

# Importance of Multidrug Efflux Pumps in the Antimicrobial Resistance Property of Clinical Multidrug-Resistant Isolates of *Neisseria gonorrhoeae*

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**The contribution of drug efflux pumps in clinical isolates of *Neisseria gonorrhoeae* that express extensively drug-resistant or multidrug-resistant phenotypes has heretofore not been examined. Accordingly, we assessed the effect on antimicrobial resistance of loss of the three gonococcal efflux pumps associated with a known capacity to export antimicrobials (MtrC-MtrD-MtrE, MacA-MacB, and NorM) in such clinical isolates. We report that the MIC of several antimicrobials, including seven previously and currently recommended for treatment was significantly impacted.**

Gonorrhoea remains a major public health concern worldwide (1). *Neisseria gonorrhoeae* has developed resistance to all antimicrobials introduced for treatment (2–5). Recently, treatment failures with the extended-spectrum cephalosporins (ESCs) cefixime and ceftriaxone were verified in several countries and some extensively drug-resistant (XDR) gonococcal strains displaying high-level ceftriaxone resistance have been described (3, 6–13). ESCs are the last options for antimicrobial monotherapy of gonorrhoea. Thus, novel antimicrobials or nonantimicrobial compounds for sustained therapy of gonorrhoea are essential.

The mechanisms of ESC resistance involve specific modifications of the target penicillin binding protein 2 and PorB1b, resulting in decreased intake, and increased efflux due to MtrC-MtrD-MtrE efflux pump overexpression (3, 5, 7, 12, 14–19). The MtrC-MtrD-MtrE efflux pump belongs to the family of resistance-nodulation-division pumps and is functionally and structurally similar to the AcrAB-TolC efflux pump in *Escherichia coli* and MexA-MexB-OprM in *Pseudomonas aeruginosa*. All three of these pumps contribute to bacterial resistance to antimicrobials, including penicillin (20) and macrolides (21). Gonococci harbor two additional efflux pumps that are known to extrude antimicrobials, i.e., the MacA-MacB and NorM pumps that export macrolides (22) and fluoroquinolones (23), respectively. However, detailed informa-

tion regarding the potential export of ESCs and other antimicrobials, especially by multiresistant clinical isolates, through these gonococcal efflux pumps is limited.

In this study, we genetically inactivated (IA) the MtrC-MtrD-MtrE, MacA-MacB, and NorM efflux pumps in XDR and multidrug-resistant (MDR) gonococcal isolates and measured the effects on susceptibility to antimicrobials previously or currently recommended for the treatment of gonorrhoea.

The isolates examined were WHO reference strains WHO F, which is considered to be a wild-type strain susceptible to all of the antimicrobials included, and WHO P (24); four isolates from ESC treatment failures, including the first XDR strain (H041) with high-level ceftriaxone resistance (6, 10, 12); one isolate displaying high-level azithromycin resistance (25); and three isolates display-

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**TABLE 1** MICs of seven antimicrobials for XDR strain H041 (12) with high-level ceftriaxone resistance preinactivation and with efflux pumps MtrC-MtrD-MtrE, MacA-MacB, and NorM IA separately and in all possible combinations<sup>a</sup>

Efflux pump(s) IA	MIC (μg/ml) (SIR category) <sup>a</sup>						
	Ceftriaxone	Cefixime	Penicillin	Azithromycin	Ciprofloxacin	Tetracycline	Solithromycin
None (preinactivation)	4 (R)	8 (R)	4 (R)	1 (R)	>32 (R)	4 (R)	0.064 (NA)
MtrCDE	1 (R)	4 (R)	0.5 (I)	0.064 (S)	16 (R)	1 (I)	0.004 (NA)
MacAB	2 (R)	4 (R)	2 (R)	0.25 (S)	>32 (R)	2 (R)	0.125 (NA)
NorM	2 (R)	8 (R)	4 (R)	0.5 (I)	>32 (R)	0.5 (I)	<0.002 (NA)
MtrCDE + MacAB	1 (R)	4 (R)	0.5 (I)	0.064 (S)	32 (R)	1 (I)	0.032 (NA)
MtrCDE + NorM	1 (R)	8 (R)	0.5 (I)	0.064 (S)	8 (R)	0.5 (I)	<0.002 (NA)
MacAB + NorM	2 (R)	8 (R)	4 (R)	0.25 (S)	>32 (R)	2 (R)	0.008 (NA)
MtrCDE + MacAB + NorM	0.5 (R)	4 (R)	0.5 (I)	0.032 (S)	16 (R)	0.5 (I)	<0.002 (NA)

<sup>a</sup> All results are reported as whole MIC dilutions and in accordance with the susceptibility (S), intermediate resistance (I), and resistance (R) breakpoints of the Clinical Laboratory and Standards Institute ([www.clsi.org](http://www.clsi.org)). For azithromycin, the breakpoints of the European Committee on Antimicrobial Susceptibility Testing ([www.eucast.org](http://www.eucast.org)) were used. Modal MICs from three repeated experiments are presented. NA, not available.

