Impact of the gonococcal FC428 penA allele 60.001 on ceftriaxone resistance and biological fitness

Ke Zhoua,b,c, Shao-Chun Chena,b,d, Fan Yangc, Stijn van der Veend,e and Yue-Ping Yinb,a

*Peking Union Medical College, Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, People’s Republic of China; aDepartment of Microbiology and Parasitology, School of Medicine, Zhejiang University, Hangzhou, People’s Republic of China; bDepartment of Dermatology, School of Medicine, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, People’s Republic of China; cState Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, School of Medicine, The First Affiliated Hospital, Zhejiang University, Hangzhou, People’s Republic of China

ABSTRACT

Global dissemination of the Neisseria gonorrhoeae ceftriaxone-resistant FC428 clone jeopardizes the currently recommended ceftriaxone-based first-line therapies. Ceftriaxone resistance in the FC428 clone has been associated with the presence of its mosaic penA allele 60.001. Here we investigated the contribution penA allele 60.001 to ceftriaxone resistance and its impact on biological fitness. Gonococcal isolates expressing penA allele 60.001 and mosaic penA allele 10.001, which is widespread in the Asia-Pacific region and associated with reduced susceptibility to ceftriaxone and cefixime, were genetic engineered to exchange their penA alleles. Subsequent antimicrobial susceptibility analyses showed that mutants containing penA allele 60.001 displayed 8- to 16-fold higher ceftriaxone and cefixime minimal inhibitory concentrations (MICs) compared with otherwise isogenic mutants containing penA allele 10.001. Further analysis of biological fitness showed that in vitro liquid growth of single strains and in the competition was identical between the isogenic penA allele exchange mutants. However, in the presence of high concentrations of palmitic acid or lithocholic acid, the penA allele 60.001-containing mutants grew better than the isogenic penA allele 10.001-containing mutants when grown as single strains. In contrast, the penA allele 60.001 mutants outcompeted the penA allele 60.001 mutants when grown in competition at slightly lower palmitic acid or lithocholic acid concentrations. Finally, the penA allele 60.001 mutants were outcompeted by their penA allele 10.001 counterparts for in vivo colonization and survival in a mouse vaginal tract infection model. In conclusion, penA allele 60.001 is essential for ceftriaxone resistance of the FC428 clone, while its impact on biological fitness is dependent on the specific growth conditions.

INTRODUCTION

Neisseria gonorrhoeae causes the widespread bacterial sexually transmitted disease gonorrhoea, which is predicted to have an annual global incidence of 87 million new cases [1]. Infections commonly manifest as urethritis or cervicitis, but asymptomatic infections of the cervix or pharynx are very frequently observed [2,3]. These untreated infections occasionally result in severe complications, including ectopic pregnancies and pelvic inflammatory disease, and they are a major source for transmission of N. gonorrhoeae [4]. N. gonorrhoeae is a multidrug-resistant pathogen that has developed resistance against all previously used antimicrobial therapies [5]. Current first-line treatment guidelines generally recommend ceftriaxone as a single drug therapy or ceftriaxone in combination with azithromycin as a dual therapy. However, many countries have reported increasing incidences of azithromycin resistance, including high-level azithromycin [6–8], and therefore the inclusion of azithromycin in the dual therapy has recently become under scrutiny [9]. Furthermore, gonococcal susceptibility to ceftriaxone is decreasing in many countries [8,10–12] and ceftriaxone treatment failures are increasingly reported globally [13–18]. Importantly, while initially ceftriaxone treatment failures were attributed to sporadic infections of unrelated strains containing mosaic penA alleles providing ceftriaxone resistance [17,19–22], in recent years many of the reported ceftriaxone treatment failures are the result of the FC428 clone identified in 2015 in Japan [23]. This clone contains the mosaic penA allele 60.001 and has successfully transmitted on a global scale, with reported cases in Japan [24], China [25–27], Denmark [28], Canada [29], Australia [30], Ireland [31], UK [32], and France [14]. In
addition, incidences where this *penA* 60.001 allele has transferred to unrelated strains and subsequently caused treatment failure have also been reported [33]. In recent years, the FC428 clone has widely transmitted throughout China, since cases have been reported from many geographically distinct regions [34], and its incidence also appears to be rapidly increasing [35].

The *mosaic penA* allele 60.001 contains the A311 V and T483S polymorphisms that were considered as essential mutations in the high-level ceftriaxone-resistant strains HO41, A8804 and GU140106 isolated previously in Japan and Australia [21,22,36], although additional I312M, F504L, N512Y and G545S polymorphisms associated with reduced cephalosporin susceptibility in mosaic *penA* alleles [37,38] are also present in *penA* allele 60.001. Importantly, the mosaic alleles *penA* 37 (HO41) and *penA* 42 (F89), which provide high-level ceftriaxone resistance, incur a biological fitness cost. Cloning of these *penA* alleles in unrelated gonococcal isolates had a negative impact on *in vitro* growth and these mutants were outcompeted by their otherwise isogenic wild-type strains for colonization in a mouse model of infection [39]. The negative impact of these *penA* alleles on biological fitness might explain why these ceftriaxone-resistant strains have thus far remained sporadic and have not widely transmitted. However, this might have changed with the occurrence of the ceftriaxone-resistant FC428 clone, which has transmitted globally. The *penA* allele of this strain might not incur a severe fitness cost as observed for other ceftriaxone-resistant *penA* alleles, which could explain its successful global transmission. Therefore, the aim of the present study was to investigate the impact of *penA* allele 60.001 on biological fitness during *in vitro* growth in cultures and *in vivo* in a mouse model of infection.

