Emergence and Spread of *Neisseria gonorrhoeae* Isolates With Decreased Susceptibility to Extended-Spectrum Cephalosporins in Argentina, 2009 to 2013

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Background: The emergence of *Neisseria gonorrhoeae* strains with decreased susceptibility to cephalosporins represents a major concern globally. The aim of this study was to examine the phenotypic and molecular characteristics of *N. gonorrhoeae* isolates with decreased susceptibility to ceftriaxone and cefixime in Argentina.

Methods: A total of 1987 isolates were collected during 2009 and 2013. The susceptibility to penicillin G, tetracycline, ciprofloxacin, cefixime, ceftriaxone, and azithromycin was determined using the agar dilution method. The major extended-spectrum cephalosporin resistance determinants (*penA*, *mtrR,* and *porB1b*) were sequenced in 42 *N. gonorrhoeae* isolates that showed decreased susceptibility to ceftriaxone (minimum inhibitory concentration [MIC], 0.06–0.125 mg/L) and cefixime (MIC, 0.125–0.25 mg/L). Genotyping by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) was performed.

Results: Between 2009 and 2013, there was a shift in the modal MICs for ceftriaxone. Among the 42 isolates exhibiting decreased susceptibility to ceftriaxone and cefixime, 95.2% were resistant to penicillin G, 95.2% to tetracycline, 97.6% to ciprofloxacin, and 33.3% to azithromycin. Thirty-five (83.3%) of the 42 isolates had a mosaic *penA* allele XXXIV, which has been previously associated with resistance to ceftriaxone and cefixime as well as treatment failures. The isolates that contained the mosaic penicillin-binding protein 2 (PBP2) XXXIV were associated with NG-MAST ST1407 or closely related genotypes.

Conclusions: In Argentina, *N. gonorrhoeae* isolates with decreased susceptibility to cefixime and ceftriaxone have now emerged, mostly due to the introduction of the internationally spread multidrug-resistant NG-MAST ST1407.

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Antimicrobial resistance in *Neisseria gonorrhoeae* represents a public health problem. In the absence of a vaccine, antibiotics are the primary treatment for gonococcal infection. An effective treatment of gonorrhea is essential for the individual patient to interrupt transmission chains and to reduce the overall disease burden.[1] Through the years, *N. gonorrhoeae* has progressively developed resistance to a wide range of antibiotics. Resistance to previously recommended first-line antimicrobials for treatment of gonorrhea is prevalent worldwide.[2,3] The extended-spectrum cephalosporin (ESC), ceftriaxone, is the only first-line option for the antimicrobial monotherapy of gonorrhea in many countries, including Argentina. However, isolates with decreased susceptibility and resistance to ESCs have been reported.[4,5] The emergence and spread of isolates with decreased susceptibility and resistance to ESCs and reports of treatment failures around the world raises the possibility that gonorrhea infections may become untreatable in certain circumstances.[6]

Decreased susceptibility and resistance to ESCs has been associated with mutations in different target genes such as *penA*, *mtrR* and *porB1b*.[7] Mutations in the *penA* gene encoding the penicillin-binding protein 2 (PBP2) is the main determinant for decreased susceptibility and resistance to ESCs. Acquisition of a *penA* mosaic gene or certain point mutation patterns in PBP2 results in a lower affinity for ESCs and consequently a decreased ESC susceptibility.[8,9] Furthermore, mutations in the promoter and/or coding sequence of the repressor gene *mtrR* increase the expression of the MtrCDE efflux pump system and further decrease susceptibility to ESCs.[7,9] Additionally, mutations in the *porB1b*

(*penB*) gene that alter amino acids G120 and A121 in the PorB1b porin result in decreased permeability and thus further decreased susceptibility to ESCs.[7,9] Alterations in *ponA* gene, which encodes PBP1, have also been observed in *N. gonorrhoeae* isolates with decreased susceptibility to ESC, but do not appear to have a role in increasing the ESC minimum inhibitory concentration [MIC] in clinical isolates.[7,9]

Molecular and genetic epidemiologic studies have been used to describe *N. gonorrhoeae* antimicrobial drug resistance to ESCs in many settings worldwide.[10,11] However, little is known about the molecular characteristics of isolates with decreased susceptibility to ESC in South America, including Argentina. This article describes phenotypic and genetic characterization of *N. gonorrhoeae* isolates with decreased susceptibility to ESCs between 2009 and 2013 in Argentina.

