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ORIGINAL ARTICLE

Neisseria gonorrhoeae antimicrobial susceptibility in Barcelona: *penA*, *ponA*, *mtrR*, and *porB* mutations and NG-MAST sequence types associated with decreased susceptibility to cephalosporins

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Abstract The aims of this study were to determine the antimicrobial susceptibility of *Neisseria gonorrhoeae* (NG) in our area, to analyze the molecular mechanisms involved in cephalosporins resistance, and to undertake molecular typing of our NG strains. Antimicrobial susceptibility was determined using the Etest. The genes *penA*, *mtrR*, *penB*, and *ponA* were studied. Molecular typing was performed by *N. gonorrhoeae* multiantigen sequence typing. Of 329 strains analyzed in 2013, none showed high-level cephalosporin resistance, but 8.2 % had resistance to cefixime [minimum inhibitory concentration (MIC) > 0.125 μg/mL] and 0.6 % to ceftriaxone (MIC > 0.125 μg/mL). Azithromycin resistance was documented in 4.3 % and ciprofloxacin resistance in 49.2 %. Among 48 strains with an MIC ≥ 0.125 μg/mL to cefixime, 58.3 % showed the *penA* mosaic pattern XXXIV, 98 % a Leu → Pro substitution at position 421 of the *ponA* gene, 100 % amino acid changes at positions 101 and 102 of the PorB1b porin, and 87.5 % of strains an adenine deletion in the promoter region of the MtrC-D-E efflux pump. A significant difference between strains with and without decreased cephalosporin susceptibility (MIC ≥ 0.125 μg/mL) was observed for these four genes. Of the 48 strains with an MIC ≥ 0.125 μg/mL to cefixime, 43.8 % belonged to the genogroup G1407 and 27.1 % belonged to the genogroup G2400. A significant association of G1407 with decreased susceptibility (MIC ≥ 0.125 μg/mL) and G2992 with susceptibility was found, and also between G1407 and mosaic pattern XXXIV and between G2400 and A501T substitution in *penA*. The NG resistance rate in our area is higher than the median of Europe. We have detected the emergence of G2400, which may be a source of antimicrobial resistance.

Introduction

Gonorrhea, a condition on the increase with high associated morbidity and a potential for sequelae, has become a major public health problem worldwide, with considerable socioeconomic consequences. Control programs are needed for this condition, based on effective prevention campaigns, adequate systems for contact tracking and notification, fast and accurate diagnostic tools, and, particularly, effective antimicrobial treatment. Antimicrobial therapy should cure individual cases, reduce the risk of complications, and end further transmission of the infection [1].

Over the years, *Neisseria gonorrhoeae* has developed resistance to all the antimicrobials used in its treatment. Currently, the extended-spectrum cephalosporins (ESCs), ceftriaxone (intramuscular) and cefixime (oral), are the only available first-line monotherapy options for gonorrhea in most settings. However, susceptibility to these antibiotics has decreased over the last decade [2–5], and clinical treatment failure has been reported with the use of cephalosporin compounds [6–10]. Of particular concern, the first three extensively drug-resistant (XDR) gonococcal isolates with high-level

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ceftriaxone resistance were recently identified in Japan [7], France [9], and Spain [11, 12].

In 2013, the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) reported resistance (>0.125 mg/L) to cefixime in 4.7 % of isolates in Europe, a value found to be much higher in Spain (15.1 %). Moreover, six of the seven isolates showing decreased susceptibility to ceftriaxone (>0.125 mg/L) were from Spain [13]. Hence, the issue of drug resistance in *N. gonorrhoeae* is of particular relevance in our country.

The development of *N. gonorrhoeae* resistance to penicillin and ESCs is a complex and multifaceted process that involves alterations in at least four known resistance determinants: *penA*, encoding modified forms of PBP2; *mtrR* mutations, resulting in overexpression of the MtrC-D-E efflux pump; *penB*, encoding mutations in the major outer membrane porin; and *ponA*, encoding an altered form of PBP1.

It has been found that *N. gonorrhoeae* strains with certain resistance profiles are grouped into specific genogroups [14]. The French strain, F89, the Spanish XDR strains, and the strains responsible for treatment failure in Norway [15], Austria [10], and the United Kingdom [6] belong to the ST1407, or to other genetically related sequence types (STs), which appear to be the most prevalent in Europe [9].

