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Pathogens associated with sexually transmitted infections: Distribution patterns of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma hominis* and *Ureaplasma urealyticum*, antimicrobial resistance and molecular characterization of *Neisseria gonorrhoeae* isolates

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ABSTRACT

Aims: The purpose of this study was to determine pathogens associated with sexually transmitted infections and the antimicrobial resistance among *Neisseria gonorrhoeae* isolates.
Methodology and results: Urethral, endocervical and anal-rectal swabs were tested for *Chlamydia trachomatis*, *N. gonorrhoeae*, *Mycoplasma hominis* and *Ureaplasma urealyticum*. The molecular characterization of some *N. gonorrhoeae* isolates was made using NG-MAST. The total positive infection rate for *C. trachomatis*, *N. gonorrhoeae* and *Mycoplasmas* on the screened samples was 13% (541/4140), 5.5% (506/9232) and 5.5% (504/9232), respectively. One of the *N. gonorrhoeae* isolates (ST 3158) was cefixime-resistant (MIC ≥ 0.25 µ/mL). Men are at more risk of *U. urealyticum* infection ($p< 0.05$) while women are at more risk of *M. hominis* infection ($p< 0.05$).
Conclusion, significance and impact of study: We identified the first *N. gonorrhoeae* isolate with reduced susceptibility to ceftriaxone in the area served by our center. Further studies are needed in order to elucidate the relation between sex and ureaplasmal and mycoplasmal infection. Our findings are useful to draw local and national strategies to control the emergence of new STs related with treatment failure.

Keywords: *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Mycoplasmas,* antimicrobial resistance, molecular characterization.

INTRODUCTION

Sexually transmitted infections (STIs) are a major global cause of acute illness, infertility, long-term disability and death in millions of men, women and infants. In 2008, the World Health Organization estimated 498 million new cases of STIs among adults that occur globally every year: *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections represent 105 and 106 million of cases, respectively (Word Health Organization, 2008; Brunner *et al.*, 2014). In Spain, chlamydial and gonorrhoeal infection cases must be notified to the surveillance systems Diseases of Compulsory Notification (DCN) and Microbiological Information System (Microbiological Information System *et al*., 2014). In 2012, *C. trachomatis* infections were notified in 1033 cases and *N. gonorrhoeae* infections were notified in 792 cases in Spain (Microbiological Information System *et al.*, 2014). *Chlamydia trachomatis* is an obligate intracellular bacterium, parasitizing eukaryotic cells (Cevenini *et al.*, 2002). *Chlamydia trachomatis* has been recognized as a pathogen of nongonococcal urethritis, salpingitis, endocervicitis, inclusion conjunctivitis of neonates, folicular conjunctivitis of adults, infantile pneumonia and associated diseases (Numazaki *et al.*, 2004). The complications of urogenital infection caused by *C. trachomatis* include tubal factor infertility, ectopic pregnancy, and chronic pelvic pain (Manavi, 2006). In 2009, the DCN reported 1954 cases of gonorrhoeae, which represents a rate of infection of 4.33 per 100.000 population (Microbiological Information System *et al.*, 2014). *Neisseria gonorrhoeae* is an intracellular Gram negative bacteria that is shed in exudates and secretions transmitted through intimate contact, such as sexual contact or vaginal delivery (Comkornruecha, 2013). Infection of the genital tract causes acute urethritis, cervicitis, proctitis or pharyngitis. Gonococcal infections in males are more likely to be symptomatic than those in females leading to serious reproductive complications in women, such as pelvic inflammatory disease, infertility and ectopic pregnancy (Brunner *et al.*, 2014; Comkornruecha, 2013). *Neisseria gonorrhoeae* has been remarkably adept at acquiring and maintaining resistance to antimicrobial drugs used for treatment (Chesson *et al*., 2014). Non-susceptible gonococci with elevated resistance to third generation extended spectrum

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cephalosporins are now being identified at increasing prevalence in many other countries and regions of the world (Lewis, 2014). Therefore, it's necessary to monitorize the antimicrobial resistance patterns in order to avoid the spread of drug resistance and treatment failure. *Mycoplasma* and *Ureaplasma* species are commonly found in the female genital tract (Aguilera-Arreola *et al.*, 2014). *Mycoplasma hominis* and *U. urealyticum* are etiologic agents of urinary calculus formation, pylonephritis, bacterial vaginosis, pelvic inflammatory disease, infertility, chorioamnionitis, spontaneous abortion, prematurity, intrauterine growth retardation, postpartum fever, and extragenital disease (Aguilera-Arreola *et al.*, 2014; Redelinghuys *et al.*, 2014).