**Materials and methods**

**Bacterial strains, mutants and culture conditions**

*N. gonorrhoeae* strains ATCC49226, SZ20 (*penA* 60.001, *mtrR* 1, *penA* 1) [26], SRRSH78 (*penA* 10.001, *mtrR* 1, *penA* 1) [8] and their derivatives were cultured on GC agar (Oxoid Ltd., Basingstoke, UK) containing 1% (v/v) Vitox (Oxoid Ltd., Basingstoke, UK) at 37°C and 5% CO₂ and stored in GC broth containing 15% glycerol (Biosharp, Hefei, China) at −80°C. The streptomycin-resistant derivatives of strains SZ20 and SRRSH78 were selected on GC agar containing 1% Vitox and streptomycin (BBI, Shanghai, China). These streptomycin-resistant derivatives were named SZ20-*penA*60 and SRRSH78-*penA*10, respectively, and contained their original *penA* alleles, but were given this name for clarity throughout the study about phenotypes associated with *penA* alleles. Strains SZ20-*penA*60.001 and SRRSH78-*penA*10.001 were genetically engineered using the dominant streptomycin-susceptible rpsl gene to exchange their *penA* alleles without leaving a selection marker [40]. Fragments of *penA* 60.001 and *penA* 10.001 were amplified from the SZ20 and SRRSH78 genomes, respectively, using primers *penA*-F (GCCAGTCGGAGTGGGAGGCTGAG), *penA*-R (GCTCTAGACGCTGTTACGAGACTTTAT), *penA*-F2 (GCCAGTCTCCTGTTTTATACGGTGATCA) and *penA*-R2 (GCCGTCGCACTCCAAACGACCACGCACTTTAT) and cloned into vector pUC57-kan and pUC57-kanA10. These vectors were subsequently linearized and transformed into strains SZ20-*penA*60 and SRRSH78-*penA*10 to generate SZ20-*penA*60 and SRRSH78-*penA*10. The chloramphenicol-resistant derivatives SZ20-*penA*60-cat2, SZ20-*penA*10-cat2, SRRSH78-*penA*10-cat2, and SRRSH78-*penA*60-cat2 were generated with the vector pUC57-lctP-cat2-aspc [41], which inserts the chloramphenicol-resistant gene cat2 in the unrelated convergent lctP-aspc locus. The kanamycin-resistant derivatives SZ20-*penA*60-kanR, SZ20-*penA*10-kanR, SRRSH78-*penA*10-kanR, and SRRSH78-*penA*60-kanR were generated with the vector pUC57-lctP-kanR-aspc [42], which inserts the kanamycin-resistant gene kanR in the lctP-aspc locus.

**Ceftriaxone and cefxime susceptibility assays**

*N. gonorrhoeae* strains were tested for ceftriaxone and cefxime susceptibility using the agar dilution method according to WHO guidelines and *N. gonorrhoeae* ATCC49226 was included for quality control. Overnight grown bacteria were suspended into GC broth containing 1% Vitox and droplets containing 10⁴ CFU were spotted onto GC agar plates containing 1% Vitox and a twofold dilution series of ceftriaxone or cefxime. Plates were incubated for 24 h at 37°C and 5% CO₂ and the minimal inhibitory concentration (MIC) was determined as the lowest concentration at which no growth was observed.

**Liquid growth and in vitro competition assays**

Overnight grown bacteria were suspended in 12 mL GC broth containing 1% Vitox at an optical density (OD₆₀₀) of 0.025. Cultures were incubated at 37°C and 200 rpm and samples were taken every two hours for OD₆₀₀ measurements. For growth in the presence of fatty acids or bile, 2 mg/L (SZ20 derivatives) or 4 mg/L (SRRSH78 derivatives) palmitic acid (Aladdin, Shanghai, China), or 10 mg/L (SZ20 derivatives) or 85 mg/L (SRRSH78 derivatives) lithocholic acid (Aladdin, Shanghai, China) were added. For competition assays, overnight grown isogenic strains containing *penA* 60.001 and *penA* 10.001 were co-cultured on GC agar (Oxoid Ltd., Basingstoke, UK) at 37°C and 5% CO₂ and stored in GC broth containing 1% (v/v) Vitox and a twofold dilution series of ceftriaxone or cefxime. Plates were incubated for 24 h at 37°C and 5% CO₂ and the minimal inhibitory concentration (MIC) was determined as the lowest concentration at which no growth was observed.
and expressing different selection markers were mixed at equal numbers and suspended in 12 mL GC broth containing 1% Vitox at an OD_{600} of 0.025. Culture was incubated at 37°C and 200 rpm and every two hours samples were taken, serially diluted and plated onto GC agar containing 1% Vitox and 100 mg/L kanamycin (Inalco SpA, Milano, Italy) or 7.5 mg/L chloramphenicol (Inalco SpA, Milano, Italy). Plates were incubated for 24–48 h at 37°C and 5% CO_{2} and colonies were enumerated. For competition assays in the presence of fatty acids or bile, 1.25 mg/L (SZ20 derivatives) or 1.5 mg/L (SRRSH78 derivatives) palmitic acid, or 5 mg/L (SZ20 derivatives) or 60 mg/L (SRRSH78 derivatives) lithocholic acid was added.