The aims of this study were to determine the antimicrobial susceptibility of *N. gonorrhoeae* in our setting, to analyze the molecular mechanisms involved in ESC resistance, and to undertake molecular typing of *N. gonorrhoeae* strains.

This would allow us to know the rate of resistance to ESCs in our area and to compare it with resistance rates from Spain and Europe. It will also allow us to know if the resistance mechanisms to these antibiotics are the same as those described previously and if the population dynamic of the resistant strains follows the same pattern as the resistant strains already characterized.

Materials and methods

Bacterial strains and antibiotic susceptibility

In total, 339 *N. gonorrhoeae* strains isolated in the Microbiology Laboratory of Hospital Vall d'Hebron (Barcelona, Spain) from January to December 2013 were studied. The strains were recovered from 321 patients who had been attended in the hospital, in the Drassanes Sexually Transmitted Infections Unit, or in some of the 150 primary care centers in our area. This represents approximately 50–60 % of known cases of gonococcal infections in Catalonia.

Urethral, endocervical, pharyngeal, or rectal specimens were collected and cultured on selective Thayer–Martin medium. Plates were incubated for 24 to 48 h at 35–37 °C in 5 % CO_2 atmosphere. Suspected *N. gonorrhoeae* colonies were identified by Gram stain, the cytochrome oxidase test, and mass spectrometry (MALDI-TOF) using the Vitek MS system (bioMérieux, Spain).

The minimum inhibitory concentrations (MICs) of penicillin, ceftriaxone, cefixime, azithromycin, ciprofloxacin, and spectinomycin were determined by the Etest method (bioMérieux, Spain) in GC II AG/W IsoVitaleX medium (Becton Dickinson, France), as specified in the product's package insert provided by the manufacturer. *Neisseria gonorrhoeae* ATCC 49226 was used as the reference strain. MICs were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [16]. In all strains, beta-lactamase production was evaluated using nitrocefin disks.

Strains were frozen at −80 °C in trypticase soy broth with 20 % glycerol for molecular studies.

DNA extraction

Bacterial DNA was extracted as follows: a colony that had undergone 24 h of incubation was resuspended in 100 μL of Milli-Q water, heated at 95 °C for 10 min, and centrifuged at 13,000 rpm for 3 min. After this process, the supernatant was diluted at 1:2 with Milli-Q water.

Molecular characterization of resistance to extended-spectrum cephalosporins

Four genes related to cephalosporin resistance were studied: *penA*, *mtrR*, *penB*, and *ponA*. Amplification and sequencing of these genes used the primers previously described and specified [17–19]. The polymerase chain reaction (PCR) parameters for *ponA*, *penB*, and *mtrR* were as follows: an enzyme activation step at 94 °C for 5 min, followed by 25 sequential heating cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and at the end of the final cycle, an extension phase of 72 °C for 7 min. The parameters for the *penA* gene amplification included an enzyme activation step at 94 °C for 12 min, followed by 40 sequential heating cycles at 94 °C for 1 min, 58 °C for 1 min, and 68 °C for 2 min, and, finally, an extension phase of 72 °C for 12 min.

The sequences of both DNA strands of the amplified products were analyzed with the SeqMan software (DNASTAR, Madison, WI, USA) to obtain a consensus sequence. The amino acid sequences were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Molecular typing

All isolates were typed using *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), as described at http://www.ng-mast.net, and using the primers described previously [20]. Briefly, after DNA extraction, internal fragments of the

porB and *tbpB* genes were PCR-amplified and sequenced in both directions. Using the NG-MAST web site, each allele was assigned a number, and the combination of the two alleles from each strain was assigned an ST. Closely related STs were clustered in genogroups (G), as described by Chisholm et al. [14], such that each genogroup included all STs that shared one allele and showed >99 % similarity to the other allele (≤5 bp difference for *porB* and ≤4 bp difference for *tbpB*).

Statistical analysis

An exploratory analysis of the data was conducted. To analyze possible differences between the responses "susceptible" or "decreased susceptibility", a test of comparisons was used in which the *p*-value (2**p* overall) corresponds to the Fisher's exact test. All analyses were performed using the R statistical program (version 3.2, Copyright (C) 2015, The R Foundation for Statistical Computing).

The funder had no involvement in the study design.

