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Emergence, spread and characteristics of *Neisseria gonorrhoeae* isolates with in vitro decreased susceptibility and resistance to extended-spectrum cephalosporins in Sweden

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Running head: Gonococci with decreased susceptibility/resistance to cephalosporins

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ABSTRACT

Neisseria gonorrhoeae has developed resistance to most therapeutic antimicrobials introduced. Most worrying, treatment failures with oral ESCs are reported, especially from WHO Western Pacific Region (WPR), and the susceptibility to all ESCs (oral and injectable), the last remaining treatment options in many settings, is decreasing globally.

Objectives To examine the emergence, spread, and characteristics of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to ESCs in Sweden.

Methods All available Swedish isolates during 1998-2009, with exposure of infection in many countries worldwide, displaying a “decreased susceptibility” to cefixime and/or ceftriaxone (MIC \geq 0.032 mg/L; n=331) were examined using antibiograms, full-length *porB* gene sequencing, *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST), and sequencing of ESC resistance determinants (*penA*, *mtrR* and *porB* [*penB* alteration]).

Results Based on the EUCAST breakpoints, 30 (9.1%) and one (0.3%) of the strains displayed in vitro resistance to cefixime and ceftriaxone, respectively. *penA* mosaic alleles and *penA* A501 alteration were detected in 24% and 11%, respectively, of the isolates, and in increasing prevalence over the years. Moreover, among these isolates 38 NG-MAST STs were detected, with ST1407 (n=29), ST1103 (n=9) and ST3378 (n=8) being most prevalent.

Conclusions The proportions of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to ESCs have substantially increased over the years in Sweden. Both *penA* mosaic allele and *penA* A501 alteration, together with *mtrR* and *penB*, are important for the decreased susceptibility and resistance to ESCs. At least one gonococcal *penA* mosaic strain (ST1407), including its evolving subtypes, with decreased susceptibility/resistance to ESCs circulates worldwide.

Neisseria gonorrhoeae is the etiological agent of gonorrhoea, which remains a global public health problem. Most worrying, *N. gonorrhoeae* has developed high-level resistance to all antimicrobials used in the traditional treatment of gonorrhoea, e.g. penicillins, tetracyclines and fluoroquinolones.[1] Furthermore, recent years gonococcal strains with resistance and/or decreased susceptibility to azithromycin, including high-level resistance, and the currently ideal treatment options extended-spectrum cephalosporins (ESCs) have emerged.[1-10] Treatment failures using oral ESCs including cefixime are occurring, especially in the WHO Western Pacific Region (WPR),[1,5,11-13] and the susceptibility to all ESCs (oral and injectable) is decreasing globally. Verified treatment failures of urogenital gonorrhoea using ceftriaxone (injectable) are still lacking. However, the emergence and spread of decreased susceptibility and resistance to ESCs are serious concerns globally and gonorrhoea may become untreatable in certain circumstances.

The first molecular mechanism for decreased in vitro susceptibility and resistance to ESCs is alteration of the *penA* gene, i.e. acquisition of a *penA* mosaic allele that encodes an altered penicillin binding-protein 2 (PBP2).[1,3-10,13-15] However, also alterations of a single amino acid, i.e. A501, of PBP2 in non-mosaic *penA* alleles may decrease the ESC susceptibility.[1,4,6,16] Furthermore, mutations in the promoter and/or coding sequence of the repressor gene *mtrR*, which cause an over-expression of the MtrCDE efflux pump system further decrease the ESC susceptibility.[1,3,5,15,17] In addition, specific *porB1b* gene mutations (the *penB* resistance determinant) that alter amino acid G101 and A102 in the outer membrane PorB1b porin result in a decreased permeability and accordingly further decreased susceptibility to ESCs.[1,3,5,15,18,19] Finally, at least one unknown resistance determinant exists,[3,15] and accordingly all mechanisms that underlay the development of ESC decreased susceptibility and resistance are far from known.