The aims of this study were therefore to investigate *C. trachomatis*, *N. gonorrhoeae* and *Mycoplasmas* distribution patterns and to determine antimicrobial resistance of the isolates obtained from patients who visited gynaecological and sexual transmitted diseases consultations during January 2000 – March 2014.

MATERIALS AND METHODS

Clinical isolates

A total of 4140 clinical isolates of *C. trachomatis* and 9232 clinical isolates of *N. gonorrhoeae* and *Mycoplasmas* were isolated from patients in Madrid attending gynaecological and sexual transmitted diseases consultations of Jimenez Diaz Foundation hospital during January 2000 to March 2014.

C. trachomatis PCR

Chlamydia trachomatis detection was made using polymerase chain reaction (PCR) according to the manufacturer´s instructions (HAIN, Lifescience).

N. gonorrhoeae culture and identification

For the identification of *N. gonorrhoeae*, urethral, endocervical and anal-rectal swabs were taken and cultured on VCA3, PVX and TSS agar (BioMérieux).Plates were incubated at 35 °C with 5% carbon dioxide (Thermo Scientific Steri-Cyle Incubator) for up to 48 h. These isolates were confirmed as *N. gonorrhoeae* using MALDI-TOF-MS (Vitek @MS Prep Station V2.3.2; BioMérieux).

N. gonorrhoeae isolates susceptibility testing

The minimum inhibitory concentrations (MICs) of penicillin G, ciprofloxacin and ceftriaxone were determined on PVX chocolate agar (BioMérieux) using E-test strips (BioMérieux); while tetracycline MIC was determined by an agar dilution method (BioMérieux). The susceptibility was interpreted according the guidelines: CLSI 2013 (Cockerill *et al.*, 2013) and EUCAST 2014 (The European Committee on Antimicrobial Susceptibility Testing, 2014).

Molecular genotyping of *N. gonorrhoeae* isolates

Nineteen gonococal isolates obtained from heterosexual Spanish/Latin American men presenting "symptoms" of urethritis (18/19) and disseminated infection (1/19) were sent to the National Center of Microbiology (CNM) for its characterization using *Neisseria* gonorrhoeae multi-antigen sequence typing (NG- MAST). Sequence-based typing, directed at the porin PorB (*porB*) and subunit B of the transferrin binding protein (*tbpB*) genes, and compared with international isolates using the NG-MAST database on the internet.

Mycoplasmas culture and identification

Samples were placed into arginine broth, incubated and on A7 solid medium (BioMérieux), incubated at 35 °C with 5% carbon dioxide for 72 h (Thermo Scientific Steri-Cycle Incubator). The A7 plates were examined microscopically under magnification of 10× for characteristic colonial morphology. *M. hominis* produced translucent colonies which looked like fried eggs, whereas *U. urealyticum* produces smaller, darker and denser colonies.

Statistical analysis

Statistical methods like Chi-square test and Fisher´s exact test were performed to determine the significant association (p<0.05) between variables of patients using the R version 3.0.1 (R-GNU Project, Free Software Foundation, USA).

Table 1: Association between *C. trachomatis* isolates and patient variables.

Variables	(Number of isolates/ Total isolates)	(%)
Age in years		
< 25	97/541	17.9
25 – 45	418/541	77.3
> 45	26/541	4.8
Sex		
Men	426/541	78.7
Women	115/541	21.3
Region/ Country		
Spain/ Latin America	462/541	85.4
North Africa	11/541	2
Sub-Saharan	2/541	0.4
Asia	12/541	2.2
Brazil	1/541	0.2
Others	50/541	9.2

Others: Turkey, Rumania, Bulgaria, Netherlands, France, Republic of Macedonia and Russia.