Results

From January to December 2013, 339 strains were isolated from 321 patients. Ten strains were excluded because, in eight patients, *N. gonorrhoeae* was recovered from two different parts of the body, and in one patient, gonococcus was recovered from three different parts. In the end, we included 329 strains isolated from 321 patients, because seven patients presented two different episodes during the study period.

Of the 321 patients included, 69.5 % were attended in the Drassanes Sexually Transmitted Infections Unit, 27.4 % in primary care centers, and the rest were attended in the hospital. Demographic, behavioral, and clinical characteristics were available for 229 patients (see Table 1). 94.3 % were men and 65.1 % of all patients were men who have sex with men (MSM). Almost 50 % were European and 22 % were coinfected with human immunodeficiency virus (HIV). More than half did not use condoms during sexual intercourse.

79.3 % of the samples were recovered from urethra, 11.6 % from rectum, 4.9 % from vagina-endocervix, 3 % from pharynx, and the remaining 1.2 % from other types of samples.

Antibiotic susceptibility

Of the 329 strains analyzed, 28 % were resistant to penicillin (MIC > 1 μg/mL) and 63.2 % showed intermediate susceptibility to this antibiotic (MIC 0.06–1 μg/mL). In total, 56 strains (17 %) were penicillinase-producing *N. gonorrhoeae* (PPNG), including 53 of 92 resistant strains (57.6 %).

In 2013, none of the isolates showed high-level cephalosporin resistance, but 27 strains (8.2 %) had resistance to cefixime (MIC > 0.125 μg/mL) and two strains (0.6 %) to ceftriaxone (MIC > 0.125 μg/mL). These two strains were also resistant to cefixime. The MIC_{50} values for cefixime and ceftriaxone were 0.032 μg/mL and 0.016 μg/mL, respectively, and the MIC_{90} values were 0.125 μg/mL and 0.064 μg/mL, respectively. The MIC distribution for ceftriaxone and cefixime is shown in Fig. 1.

Azithromycin resistance was documented in 4.3 % of strains (MIC > 0.5 μg/mL). One hundred and sixty-two strains (49.2 %) were resistant to ciprofloxacin (MIC > 0.06 μg/mL), and, of them, 110 (68 %) showed high-level resistance to this antibiotic (MIC ≥ 8 μg/mL). All the strains studied were susceptible to spectinomycin.

Molecular characterization of the cephalosporin resistance determinants

Molecular characterization was performed for 48 strains, 27 of which were resistant to cefixime (MIC > 0.125 μg/mL) and 21 had decreased susceptibility (MIC 0.125 μg/mL) to this antibiotic. To enable a comparison of the results, 40 strains susceptible to this antimicrobial, with different MIC ranges, were selected. The findings on the characterization of the *penA*, *ponA*, *penB*, and *mtrR* genes in these 88 strains are shown in Table 2.

Regarding the *penA* gene, which codes for PBP2, among the 48 studied strains with decreased susceptibility (DS) to cephalosporin, 28 (58.3 %) showed the *penA* mosaic pattern XXXIV and 19 (39.6 %) showed the non-mosaic pattern XXXVI, in accordance with the established nomenclature [7, 9, 18, 21]. Furthermore, among the 19 strains with pattern XXXVI, 14 (73.7 %) showed mutations at position A501. In contrast, among the 40 cephalosporin-susceptible strains, only one (ceftriaxone MIC 0.016 μg/mL and cefixime MIC 0.047 μg/mL) had mosaic pattern XXXIV; the remainder presented pattern XXXVI. In contrast to DS strains, only 8 (20.5 %) of the susceptible strains showed a mutation at A501.

As to the *ponA* gene, which codes for PBP1, all DS strains except one (98 %) showed a Leu → Pro substitution at position 421 of the protein, in contrast to the susceptible strains, in which only 22 (55 %) had this substitution.

To characterize the *penB* gene, which codes for the major outer membrane porin, PorB1b, we assessed the amino acids at positions 101 and 102, as it is known that mutations in these positions confer resistance to certain antimicrobial agents. All 48 strains with decreased susceptibility to cephalosporins showed amino acid changes in these positions: 29 (60.4 %) presented a Gly → Lys substitution at 101 and Ala → Asn at 102, 18 (37.5 %) had the same Gly → Lys substitution at 101 with an Ala → Asp substitution at 102, and 1 one strain showed a single change, Ala → Ser, at position 102. In contrast, among the susceptible strains, 32 (80 %) showed mutations at these positions, but the substitutions observed were much more variable than in the DS strains (Table 2); the