MATERIALS AND METHODS

N. gonorrhoeae Isolates

A total of 1987 *N. gonorrhoeae* isolates from consecutive patients (one isolate per patient) were collected between 2009 and 2013 from the Gonococcal Antimicrobial Susceptibility Surveillance Programme-Argentina. This surveillance program was initiated in early 1983 with the purpose of monitoring patterns of antimicrobial resistance in *N. gonorrhoeae*. Currently, the program includes 70 hospital laboratories distributed throughout Argentina. The isolates recovered from each hospital are sent to the National Reference Laboratory for antimicrobial susceptibility testing and additional studies. In this study, the isolates were obtained from 20 of the 24 provinces in Argentina. The strains were identified as *N. gonorrhoeae* based on Gram stain, oxidase test, superoxol test (30% hydrogen peroxide), carbohydrate utilization reactions, the Phadebact GC Monoclonal Test (MKL Diagnostic AB, Sollentuna, Sweden), and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (Microflex LT, Bruker Daltonik, Bremen, Germany).[12,13] Isolates were stored at −80°C in trypticase soy broth containing 20% glycerol.

Isolates exhibiting decreased susceptibility to ceftriaxone (MIC, 0.06–0.25 mg/L) and cefixime (MIC, 0.125–0.25 mg/L) were selected for molecular characterization.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *N. gonorrhoeae* to cefixime (Bagó Laboratories, Buenos Aires, Argentina); penicillin G, tetracycline, ciprofloxacin, ceftriaxone, and azithromycin (Richet Laboratories, Buenos Aires, Argentina) were determined by the agar dilution method following the Clinical and Laboratory Standards Institute guidelines.[14] Concentration of antibiotics used were as follows: penicillin G, 0.016 to 128 mg/L; tetracycline, 0.06 to 32 mg/L; ciprofloxacin, 0.001 to 32 mg/L; ceftriaxone and cefixime, 0.001 to 0.5 mg/L; azithromycin, 0.016 to 4 mg/L. Isolates were subcultured onto Difco GC Medium Base agar (BD, Franklin Lakes, NJ) supplemented with 1% Britalex enrichment supplement (Britania Lab. Argentina) for 18 to 24 hours at 35°C in a humidified environment and enriched with 5% CO_2 before testing. The Clinical and Laboratory Standards Institute guidelines (M100-S25) were used to interpret the results, except in the cases of azithromycin, for which the European Committee on Antimicrobial Susceptibility Testing were applied.[15,16] *N. gonorrhoeae* American Type Culture Collection 49226 and the 2008 World Health Organization *N. gonorrhoeae* reference strains panel were used as quality control strains in the testing.[17] β-lactamase production was tested using Nitrocefin discs (BBL; BD, Franklin Lakes, NJ).

Molecular Studies

DNA was extracted from all isolates with decreased susceptibility to ESC, as described previously.[18] Polymerase chain reaction (PCR) using previously described primers were performed to amplify *penA*, *porB1b* (*penB*) and the promoter and coding regions of *mtrR* gene.[19] All PCR amplifications were performed on a MyCycler thermal cycler (Bio-Rad, Irvine, Calif). The cycling parameters for *penA* amplification were as follows: after an initial denaturation step at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 60 seconds, annealing at 52°C for 60 seconds, and elongation at 72°C for 2.30 minutes; and a final extension for 5 minutes at 72°C. The same cycling parameters were applied for the amplification of *mtrR* and *porB1b* genes with the exception of annealing temperature, which was 60°C (*mtrR*) or 50°C (*porB1b*) for 60 seconds, and elongation temperature at 72°C for 60 seconds. The size of the amplified products was confirmed by electrophoresis on a 1.5% agarose gel. PCR products were purified using AccuPrep PCR Purification Kit (Bioneer, Daejeon, Republic of Korea) and sequenced using the BigDye terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, Calif) on an ABI 3500 Genetic Analyzer (Applied Biosystems). The resulting sequences were aligned with reference sequences using BioEdit Sequence Alignment Editor software (version 7.2.5). The GenBank accession number of the reference sequence used are M32091 (*penA*), Z25796 and AE004969.1 (*mtrR*), and J03017 (*porB1b*). The amino acid sequence patterns of PBP2 (*penA*) were classified as described previously.[8,20]

