by the mtr system. *Mol Microbiol* 1994; 11(4): 769–75. doi:10.1111/j.1365-2958.1994.tb00354.x

53 Ropp PA, Hu M, Olesky M, Nicholas RA. Mutations in ponA, the gene encoding penicillin-binding protein 1, and a novel locus, penC, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2002; 46(3): 769–77. doi:10.1128/AAC.46.3.769-777.2002

54 Zhao S, Tobiason DM, Hu M, Seifert HS, Nicholas RA. The penC mutation conferring antibiotic resistance in *Neisseria gonorrhoeae* arises from a mutation in the PilQ secretin that interferes with multimer stability. *Mol Microbiol* 2005; 57(5): 1238–51. doi:10.1111/j.1365-2958.2005.04752.x

55 Olesky M, Zhao S, Rosenberg RL, Nicholas RA. Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with penB mutations. *J Bacteriol* 2006; 188(7): 2300–8. doi:10.1128/JB.188.7.2300-2308.2006

56 Harris SR, Cole MJ, Spiteri G, Sanchez-Buso L, Golparian D, Jacobsson S, Goater R, Abudahab K, Yeats CA, Bercot B, Borrego MJ, Crowley B, Stefanelli P, Tripodo F, Abad R, Aanensen DM, Unemo M, Azevedo J, Balla E, Barbara C. Public health surveillance of multidrug-resistant clones of *Neisseria gonorrhoeae* in Europe: a genomic survey. *Lancet Infect Dis* 2018; 18(7): 758–68. doi:10.1016/S1473-3099(18)30225-1

57 Shimuta K, Watanabe Y, Nakayama S, Morita-Ishihara T, Kuroki T, Unemo M, Ohnishi M. Emergence and evolution of internationally disseminated cephalosporin-resistant *Neisseria gonorrhoeae* clones from 1995 to 2005 in Japan. *BMC Infect Dis* 2015; 15: 378. doi:10.1186/s12879-015-1110-x

58 Grad YH, Kirkcaldy RD, Trees D, Dordel J, Harris SR, Goldstein E, Weinstock H, Parkhill J, Hanage WP, Bentley S, Lipsitch M. Genomic epidemiology of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime in the USA: a retrospective observational study. *Lancet Infect Dis* 2014; 14(3): 220–6. doi:10.1016/S1473-3099(13)70693-5

59 Lo JY, Ho KM, Leung AO, Tiu FS, Tsang GK, Lo AC, Tapsail JW. Ceftibuten resistance and treatment failure of *Neisseria gonorrhoeae* infection. *Antimicrob Agents Chemother* 2008; 52(10): 3564–7. doi:10.1128/AAC.00198-08

60 Unemo M, Nicholas RA, Jerse AE, Davies C, Shafer WM. Molecular mechanisms of antibiotic resistance expressed by the pathogenic *Neisseria*. In Genco C, Wetzler L, editors. *Neisseria*: molecular mechanisms of pathogenesis, 2nd edn. Norfolk, UK: Caister Academic Press; 2014. pp. 161–192.

61 Doná V, Kasraian S, Lupo A, Guilarte YN, Hauser C, Furrer H, Unemo M, Low N, Endimiani A. Multiplex real-time PCR assay with high-resolution melting analysis for characterization of antimicrobial resistance in *Neisseria gonorrhoeae*. *J Clin Microbiol* 2016; 54(8): 2074–81. doi:10.1128/JCM.03354-15

62 Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas RA. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2009; 53(9): 3744–51. doi:10.1128/AAC.00304-09

63 Whiley DM, Jacobsson S, Tapsall JW, Nissen MD, Sloots TP, Unemo M. Alterations of the pilQ gene in *Neisseria gonorrhoeae* are unlikely contributors to decreased susceptibility to ceftriaxone and cefixime in clinical gonococcal strains. *J Antimicrob Chemother* 2010; 65(12): 2543–7. doi:10.1093/jac/dkq377

64 Lee SG, Lee H, Jeong SH, Yong D, Chung GT, Lee YS, Chong Y, Lee K. Various penA mutations together with mtrR, porB and ponA mutations in *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime or ceftriaxone. *J Antimicrob Chemother* 2010; 65(4): 669–75. doi:10.1093/jac/dkp505

65 World Health Organization. Global action plan on antimicrobial resistance. Geneva: World Health Organization; 2015.

66 Buono SA, Watson TD, Borenstein LA, Klausner JD, Pandori MW, Godwin HA. Stemming the tide of drug-resistant *Neisseria gonorrhoeae*: the need for an individualized approach to treatment. *J Antimicrob Chemother* 2015; 70(2): 374–81. doi:10.1093/jac/dku396

67 Klausner JD, Kerndt P. Cephalosporin resistance in *Neisseria gonorrhoeae* infections. *JAMA* 2013; 309(19): 1989–91. doi:10.1001/jama.2013.4078

68 Wong LKHP, Soge OO, Humphries RM, Klausner JD. Real-time PCR targeting the penA mosaic XXXIV type for prediction of extended-spectrum-cephalosporin susceptibility in clinical *Neisseria gonorrhoeae* isolates. *Antimicrob Agents Chemother* 2017; 61(11): e01339-17. doi:10.1128/AAC.01339-17

69 Lo JY, Ho KM, Lo AC. Surveillance of gonococcal antimicrobial susceptibility resulting in early detection of emerging resistance. *J Antimicrob Chemother* 2012; 67(6): 1422–6. doi:10.1093/jac/dks036