Table 1 Demographic, behavioral, and clinical characteristics of the patients

		Patients ($n = 229$)	%
Median age in years (IQR)		31 (15–57)	
Gender			
	Male	216	94.3
	Female	10	4.4
	Unknown	3	1.3
Sexual orientation			
	MSM	149	65.1
	HTS	64	27.9
	Bisexual	8	3.5
	Unknown	8	3.5
Ethnicity			
	European	112	48.9
	American	43	18.8
	African	12	5.2
	Asian	1	0.4
	Unknown	61	26.6
Educational level			
	Without studies	1	0.4
	Elementary	51	22.3
	Secondary	30	13.,1
	Higher	40	17.5
	Unknown	107	46.7
Symptomatology			
	Yes	188	82.1
	No	28	12.2
	Unknown	13	5.7
Coinfections			
HIV status	Positive	50	21.8
	Negative	126	55.0
	Unknown	53	23.1
Other STI in the previous 12 months	*N. gonorrhoeae*	28	12.2
	C. trachomatis	10	4.4
	LGV	5	2.2
	T. pallidum	28	12.2
	Genital warts	2	0.9
	HSV	1	0.4
Contact tracing			
No		126	55.0
Yes		103	45.0
	Median no. of partners in the previous 2 months	2 (1–50)	
Sexual behavior			
	Without condom	117	51.1
	With condom	30	13.1
	VS or AS with condom and OS without condom	10	4.4
	Unknown	72	31.4

remaining seven strains (17.5 %) had no mutations in these positions.

With regard to the MtrC-D-E efflux pump repressor, a deletion of one adenine was seen in the promoter region of the repressor in 42 of the 48 DS strains (87.5 %). Furthermore, 38 of these 42 strains (90.5 %) showed the H105Y mutation in the repressor coding region, and one strain the A39T mutation. Only four of the 48 DS strains did not show the promoter deletion, and two strains were not viable for characterization of this gene. By comparison, only 10 (25 %) of the susceptible strains showed the promoter deletion. In the remainder, the deletion was not found, and the susceptible strains were seen to present a much more variable mutation profile in the repressor coding region.

A significant difference between the two groups was seen for the four genes studied. For *penA*, univariate analysis with the Fisher's exact test yielded a statistically significant association of the mosaic pattern XXXIV with the DS group and the non-mosaic pattern XXXVI with the susceptible group ($p = 0.0001$). Statistical analysis within the DS group taking into consideration the MIC value yielded a significant association of strains with an MIC ≥ 0.25 μg/mL with the mosaic pattern XXXIV ($p = 0.0046$). Examination of the relationship between

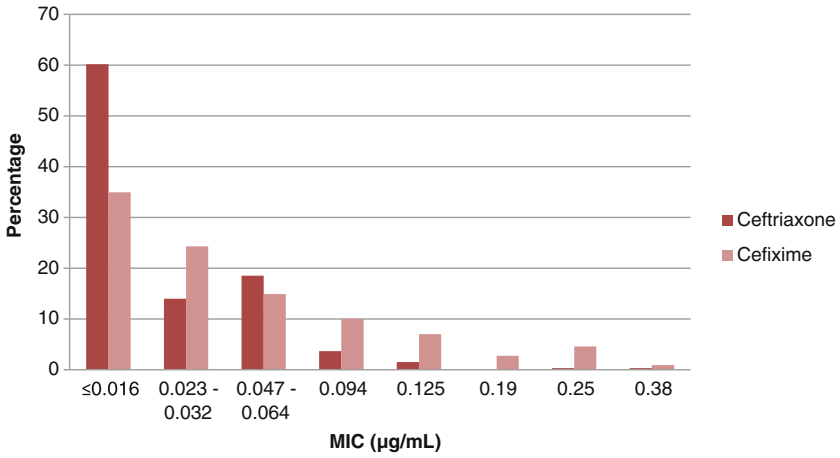

Fig. 1 Minimum inhibitory concentration (MIC) distribution for ceftriaxone and cefixime

cephalosporin susceptibility and the S501T substitution also showed a significant association of the DS group with this substitution ($p = 0.0003$).

For the *ponA* gene, there was an association of the L421P substitution with decreased susceptibility to ESCs ($p = 0.0001$) and with an adenine deletion in the mtrR promoter ($p = 0.0001$).