Molecular Typing of Isolates

For molecular genotyping, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) was performed on all gonococcal isolates with decreased susceptibility to ESC, as previously described.[21] The *porB* and *tbpB* gene numbers as well as sequence types (STs) were assigned in accordance with the NG-MAST website (http://www.ng-mast.net). The partial *porB* gene sequences (490 bp) of the 42 strains in this study and the first isolate resistant to ESCs reported in our country[22] were aligned using BioEdit (version 7.2.5), and a neighbor-joining tree was generated using this alignment and MEGA version 6 software. Similarity of alleles was evaluated by individual pairwise alignment. Alleles of *porB* showing 99% or greater identity (<5-bp difference) were grouped.[23] The groups were named according to the majority allele in each group.

Statistical Analysis

A χ^2 test was used to detect significant difference between proportions. Significance was set at a P value less than 0.05.

Ethical Approval

No institutional review board approval was necessary for this study, because no personal information of the patients was collected during the investigation.

RESULTS

Antibiotic Susceptibility

In the period of study, the proportion of isolates with ceftriaxone MIC ≤ 0.004 mg/L decreased from 51.3% and 50.0% in 2009 and 2010 to 25.4%, 34.2% and 26.4% in 2011, 2012, and 2013 (P <0.05), and a right shift in the distribution of ceftriaxone MIC was observed (Fig. 1). Although no ceftriaxone-resistant isolates were found, 3.1% (42/1361) of strains between 2011 and 2013 showed MICs values in the range of 0.06 to 0.125 mg/L.

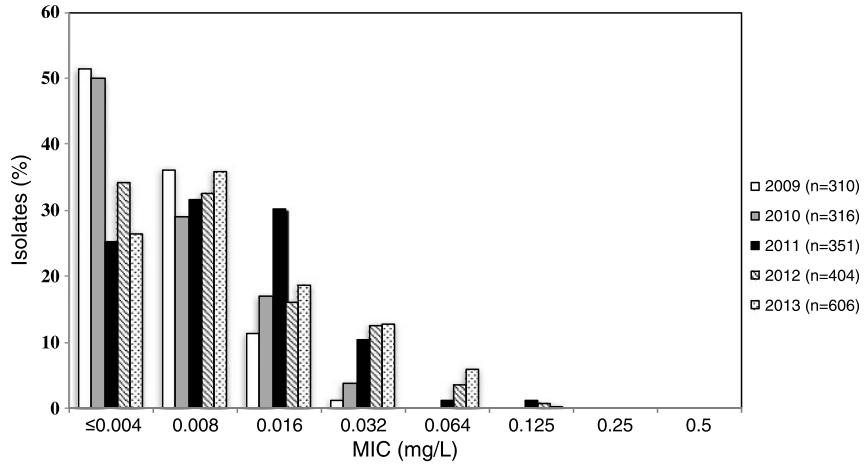

Figure 1. Distribution of ceftriaxone MICs for *N. gonorrhoeae* isolates (n = 1987) obtained in Argentina during 2009 and 2013.

The proportion of isolates with ceftriaxone MIC value of 0.06 to 0.125 mg/L increased from 2.3% in 2011 to 4.3% in 2013. The MICs of the isolates with decreased susceptibility to ceftriaxone are described in Supplementary Table 1, http://links.lww.com/OLQ/A164. All of these isolates were β-lactamase negative and showed decreased susceptibility to cefixime. Moreover, the rates of resistance to penicillin G, tetracycline, ciprofloxacin, and azithromycin were 95.2% (40/42), 95.2% (40/42), 97.6% (41/42), and 33.3% (14/42), respectively.

