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Typing and antimicrobial susceptibility of 134 *Neisseria gonorrhoeae* strains from Southern Spain

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ABSTRACT

Introduction. Last guidelines have recommended the introduction of dual antimicrobial therapy in order to avoid treatment failure. In the present report, the susceptibility to some antibiotics was evaluated, and the typing of *Neisseria gonorrhoeae* strains was performed.

Material and methods. Gonococcal isolates were tested for susceptibility according to the recommendations of both CLSI and EUCAST. A total of 134 isolates were typed by the NG-MAST technique.

Results. Seventy-two different *N. gonorrhoeae* types were found, and the most frequent types obtained were ST 1407, ST 14958, ST 7192, ST 13251 and ST 5405. If CLSI/EUCAST criteria were applied, a ST 9807 type was found nonsusceptible to ceftriaxone and cefixime (MIC 0.5 mg/L), and a ST 12800 type was found nonsusceptible only to cefixime (MIC 0.25 mg/L). When only EUCAST breakpoints were taken into account, three strains were also resistant to cefixime (MIC 0.25 mg/L) and three isolates were resistant to ceftriaxone (MIC 0.19, 0.16 and 0.25 mg/L, respectively). The majority of strains were resistant to ciprofloxacin (68.6%), and all *N. gonorrhoeae* strains were susceptible to spectinomycin; 9.7% of isolates were resistant to azithromycin.

Conclusions. Molecular typing may be a useful tool to predict antimicrobial resistance. High rates of resistance to penicillin, tetracycline and ciprofloxacin were found in this area. It is highly recommended to carry out antimicrobial susceptibility in all gonorrhoea cases and to identify treatment failures to verify emerging resistance.

Keywords: *Neisseria gonorrhoeae*; susceptibility testing; molecular typing; epidemiology; resistance

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Tipado y sensibilidad a antimicrobianos de 134 cepas de *Neisseria gonorrhoeae* en el sur de España

RESUMEN

Introducción. Las últimas guías recomiendan la introducción de tratamiento antimicrobiano doble para evitar fallos de tratamiento. En esta publicación, se evaluó la sensibilidad a algunos antibióticos, y se realizó el tipado de las cepas de *Neisseria gonorrhoeae*.

Material y métodos. La determinación de sensibilidad fue realizada según las recomendaciones tanto del CLSI como del (EUCAST). Un total de 134 cepas fueron tipadas mediante la técnica de NG-MAST.

Resultados. Se encontraron 72 tipos diferentes de *N. gonorrhoeae*, siendo los más frecuentes el ST 1407, 14958, 7192, 13251 y 5405. Aplicando los criterios del CLSI/EUCAST, un tipo ST 9807 fue no sensible a ceftriaxona y cefixima (CMI 0,5 mg/L), y un tipo ST 12800 fue no sensible solo a cefixima (CMI 0,25 mg/L). Al aplicar solamente los puntos de corte de EUCAST, tres cepas fueron también resistentes a cefixima (CMI 0,25 mg/L) y tres lo fueron también a ceftriaxona (CMI 0,19, 0,16 y 0,25 mg/L, respectivamente). La mayoría de cepas fueron resistentes a ciprofloxacino (68,6%), y todas las cepas de *N. gonorrhoeae* fueron sensibles a espectinomicina; el 9,7% de aislamientos fueron resistentes a azitromicina.

Conclusiones. El tipado molecular puede ser una herramienta útil para predecir resistencia a antimicrobianos. En esta área se encontraron altas tasas de resistencia a penicilina, tetraciclina y ciprofloxacino. Se recomienda encarecidamente realizar estudio de sensibilidad a antibióticos en todos los casos de gonorrea e identificar fallos de tratamiento para verificar la aparición de resistencias.

Palabras clave: *Neisseria gonorrhoeae*; estudio de sensibilidad; tipado molecular; epidemiología; resistencia

INTRODUCTION

Infections due to *Neisseria gonorrhoeae* (NG) are the second most commonly reported sexually transmitted infections (STIs) worldwide caused by bacteria [1]. The prevalence of this infection varies among populations but it continues to present a serious public health problem in many countries [2], although the vast majority of the gonorrhoea burden globally is in low and middle-income countries [2]. Thus, an appropriate diagnosis and an effective treatment of this infection are important factors contributing to public health control and to prevent serious complications.