**TABLE 2** Impact of seven antimicrobials that inactivate the MtrC-MtrD-MtrE, MacA-MacB, and NorM efflux pumps on MICs for the nine *N. gonorrhoeae* isolates investigated pre- and postinactivation

Isolate and efflux pump IA <sup>a</sup>	MIC (μg/ml) <sup>b</sup>						
	CRO	CFM	PEN	AZM	CIP	TET	SOM
<b>WHO F (24)</b>							
None (preinactivation)	<0.002	<0.016	0.032	0.125	0.004	0.25	0.064
MtrCDE	<0.002	<0.016	0.016	0.032	0.004	0.25	0.004
MacAB	<0.002	<0.016	0.032	0.125	0.004	0.25	0.016
NorM	<0.002	<0.016	0.032	0.125	0.004	0.25	<0.002
<b>WHO P (24)</b>							
None (preinactivation)	0.004	<0.016	0.25	2	0.004	1	0.5
MtrCDE	0.002	<0.016	0.125	0.064	0.004	1	0.008
MacAB	0.008	<0.016	0.25	2	0.008	1	0.25
NorM	0.004	<0.016	0.5	4	0.008	1	0.25
<b>NOR case 1 (6)</b>							
None (preinactivation)	0.125	0.25	4	0.5	>32	4	0.125
MtrCDE	0.016	0.125	0.125	0.064	8	0.5	<0.002
MacAB	0.125	0.25	4	0.5	>32	4	0.125
NorM	0.032	0.25	2	0.125	16	1	0.016
<b>NOR case 2 (6)</b>							
None (preinactivation)	0.125	0.25	2	0.5	>32	4	0.125
MtrCDE	0.016	0.064	0.25	0.064	4	0.5	0.016
MacAB	0.064	0.125	1	0.5	32	1	0.064
NorM	0.064	0.064	2	0.5	16	2	0.032
<b>SWE case (10)</b>							
None (preinactivation)	0.125	0.5	4	0.25	>32	2	0.125
MtrCDE	0.016	0.125	0.25	0.032	4	0.5	<0.002
MacAB	0.125	0.5	4	0.5	>32	2	0.064
NorM	0.125	0.25	2	0.5	16	2	0.016
<b>AZM HLR (25)</b>							
None (preinactivation)	0.008	<0.016	0.25	4096	0.016	2	32
MtrCDE	0.002	<0.016	0.125	128	0.004	1	0.5
MacAB	0.004	<0.016	0.25	4096	0.016	2	32
NorM	0.002	<0.016	0.25	4096	0.016	2	16
<b>AZM LLR 1</b>							
None (preinactivation)	0.008	<0.016	0.25	8	0.004	1	0.125
MtrCDE	0.004	<0.016	0.125	1	0.004	1	<0.002
MacAB	0.008	<0.016	0.125	2	0.004	0.5	0.064
NorM	0.008	<0.016	0.25	4	0.004	1	<0.002
<b>AZM LLR 2</b>							
None (preinactivation)	0.004	0.032	0.25	8	0.002	1	0.125
MtrCDE	<0.002	<0.016	0.064	1	0.004	0.5	<0.002
MacAB	0.004	<0.016	0.25	2	0.004	1	0.064
NorM	0.004	<0.016	0.25	4	0.004	1	0.032
<b>AZM LLR 3</b>							
None (preinactivation)	<0.002	<0.016	0.032	8	0.004	1	0.5
MtrCDE	<0.002	<0.016	0.016	1	0.004	1	0.016
MacAB	<0.002	<0.016	0.032	4	0.004	1	0.25
NorM	<0.002	<0.016	0.032	8	0.004	1	0.064

<sup>a</sup> Includes 2008 WHO *N. gonorrhoeae* reference strains F and P (24), three isolates from treatment failures with ESCs (NOR cases 1 and 2 [6] and an SWE case [10]), three isolates with low-level resistance (LLR) to azithromycin, and one isolate displaying high-level resistance (HLR) to azithromycin (25).

<sup>b</sup> CRO, ceftriaxone; CFM, cefixime; PEN, penicillin; AZM, azithromycin; CIP, ciprofloxacin; TET, tetracycline; SOM, solithromycin. All results are reported in whole MIC dilutions and susceptibility (S) and resistance (R) breakpoints of the Clinical Laboratory and Standards Institute ([www.clsi.org](http://www.clsi.org)). For azithromycin, the breakpoints of the European Committee on Antimicrobial Susceptibility Testing ([www.eucast.org](http://www.eucast.org)) were used. Modal MICs from three repeated experiments are presented.