The 42 isolates with decreased susceptibility to ESC were distributed within the 7 health regions of Argentina, and identified in the following provinces: Buenos Aires (n = 2), Ciudad Autónoma de Buenos Aires (n = 10), Chaco (n = 2), Córdoba (n = 19), Formosa (n = 2), Mendoza (n = 1), Rio Negro (n = 3), Salta (n = 1), San Luis (n = 1), and Santa Cruz (n = 1).

Molecular Characterization of the Resistance Genes

Sequencing of the *penA* gene from the 42 *N. gonorrhoeae* isolates with decreased susceptibility to ESC revealed that 90.5% (n = 38) of the isolates contained a mosaic *penA* allele. Thirty-five (92.1%) and three (7.9%) of these isolates possessed the mosaic *penA* allele XXXIV and X, respectively, which have been associated with decreased susceptibility or resistance to ESCs. The proportion of isolates with *penA* mosaic allele XXXIV increased from 37.5% in 2011 to 96.1% in 2013. The remaining 9.5% (n = 4) of the isolates revealed a *penA* nonmosaic allele. Four nonmosaic PBP2s were found among these 4 isolates, such as PBP2-V, IX, XII, and XIII. These 4 nonmosaic PBP2s had the following mutation patterns: PBP2-V (D345a, F504L, A510V, A516G, G542S, I566V, N574a and A575V), PBP2-IX (D345a, F504L, A510V, A516G and P551L), PBP-XII (D345a, F504L, A510V, A516G and P551S) and PBP2-XIII (D345a, A501V, F504L, A510V, A516G, and P551S).

Mutations in the promoter region or coding sequence of the *mtrR* gene were observed in 42 (100%) of the isolates studied. All isolates harbored a single nucleotide (A) deletion in the 13-bp inverted repeat located between the −10 and −35 sequences of the promoter combined with an H105Y substitution in the multimeric region of MtrR.

PorB1b amino acid substitutions were observed in all isolates, suggesting a decreased intake of antimicrobials. Forty of the 42 *N. gonorrhoeae* isolates carried double mutation at amino acid residues G120 and A121 of *porB1b* gene, which included G120K/A121N 85.7% (n = 36) followed by G120K/A121D 9.5% (n = 4). Two isolates were represented by A121G and A121S/N122K alteration, respectively. Alterations in *penA*, *mtrR* and *porB1b* genes for all isolates are shown in Supplementary Table 1, http://links.lww.com/OLQ/A164.

N. gonorrhoeae Multiantigen Sequence Typing and Phylogenetic Analysis

In this study, a total of 16 NG-MAST STs were identified among the 42 isolates, of which 5 were new. *N. gonorrhoeae* ST1407 (n = 22/42, 52.4% of isolates) was the most prevalent ST, followed by ST3431 (n = 4/42, 9.6%) and ST925 (n = 3/42, 7.1%). Thirteen STs (30.9%) were represented by only one isolate (Supplementary Table 1, http://links.lww.com/OLQ/A164). A phylogenetic tree using neighbor-joining method was generated using the *porB* gene sequences (490 bp) identified from the isolates in the study. The analysis of *porB* genes revealed a group highly related, in which 83.3% (n = 35/42) of isolates were represented in the *porB908* group (the *porB* allele in NG-MAST ST1407) (Fig. 2). Thirty-three of these 35 isolates had *tbpB* allele 110. The remaining 2 isolates had *tbpB* allele 1551 and 32, which differ by 4 and 15 nucleotides from *tbpB* allele 110, respectively. All isolates in the *porB908* group contained *penA* mosaic allele XXXIV, the *porB1b* G120K/A121N mutations and an A deletion combined with an H105Y substitution in the multimeric region of MtrR. Interestingly, the NG-MAST ST3620 isolate (*porB* allele164 and *tbpB* allele 33) that contained PBP2-IX was related with the ST13064 (*porB* allele 7592 and *tbpB* allele 33) isolate resistant to ESCs in Argentina (Fig. 2). ST13064 differs from ST3620 by 8 nucleotides in the *porB* allele.