RESULTS

Out of the 4140 samples screened during January 2009 to March 2014, a total of 541 of the cases were related to *C. trachomatis* infection. LGV and gonorrhea concurrent infection were found in 2.8% and 6.7% of screened

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samples, respectively. Table 1 describes the association between *C. trachomatis* isolates and patient variables.

Screening of *N. gonorrhoeae*

Neisseria gonorrhoeae culture was positive in 5.5% (506/9232). Table 2 shows the association between *N. gonorrhoeae* isolates and patient variables.

Antimicrobial susceptibility of *N. gonorrhoeae* isolates

Susceptibilities of 99.4% (503/506) and 54.7% (277/506) were found in the *N. gonorrhoeae* isolates for ceftriaxone and ciprofloxacin respectively. Sixty nine percent (349/506) of *N. gonorrhoeae* isolates were resistant to penicillin, whereas resistance to tetracycline was detected in 51.8% (262/ 506) of cases. The antimicrobial susceptibility of all isolates are summarised in Table 3.

Table 2: Association between *N. gonorrhoeae* isolates and patient variables.

Variables	(Number of isolates/ Total isolates)	(%)
Age in years		
< 25	58/506	11.5
25 – 45	395/506	78
> 45	53/506	10.5
Sex		
Men	499/506	98.6
Women	7/506	1.4
Region/ Country		
Spain/ Latin America	423/506	83.6
North Africa	24/506	4.7
Sub-Saharan	9/506	1.8
Asia	9/506	1.8
Brazil	11/506	2.2
Others	30/506	5.9

Others: Turkey, Rumania, Bulgaria, Netherlands, France, Republic of Macedonia and Russia.

Table 3: Susceptibility of *N. gonorrhoeae* isolates to penicillin, tetracycline and ciprofloxacin, according to sex and age.

	Susceptibility to penicillin				
	S	R	OR	CI 95%	p value
Sex					
Men	120 (29.7)	284 (70.3)			
Women	1 (14.3)	6 (85.7)	2.54	0.30-21.3	0.68
Age in years					
<25	11(22.9)	37 (77.1)			
25-45	90 (28.6)	225 (71.4)	0.74	0.36-1.52	0.42
>45	20 (41.7)	28 (58.3)	0.42	0.17-1.01	0.05
	Susceptibiliy to tetracycline				
	S	R	OR	CI 95%	p value
Sex					
Men	202 (48.9)	211 (51.1)			
Women	2 (28.6)	5 (71.4)	2.49	0.46-12.48	0.45
Age in years					
<25	23 (47.9)	25 (52.1)			
25-45	154 (47.4)	171 (52.6)	1,02	0.56-1.87	0.95
>45	27 (57.4)	20 (42.6)	0.68	0.30-1.53	0.35
	Susceptibility to ciprofloxacin				
	S	R	OR	CI 95%	p value
Sex					
Men	226 (55.0)	185 (45.0)			
Women	4 (57.1)	3 (42.9)	0.92	0.20-4.15	1.00
Age in years					
<25	23 (46.9)	26 (53.1)			
25-45	180 (55.9)	142 (44.1)	0.70	0.38-1.28	0.24
>45	27 (57.4)	20 (42.6)	0.66	0.29-1.47	0.30

S, susceptible; R, resistant; OR, Odds ratio; CI, confidence interval. Number of isolates (Percent).

Molecular genotyping of *N. gonorrhoeae* isolates

Of the 19 isolates sequence typed, 16 different STs were identified, representing 13 *porB* alleles and 10 *tbpB* alleles. Ten (5.9%) of these STs have not been previously described. The most frequently observed types (represented by a ≥ 2 isolates) were ST21 (n=3) and ST4995 (n=2). Eighteen isolates (94.7%) were susceptible

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to ceftriaxone, but one isolate was cefixime-resistant, defined as an cefixime MIC ≥0.25 µg/mL. The cefixime-resistant isolate was in NG-MAST type 3158 which shared the same *porB* allele (1914) with ST9808. None of the *N. gonorrhoeae* isolates showed decreased susceptibility to gentamycin and spectinomicin. Results are shown in the Table 4.