**Spot assays**

Overnight grown bacteria were suspended in GC broth containing 1% Vitox and 5 μL droplets of a tenfold dilution series were applied on GC agar containing 1% Vitox and GC agar containing 1% Vitox and 12 mg/L (SZ20 derivatives) or 160 mg/L (SRRSH78 derivatives) palmitic acid, or 10 mg/L (SZ20 derivatives) or 70 mg/L (SRRSH78 derivatives) lithocholic acid. Plates were incubated for 24–48 h at 37°C and 5% CO_{2} and colonies were enumerated. For competition assays in the presence of fatty acids or bile, 1.25 mg/L (SZ20 derivatives) or 1.5 mg/L (SRRSH78 derivatives) palmitic acid, or 5 mg/L (SZ20 derivatives) or 60 mg/L (SRRSH78 derivatives) lithocholic acid was added.

**In vivo competition assays in a mouse vaginal tract model of infection**

Competition assays in a mouse vaginal tract infection model were performed as described previously [40,41,43]. Dioestrus stage female BALB/c mice (Shanghai SLAC Laboratory Animal Company, Shanghai, China) at six to eight weeks of age were injected subcutaneously with 0.1 mg of β-estradiol (Aladdin, Shanghai, China) in sesame oil (Sigma-Aldrich Co., St Louis, USA) on days −2, 0 and 2. In addition, mice also received two doses of 0.6 mg vancomycin (Meilunbio, Dalian, China) and 1.2 mg streptomycin every day and drinking water was spiked with 0.4 g/L trimethoprim (Meilunbio, Dalian, China). Mixed bacterial suspensions containing equal numbers of strain SZ20-penA60-catA2 and strain SZ20-penA10-kanR or equal numbers of strain SRRSH78-penA10-kanR and strain SRRSH78-penA60-catA2 were formulated in PBS with 0.5 mM CaCl_{2} (Sigma-Aldrich Co., St Louis, USA), 1 mM MgCl_{2} (Sigma-Aldrich Co., St Louis, USA) and 1% (w/v) gelatin (Aladdin, Shanghai, China) and inoculated intravaginally on day 0 at a total dose of 2×10^{7} CFU. Daily bacterial load in the vaginal tract were monitored by swabbing and plating on GC agar containing with 1% Vitox, 3 mg/L vancomycin, 7.5 mg/L colistin (Meilunbio, Dalian, China), 2.8 mg/L nystatin (Meilunbio, Dalian, China), 5 mg/L trimethoprim, 100 mg/L streptomycin and 100 mg/L kanamycin or 7.5 mg/L chloramphenicol. The competition index (CI) was calculated as (penA10/pemA60)_{output}/(penA10/pemA60)_{input}. All animal experiments were approved by the Zhejiang University Animal Care and Use Committee under project license number ZJU2015-032-01. Procedures followed the guidelines of the Administration of Affairs Concerning Experimental Animals of the People’s Republic of China and adhered to the principles of the Declaration of Helsinki.

**Results**

**Contribution of penA allele 60.001 to cephalosporin resistance**

Ceftriaxone resistance in the FC428 clone has been widely attributed to the presence of penA allele 60.001, although its specific contribution has never been experimentally verified. Therefore, penA allele replacement mutants were generated for N. gonorrhoeae strains SZ20 and SRRSH78, which contain penA allele 60.001 and penA allele 10.001, respectively. Strain SZ20 was isolated in 2016 from a patient in Suzhou [26], and is closely related to the FC428 clone because besides an identical penA allele, it also shows identical MLST (ST1903), NG-MAST (ST3435) and NG-STAR (ST233) sequence types. Strain SRRSH78 was isolated in 2016 from a patient in Hangzhou [8], and contains penA allele 10.001. This penA allele is the most widespread mosaic penA allele in China and other Asia-Pacific countries and is generally mostly associated with low-level cefixime resistance or reduced susceptibility (MIC ≤0.25 mg/L) [8,44,45]. Furthermore, penA allele 10.001 contains the I312M, F504L and N512Y and G545S polymorphisms associated with reduced cephalosporin susceptibility, similar to penA allele 60.001, but not the A311 V and T483S polymorphism associated with high-level resistance. Ceftriaxone and cefixime susceptibility analysis showed that strain SZ20 was indeed resistant against ceftriaxone (MIC=0.5 mg/L) and cefixime (MIC=2 mg/L),