Treatment is usually given empirically following recommendations in evidence-based treatment guidelines. Ceftriaxone or dual antimicrobial therapy of ceftriaxone plus azithromycin is currently the only options for empirical first-line therapy in most countries [3, 4]. However, the increase of resistance to recommended treatments for gonorrhoea may seriously affect to infection control [5].

Recently, some authors have described failure of dual antimicrobial therapy in treatment of gonorrhoea [6] and during the last years several NG isolates exhibited decreased susceptibility and resistance to the third generation cephalosporins have been reported [7, 8]. In 2015, 1.7% of isolates from European countries showed decreased susceptibility to cefixime, and there was a high prevalence of resistance to ciprofloxacin (49.4%) and azithromycin (7.1%) [9].

Due to these facts, antimicrobial surveillance programs are necessary to detect patterns of resistance at international, national, but also at regional level to ensure treatment efficacy against this infection. In this sense, molecular epidemiology surveillance can help to provide additional information on the emergence and dissemination of antimicrobial resistance. Molecular studies for epidemiological surveillance are necessary to detect association between genotype and antimicrobial resistance and to know how resistant strains emerge and disseminate [10].

The main objective of the present study was to analyze the antimicrobial susceptibility of *N. gonorrhoeae* strains isolated from genital specimens in patients belonging to a health area of Southern Spain, as well as to perform the typing of these samples in order to detect epidemiological clusters of infection and possible modifications of the susceptibility patterns.

MATERIALS AND METHODS

Collection of NG strains. All NG strains isolated from January 2012 to December 2016 were included in this study. The genital samples were obtained from patients with STIs belonging to the health area of the Hospital of Poniente (El Ejido, Almería, Spain) composed by primary health care and the reference hospital, and were sent to the microbiology laboratory for culture. A total of 134 isolates were included for both antimicrobial susceptibility study and molecular

typing. All samples were obtained from genital sites (urethral or endocervical/vaginal exudates). No extragenital samples (rectal and pharyngeal specimens) were included in this study because these kind of samples were not sent to our laboratory. All samples were cultured in VCA agar (BioMérieux, France). The identification of NG suspected strains was performed by Gram stain, oxidase and catalase production, and finally with both biochemical analysis by the API NH system (BioMérieux, France) and proteomic analysis by MALDI-TOF technology (Vitek MS, bioMérieux, France). The identification of all strains was confirmed at the National Centre of Microbiology (Instituto de Salud Carlos III, Madrid, Spain).

Antimicrobial susceptibility testing. Gonococcal isolates were tested for susceptibility according the recommendations of Clinical and Laboratory Standards Institute (CLSI) [11] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [12]. All strains were tested for susceptibility to penicillin, ceftriaxone, cefixime, tetracycline, ciprofloxacin, azithromycin and spectinomycin by agar dilution tests. All isolates were also tested for penicillinase production using the Cefinase test (bioMérieux, Marcy-l'Etoile, France). MIC interpretation was performed according to both CLSI and EUCAST, and then compared.

Molecular typing. All NG strains (n= 134) were typed by the *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) technique [13], which differentiates strains on the basis of sequence variation in fragments of two hypervariable genes, the subunit B of the transferring binding protein (*tbpB*) and the porin Por B (*porB*). The typing was performed at the National Centre of Microbiology (Instituto de Salud Carlos III, Madrid, Spain). Allele numbers and STs were assigned using NG-MAST databases (www.ng-mast.net). Moreover, all NG strains were also serotyped at this center by means of the Phadebact® Monoclonal GC Test (MKL Diagnostics AB, Sollentuna, Sweden).

RESULTS

One hundred and four samples were taken from men, and the remaining from women. Finally, 133 patients were included in this study. The age of patients included in this study range from 20 to 50 years old (median age= 32 years old). Sixty-nine (51.4%) samples were obtained from immigrant population and 65 (48.6%) from indigenous patients. The vast majority of strains were obtained from urethral samples (104/77.7%) whereas 30 (22.3%) strains were obtained from vaginal or endocervical fluid. From the 134 typed strains, 72 different STs were obtained and all NG strains included in this study belonged to the IB serotype. It can see the distribution of main types in table 1. The most frequent types found were STs 1407 (n=8), STs 14958 (n=8), STs 7192 (n=7), STs 13251 (n=7), STs 5405 (n=5), STs 2992 (n=4), STs 387 (n=4), STs 7232 (n=4), STs 5624 (n=4), and STs 5120 (n=4). An important finding is that a ST 9807 strain was nonsusceptible (according to CLSI and EUCAST criteria) to ceftriaxone and

