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Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain

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Objectives: The aim of this study was to characterize the first two extended-spectrum cephalosporin-resistant and multidrug-resistant (MDR) *Neisseria gonorrhoeae* isolates collected from two sexually related patients (men who have sex with men) in Spain.

Methods: Antimicrobial susceptibility was studied by Etest. Genes involved in quinolone, ceftriaxone and multidrug resistance were amplified by PCR and sequenced in both directions. The isolates were typed by *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST).

Results: The two isolates had the same MDR profile, showing resistance to penicillin (MIC 0.094 mg/L; β-lactamase negative), ceftriaxone (MIC 1.5 mg/L), cefixime (MIC 1.5 mg/L), cefotaxime (MIC 1 mg/L), ciprofloxacin (MIC >32 mg/L) and tetracycline (MIC 1.5 mg/L). NG-MAST showed that both isolates belonged to sequence type (ST) 1407 (*porB*-908 and *tbpB*-110). Ciprofloxacin resistance was due to amino acid substitutions in GyrA (S91F and D95G) and ParC (S87R). An A deletion in the promoter of the MtrCDE efflux pump (*mtrR*) was detected. No changes were detected in the *pilQ* gene. The outer membrane protein PorB showed two substitutions at G120K and A121N. An L421P substitution was observed in the PBP1A (*ponA*) sequence. The sequence of PBP2 (*penA*) showed a mosaic structure related to genotype XXXIV with a single additional amino acid substitution (A501P). This genotype was identical to a recently described French isolate (F89).

Conclusions: This is the first reported case of high-level extended-spectrum cephalosporin-resistant *N. gonorrhoeae* transmission. The molecular typing and MDR genotype suggest possible European spread of this strain, highlighting the need for surveillance and the importance of testing the susceptibility of *N. gonorrhoeae* to extended-spectrum cephalosporins.

Keywords: *N. gonorrhoeae*, NG-MAST, multidrug resistant

Introduction

Neisseria gonorrhoeae remains a major cause of sexually transmitted infection. Extended-spectrum cephalosporins (ESCs; cefixime and ceftriaxone, for oral and parenteral use, respectively) are the first-line treatment in many countries, and resistance is currently rare.[1] However, two unrelated *N. gonorrhoeae* strains with high-level resistance to third-generation cephalosporins were recently described.[2,3] The first, with a ceftriaxone MIC of 2 mg/L, was described in Japan, and molecular typing using *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) showed a novel sequence type (ST), ST4220.[2] To our knowledge, this genotype has not been described elsewhere, suggesting this was a sporadic event. The second strain, also with a ceftriaxone MIC of 1.5–2 mg/L, was described later in France, and belonged to ST1407, a well-known European clone with diminished susceptibility to ESCs.[3] Transformation studies demonstrated that the development of new mutations in the *penA* gene (PBP2) were responsible for the development of high-level cephalosporin resistance in both strains. The *penA* gene of the Japanese strain was similar to mosaic allele X with 12 amino acid substitutions, whereas the *penA* gene of the French strain was similar to mosaic allele XXXIV with an additional amino acid substitution (A501P). In addition, both strains had alterations in other

genes responsible for resistance to several groups of antimicrobials (*gyrA*, *parC*, *mtrR*, *ponA* and *porB1b*).

Subsequently, the first two *N. gonorrhoeae* isolates in Spain with high-level resistance to ESCs were detected in two sexually related patients [men who have sex with men (MSM)].[4] The aims of the current study were to analyse the molecular mechanisms involved in β-lactam and multidrug resistance, and to undertake molecular typing of these *N. gonorrhoeae* isolates.

Materials and methods

Bacterial strains, antibiotic susceptibility and molecular typing

Two gonococcal isolates obtained from urethral and rectal swabs of two sexually related patients (MSM) were used for the study.[4] Antimicrobial susceptibility was studied by Etest (bioMérieux) on a GC medium (Becton Dickinson) following the recommendations and criteria of EUCAST.[5] The current EUCAST MIC breakpoints for penicillin are ≤0.06 mg/L (susceptible) and >1 mg/L (resistant) and for cefixime and ceftriaxone the breakpoints are ≤0.12 mg/L (susceptible) and >0.12 mg/L (resistant).[5] The production of β-lactamase was tested using the nitrocefin method (Becton Dickinson).

NG-MAST was used for molecular typing, following methodology described previously.[6] Briefly, bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Internal fragments of *porB* and *tbpB* genes were amplified by PCR, and sequenced in both directions using a BigDye Terminator v3.1 in an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). Allele number and *N. gonorrhoeae* STs were assigned using the NG-MAST web site (http://www.mlst.net).