| Table 1. Ceftriaxone and cefixime susceptibility of gonococcal wild-type strains and penA allele exchange mutants. |
|---------------------------------------------------------------|
| **Strain** | **Ceftriaxone** | **Cefixime** |
| ATCC49226 | 0.016 | 0.03 |
| SZ20 | 0.5 | 2 |
| SZ20-penA60 | 0.5 | 2 |
| SRRSH78-penA10 | 0.06 | 0.25 |
| SRRSH78-penA60 | 0.5 | 2 |
while strain SRRSH78 was susceptible to ceftriaxone and low-level resistant to cefixime (Table 1). Importantly, isogenic strains in which the penA 60.001 and 10.001 alleles were exchanged showed an inversion of susceptibility, highlighting that penA allele 60.001 provides higher resistance to cephalosporins compared with penA allele 10.001. The strains containing penA60 showed eight- to sixteen-fold higher ceftriaxone MIC values compared with isogenic strains containing penA10. Similarly, penA60 strains showed eightfold higher cefixime MIC values compared with penA10 strains. These results highlight the important

Figure 1. In vitro growth curves of the gonococcal penA60/penA10 allele exchange mutants in liquid culture. (A) Growth of single-strain SZ20 penA mutants (SZ20-penA60 and SZ20-penA10) determined by absorbance measurements (OD₆₀₀) in liquid culture. (B) Growth of single-strain SRRSH78 penA mutants (SRRSH78-penA60 and SRRSH78-penA10) determined by absorbance measurements (OD₆₀₀) in liquid culture. (C) Growth of strains SZ20-penA60-catA2 and SZ20-penA10-kanR in competition in liquid culture determined by CFU counts on selective agar plates. (D) Growth of strains SRRSH78-penA60-catA2 and SRRSH78-penA10-kanR in competition in liquid culture determined by CFU counts on selective agar plates. (E) Growth of strains SZ20-penA60-kanR and SZ20-penA10-catA2 in competition in liquid culture determined by CFU counts on selective agar plates. (F) Growth of strains SRRSH78-penA60-kanR and SRRSH78-penA10-catA2 in competition in liquid culture determined by CFU counts on selective agar plates. The graphs represent the average and standard deviation of three biological independent experiments.
contribution of penA allele 60.001 to both ceftriaxone and cefixime resistance.

Impact of penA allele 60.001 on in vitro biological fitness

The in vitro biological fitness of the penA allele exchange mutants was determined during in vitro growth both in the presence and absence of the host antimicrobial compounds palmitic acid and lithocholic acid, which are highly abundant in the mucosal epithelia and rectum, respectively. In the absence of antimicrobial compounds, liquid growth of single strains was indistinguishable for both SZ20 (Figure 1(A)) and SRRSH78 (Figure 1(B)) when comparing the penA mutants expressing penA60 and penA10. To compare growth in competition, the chloramphenicol-resistance selection marker catA2 was inserted in the penA60 strains and the kanamycin-resistance marker kanR in the penA10 strains and competitive growth was evaluated by CFU determination on agar plates containing chloramphenicol or kanamycin. Again, no differences in growth between the penA60- and penA10-containing mutants were observed for both SZ20 (Figure 1(C)) and SRRSH78 (Figure 1(D)). To ensure results were not affected by the respective selection markers, selection markers were changed, which gave similar results (Figure 1(E,F)). Subsequently, penA mutants were tested for their ability to grow in liquid cultures as single strains in the presence of high palmitic acid and lithocholic acid concentrations. Interestingly, for both strains the mutants containing penA60 grew significantly better at the highest permissive palmitic acid concentrations than the mutants containing penA10 (Figure 2(A,B)). Similar results were obtained in spot assays where penA60-containing mutants displayed a higher growing fraction on agar plates containing high palmitic acid concentrations (Figure 2(C)). Also, penA60 mutants grew significantly better in liquid culture containing the highest permissive lithocholic acid concentrations than the penA10-containing mutants (Figure 2(D,E)) and they showed a higher growing fraction in spot assays on plates with elevated lithocholic acid concentrations (Figure 2(F)). Subsequently, competition assays were performed with the penA exchange mutants for liquid growth in the presence of palmitic acid and lithocholic acid using slightly lower concentrations than for the single-strain growth experiments, which was more permissive for growth of the penA10 strains. Interestingly, under these slightly
less stressful conditions, the mutants containing penA10 were actually outcompeting the mutants containing penA60 for growth in the presence of palmitic acid, since the penA10-containing strains reached higher CFU counts after four to six hours growth (Figure 3). After six hours of growth, a decline in CFU counts was observed for most experiments. Similar results were obtained when selection markers were changed. Finally, competition experiments were performed in the presence of elevated lithocholic acid concentrations. Again, mutants containing penA10 outcompeted penA60-containing mutants and reached higher CFU counts after six hours of growth and CFU counts remained higher in the decline phase after eight hours incubation (Figure 4). Also under these conditions, the selection markers did not affect the final outcome, since similar differences between the penA60 and penA60 mutants were observed when selection markers were changed. Overall, these data provide a mixed picture on the impact of penA allele 60.001 on in vitro biological fitness.