Table 4: *N. gonorrhoeae* multi-antigen sequence type and antimicrobial susceptibility of nineteen isolates. Madrid, 2013-2014.

ST (n)	Antimicrobial susceptibility[(a)]						allele	
	CRO	CFX	PNG	TET	CIP	AZT	*porB*	*tbpB*
1780 (1)	S	S	I	R	S	I	1143	39
2992 (1)	S	S	I	R	S	I	1808	29
8433 (1)	S	S	I	R	S	S	1489	263
4995 (1)	S	S	R	S	S	S	3031	33
2 (1)	S	S	I	S	R	S	2	16
3158 (1)	S	R	R	S	R	I	1914	110
9973 (1)	S	S	I	R	S	I	28	138
21 (1)	S	S	S	S	S	S	14	33
21 (1)	S	S	I	R	R	S	14	33
21 (1)	S	S	R	S	S	S	14	33
9974 (1)*	-	-	-	-	-	-	3957	1388
9808 (1)	S	S	I	S	R	S	1914	33
10026 (1)	S	S	R	S	R	S	5877	110
292 (1)	S	S	I	R	I	S	28	4
9971 (1)	S	S	R	S	R	S	5878	137
3378 (1)	S	S	R	S	R	I	2043	110
7232 (1)	S	S	I	S	R	S	1489	1388
2018 (1)	S	S	R	S	R	I	182	29
4995 (1)	S	S	R	S	R	S	3031	33

R, resistant; S, susceptible; I, intermediate.
CRO, ceftriaxone; CFX, cefixime; PNG, penicillin G; TET, tetracycline; CIP, ciprofloxacin; AZT, azithromycin.
[(a)] Breakpoints according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org) and the Clinical and Laboratory Standards Institute (CLSI; http://www.clsi.org) were used, when available.
*Antimicrobial susceptibility not tested.

Screening of *Ureaplasma urealyticum* and *Mycoplasma hominis*

The total positive rate of infection was 5.5% (504/9232) for the screened samples. The results of *U. urealyticum*, *M. hominis* and coinfection with both pathogens, according to sex and region/ country origin, are presented in Table 5.

DISCUSSION

Chlamydia trachomatis infection is a public health problem due to its impact on untreated diseases of reproductive system, transmission of other sexually acquired infections and costs to health systems (Shaw *et al.*, 2011). Across Europe, the incidence of *C. trachomatis* infection has generally increased over the past years, with rates of 100 cases per 100,000 (Piñeiro *et al.*, 2009). We found that the percentage of men infected with *C. trachomatis* was higher compared to women, which can be due to the fact of chlamydiasis is often asymptomatic in women. This finding is not consistent with those of other studies conducted in Asturias (Fernández-Benítez *et al.*, 2013). However, a plausible explanation to this may be the difference in methodology and profile of patients studied in our study and that of Fernández-Benítez *et al.* (2013). We also reported an increase in the number of cases of chlamydiasis diagnosed. However, those results do not imply an increase in real number of cases because PCR technique was slowly implemented in this center, reducing the number of non-diagnosed cases compared with the previous years. Higher percentage of cases (77.3%) was found among individuals between 25 and 45 years of age

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compared to other age groups. Though a high percentage of cases have been reported in Catalonia among individuals ≤25 years of age (Corbeto *et al.*, 2011), no significant differences were found in both studies among individuals between 25 and 45 years of age. Our study supports other conclusions (Orellana *et al.*, 2009) that concurrent infection with both gonorrhea and lymphogranuloma venereum is common in some areas of Community of Madrid. In the current study, Spanish/Latin American and individuals from other nationalities (Turkey, Rumania, Bulgaria, Netherlands, France, Republic of Macedonia and Russia) presented the highest percentage of cases of chlamydiasis compared to individuals of other region/country groups (North Africa, Sub-Saharan Africa, Asia and Brazil). To our knowledge, this study is the first one in Spain that has explored the percentage of cases of chlamydiasis and region/country, sex and age profiles of individuals infected with *C. trachomatis*.

Table 5: Association between *U. urealyticum* and *M. hominis* isolates and patient variables.