Most, if not all, resistance in *N. gonorrhoeae* to different antimicrobials has historically

emerged in the WHO Western Pacific Region (WPR). This resistance has subsequently and rapidly spread globally, by sex tourists and other travellers.[1] The emergence and spread of ESC decreased susceptibility and resistance globally will most probably follow the same pattern. Accordingly, already in 2001 two cases of treatment failure with oral cephalosporins were reported in Japan,[20] and in 2006 all oral ESCs were excluded in the treatment guidelines for gonorrhoea in Japan, due to the high level of resistance.[1,5] In Sweden, the first gonococcal isolate with decreased susceptibility to ESCs, which also contained the characteristic *penA* mosaic allele X,[10] and was indistinguishable to the Japanese isolates causing treatment failures, was found in 2002.[3]

The aims of this study were to examine the emergence, spread, and phenotypic as well as genotypic characteristics of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to extended-spectrum cephalosporins (ESCs) in Sweden.

METHODS

***Neisseria gonorrhoeae* isolates**

All examined isolates were obtained at the National Reference Laboratory for Pathogenic Neisseria, Örebro University Hospital, Sweden from 1998 through 2009. All *N. gonorrhoeae* clinical isolates displaying a minimum inhibitory concentration [MIC] of ≥ 0.032 to cefixime and/or ceftriaxone were included (n=331). These isolates were from 331 patients (females 18% and males 82%). The mean age of the males were 34 years (median age: 31 years; and range: 16 to 68 years) and the mean age of the females were 34 years (median age: 32 years; and range: 15 to 59 years). The gender distribution and age distribution were relatively similar during the years examined. Unfortunately, complete data regarding sexual orientation of the patients and country of exposure to infection were not available. However, data available showed that the patients belonged to both heterosexual and men who have sex with men

(MSM) networks. Furthermore, especially the heterosexuals were exposed to the infection in many countries worldwide, e.g. European countries (n=14, spread all over Europe), African countries (n=3), Asian countries (n=6), Australia, and USA. The isolates were species confirmed with sugar utilization test and/or Phadebact GC Monoclonal Test (Boule Diagnostics AB, Huddinge, Sweden), and preserved as previously described.[21] The WHO 2008 *N. gonorrhoeae* reference strains were included as control strains.[22]

Antimicrobial susceptibility testing

The susceptibility (mg/L) to cefixime, ceftriaxone, ampicillin, azithromycin, ciprofloxacin, and spectinomycin was determined using the Etest method (AB Biodisk, Solna, Sweden), as previously described.[23] The breakpoints used for susceptibility (S) and resistance (R) were as follows: cefixime ($S \leq 0.125$ mg/L, $R > 0.125$ mg/L; only tentative in vitro breakpoint due to the lack of appropriate correlates between in vitro findings and clinical outcome), ceftriaxone ($S \leq 0.125$ mg/L, $R > 0.125$ mg/L; only tentative in vitro breakpoint due to the lack of appropriate correlates between in vitro findings and clinical outcome), ampicillin ($S \leq 0.125$ mg/L, $R > 2$ mg/L), azithromycin ($S \leq 0.25$ mg/L, $R > 0.5$ mg/L), ciprofloxacin ($S \leq 0.032$ mg/L, $R > 0.064$ mg/L) and spectinomycin ($S \leq 64$ mg/L, $R > 64$ mg/L), according to The European committee on antimicrobial susceptibility testing (EUCAST [www.eucast.org]). β -lactamase production was detected using nitrocefin discs.

Isolation of genomic DNA

Bacterial DNA was isolated in the robotized NorDiag Bullet instrument (NorDiag ASA Company, Oslo, Norway) using the BUGS'n BEADS™ STI-*fast* kit (NorDiag ASA Company), according to the instructions from the manufacturer.

Detection of *penA* mosaic alleles and alteration of amino acid A501 in PBP2

All gonococcal isolates (n=331) were screened for the presence of a *penA* mosaic allele using a previously described *penA* mosaic allele conventional PCR,[24] as well as an earlier depicted method based on real-time PCR and pyrosequencing.[25] Both these methods were used in order to identify most/all types of *penA* mosaic alleles, and the pyrosequencing method also identifies alterations of A501 in PBP2. DNA of the WHO 2008 *N. gonorrhoeae* WHO reference strain K,[22] which contains the characteristic *penA* mosaic allele X,[10] and sterile water were included in each run as positive control and negative control, respectively.