Impact of penA allele 60.001 on in vivo biological fitness in a mouse vaginal tract infection model

The in vivo biological fitness of the penA allele exchange mutants was investigated by competition assays for colonization of the mouse vaginal tract. Bacterial suspensions containing equal numbers of the SZ20 or SRRSH78 mutants expressing catA2 (penA60) or kanR (penA10) were used to inoculate the mouse vaginal tract and colonization was monitored for three days by daily swabbing. For both SZ20 and SRRSH78 in vivo competition assays, the penA10-containing mutants showed higher recovery of CFU counts for all three days compared with the

Figure 3. In vitro competition assays of the gonococcal penA60/penA10 allele exchange mutants in the presence of palmitic acid. (A) Growth of strains SZ20-penA60-catA2 and SZ20-penA10-kanR in competition in liquid culture containing 1.25 mg/L palmitic acid. (B) Growth of strains SRRSH78-penA60-catA2 and SRRSH78-penA10-kanR in competition in liquid culture containing 1.5 mg/L palmitic acid. (C) Growth of strains SZ20-penA60-kanR and SZ20-penA10-catA2 in competition in liquid culture containing 1.25 mg/L palmitic acid. (D) Growth of strains SRRSH78-penA60-kanR and SRRSH78-penA10-catA2 in competition in liquid culture containing 1.5 mg/L palmitic acid. Competitive growth was determined by CFU counts on selective agar plates. The graphs represent the average and standard deviation of three biological independent experiments. Significant differences between the penA60/penA10 mutants at corresponding time-points were identified by Student’s two-tailed unpaired t-test (GraphPad Prism). *P<0.05; **P<0.01; ***P<0.001.
penA60-containing mutants (Figure 5). Also, the calculated CI-values for all colonized mice ranged between five and two thousand, indicating that mutants containing penA10 outcompeted the penA60 mutants (Figure 5). Therefore, these data indicate that penA allele 60.001 has a negative impact on in vivo biological fitness in a mouse vaginal tract infection model.

**Discussion**

The emergence and global transmission of the gonococcal FC428 clone over the past few years has become a major threat to ceftriaxone-based therapy, which is currently the last-remaining first-line treatment. Ceftriaxone resistance in the FC428 clone has been attributed to the presence of mosaic penA allele 60.001, although thus far its contribution to ceftriaxone resistance has been established only by association. In the current study, we showed by genetic engineering strategies that otherwise isogenic strains expressing penA allele 60.001 showed up to sixteen-fold higher ceftriaxone MIC values compared with strains expressing mosaic penA allele 10.001. Allele penA 10.001 is frequently encountered in gonococcal isolates in the Asia-Pacific region and has been associated with cefixime resistance or reduced susceptibility, and less abundantly with lower-level ceftriaxone resistance (up to ceftriaxone MIC = 0.25 mg/L) [8,44,45]. However, even though penA allele 10.001 is able to provide a major reduction in ceftriaxone susceptibility compared with many other mosaic and non-mosaic penA alleles, likely because it contains the I312M, F504L, N512Y and G545S polymorphisms previously associated with reduced susceptibility [37,38], penA allele 60.001 was still able to further reduce susceptibility over penA allele 10.001. The key polymorphisms in penA allele 60.001 that are associated with higher-level ceftriaxone resistance are A311 V and T483S. These
polymorphism were also present in the ceftriaxone-resistant Australian strain A8804 [21]. This strain displayed a ceftriaxone MIC of 0.5 mg/L, which is similar to the MIC observed for the penA 60.001-expressing strains in our study. A previous study on the contribution of these polymorphisms showed that the introduction of individual A311 V and T483S mutations in the mosaic penA allele of strain 35/02 provided a 2- and 4-fold increase in ceftriaxone MICs, respectively [36]. Since that study focused on the identification of essential polymorphisms for ceftriaxone resistance in strain HO41, which contains and additional key T316P polymorphism, the combined A311 V and T483S mutations were not tested [22]. However, combining the A311 V, T316P and T483S mutations in the penA allele of strain 35/02 resulted in similar ceftriaxone susceptibility levels as isogenic strains expressing the HO41 penA allele [36].

It is often assumed that mutations providing antibiotic resistance are costly and reduce biological fitness [46,47]. Therefore, susceptible bacteria are able to outcompete resistant bacteria in the absence of antibiotic pressure, which might prevent widespread transmission of some of the most resistant strains. Indeed, it has been shown that mosaic penA alleles of the ceftriaxone-resistant gonococcal strains HO41 and F89 reduced biological fitness during in vitro liquid growth and in vivo in a mouse model of infection [39], which would explain why these strains have not shown widespread transmission. Interestingly, in vivo competition assays with isogenic strains expressing the ceftriaxone-resistant penA alleles of HO41 (allele 37) and F89 (allele 42) allowed for the rapid arise of compensatory mutations for the HO41 penA allele, but not the F89 penA allele for which resistance was dependent.