ST	Antimicrobial susceptibility			Allele	
	Ceftriaxone	Cefixime	Azithromycin	por B	tbpB
1407	S	S	S	908	110
1407	S	S	S	908	110
1407	R	S	S	908	110
1407	R	S	S	908	110
1407	S	R	R	908	110
1407	S	S	R	908	110
1407	S	S	S	908	110
1407	S	S	S	908	110
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
14958	S	S	S	8692	137
7192	S	S	S	90	138
7192	S	S	S	90	138
7192	S	S	S	90	138
7192	S	S	S	90	138
7192	S	S	S	90	138
7192	S	S	S	90	138
7192	S	S	S	90	138
13251	S	S	S	7696	137
13251	S	S	S	7696	137
13251	S	S	S	7696	137
13251	S	S	S	7696	137
13251	S	S	S	7696	137
13251	S	S	S	7696	137
13251	S	S	S	7696	137
13251	S	S	S	7696	137
5405	S	S	S	3279	1139
5405	S	S	S	3279	1139
5405	S	S	S	3279	1139
5405	S	S	S	3279	1139
5405	S	S	S	3279	1139
387	S	S	S	266	118
387	S	S	S	266	118
387	S	S	S	266	118
387	S	S	S	266	118
2992	S	S	S	1808	29
2992	S	R	R	1808	29
2992	S	S	S	1808	29
2992	S	S	S	1808	29
5120	S	S	S	3105	118
5120	S	S	S	3105	118
5120	S	S	S	3105	118
5120	S	S	S	3105	118
7232	S	S	S	1489	1388
7232	S	S	S	1489	1388
7232	S	S	S	1489	1388
7232	S	S	S	1489	1388
5624	S	S	S	90	953
5624	S	S	S	90	953
5624	S	S	S	90	953
5624	S	S	S	90	953
9807	R	R	S	5785	137
7072	S	R	S	4259	10

S: susceptible, R: resistant

Table 2 Antimicrobial susceptibility comparison between CLSI and EUCAST recommendations.

	CLSI				EUCAST				
	R	S	I	%NS	R	S	I	%NS	
Penicillin	55	41	38	69.4	Penicillin	69	22	43	83.5
Ceftriaxone	1	133	0.7		Ceftriaxone	4	130	2.9	
Cefixime	2	132	1.4		Cefixime	5	129	3.7	
Tetracycline	74	35	25	73.8	Tetracycline	74	32	28	76.1
Ciprofloxacin	92	42	68.6		Ciprofloxacin	92	42	68.6	
Spectinomycin	134	0			Spectinomycin	134	0		
Azithromycin					Azithromycin	13	121	9.7	

R: resistant; S: susceptible; I: intermediate; NS: nonsusceptible; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing

Table 3 Susceptibility percentage to tested antibiotics comparing CLSI and EUCAST.

Antibiotic	CLSI percentage	EUCAST percentage
Penicillin	30.6	16.5
Ceftriaxone	99.3	97.1
Cefixime	98.6	96.3
Tetracycline	26.2	23.9
Ciprofloxacin	31.4	31.4
Spectinomycin	100	100
Azythromycin		90.3

cefixime (MIC 0.5 mg/L for both antibiotics). Moreover, according to EUCAST criteria, four strains were also resistant to cefixime (ST 2992, ST 7072, ST 12800 and ST 1407; MIC 0.25 mg/L) and three more strains were resistant to ceftriaxone [ST 1407 (MIC 0.19 and 0.25 mg/L, respectively), and ST 225 (MIC 0.16 mg/L)]. All STs 1407 and 14958 included in this study were non-susceptible to ciprofloxacin.

Forty-seven isolates (35%) demonstrated penicillinase production. If only CLSI criteria are applied, only a gonococcal strain (ST 9807) was nonsusceptible to ceftriaxone and cefixime, whereas according to EUCAST criteria five gonococcal strains were resistant to cefixime and four were resistant to ceftriaxone. According to EUCAST criteria, only 22 (16.4%) gonococcal strains were susceptible to penicillin, but if CLSI criteria are applied 41 (30.5%) isolates were susceptible to this antibiotic. The majority of isolates in this study were resistant to ciprofloxacin (92/68.6%). All NG strains were susceptible to spectinomycin. Regarding to azithromycin, 13 (9.7%) were resistant to this drug. MIC for 7 strains resistant to azithromycin was 1 mg/L, and the MIC for the remaining cases was 2 mg/L. Unfortunately, no data about previous treatment and co-infections could be obtained. The complete data corresponding to gonococcal strains susceptibility and resistance are shown in table 2. Comparison data of antimicrobial susceptibility between CLSI and EUCAST are included in table 3.