***mtrR* and *porB1b* sequencing**

The promoter and coding sequence of *mtrR*, and the full-length *porB1b* gene were sequenced in all isolates possessing a *penA* mosaic allele or any alteration of A501 in PBP2 (n=115), as previously described.[3,22] DNA of the WHO 2008 *N. gonorrhoeae* reference strain M and sterile water were included in all runs as positive control and negative control, respectively.[22]

***Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST)**

NG-MAST, was performed on all isolates possessing a *penA* mosaic allele or any alteration of A501 in PBP2 (n=115), as previously described.[26] NG-MAST allele numbers of *porB* and *tbpB*, and sequence types (STs) were assigned using the NG-MAST (www.ng-mast.net) database.

Sequence alignments and phylogenetic analysis

Multiple-sequence alignments of nucleotide sequences and the deduced corresponding amino acid sequences were performed in the software BioEdit Sequence Alignment Editor version

7.0.9.0 with manual adjustment.

Phylogenetic analysis of the full-length *porB1b* gene encoding the mature PBP2 was performed with the software TREECON (version 1.3b) by using the Jin and Nei substitution model, the Kimura evolutionary model, an α -value of 0.5 and the neighbor-joining method, as previously described.[26]

RESULTS

Longitudinal trends in the MICs of cefixime and ceftriaxone

The distributions of MICs of cefixime (2003, which was the first year all isolates were examined, and 2009) and ceftriaxone (1998 and 2009) displayed distinct shifts towards higher MICs over the years for both antimicrobials. An increased proportion of isolates with MICs above the tentative in vitro resistance breakpoints, cefixime 5.2% (n=12) in 2009 and ceftriaxone 0.4% (n=1) in 2009, were identified (Fig. 1).

Antimicrobial susceptibility of all selected *N. gonorrhoeae* isolates from 1998 to 2009 (n=331)

Based on the EUCAST breakpoints, 30 (9.1%) of the strains displayed in vitro resistance to cefixime. However, 330 (99.7%) of the isolates were susceptible to ceftriaxone, and accordingly only one isolate showed resistance (MIC=0.250 mg/L). Among all the isolates, 8% were resistant to ampicillin, 17% to azithromycin, and 91% to ciprofloxacin, however, no isolates resistant to spectinomycin were found. Beta-lactamase production was identified in 41 (12%) of the isolates.

The year of collection, number of isolates, presence of ESC resistance determinants, and susceptibility to ESCs of all isolates are summarized in Table 1.

Table 1. *Neisseria gonorrhoeae* displaying increased MICs of cefixime and/or ceftriaxone (≥ 0.032 mg/L) isolated during 1998-2009 in Sweden (n=331)

Yr of isolation (No. of isolates; % of isolates) ^a	<i>penA</i> alteration (% of isolates)	<i>mtrR</i> alteration ^b		PorB1b (<i>penB</i> alteration)		MIC (mg/L) range ^c	
	Mosaic allele	Promoter region	Coding region	G101	A102	Cefixime	Ceftriaxone
	Alteration of A501 in PBP2 ^d						
1998 (0; 0%), 1999 (2; 2%), 2000 (0; 0%), 2001 (2; 1%), 2005 (32; 6%)	None
	None
2002 (21; 17%) ^e	6 (5.0) ^e	6	6	6	6	0.25-0.5	0.064-0.125
	None
2003 (27; 21%) ^e	5 (3.8) ^e	5	None	5	5	0.25-0.5	0.125
	3 (2.3)	None	3	3	3	0.064-0.094	0.064-0.125
2004 (17; 11%)	None
	3 (2.0)	1	2	3	3	0.032-0.064	0.064-0.125
2006 (49; 11%)	2 (0.5)	2	None	2	2	0.047-0.094	0.064-0.125
	3 (0.7)	3	None	2	3	0.032-0.047	0.064
2007 (43; 9%)	10 (2.1)	10	None	10	10	0.032-0.25	0.032-0.125
	9 (1.8)	5	6	9	9	0.016-0.047	0.032-0.064
2008 (81; 15%)	20 (3.8)	20	None	19	19	0.023-0.25	0.023-0.25
	12 (2.3)	11	None	12	10	0.023-0.19	0.023-0.125
2009 (57; 13%)	35 (8.1)	33	None	34	34	0.032-0.38	0.008-0.125
	7 (1.6)	6	None	7	7	0.023-0.064	0.023-0.094

MIC, minimum inhibitory concentration

^aThe selection of isolates with increased MICs of cefixime and ceftriaxone was performed from a collection of 348 isolates (1998), 85 (1999), 131 (2000), 141 (2001), 120 (2002), 130 (2003), 149 (2004), 486 (2005), 431 (2006), 487 (2007), 523 (2008) and 434 (2009).