Figure 5. In vivo competition assays of the gonococcal penA60/penA10 allele exchange mutants in a mouse vaginal tract infection model. (A) Recovery of SZ20-penA60-catA2 and SZ20-penA10-kanR CFUs from the mouse vaginal tract after competitive colonization. (B) Competition indices (CIs) between SZ20-penA60-catA2 and SZ20-penA10-kanR based on recovered CFU counts from the mouse vaginal tract. (C) Recovery of SRRSH78-penA60-catA2 and SRRSH78-penA10-kanR CFUs from the mouse vaginal tract after competitive colonization. (D) CIs between SRRSH78-penA60-catA2 and SRRSH78-penA10-kanR based on recovered CFU counts from the mouse vaginal tract. The CIs were calculated as (penA10/penA60)output/(penA10/penA60)input. Significant differences in recovered CFUs between penA60/penA10 mutants and between CIs calculated for the in vivo mouse vaginal tract infection model and in vitro growth in liquid culture at corresponding time-points were identified by Student’s two-tailed unpaired t-test (GraphPad Prism). *P<0.05; **P<0.01; ***P<0.001.
on an A501P polymorphism [17,39,48]. Importantly, for *N. gonorrhoeae* several mutations have already been described that provide antibiotic resistance and also improve biological fitness. Mutations in mtrR and its promoter, which alleviate repression of the MtrCDE multidrug efflux pump, are advantageous for fitness during colonization of a mouse model of infection [49,50]. Multidrug-resistant strains generally contain one or more mtrR mutations to increase efflux of hydrophobic or amphipathic antibiotics, while increased efflux of host-derived antimicrobial compounds such as fatty acids, bile and antimicrobial peptides allows for increased *in vivo* fitness [41,49,50]. Similarly, the 23S rRNA A2059G polymorphism is the sole mutation providing high-level azithromycin resistance and furthermore enhances *in vivo* biological fitness in a mouse vaginal tract infection model [40]. Our current results provide a more mixed picture on the impact of penA allele 60.001 on biological fitness. Strains expressing penA allele 60.001 were outcompeted by isogenic strains expressing penA allele 10.001 for *in vivo* colonization in a mouse model of infection and for *in vitro* liquid growth in the presence of additional stress (fatty acid/bile). However, both single-strain growth and competitive growth *in vitro* in liquid cultures in the absence of additional stress was identical between the penA 60.001 and penA 10.001 isogenic strains. Furthermore, during single-strain *in vitro* growth experiments in the presence of additional stress, which was at a higher stress level than during the competitive growth experiments, the penA 60.001 strains actually grew better. Therefore, it seems that penA allele 60.001 actually allows *N. gonorrhoeae* to grow at a higher stress level, even though it negatively impacts competitive growth at a lower stress level. Translation of these results to fitness during colonization of the human host will be difficult. Whether strains containing penA allele 60.001 will show reduced fitness in the human host might really be dependent on the combination of stresses encountered, but given that the FC428 clone has already shown global transmission, its fitness defects in the human host are likely very limited.

In conclusion, here we showed that penA allele 60.001 of the ceftriaxone-resistant gonococcal FC428 clone reduces ceftriaxone susceptibility by eight- to sixteen-fold compared with mosaic penA allele 10.001. Further analysis of the impact of penA allele 60.001 on biological fitness provided a mixed picture where penA 60.001 negatively impacts *in vivo* fitness in a mouse vaginal tract infection model, while *in vitro* liquid growth in the absence of additional stress seemed unaffected. Therefore, the negative impact of penA allele 60.001 on biological fitness might not be very severe, which would explain the successful global transmission of the FC428 clone in recent years.

Acknowledgements

This work was supported by the Chinese Academy Medical Sciences Initiative for Innovative Medicine under Grant 2016-12M-3-021; the National Natural Science Foundation of China under Grant 81871695; the Zhejiang Provincial Natural Science Foundation of China under Grant LR16H190001; and the Jiangsu Natural Science Foundation under Grant BK20171133.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Chinese Academy Medical Sciences Initiative for Innovative Medicine [grant number 2016-12M-3-021], the National Natural Science Foundation of China [grant number 81871695]; the Zhejiang Provincial Natural Science Foundation [grant number LR16H190001]; and the Jiangsu Natural Science Foundation [grant number BK20171133].

ORCID

Stijn van der Veen https://orcid.org/0000-0002-8176-391X

References

[1] Unemo M, Lahra MM, Cole M, et al. World Health Organization Global Gonococcal Antimicrobial Surveillance Program (WHO GASP): review of new data and evidence to inform international collaborative actions and research efforts. Sex Health. 2019 Sep;16(5):412–425.

[2] Edwards JL, Apicella MA. The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. Clin Microbiol Rev. 2004 Oct;17(4):965–981.

[3] Farley TA, Cohen DA, Elkins W. Asymptomatic sexually transmitted diseases: the case for screening. Prev Med. 2003 Apr;36(4):502–509.

[4] Hook EW, Bernstein K. Kissing, saliva exchange, and transmission of *Neisseria gonorrhoeae*. Lancet Infect Dis. 2019 Oct;19(10):e367–e369.

[5] Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev. 2014 Jul;27(3):587–613.