	Number of isolates/Total of isolates	OR	CI 95%	p value
U. urealyticum				
Sex				
Men	167/192			
Women	202/312	0.28	0.17-0.44	<0.05*
Region/ Country				
Spain/ Latin America	328/450			
Asia	3/5	0.56	0.10-3.38	0.62
North Africa/ Magreb	13/15	2.42	0.54-10.87	0.37
Others	22/29	1.17	0.49-2.81	0.63
Subsaharan	2/4	0.37	0.04-2.97	0.30
Brazil	1/1			
M. hominis				
Sex				
Men	11/192			
Women	63/312	4.16	2.13-8.12	<0.05*
Region/ Country				
Spain/ Latin America	68/450			
Asia	2/5	3.75	0.61-22.83	0.17
North Africa/ Magreb	1/15	0.40	0.05-3.10	0.71
Others	3/29	0.65	0.19-2.20	0.60
Subsaharan	0/4			
Brazil	0/1			
U.urealyticum concurrent wit*h M. hominis*				
Sex				
Men	14/192			
Women	47/312	2.26	1.21-4.22	<0.05*
Region or country origin				
Spain/ Latin America	54/450			
Asia	0/5			
North Africa/ Magreb	1/15	0.52	0.07-4.06	1.00
Others	4/29	1.17	0.40-3.50	0.77
Subsaharan	2/4	7.33	1.01-53.14	0.08
Brazil	0/1			

OR, Odds ratio; CI, confidence interval.
Others: Turkey, Rumania, Bulgaria, Netherlands, France, Republic of Macedonia and Russia.
(*) Statistically significant.

As compared to other studies in Brazil by Costa *et al*. (2013), the percentage of cases of gonorrhea in Madrid that we estimated was unique (5.5%). Our findings are supported by those of other studies conducted by Orellana *et al.* (2009) showing about 7.4% of cases of gonorrhea in Pontones, Madrid. We found that the percentage of men infected with *N. gonorrhoeae* was higher compared to women, because men are more likely to have symptoms compared to women. This finding is consistent with other studies conducted in Zimbabwe, Peru, China and Russia (Detels *et al.*, 2011). We observed a high percentage of cases of gonorrhea in

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Spanish/Latin American individuals (83.6%) compared to other region/country groups. Those results might be related to the fact that we have a relatively large number of Latin American individuals which are ascribed to our center. This was the first study in Madrid, which described the percent of cases of gonorrhea and the epidemiological profile of patients infected with *N. gonorrhoea*e. We also identified the first *N. gonorrhoeae* isolate (0.6%) with reduced susceptibility to ceftriaxone (MIC≥0.25 mg/L) in the area served by our center in Madrid. A similar rate of resistance to ceftriaxone has been observed in clinical isolates of *N. gonorrhoeae* in Barcelona (Serra-Pladevall *et al.*, 2013). However, a high rate of resistance to ceftriaxone was observed for *N. gonorrhoeae* isolates in Vietnam (Olsen *et al.*, 2013), China (Zhang *et al.*, 2013) and Ethiopia (Tadesse *et al.*, 2001). Through ceftriaxone resistance has been reported worldwide (Casco *et al.*, 2011), ceftriaxone is still the last remaining option for empirical first-line treatment of gonorrhea (Ohnishi *et al.*, 2011). High-level resistance to penicillin has been observed in sixty-nine percent of *N. gonorrhoeae* isolates. Similar to our findings, high-level penicillin resistant isolates have been reported in Vietnam (Olsen *et al.*, 2013), Mozambique (Apalata *et al*., 2009) and India, Pakistan and Bhutan (Sethi *et al.*, 2013). We found that the risk of penicillin-resistant isolates in Spanish/Latin American is forty percent lower in individuals older than 45 years of age compared to individuals <25 years of age (OR=0.42;CI 95%, 0.17-1.0, p=0.05). A high rate of resistance to ciprofloxacin (44.3%) was also observed for *N. gonorrhoeae* isolates. Our results are in agreement with previous data obtained in an earlier study made in Barcelona (Serra-Pladevall *et al.*, 2013). On other hand, lower resistance rates have been observed in studies conducted in Pontones-Madrid (Orellana *et al*., 2009), Kenia (Lewis *et al*., 2011) and Brazil (Costa *et al*., 2013). In addition, we also found high rate of resistance to tetracycline (51.8%). A similar rate of resistance to tetracycline has been observed in clinical isolates of *N. gonorrhoeae* in studies made in Brazil (Costa *et al*., 2013), Mozambique (Apalata *et al*., 2009), India, Pakistan and Bhutan (Sethi *et al*., 2013). No statistically significant associations were finally observed between the resistance of *N. gonorrhoeae* isolates and sex, age and region/country (p>0.05).