^bNumber of isolates that contained the characteristic single nucleotide (A) deletion in the inverted repeat of the promoter region and alteration of G45 in MtrR, respectively.

^cMIC-values for isolates that possessed a *penA* mosaic allele or an alteration of A501 in PBP2.

^dThe alterations of A501 in PBP2 were A501V (n=33) and A501T (n=4).

^eEight of the 11 isolates with decreased susceptibility and also *penA* mosaic allele in 2002 (n=6) and 2003 (n=5) were received from the USA (n=5) and UK (n=3) for a specific study regarding ESC decreased susceptibility.

***penA* mosaic allele and alteration of A501 in PBP2**

Seventy-eight (24%) isolates contained a *penA* mosaic allele, identified using the conventional PCR (n=77) and the pyrosequencing method (n=78), and 37 (11%) isolates comprised an alteration of A501 (A501V [n=33] and A501T [n=4]) in PBP2 (Table 1 and 2). However, no isolates contained a *penA* mosaic allele including any mutations causing an alteration of A501 in PBP2.

The proportion of isolates with *penA* mosaic allele substantially increased from the year 2006 (0.5%) to 2009 (8.1%), while there were no isolates with *penA* mosaic alleles during 1998-2001, in 2004 or in 2005. There were in total 11 isolates with *penA* mosaic allele already in 2002 (n=6) and 2003 (n=5), however, eight of those were received from USA (n=5) and UK (n=3) for a specific study regarding ESC decreased susceptibility. The prevalence of isolates with PBP2 A501 alteration ranged from 0.5% to 2.3% between 2003, when these were firstly detected, and 2009. The MICs of cefixime and ceftriaxone for the *penA* mosaic allele isolates ranged from 0.023-0.500 mg/L (mean MIC: 0.064 mg/L) and 0.008-0.250 mg/L (mean MIC: 0.047), respectively. The MICs of the isolates with PBP2 A501 alteration were 0.016-0.19 mg/L (cefixime, mean MIC: 0.064 mg/L) and 0.023-0.125 mg/L (ceftriaxone, mean MIC: 0.094 mg/L), respectively (Table 1 and 2). The majority of isolates, 62 (80%) out of 79, that displayed an increased MIC (>0.032) to both cefixime and ceftriaxone, also possessed either a *penA* mosaic allele or an alteration of A501 in PBP2.

Table 2. The NG-MAST STs, year of isolation, and MICs of cefixime and ceftriaxone for all *Neisseria gonorrhoeae* with a *penA* mosaic allele (n=78) or an alteration of A501 in PBP2 (n=37) isolated during 2002-2009 in Sweden

			No. of isolates with MIC (mg/L)								
NG-MAST ST (No. of isolates)	Year		≤0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5
<i>penA</i> mosaic allele isolates (n=78)											
1407 (29)	2007-2009	IX	2	4	7	3	7	1	4	1	
		TX	14	5	4	1	4		1		
3378 (8)	2008-2009	IX			2	2	1	1	1	1	
		TX	5	1		1	1				
326 (6)	2002	IX							5		1
		TX			1		5				
925 (5)	2003	IX							2		3
		TX					5				
4272 (3) ^a	2007-2008	IX							3		
		TX		1			2				
4275 (3) ^a	2008	IX	1				2				
		TX			3						
4269 (3) ^a	2008-2009	IX				1			1	1	
		TX				1	2				
3431 (3)	2009	IX		1	2						
		TX	3								
4274 (2) ^a	2008-2009	IX		1	1						
		TX	2								
2212 (2)	2009	IX		2							
		TX	2								
3158 (2)	2009	IX			2						
		TX	2								
4268 (2) ^a	2009	IX					1		1		
		TX	1				1				
Unique STs (10) ^b	2006-2009	IX	1	1	2	2	1	2	1		
		TX	3	2	1	1	3				
A501 alteration in PBP2 (n=37)											
1103 (9)	2008-2009	IX	2	2	3	1	1				
		TX		1	3	2	3				
1619 (6)	2003, 2007	IX	1	2	2	1					
		TX			4	1	1				
437 (3)	2006, 2008	IX		3							
		TX			3						
2943 (3)	2007	IX	3								
		TX	1	1	1						
1288 (3)	2009	IX	3								
		TX	3								
1723 (2)	2004	IX			2						
		TX			1		1				
1405 (2)	2007	IX	2								
		TX	2								
Unique STs (9) ^c	2003-2009	IX	7		1			1			
		TX	4	1	1	3					

NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing; ST, sequence type; MIC, minimum inhibitory concentration; IX, cefixime; TX, ceftriaxone.