[6] Fifer H, Cole M, Hughes G, et al. Sustained transmission of high-level azithromycin-resistant *Neisseria gonorrhoeae* in England: an observational study. Lancet Infect Dis. 2018 May;18(5):573–581.

[7] Katz AR, Komeya AY, Kirkcaldy RD, et al. Cluster of *Neisseria gonorrhoeae* isolates with high-level azithromycin resistance and decreased ceftriaxone susceptibility, Hawaii, 2016. Clin Infect Dis. 2017 Sep 15;65(6):918–923.

[8] Yan J, Xue J, Chen Y, et al. Increasing prevalence of *Neisseria gonorrhoeae* with decreased susceptibility to ceftriaxone and resistance to azithromycin in Hangzhou, China (2015–17). J Antimicrob Chemother. 2019 Oct 16;74(1):29–37.
[9] Kong FYS, Horner P, Unemo M, et al. Pharmacokinetic considerations regarding the treatment of bacterial sexually transmitted infections with azithromycin: a review. J Antimicrob Chemother. 2019 May;1(74)(5):1157–1166.

[10] Ison CA, Town K, Obi C, et al. Decreased susceptibility to cefalosporins among gonococci: data from the gonococcal resistance to Antimicrobials Surveillance Programme (GRASP) in England and Wales, 2007–2011. Lancet Infect Dis. 2013 Sep;13(9):762–768.

[11] Shimuta K, Watanabe Y, Nakayama S, et al. Emergence and evolution of internationally disseminated cephalosporin-resistant Neisseria gonorrhoeae clones from 1995 to 2005 in Japan. BMC Infect Dis. 2015 Sep;17;15:378.

[12] Yin YP, Han Y, Dai XQ, et al. Susceptibility of Neisseria gonorrhoeae to azithromycin and ceftriaxone in China: A retrospective study of national surveillance data from 2013 to 2016. PLoS Med. 2018 Feb;15(2);e1002499.

[13] Chen MY, Stevens K, Tideman R, et al. Failure of 500 mg of ceftriaxone to eradicate pharyngeal gonorrhoea, Australia. J Antimicrob Chemother. 2013 Jun;68(6):1445–1447.

[14] Poncin T, Fouere S, Braille A, et al. Multidrug-resistant Neisseria gonorrhoeae failing treatment with ceftriaxone and doxycycline in France, November 2017. Euro Surveill. 2018 May;23(21):1800264.

[15] Tapsall J, Read P, Carmody C, et al. Two cases of failed ceftriaxone treatment in pharyngeal gonorrhoea verified by molecular microbiological methods. J Med Microbiol. 2009 May;58(Pt 5):683–687.

[16] Unemo M, Golparian D, Hestner A. Ceftriaxone treatment failure of pharyngeal gonorrhoea verified by international recommendations, Sweden, July 2010. Euro Surveill. 2011 Feb 10;16(6):19792.

[17] Unemo M, Golparian D, Nicholas R, et al. 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 Mar;56(3):1273–1280.

[18] Unemo M, Golparian D, Potocnik M, et al. Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line cefixime verified in Slovenia, September 2011. Euro Surveill. 2012 Jun 21;17(25):20200.

[19] Camara J, Serra J, Ayats J, et al. Molecular characterization of two high-level ceftriaxone-resistant Neisseria gonorrhoeae isolates detected in Catalonia, Spain. J Antimicrob Chemother. 2012 Aug;67(8):1858–1860.

[20] Deguchi T, Yasuda M, Hatazaki K, et al. New clinical strain of Neisseria gonorrhoeae with decreased susceptibility to ceftriaxone, Japan. Emerg Infect Dis. 2016 Jan;22(1):142–144.

[21] Lahra MM, Ryder N, Whiley DM. A new multidrug-resistant strain of Neisseria gonorrhoeae in Australia. N Engl J Med. 2014 Nov 6;371(19):1850–1851.

[22] Ohnishi M, Golparian D, Shimuta K, et al. Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhoea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob Agents Chemother. 2011 Jul;55(7):3538–3545.

[23] Nakayama S, Shimuta K, Furubayashi K, et al. New ceftriaxone- and multidrug-resistant Neisseria gonorrhoeae strain with a novel mosaic penA gene isolated in Japan. Antimicrob Agents Chemother. 2016 Jul;60(7):4339–4341.

[24] Lee K, Nakayama SI, Osawa K, et al. Clonal expansion and spread of the ceftriaxone-resistant Neisseria gonorrhoeae strain FC428, identified in Japan in 2015, and closely related isolates. J Antimicrob Chemother. 2019 Jul;1(74):1812–1819.

[25] Chen SC, Han Y, Yuan LF, et al. Identification of internationally disseminated ceftriaxone-resistant Neisseria gonorrhoeae strain FC428, China. Emerg Infect Dis. 2019 Jul;25(7):1427–1429.

[26] Yang F, Zhang H, Chen Y, et al. Detection and analysis of two cases of the internationally spreading ceftriaxone-resistant Neisseria gonorrhoeae FC428 clone in China. J Antimicrob Chemother. 2019 Dec;1(12):3635–3636.