DISCUSSION

The emergence and spread of *N. gonorrhoeae* isolates with decreased susceptibility to ESCs has become a global concern. The present study represents the first nationwide study to identify the genetic determinants for ESCs resistance and molecular epidemiology of *N. gonorrhoeae* isolates with decreased susceptibility to ceftriaxone and cefixime between 2009 and 2013 in Argentina. As observed in other countries worldwide,[11] an upward shift of ceftriaxone MICs was observed during the period study. The results show the presence of *N. gonorrhoeae* isolates with ceftriaxone MIC of 0.06 to 0.125 mg/L circulating in our population since 2011. The percentage of isolates with decreased

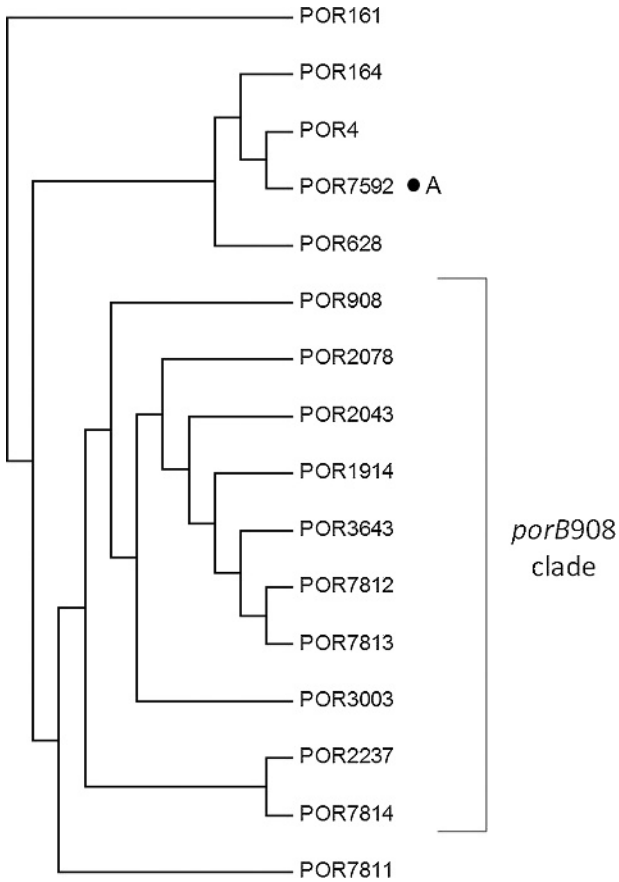

Figure 2. Phylogenetic tree of *porB* alleles (490 bp) from *N. gonorrhoeae* isolates with decreased susceptibility and resistance to ESCs (n = 43) in Argentina. A, Isolate resistant to ceftriaxone and cefixime.