^aNew sequence types found in the present study.

^bUnique STs represented by single isolates; ST3128, ST3499, ST3709, ST3787, ST4265^a, ST4266^a, ST4267^a, ST4273^a, ST4276^a and ST4642^a.

^cUnique STs represented by single isolates; ST1493, ST1582, ST1724, ST1766, ST3755, ST4271^a, ST4277^a, ST4278^a, and ST4279^a.

Specific mutations in *mtrR* and *porB1b* (*penB* alteration)

Mutations in the promoter region or coding sequence of the *mtrR* gene that evidently cause an

over-expression of the MtrCDE efflux pump were observed in 110 (96%) of the isolates examined (n=115). Ninety-three (81%) had the characteristic single nucleotide (A) deletion in the inverted repeat of the promoter region and eight isolates (7%) displayed alterations of amino acid G45 in MtrR (Table 1). Nine (8%) of the isolates contained both these alterations. *penB* alterations in amino acid 101 and/or 102 were detected in 113 (98%) of the isolates. One of these isolates lacked the alteration in amino acid position 101 and two isolates did not possess the alteration in 102. In only five (4%) of the isolates a mutation in *mtrR* occurred in the absence of the *penB* alteration. All the 31 isolates displaying in vitro resistance to cefixime (n=30) and ceftriaxone (n=1) according to EUCAST breakpoints contained *penA* alterations (mosaic allele [n=30] and A501 [n=1]) and *penB* alteration, and 30 of these isolates also comprised the single nucleotide (A) deletion in the promoter region of *mtrR*.

***N. gonorrhoeae* multiantigen sequence typing and phylogenetic analysis**

Among the isolates containing a *penA* mosaic allele (n=78), 22 different NG-MAST STs were identified, of which 11 had not been previously described (Table 2). However, the phylogenetic analysis of the full-length *porB* gene sequences (25 different sequences) showed that 60 (77%; 90% [60/67] of all found after 2003) of these *penA* mosaic allele isolates belonged to a cluster comprising all ST1407 (n=29) and very closely related genotypes (n=31), i.e. differing by a maximum of 14 nucleotides in the full-length *porB* gene (Fig. 2). The isolates comprising this cluster were from patients infected in Sweden, but also many other European Union (EU) as well as East-European countries, USA, South-East Asia (Thailand), and WHO WPR (Japan, The Philippines, China, Korea, Australia). Among the isolates comprising an alteration of A501 in PBP2 (n=37), a total of 16 different STs were observed, of which 4 had not been previously described. These isolates were phylogenetically more heterogeneous (data not shown), however, nine isolates belonged to the identical ST

(ST1103) (Table 2).

DISCUSSION

In the present study, it was clearly depicted that the susceptibility to extended-spectrum cephalosporins (both oral and injectable), the last remaining options for treatment of gonorrhoea, is rapidly decreasing and, according to the tentative EUCAST breakpoints [www.eucast.org], in vitro resistance to both cefixime and ceftriaxone has already been identified in Sweden. Furthermore, it was clearly shown that the decreased susceptibility and resistance to extended-spectrum cephalosporins such as cefixime and ceftriaxone is associated with both *penA* mosaic allele and alteration of A501 in PBP2, in synergy with the *mtrR* and *penB* resistance determinants, which has been previously reported.[1,3,15] However, the effects of the different resistance determinants on the fold-increase of the MICs differ for cefixime and ceftriaxone.[15] Interestingly, no clinical *N. gonorrhoeae* isolate with a *penA* mosaic allele that also contains mutations causing an alteration of A501 in PBP2 has ever been reported. The alteration of A501 in PBP2 still seems to be a gonococcal specific alteration, perhaps due to ESC selection pressure instead of transformation, which may reflect the lower extent of clonal spread observed in the present study. It cannot be excluded that an isolate with a *penA* mosaic allele that develops mutations resulting in an alteration of A501 comprises a lower biological fitness. However, probably this is not the case and instead it is only a matter of time before these type of strains will emerge, which may even be resistant to both cefixime and ceftriaxone.