From 134 samples, only 10 (7.4%) strains were susceptible to all antibiotics tested; 41 (30.5%) strains were resistant to more than three antibiotic (multiresistant), 28 (20.8%) were resistant to three antibiotics, 17 (12.6%) resistant to two antibiotics and finally 32 (23.8%) were resistant to only one antibiotic.

Regarding to isolates distribution in our health area, an epidemiological relationship could be observed. Three strains of ST1407 type, three strains of ST 5405 type, two strains of ST 2992 type and two strains of ST 5120 type are grouped and located in the same geographical area (health basic zone), so a presumptive relationship could be observed. Moreover, the strain ST 9807 (nonsusceptible to ceftriaxone and cefixime) was also located in the main epidemiological focus, considered as the geographical area in which the majority of strains were obtained.

A patient had two different isolates (ST 6715 and ST 4260) in two different cultures separated by 1 month. After a correct treatment, this patient had again the strain ST 6715 one year later in a new urethral sample.

From the 35 strains belonging to types ST 1407, ST 14958, ST 7192, ST 13251, and ST 5405, 25 of them were found in immigrant population and these strains appear to have had epidemiological relationship because they were located in the same health basic zone.

During the follow-up period, test of cure was not carried out in the majority of patients (86/64.1%), whereas test of cure was performed in the remaining patients at least 72 hours after completion of treatment. From these patients the culture was negative in 47, but in one patient, a new type of *N. gonorrhoeae* was found.

DISCUSSION

Gonorrhoea remains an important public health problem because of untreated infections may lead to severe sequelae. In 2012, the World Health Organization (WHO) estimated 78.3 million new global cases among adults worldwide, although the true incidence is probably underestimated [2]. Thus, effective

treatment of gonorrhoea is crucial to the disease control, but the progressive increase of resistance to recommended treatment regimens has compromised infection control efforts. Over the past decades, many countries already have high prevalence of gonococcal resistance to all antibiotics that have been used for treatment such as penicillins, sulphonamides, tetracyclines, quinolones and early generation macrolides and cephalosporins [3, 5, 14]. The results of the present study also show a high percentage of isolates non-susceptible to these antibiotics such as penicillin [69.4% (CLSI); 83.5% (EUCAST)], tetracycline [73.8% (CLSI); 76.1% (EUCAST)], and ciprofloxacin (68.6%), so these antimicrobials cannot be used as empirical treatment.

When any laboratory results are available, the empirical first-line therapy guidelines recommend the use of extended spectrum cephalosporins (ceftriaxone or cefixime) [15] and, recently, European guidelines as well as most of therapeutic guidelines recommend the use of dual therapy with ceftriaxone plus azithromycin [16]. However, some gonococcal strains with low-level ceftriaxone and cefixime resistance have been described [3, 17]. Some of these reductions on susceptibility were accompanied with subsequent treatment failures [18]. In addition, multidrug-resistant (MDR) gonococcal strains have been detected [3, 5, 14], and highly ceftriaxone-resistant NG strains have been recently reported [7, 8, 19-21], and this fact shows that susceptibility to ceftriaxone is progressively decreasing.

In our study, the majority of strains were susceptible to extended spectrum cephalosporins, but according to EUCAST four isolates were resistant to ceftriaxone and five strains were resistant to cefixime. On the other hand, 13 (9.7%) strains were resistant to azithromycin. A recent study reported 32% of resistance to azithromycin from 384 NG isolates [22]. In view of these results, the best empirical treatment for gonococcal infections in our health area should be the administration of dual therapy as it is recommended, although it is strongly recommended the susceptibility testing to antibiotics in order to detect resistance.

A similar study performed in Spain [23] with 100 NG strains shows three NG strains nonsusceptible to ceftriaxone and ten NG strains resistant to cefixime according to EUCAST criteria. The percentage of resistance to ceftriaxone was similar to the present study (4% vs 2.9%), although the percentage of resistance to cefixime was higher than in our study (11% vs. 3.7%). However, if CLSI criteria were applied, the percentages of resistance to ceftriaxone and cefixime were low and similar in both studies.