Lastly, analysis of the relationships between porin PorB1b mutations and cephalosporin susceptibility showed a significant association of decreased susceptibility to ESCs with mutations at positions 101 and/or 102 ($p = 0.0026$).

Molecular typing

Analysis of the NG-MAST profiles in the 88 *N. gonorrhoeae* strains is shown in Table 3. Among the 48 DS strains, the most prevalent genogroup was G1407 (43.8 %), followed by G2400 (27.1 %). Two strains belonged to ST7228, which was previously described by our group [22]. In contrast, among susceptible strains, the ST distribution was much more variable.

Statistical analysis showed a significant association of G1407 with DS strains ($p < 0.0001$) and G2992 with susceptible strains ($p = 0.0153$).

Furthermore, evaluation of the relationships between the two main genogroups of DS strains and the PBP2 structure showed a statistically significant association of G1407 with the mosaic pattern XXXIV ($p = 0.0001$) and G2400 with A501T substitution in the non-mosaic pattern XXXVI ($p = 0.0001$).

Discussion

This study shows that: (1) the rate of resistance in our area in 2013 was higher than the average for European countries, but less than the overall average for Spain [13], (2) the molecular mechanisms that give rise to a reduction in susceptibility to ESCs are those previously described, and (3) in what would be the greatest contribution of the study, the population dynamic of the strains with decreased susceptibility to ESCs is very different from that of the susceptible strains, and that we have detected the emergence of the genogroup G2400 that, like G1407, could more easily develop antimicrobial resistance.

The most important factor in decreased cephalosporin susceptibility is the changes that occur in the *penA* gene. Acquisition of *penA* mosaic and non-mosaic alleles with mutations at A501 is associated with enhanced cephalosporin MICs [1, 7, 9, 12]. For example, the XDR isolated in France and Spain [9, 12] contained the *penA* mosaic allele XXXIV with an additional A501P change. In our study, 28 of the 48 DS strains showed the mosaic XXXIV allele, which has been related to the MLST ST1901/NG-MAST ST1407 clone, one of the most prevalent in Europe associated with decreased cephalosporin susceptibility [14]. In contrast to the XDR strains from France and Spain, none of the study strains with the XXXIV allele showed an amino acid change at A501. The remaining 19 strains contained the non-mosaic XXXVI allele, of which 14 strains had the A501T substitution.

Although the mutation in the *ponA* gene was present in most of the DS isolates in our study, it is believed to be involved in conferring high-level penicillin resistance in strains containing *penA*, *mtrR*, and *penB* changes, but it does not seem to raise cephalosporin MICs in clinical isolates [1, 23, 24].

Another mechanism implicated in *N. gonorrhoeae* drug resistance is a change in the membrane porin (porB1b) encoded by the *penB* gene, which decreases cell wall permeability. In our series, 47 of the 48 DS strains showed changes in the amino acids G101 and A102 located in loop 3 of the protein. These mutations have been associated with gonococcal strains showing decreased cephalosporin susceptibility [23, 25, 26] and have been described in highly resistant strains in Japan [7], France [9], and Spain [12]. Even so, these two

Table 2 Characterization of resistance determinants in 48 *Neisseria gonorrhoeae* strains with decreased susceptibility to extended-spectrum cephalosporins (ESCs) and 40 susceptible strains

		ESC decreased susceptibility	ESC susceptible
PBP2			
Mosaic XXXIV		28	1
Non-mosaic XXXVI		19	38
	XXXVI	0	3
	XXXVI (S551L)	2	1
	XXXVI (S551P)	0	12
	XXXVI (A501T and S551L)	13	8
	XXXVI (H541N and S551P)	2	10
	XXXVI (G542S and S551P)	1	2
	XXXVI (A540X and S551P)	0	1
	XXXVI (A501T, G542S, and S551P)	1	0
	XXXVI (H541N, S551P, P552V, K555Q, and I556V)	0	1
n/d		1	1
PBP1			
wt		1	18
L421P		47	22
n/d		0	0
PorB1b			
Wt		0	7
101 and 102 mutation		48	32
	G101K, A102N	29	5
	G101K , A102D	18	12
	G101, A102S	1	7
	G101D, A102G	0	4
	G101K , A102G	0	2
	G101N, A102D	0	2
n/d		0	1
MtrC-D-E			
mtrR promoter	MtrR		
With A deletion		42	10
	wt	3	1
	A39T	1	0
	H105Y	38	9
Without A deletion		4	25
	wt	1	3
	A39T	3	10
	A39T, R44H	0	8
	G45T	0	2
	H105Y	0	2
n/d		2	5

wt wild type; *n/d* not determined

mutations need synergy with the other resistance determinants examined in this study to raise cephalosporin MICs.