The selection of isolates with MIC-values of ≥ 0.032 for cefixime and/or ceftriaxone in the present study made it possible to detect diverse combinations of the resistance determinants mentioned above. Accordingly, in order to detect isolates containing the initial ESC resistance determinants, i.e. in isolates that cannot be considered as fully susceptible to ESCs, the

breakpoints stated by Clinical and Laboratory Standards Institute (CLSI [www.clsi.org]) and EUCAST [www.eucast.org] are too high. Nevertheless, detection of the initial resistance determinants (*penA* mosaic allele or alteration of A501 in PBP2, *mtrR* and *penB* alterations) is currently only for surveillance purposes, i.e. to monitor the emergence and spread of isolates with decreased ESC susceptibility, and not for identification of isolates with clinical resistance causing treatment failures. However, especially for emergence and spread of decreased susceptibility and resistance to ESCs, especially ceftriaxone, this type of surveillance (using lower MIC breakpoints), if possible, supplemented by genetic screening for detection of *penA* mosaic alleles as well as alterations of A501 in PBP2, in addition to the *mtrR* and *penB* determinants, and monitoring of longitudinal trends in ESC MICs over time would be exceedingly valuable. Alterations in additional genes have been previously suggested to contribute to the decreased susceptibility and resistance to ESCs such as mutations in *pilQ* (previously known as *penC*),[27] and mutations in *ponA* encoding the PBP1.[3] However, recent studies have shown that, in contrast to the penicillins, *pilQ* and *ponA* do not significantly affect the susceptibility to ESCs in *N. gonorrhoeae*,[15,28] therefore these two genes were not examined in the present study. Most important, in many cases it remains difficult to accurately correlate the known resistance determinants (alterations in *penA*, *mtrR*, and *penB*) to the ESC MICs of the isolates.[1,3,5,15] Partly this is due to the fact that there are different types of *penA* mosaic and PBP2 A501 altered alleles, which continue to evolve, and their effects on the ESC MICs of the corresponding gonococcal isolates highly vary, e.g. some A501 altered alleles do not significantly increase the ESC MICs. In addition, the presence or absence of the *mtrR* and *penB* resistance determinants further affects the MICs of the ESCs. However, it is also clear that at least one unknown resistance determinant exists and accordingly the mechanisms and their interplay that underlay the development of ESC decreased susceptibility and resistance are far from

known.[1,3,15] Accordingly, an enhanced understanding and better correlates of the genetic resistance determinants, the *in vitro* ESC MICs of the corresponding isolates, treatment outcome, and spread of isolates with decreased susceptibility and resistance to ESC are essential.

Finally, the present study revealed, using NG-MAST and full-length *porB* gene sequencing, that several different gonococcal strains with *penA* mosaic allele or alterations of A501 in PBP2 are circulating in Sweden and other countries.[www.ng-mast.net; present study; 2,3,4,6,29,30] However, specific gonococcal strains such as ST1407, and its evolving genetically highly similar subtypes, containing a *penA* mosaic allele mainly identical to the *penA* mosaic allele X associated with treatment failures using oral ESCs in e.g. Japan,[10] are now spreading in increasing prevalence in the WHO WPR (Japan, The Philippines, China, Korea, Australia), South-East Asia (Thailand), many EU as well as East-European countries, USA, etc.[www.ng-mast.net; present study; 2,4,29,30] Accordingly, a more or less global spread, after initial emergence in the WHO WPR, of some gonococcal *penA* mosaic allele strains with decreased susceptibility and resistance to ESCs can already be seen.

In conclusions, the proportions of *N. gonorrhoeae* isolates with decreased susceptibility and resistance to cefixime and ceftriaxone have substantially increased over the years in Sweden. Several different gonococcal strains containing *penA* mosaic allele or alterations of A501 in PBP2 with decreased susceptibility to ESCs are circulating in Sweden and other countries. However, specific gonococcal strains such as ST1407, and its evolving genetically highly similar subtypes, account for the majority of the isolates spreading in increasing prevalence in Sweden and many other countries worldwide. Nevertheless, the magnitude of this spread as well as the presence of treatment failures are far from known, and it is essential to more effectively monitor and also consider this in a global context. Finally, an enhanced surveillance, understanding and better correlates of the genetic resistance determinants, the *in*

vitro ESC MICs of the corresponding isolates, treatment outcome, and spread of isolates with decreased susceptibility and resistance to ESC are essential.