ing low-level azithromycin resistance. All of the strains except WHO F contained *mtrR* mutations known to increase the expression of *mtrCDE* (21, 26) and possessed the *mtrCDE*-, *macAB*-, and *norM*-encoded pumps. None of the strains produced  $\beta$ -lactamase or contained any *erm* genes (data not presented). The MICs ( $\mu\text{g/ml}$ ) of seven antimicrobials pre- and postinactivation of the efflux pump(s) were determined by the Etest method (bioMérieux, Solna, Sweden) on Difco GC Medium Base agar (BD Diagnostics, Sparks, MD) supplemented with 1% IsoVitalEx (BD Diagnostics) according to the manufacturer's instructions (Tables 1 and 2). The *mtrD*, *macA*, and *norM* genes were IA in the isolates by spot transformation (27) with 0.1  $\mu\text{g}$  of chromosomal DNA from genetic derivatives of strain FA19 or wild-type strain FA19 bearing insertions in *mtrD* (strain KH14; *mtrD::kan*), *macA* (strain BR54; *macA::spc*), or *norM* (strain FA19; *norM::kan*). Transformants resistant to kanamycin (100  $\mu\text{g/ml}$ ) or spectinomycin (60  $\mu\text{g/ml}$ ) were selected as previously described (22, 23, 28). MIC differences between the mutants and the parent strain were considered significant if they were  $>2$ -fold. Inactivation of efflux pump genes in representative transformants was confirmed by PCR and pump gene-specific oligonucleotide primers as described previously (22, 23, 28).

Loss of the MtrC-MtrD-MtrE pump had the highest impact on the XDR phenotype of H041 (Table 1) and on MDR phenotypes (Table 2). The H041-IA<sub>MtrCDE</sub> transformant showed significantly increased susceptibility to penicillin, ceftriaxone, azithromycin, tetracycline, and solithromycin. The H041 transformant with MacAB IA had 4- and 2-fold increased susceptibility to azithromycin and ceftriaxone, respectively, while the transformant with NorM IA showed significantly ( $>32$ -fold) increased solithromycin susceptibility. Loss of multiple pumps did not significantly increase susceptibility compared to that of mutants lacking only MtrC-MtrD-MtrE (Table 1).

Loss of the MtrC-MtrD-MtrE pump by the three additional clinical ESC-resistant isolates significantly increased susceptibility to ESCs, penicillin, azithromycin, ciprofloxacin, and solithromycin (Table 2). Loss of the MacA-MacB or NorM pump by these strains did not consistently and significantly change the MICs of antimicrobials. Since azithromycin is used in the dual therapy with ceftriaxone recommended in the United States (29) and Europe (30), we examined four strains with low-level azithromycin resistance (MIC = 2 to 8  $\mu\text{g/ml}$ ) and one strain with high-level azithromycin resistance (MIC = 4,096  $\mu\text{g/ml}$ ). In all four strains with low-level resistance, susceptibility to azithromycin was increased 8- to 32-fold by loss of the MtrC-MtrD-MtrE pump, whereas loss of the MacA-MacB or NorM pump had no significant effect on azithromycin resistance. Similarly, loss of the MtrC-MtrD-MtrE pump by the strain with high-level resistance (MIC = 4,096  $\mu\text{g/ml}$ ) decreased its susceptibility to azithromycin 5-fold (Table 2).

In gonococci, the main focus of past research regarding efflux pump-mediated antimicrobial resistance has been the MtrC-MtrD-MtrE pump, which can be overexpressed because of specific mutations in *mtrR* (promoter or coding sequence), which encodes the repressor MtrR (16, 17, 20, 31–33). Overexpression is known to affect the susceptibility of macrolides, penicillins, and ESCs (3, 5, 15, 20, 32). Here we showed that susceptibility to ciprofloxacin and solithromycin can also be affected. The MtrC-MtrD-MtrE pump also exports fatty acids, bile salts, and endogenous antibacterial peptides, and accordingly, overexpression

might cause enhanced fitness of the gonococcal strains (34–36). The MacA-MacB and NorM pumps are known to export macrolides (22) and fluoroquinolones (23), respectively. We also showed that ESCs, penicillin, and solithromycin might be exported by NorM and/or MacA-MacB. However, since  $\beta$ -lactam antimicrobials and solithromycin have not previously been described as substrates of the MacA-MacB or NorM efflux pump (31), we cannot discount the possibility that loss of these pumps has secondary effects that increase cell envelope permeability to these antimicrobials.

In conclusion, we found that inactivation of efflux pumps in antimicrobial-resistant clinical gonococcal isolates influenced their susceptibility to many antimicrobials, even reverting strains to clinical susceptibility according to MIC breakpoints. This was most notable for the MtrC-MtrD-MtrE pump in H041, the first clinical XDR isolate with high-level resistance to ceftriaxone, which was obtained from a ceftriaxone treatment failure (12). However, this was also observed for many MDR clinical gonococcal strains (6, 10, 24, 25), including strains that resulted in ESC treatment failures. Accordingly, a specific efflux pump inhibitor (EPI) of the gonococcal MtrC-MtrD-MtrE pump, particularly co-administered with appropriate antimicrobials, might be a future treatment option for gonorrhoea. This could be a novel way to retain antimicrobials for longer use, e.g., ceftriaxone, or even restore antimicrobials no longer in use because of high-level resistance, e.g., penicillin, and could also mitigate the emergence of resistance. Nevertheless, many challenges remain in the development of a gonococcal EPI, e.g., selection of an ideal target and avoidance of toxicity to human cells (37–39).

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