[27] Yuan Q, Li Y, Xi L, et al. Identification of multidrug-resistant Neisseria gonorrhoeae isolates with combined resistance to both ceftriaxone and azithromycin, China, 2017–2018. Emerg Microbes Infect. 2019;8(1):1546–1549.

[28] Terkelsen D, Tolstrup J, Johnsen CH, et al. Multidrug-resistant Neisseria gonorrhoeae infection with ceftriaxone resistance and intermediate resistance to azithromycin, Denmark, 2017. Euro Surveill. 2018 Oct;22(42):1700659.

[29] Lefebvre B, Martin I, Demczuk W, et al. Ceftriaxone-resistant Neisseria gonorrhoeae, Canada, 2017. Emerg Infect Dis. 2018 Feb;24(2):381–383.

[30] Lahra MM, Martin I, Demczuk W, et al. Cooperative recognition of internationally disseminated ceftriaxone-resistant Neisseria gonorrhoeae strain. Emerg Infect Dis. 2018 Apr;24(4):735–740.

[31] Golparian D, Rose L, Lynam A, et al. Multidrug-resistant Neisseria gonorrhoeae isolate, belonging to the internationally spreading Japanese FC428 clone, with ceftriaxone resistance and intermediate resistance to azithromycin, Ireland, August 2018. Euro Surveill. 2018 Nov;23(47):1800617.

[32] Eyre DW, Town K, Street T, et al. Detection in the United Kingdom of the Neisseria gonorrhoeae FC428 clone, with ceftriaxone resistance and intermediate resistance to azithromycin, October to December 2018. Euro Surveill. 2019 Mar;24(10):1900147.

[33] Eyre DW, Sanderson ND, Lord E, et al. Gonorrhoea treatment failure caused by a Neisseria gonorrhoeae strain with combined ceftriaxone and high-level azithromycin resistance, England, February 2018. Euro Surveill. 2018 Jul;23(27):1800323.

[34] Chen SC, van der Veen S, Yin YP. Widespread transmission of the ceftriaxone-resistant Neisseria gonorrhoeae FC428 clone in China. Submitted.

[35] Yan J, Chen Y, Yang F, et al. High incidence of the ceftriaxone-resistant Neisseria gonorrhoeae FC428 clone in Hangzhou, China. Submitted.

[36] Tomberg J, Unemo M, Ohnishi M, et al. 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 Jul;57(7):3029–3036.

[37] Singh A, Tomberg J, Nicholas RA, et al. Recognition of the beta-lactam carbonylate triggers acylation of Neisseria gonorrhoeae penicillin-binding protein 2. J Biol Chem. 2019 Sep;20;294(38):14020–14032.

[38] Tomberg J, Unemo M, Davies C, et al. 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 Sep 21;49(37):8062–8070.

[39] Vincent LR, Kerr SR, Tan Y, et al. In vivo-selected compensatory mutations restore the fitness cost of mosaic penA alleles that confer ceftriaxone resistance in Neisseria gonorrhoeae. MBio. 2018 Apr 3;9(2):e01905-17.

[40] Zhang J, van der Veen S. Neisseria gonorrhoeae 23S rRNA A2059G mutation is the only determinant necessary for high-level azithromycin resistance and improves in vivo biological fitness. J Antimicrob Chemother. 2019 Feb 1;74(2):407–415.

[41] Wang S, Xue J, Lu P, et al. Gonococcal MtrE and its surface-expressed Loop 2 are immunogenic and elicit bactericidal antibodies. J Infect. 2018 Sep;77(3):191–204.

[42] Wang Z, Wang X, Lu P, et al. Identification and characterization of the Neisseria gonorrhoeae MscS-like mechanosensitive channel. Infect Immun. 2018 Jun;86(6):e00090-18.

[43] Jerse AE, Sharma ND, Simms AN, et al. A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. Infect Immun. 2003 Oct;71(10):5576–5582.

[44] 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 Jan;49(1):137–143.

[45] Lee H, Suh YH, Lee S, et al. Emergence and spread of cephalosporin-resistant Neisseria gonorrhoeae with mosaic penA alleles, South Korea, 2012-2017. Emerg Infect Dis. 2019 Mar;25(3):416–424.

[46] Andersson DI, Levin BR. The biological cost of antibiotic resistance. Curr Opin Microbiol. 1999 Oct;2(5):489–493.

[47] Nilsson AI, Zorzet A, Kanth A, et al. Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. Proc Natl Acad Sci U S A. 2006 May 2;103(18):6976–6981.

[48] Tomberg J, Fedarovich A, Vincent LR, et al. Alanine 501 mutations in penicillin-binding protein 2 from Neisseria gonorrhoeae: structure, mechanism, and effects on cephalosporin resistance and biological fitness. Biochemistry. 2017 Feb 28;56(8):1140–1150.

[49] Warner DM, Folster JP, Shafer WM, et al. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. J Infect Dis. 2007 Dec 15;196(12):1804–1812.

[50] Warner DM, Shafer WM, Jerse AE. Clinically relevant mutations that cause derepression of the Neisseria gonorrhoeae MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol. 2008 Oct;70(2):462–478.