Molecular characterization of the resistance profile

Bacterial DNA was extracted and genes related to quinolone resistance (*gyrA* and *parC*), β-lactam resistance (*ponA* and *penA*) and multidrug resistance (promoter of *mtrR*, *pilQ* and *porB*) were amplified by PCR and sequenced as described previously.[7,8]

Results and discussion

The two isolates displayed the same antimicrobial susceptibility profile, with the following MICs: penicillin, 0.094 mg/L; cefixime, 1.5 mg/L; ceftriaxone, 1.5 mg/L; cefotaxime, 1 mg/L; tetracycline, 1.5 mg/L; and ciprofloxacin, >32 mg/L. The β-lactamase test was negative. By disc diffusion, both isolates were susceptible to azithromycin and spectinomycin. The two isolates reported here showed two amino acid substitutions in GyrA (S91F and D95G) and one in ParC (S87R), all of them previously shown to be involved in quinolone resistance.[8] No changes were detected in the *pilQ* gene. Both isolates showed an A deletion in the promoter of the MtrCDE efflux pump (*mtrR*), two amino acid substitutions (G120K and A121N) in the outer membrane protein PorB and an L421P change in the PBP1A (*ponA*) sequence. The sequence of PBP2 (*penA*) showed a mosaic structure related to genotype XXXIV with a single additional amino acid change (A501P).[9] The genotype of *mtrR*, *porB* and *ponA* with mosaic XXXIV at PBP2 has previously been associated with diminished susceptibility to cefixime in European isolates belonging to ST1407. Moreover, the F89 strain of *N. gonorrhoeae* isolated in France, which had high-level resistance to ESCs, showed the same resistance genotype, including the A501P substitution in the PBP2 gene with a mosaic structure (XXXIV).

The patient with symptomatic urethritis was cured after doxycycline therapy (100 mg, twice a day for 7 days) in spite of the isolate showing borderline resistance to tetracycline (MIC 1.5 mg/L), probably mediated by overexpression of a multidrug resistance efflux pump, as the *tet*(M) gene was not detected by PCR (data not shown). The second patient remained asymptomatic, but azithromycin treatment (500 mg/day) for 3 days eradicated the microorganism from the rectum, as confirmed by culture.[4]

NG-MAST showed that both isolates belonged to ST1407 (*porB*-908 and *tbpB*-110), which is the most prevalent clone in Europe. ST1407 is a successful clone that has spread worldwide and is associated with decreased susceptibility to cefixime and treatment failure. Moreover, the first European strain (F89) with high-level resistance to ESCs, recently identified in France, also belonged to this ST.[3]

The two isolates described here are only the third and fourth ceftriaxone-resistant *N. gonorrhoeae* to have been reported to date. Ceftriaxone resistance in *N. gonorrhoeae* thus currently remains rare, having previously only been detected in two sporadic and epidemiologically unrelated strains from Japan and France. This may reflect the fact that ESCs have only recently been widely used in the treatment of gonorrhoea. It may also be the case that the biological cost caused by the accumulation of mutations among structural genes could affect the fitness of this pathogen, reducing its potential to spread as a successful clone.

The fact that the two patients reported here were sexual partners, coupled with the fact that the two isolates are genetically related, makes this the first documented case of inter-patient transmission of ceftriaxone-resistant *N. gonorrhoeae*. In addition, our results showed that these two resistant isolates were genetically related to the F89 strain isolated in France. All three isolates (Spanish and French) were collected from clinical samples of MSM patients, suggesting the presence of circulating multidrug-resistant clones in this community. Two hypotheses could explain these results. Firstly, since isolates belonging to ST1407 comprise a widespread European clone with diminished susceptibility to cefixime, development of ceftriaxone resistance due to an A501P substitution in a PBP2 XXXIV allele may have occurred. This hypothesis is supported by the recent report of an Austrian isolate also belonging to 1407 and with an amino acid substitution (T534A) in the same *penA* allele, XXXIV, that was resistant to cefixime and which caused a treatment failure.[10] The second and more worrisome hypothesis is the continental spread of an ESC-resistant strain among the MSM community, a population at high risk of transmission of gonococcal infection. However, the epidemiological relationship between the Spanish and French patients is unknown, hence this hypothesis cannot be confirmed on the basis of currently available data. Nonetheless, the transmission between the two sexually related patients in Spain lends support to this idea. If this hypothesis is confirmed, this presages a new era in gonococcal infections, with the presence of a multidrug- and ceftriaxone-resistant strain capable of spreading successfully. However, more studies analysing the biological fitness of this new PBP2 mosaic structure are needed, since it has been suggested to be the limiting factor for the spread of ESC-resistant *N. gonorrhoeae*.

This report highlights the importance of undertaking surveillance of the susceptibility of *N. gonorrhoeae* to ESCs in order to detect the possible spread of this new variant of the European clone. Molecular characterization of isolates with reduced susceptibility to ESCs will be a vital component of such surveillance if we are to establish the role of this new clone in the epidemiology of sexually transmitted infections. The observation that one of the patients described here was asymptomatic emphasizes the need to sample both symptomatic and asymptomatic patients. Finally, if high-level resistance to ESCs coupled with resistance to other antibiotic classes becomes widespread, it will be essential to establish new and effective antibiotic therapy if gonorrhoea is to remain a treatable infection.

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Transparency declarations

None to declare.

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