susceptibility to ESCs increased from 2.3% in 2011 to 4.3% in 2013. Moreover, data from Gonococcal Antimicrobial Susceptibility Surveillance Programme-Argentina showed a continuous increase of these isolates until 7.9% in 2015 (unpublished data). In Argentina, national guidelines recommend ceftriaxone (125–250 mg) monotherapy as the first-line treatment for uncomplicated gonorrhea.[24,25] Although no failure to treat urogenital gonorrhea with ceftriaxone (250 mg–1 g) has been detected to date, failures to treat pharyngeal gonorrhea with ceftriaxone have been verified in some countries.[26,27] Isolates obtained from these treatment failures showed ceftriaxone MIC ranged from 0.016 to 4 mg/L.[5] *Neisseria gonorrhoeae* infection of the pharynx is more difficult to eradicate than infections at urogenital and anorectal sites. Moreover, it has been suggested that occupation of pharyngeal niche for gonococci provided an opportunity to exchange DNA, through transformation, with commensal *Neisseria* spp. which act as a reservoir of AMR genes.[2,9] Dual antimicrobial therapy with ceftriaxone and azithromycin has been implemented in Europe and United States as a strategy to stem the development of *N. gonorrhoeae* AMR.[28,29] Surprisingly, in this study, 33% of isolates with decreased susceptibility to ceftriaxone and cefixime showed resistance to Azithromycin. In Argentina, macrolides are not used to treat gonorrhea. However, Azithromycin (1 g) monotherapy is recommended for the treatment of *Chlamydia trachomatis* or *Mycoplasma genitalium* infections. In addition, national guidelines recommend treat these infections when they are suspected but cannot be confirmed.[24,25] This practice may represent a risk of selecting azithromycin-resistant *N. gonorrhoeae* isolates. Consequently, the results of the present study and the recent report of a ceftriaxone-resistant *N. gonorrhoeae* isolate in our country[22] suggest that revision of the empirical treatment guidelines should be considered. Furthermore, in Argentina, the STI syndromic management approach,[30] results in suboptimal diagnosis, principally of pharyngeal and anorectal gonococcal infections which are typically asymptomatic. Therefore, there is an urgent need to strengthen the *N. gonorrhoeae* surveillance, to support detection of asymptomatic infections, to establish criteria for defining cases of treatment failure, to monitor AMR trends (with focus on ceftriaxone, azithromycin and multidrug resistance), to develop evidence-based guidelines and design and implement strategies to prevent and mitigate emergence and spread of *N. gonorrhoeae* resistance.

The molecular study of the isolates with decreased susceptibility to ceftriaxone and cefixime showed the emergence of *N. gonorrhoeae* isolates harboring the *penA* mosaic allele XXXIV within Argentina. All *N. gonorrhoeae* isolates with the mosaic *penA* XXXIV allele were assigned to NG-MAST ST1407 or closely related genotypes. ST1407 has been previously shown to account for most of the decreased susceptibility or resistance to ESCs, and to be responsible for treatment failures in many countries.[5] In this study, at least four STs that are highly related to ST1407, including ST3431, ST3158, ST3378 and ST3709, have been reported in others countries.[11] The emergence of ST1407 and its related STs in our country may be due to the introduction of gonorrhea from other countries and subsequently spread within Argentina. Moreover, the significantly increased of ST1407 and clonally related isolates from 2011 (37.5%) to 2013 (96.1%) suggests a continuous transmission of this clone. Hence, it is pivotal not only to monitor the dissemination of ST1407 and related STs, as well as the emergence of new variants, but also to monitor their potential to become untreatable.

Although, the majority of the isolates with decreased susceptibility to ESC in this study showed a *penA* mosaic allele, four isolates had a different *penA* nonmosaic allele. These four nonmosaic PBP2s contained G542S, P551S, P551L and/or A501V substitutions, which have been associated with increased ESC MICs.[9] Recently, a *N. gonorrhoeae* strain resistant to ceftriaxone and cefixime, which contained a *penA* nonmosaic allele (PBP2 pattern IX), was reported in Argentina.[22] Notably, the phylogenetic analysis showed that this *N. gonorrhoeae* isolate ST13064 was related to a strain ST3620 from 2011. The isolates showed the same *penA* and *mtrR* allele, but a different *porB1b* allele (data not shown). Moreover, both isolates had the NG-MAST *tbpB* 33 allele. Accordingly, the *N. gonorrhoeae* strain resistant to ceftriaxone and cefixime ST13064 might be an evolved subtype of the gonococcal clone ST3620. However, additional phylogenetic studies are needed to reinforce this hypothesis. A limitation of this study was the lack of demographic and epidemiological data to identify index case, high-risk population, potential transmission networks or core groups.

In conclusion, isolates with decreased susceptibility and resistant to cefixime and ceftriaxone have now emerged in Argentina. Enhanced phenotypic and molecular understanding of the evolution and spread of *N. gonorrhoeae* resistance to different antimicrobials in our country is imperative. Moreover, there is a need to strengthen the surveillance system integrating epidemiologic, treatment and laboratory information to detect treatment failures, identifies communities at high risk, and trace sexual contacts. These efforts should be used in public health responses to mitigate emergence and spread of multidrug-resistant gonococci.

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