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Figures: 2

Key messages

- Antimicrobial resistance (AMR) in gonococci is a major public health problem worldwide, and gonorrhoea may become untreatable in certain circumstances.
- The gonococcal susceptibility to extended-spectrum cephalosporins (ESCs), the last remaining treatment options in many settings globally, is rapidly decreasing in Sweden as observed worldwide.
- *penA* mosaic alleles and A501 alterations, together with *mtrR* and *penB*, are important for decreased susceptibility/resistance to ESCs, however, unknown resistance determinant(s) also exist(s).
- At least one gonococcal *penA* mosaic strain (ST1407), including its evolving genetically related subtypes, with decreased susceptibility/resistance to ESCs seems to be circulating worldwide.

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Contributors

MU in collaboration with DG, BH and HF initiated and designed the study. DG performed all the laboratory work. DG and MU analysed all the data and wrote the manuscript, in collaboration with BH and HF.

Competing Interest: None declared.

EXCLUSIVE LICENCE

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Conflict of interest None.

REFERENCES

1. **Tapsall JW**, Ndowa F, Lewis DA, *et al.* Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. *Expert Rev Anti Infect Ther* 2009;**7**:821-34.
2. **Chisholm SA**, Neal TJ, Alawattagama AB, *et al.* Emergence of high-level azithromycin resistance in *Neisseria gonorrhoeae* in England and Wales. *J Antimicrob Chemother* 2009;**64**:353-8.

3. **Lindberg R**, Fredlund H, Nicholas R, *et al.* Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime and ceftriaxone: Association with genetic polymorphisms in penA, mtrR, porB1b, and ponA. *Antimicrob Agents Chemother* 2007;**51**:2117-22.
4. **Whiley DM**, Limnios A, Ray S, *et al.* Diversity of penA alterations and subtypes in Neisseria gonorrhoeae strains from Sydney, Australia, that are less susceptible to Ceftriaxone. *Antimicrob Agents Chemother* 2007;**51**:3111-6.
5. **Barry PM**, Klausner JD. The use of cephalosporins for gonorrhea: the impending problem of resistance. *Expert Opin Pharmacother* 2009;**10**:555-77.
6. **Lee SG**, Lee H, Jeong SH, *et al.* 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**:669-75.
7. **Osaka K**, Takakura T, Narukawa K *et al.* Analysis of amino acid sequences of penicillin-binding protein 2 in clinical isolates of Neisseria gonorrhoeae with reduced susceptibility to cefixime and ceftriaxone. *J Infect Chemother* 2008;**14**:195-203.
8. **Muratani T**, Akasaka S, Kobayashi T, *et al.* Outbreak of Cefozopran (penicillin, oral cephems, and aztreonam)-resistant Neisseria gonorrhoeae in Japan. *Antimicrob Agents Chemother* 2001;**45**:3603-6.
9. **Ameyama S**, Onodera S, Takahata M, *et al.* 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**:3744-9.
10. **Ito M**, Deguchi T, Mizutani KS, *et al.* 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**:137-43.
11. **Yokoi S**, Deguchi T, Ozawa T, *et al.* Threat to cefixime treatment of gonorrhea.