All isolates of *N. gonorrhoeae* tested at the National Center of Microbiology (CNM) were susceptible to gentamicin and spectinomycin. Those findings are in agreement with results reported in Barcelona (Serra-Pladevall *et al*., 2013), Vietnam (Olsen *et al.*, 2013), India, Pakistan and Bhutan (Sethi *et al*., 2013). Although aminoglycosides are still a good option of treatment in those countries, it has reported gentamicin-resistant isolates in Mozambique and Ethiopia, which may be due to its continued empiric use in these countries. Interestingly, 63.2% of the isolates tested at the CNM were susceptible to azithromycin. Despite azithromycin not being recommended as monotherapy for gonorrhoea, it is recommended as co-treatment in combination with ceftriaxone at a dose of either 1 g or 2 g (Berçot *et al*., 2014). Our findings are in agreement with previously published studies (Serra-Pladevall *et al*., 2013) which suggest that azithromycin could be considered an alternative in the treatment of uncomplicated urogenital gonorrhea in those persons with a history of penicillin or cephalosporin allergy. High-levels of resistance to penicillin (47.4%) and ciprofloxacin (57.9%) were observed in isolates of *N. gonorrhoeae* which were tested at the CNM. These results are consistent with the rates of resistance obtained at our center.The molecular characterization of 19 *N. gonorrhoeae* isolates showed a total of 16 different NG-MAST STs. The ST21 (n=3) and ST4995 (n=2) were the most prevalent STs, while the remaining 14 isolates were represented by single isolates. The high number of unique STs may be a consequence of random sampling, suboptimal diagnostic, non-effective contact tracing, local emergence of new STs and import of strains. All isolates of ST21 in the current study were sensitive to ceftriaxone, cefixime and azithromycin. Similar results were found by Serra-Pladevall *et al*. (2013). Decreased cefixime susceptibility was observed in one isolate from ST3158, which shares the *tbpB* allele (allele 110) con 12 isolates from ST1407 identified by Serra-Pladevall *et al*. (2013). ST1407 has been previously reported in a high proportion of men who have sex with men (Ison *et al*., 2013). Sixty eight percent of the isolates were susceptible to azithromycin. This finding supports the use of azithromycin as a second-line option in the area served by our center. The molecular characterization of *N. gonorrhoeae* isolates may be useful to draw local and national strategies to control the emergence of new STs which may be associated with the increase of antimicrobial resistance and treatment failure in this region.

The percentage of cases of *U. urealyticum* and *M. hominis* infection (73.2% and 14.7%, respectively) reported by this study is similar to others conducted in Netherlands (Bayraktar *et al*., 2010) and lower when compared to others conducted in Sudan (Abdelaziz *et al*., 2014). Our findings reveal that the risk of *U. urealyticum* infection is 20% lower in women compared to men (OR=0.28;CI 95%, 0.17-0.44, p<0.05). However, women are at more risk of *M. hominis* infection compared to men (OR=4.2;CI 95%, 2.13-8.12, p<0.05). Also, women were at more risk of mixed infection by both pathogens compared to men (OR=2.26; CI 95%, 1.21-4.22, p<0.05). This study suggest that there is an association between sex and the risk of mycoplasmal and ureaplasmal infection. However, further studies are needed to clarify this issue.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest relevant to this paper.

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gonorrhoeae isolates, using NG- MAST technique. The CNM was not involved in the development of the study design, the collection, analysis and interpretation of the data, in the writing or decision to submit the paper for publication.

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