Finally, a specific mutation in the promoter or coding sequence of mtrR markedly increases expression of the MtrC-D-E efflux pump, which enhances efflux of cephalosporins, particularly ceftriaxone [1]. The determinant most strongly associated with increased cephalosporin MICs is an adenine deletion in the 13 base-pair inverted repeat of the promoter region, which was present in 42 of the 48 DS strains in our study. Amino acid changes in the MtrR repressor, specifically at positions A39, R44, G45, and H105, may also contribute to the MIC increases [18, 27]. In our series, the most commonly observed profile in DS strains was the adenine deletion and H105Y mutation, previously described by Thakur et al. [25]. The deletion is believed to impede binding of the repressor to the MtrC-D-E promoter and the H105Y substitution could inhibit MtrR dimerization.

Other authors have suggested an association of the *penA* mosaic structure and *mtrR*, *penB*, and *ponA* changes with decreased cephalosporin susceptibility [27]. In this same line, we found a statistically significant relationship between the various resistance determinants and increases in cephalosporin MICs.

In this study, the most prevalent genogroup in strains showing DS to cephalosporin compounds was G1407, followed by G2400. According to data reported by Chisholm et al. in 2010 [14], G1407 was the most prevalent genogroup (23 %) among *N. gonorrhoeae* isolated in the 21 participating European

Table 3 *Neisseria gonorrhoeae* multiantigen sequence types (STs) by genogroup

ESC decreased susceptibility					ESC susceptible				
ST	*n*	Genogroup	*n*	%	ST	*n*	Genogroup	*n*	%
1407	11	1407	21	43.8*	1407	0	1407	1	2.6*
3149	1				3149	0			
3378	6				3378	0			
4120	2				4120	0			
10022	0				10022	1			
10426	1				10426	0			
2400	11	2400	13	27.1	2400	5	2400	7	18.4
6360	1				6360	1			
10459	1				10459	0			
12564	0				12564	1			
5	2	1034	2	4.2	5	0	1034	0	0
2992	0	2992	0	0*	2992	5	2992	5	13.2*
289	0	225	0	0	289	1	225	3	7.9
346	0				346	1			
9433	0				9433	1			
7228	2	–	2	4.2	7228	4	–	4	10.5
5624	0	–	0	0	5624	5	–	5	13.2
Other ST	9	–	9	18.8	Other ST	13	–	13	34.2

*p-Value <0.05

countries. The NG-MAST ST1407, together with the MLST ST7363, were responsible for all confirmed cases of therapeutic failure with cefixime, and three of the five cases of failure with ceftriaxone.

Our findings concur with the idea that G1407 is a highly prevalent clone and, as seen in other studies [1, 10, 14, 15, 28–30], it is associated with strains having decreased susceptibility to cephalosporins and the mosaic XXXIV allele in the *penA* gene. It is important to note that this study has clearly shown that the genogroup G1407 is significantly more prevalent among the strains with DS to ESCs than among sensitive strains.

Another important finding is that G2400 was the second most prevalent genogroup among DS strains (27.1 %) and the most prevalent among sensitive strains (18.4 %), accounting for 23.3 % of the total number of strains. Furthermore, G2400 was associated with the A501T substitution in non-mosaic pattern XXXVI ($p = 0.0001$) and an elevated cefixime MIC. Although this genogroup had already been described, to our knowledge, this is the first reported observation of a high prevalence of G2400 among strains with decreased susceptibility to cephalosporins and its clear association with the A501T substitution in the *penA* gene.

The main limitation of this study is that most of the strains examined were from a small geographic area. Therefore, the results are only representative of the situation in that specific area and cannot be generalized. Furthermore, because of the small number of isolates in some groups, logistic regression analysis could not be performed.

In conclusion, the results of this study highlight the high antimicrobial resistance rate in our area and support the known association between a highly prevalent clone worldwide (G1407) and the ability to develop cephalosporin resistance. The most important finding is the emergence of a new clone, G2400, which may also be a source of antimicrobial resistance.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study, formal consent is not required.

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