- Emerg Infect Dis* 2007;**13**:1275-7.
12. **Lo JYC**, Ho KM, Leung AOC, *et al.* Ceftibuten resistance and treatment failure of *Neisseria gonorrhoeae* infection. *Antimicrob Agents Chemother* 2008;**52**:3564-7.
 13. **Tapsall JW**. Implications of current recommendations for third-generation cephalosporin use in the WHO Western Pacific region following the emergence of multiresistant gonococci. *Sex Transm Infect* 2009;**85**:256-8.
 14. **Ohnishi M**, Watanabe Y, Ono E, *et al.* Spread of a chromosomal cefixime-resistant penA gene among different *Neisseria gonorrhoeae* lineages. *Antimicrob Agents Chemother* 2010;**54**:1060-7.
 15. **Zhao S**, Duncan M, Tomberg J, *et al.* Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2009;**53**:3744-51.
 16. **Powell AJ**, Tomberg J, Deacon AM, *et al.* Crystal structures of penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of *Neisseria gonorrhoeae* reveal an unexpectedly subtle mechanism of antibiotic resistance. *J Biol Chem* 2009;**284**:1202-12.
 17. **Folster JP**, Johnson PJ, Jackson L, *et al.* MtrR modulates rpoH expression and levels of antimicrobial resistance in *Neisseria gonorrhoeae*. *J Bacteriol* 2008;**191**:287-97.
 18. **Olesky M**, Zhao S, Rosenberg RL, *et al.* Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with penB mutations. *J Bacteriol* 2006;**188**:2300-8.
 19. **Olesky M**, Hobbs M, Nicholas RA. Identification and analysis of amino acid mutations in Porin IB that mediate intermediate-level resistance to penicillin and tetracycline in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* 2002;**46**:2811-20.

20. **Akasaka S**, Muratani T, Yamada Y, *et al.* Emergence of cephem- and aztreonam-high-resistant *Neisseria gonorrhoeae* that does not produce beta-lactamase. *J Infect Chemother* 2001;**1**:49-50.
21. **Unemo M**, Olcén P, Berglund T, *et al.* Molecular epidemiology of *Neisseria gonorrhoeae*: sequence analysis of the *porB* gene confirms presence of two circulating strains. *J Clin Microbiol* 2002;**40**:3741-9.
22. **Unemo M**, Fasth O, Fredlund H, *et al.* 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* 2009;**63**:1142-51.
23. **Berglund T**, Unemo M, Olcén P, *et al.* One year of *Neisseria gonorrhoeae* isolates in Sweden: the prevalence study of antibiotic susceptibility shows relation to the geographic area of exposure. *Int J STD AIDS* 2002;**13**:109-14.
24. **Whiley D**, Bates J, Limnios A, *et al.* Use of a novel screening PCR indicates presence of *Neisseria gonorrhoeae* isolates with a mosaic *penA* gene sequence in Australia. *Pathology* 2007;**39**:445-6.
25. **Unemo M**, Olcén P, Fredlund H, *et al.* Real-time PCR and subsequent pyrosequencing for screening of *penA* mosaic alleles and prediction of reduced susceptibility to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae*. *APMIS* 2008;**116**:1004-8.
26. **Unemo M**, Sjöstrand A, Akhras M, *et al.* Molecular characterization of *Neisseria gonorrhoeae* identifies transmission and resistance of one ciprofloxacin-resistant strain. *APMIS* 2007;**115**:231-41.
27. **Zhao S**, Tobiason DM, Hu M, *et al.* 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**:1238–51.
28. **Whiley DM**, Jacobsson S, Tapsall JW, *et al.* The *Neisseria gonorrhoeae* pilQ gene is an unlikely contributor to reduced susceptibility to extended spectrum cephalosporins in clinical strains. Submitted for publication.
29. **Pandori M**, Barry PM, Wu A, *et al.* Mosaic penicillin-binding protein 2 in *Neisseria gonorrhoeae* isolates collected in 2008 in San Francisco, California. *Antimicrob Agents Chemother* 2009;**53**:4032-4.
30. **Tapsall JW**, Ray S, Limnios A. Characteristics and population dynamics of mosaic penA allele-containing *Neisseria gonorrhoeae* isolates collected in Sydney, Australia, in 2007-2008. *Antimicrob Agents Chemother* 2010;**54**:554-6.

Figure legends

Figure 1 The distribution of *Neisseria gonorrhoeae* MICs of **A.** ceftriaxone (1998 and 2009) and **B.** cefixime (2003, which was the first year all isolates were examined, and 2009). The tentative in vitro breakpoints for resistance (www.eucast.org) are indicated as a dashed line.

1998, 348 isolates; 2003, 130 isolates; and 2009, 230 isolates

Figure 2 Phylogenetic tree describing the evolutionary relationships of full-length *porB* gene sequences of *Neisseria gonorrhoeae* penA mosaic isolates (n=78) cultured in Sweden during 2002-2009. The 2000 WHO K reference strain,[22] comprising a *penA* mosaic allele X and cultured in Japan in 2001, was used to root the tree. The *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) sequence type (ST) and number of isolates are indicated.