Regarding to the susceptibility to antimicrobials, there are some differences in the breakpoint criteria between CLSI and EUCAST. According to CLSI, susceptibility to ceftriaxone and cefixime is defined when MIC is ≤ 0.25 mg/L, whereas strains with MIC > 0.25 mg/L are defined as nonsusceptible. According to EUCAST, however, susceptibility to these antibiotics is defined when MIC is ≤ 0.125 mg/L, and those with MIC > 0.125 mg/L are classified as resistant. On the other hand, only EUCAST

has defined resistance to azithromycin (MIC > 0.5 mg/L). Due to these differences, a standardization of the breakpoints for the study of susceptibility to these antimicrobials according to clinical and microbiological criteria is necessary.

Regarding the empirical treatment applied to our patients with resistance to ceftriaxone and/or cefixime, all patients were correctly treated with ceftriaxone (250 mg, intramuscularly) and azithromycin 1 g orally. Test of cure was only performed in one of these patients, but no failure in the treatment was clinically demonstrated. Although it is not universally recommended to perform test of cure, some guidelines recommend it in order to ensure eradication of infection and identify emerging resistance [24]. Overall, test of cure was carried out in 48 (35.8%) patients in this study, so efforts should be taken to warn the physicians about the importance of the follow-up to the patients.

Seventy-two different types were isolated in this study, although both ST 1407 and ST 14958 were the most frequent. While considerable diversity was observed through the medical literature, ST 1407 was the most common type encountered in the recent report from Barcelona [23] as well as in some European countries [25, 26], being the wide dissemination of this type in Europe a recent phenomenon [27]. Due to some types have been associated with specific resistance phenotypes, it is highly recommended to perform molecular typing of the isolates in order to detect this association. Thus, ST 1407 is the clone that has been associated with resistance to ceftriaxone and cefixime [28, 29] and with treatment failures with extended spectrum cephalosporins [28]. Our data also support the evidence that all ST 1407 isolates in Europe are ciprofloxacin resistant, strongly associated with decreased susceptibility to cefixime [30]. All our STs 1407 strains were resistant to ciprofloxacin, as well as all isolates published recently by Chisholm et al coming from different countries of the European Union [30]. This fact enhances the usefulness of molecular typing techniques as a method to predict antimicrobial resistance. Moreover, the results of the present study demonstrate that other types such as ST 9807, ST 7072, ST 225, ST 12800 and ST 2992 showed decreased susceptibility to ceftriaxone and cefixime. To our knowledge, these strains that showed decreased susceptibility to cephalosporins have not been frequently reported and, until now, they have not been linked with resistance to cephalosporins.

The Centers for Disease Control and Prevention [31] recommends nucleic acid amplification tests to detect gonorrhoea in all laboratories, but it does recommend culture as well to monitor the antimicrobial susceptibility. Moreover, the introduction of correct empirical treatment based on antimicrobial susceptibility data at regional level as well as the adequate follow-up of patients in order to ensure the definitive eradication of the infection, are the other cornerstones of infection control.

Due to the increasing number of NG multiresistant strains, the introduction of antimicrobial surveillance programs should be mandatory, not only at national or international

level but also at regional level. The presence of quality regional surveillance data about antimicrobial resistance to *N. gonorrhoeae* is a good approach to estimate the global burden of resistance to this microorganism. The subsequent communication of data to physicians working in this health area is crucial to improve the control of gonococcal infections and to know the appropriate empirical treatment at each moment.

Finally, main limitation of this study was the absence of data about extragenital samples (rectal or pharyngeal samples), as well as data about sexual orientation, sexual practice and HIV status of the patients included here. An additional limitation was the absence of data about previous treatment and co-infections of the patients with resistance to azithromycin.

The results of this study show that ST 1407, ST 14958, ST 7192, and ST 13251 are the main types found in our health area. Some NG types such as ST 1407, ST 225, ST 9807, ST 7072, ST 2992 and ST 12800 were associated with decreased susceptibility to third generation cephalosporins. Due to some types are associated to decreased susceptibility to these antibiotics, molecular typing may be a useful tool to predict antimicrobial resistance in absence of antimicrobial testing. Resistance to penicillin, tetracycline, and ciprofloxacin were high in this study, but resistance to azithromycin was lower than in other studies. Due to the low rates of resistance to cefixime and ceftriaxone in this population, these antimicrobials may be still used as empirical treatment of gonorrhoea in our patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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