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Molecular characterization of *Neisseria gonorrhoeae* on non-cultured specimens from multiple anatomic sites

Anna Carannante[1], Valeria Ghisetti[2], Ivano Dal Conte[3], Gabriella Gregori[2], Maria Laura Stella[4], Paola Vacca[1], Simonetta Del Re[2] and Paola Stefanelli[1]

[1]*Dipartimento Malattie Infettive, Istituto Superiore di Sanità, Rome, Italy*
[2]*Dipartimento di Malattie Infettive, Laboratorio di Microbiologia e Virologia, Ospedale Amedeo di Savoia, Turin, Italy*
[3]*Dipartimento di Malattie Infettive, Ambulatorio IST, Ospedale Amedeo di Savoia, Turin, Italy*
[4]*Dipartimento di Malattie Infettive, Università di Torino, Turin, Italy*

Abstract

Introduction. The aim of this study was to molecularly characterize *Neisseria gonorrhoeae* on non-cultured specimens collected from multiple anatomic sites. *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) together with the gene sequence analysis of antimicrobial resistance (AMR) target genes were used.

Materials and methods. Seventeen genital and extra-genital samples from eight patients (7 were men who have sex with men, MSM, and 1 women who have sex with men, WSM) with gonorrhoea symptoms were analyzed. For 7, of the 8 patients, conventional culture method has been used to identify gonorrhoea. All the samples were tested with the rapid molecular method CEPHEID. Amplification and sequencing of *porB* and *tbpB,* to identify the Sequence Type (ST) by NG-MAST, and *penA, mtrR, porB1b, ponA* genes were also performed. Antimicrobial susceptibility by Etest, for the available culture positive samples, was carried out.

Results. For 7 patients the ST was obtained and for 6 the complete sequence analysis of the AMR target genes was also defined. For the majority of them, samples collected from multiple sites (oropharynx, rectum, vaginal and urethra) confirm the presence of the same gonorrhoea strain. In particular, for 5 patients the same STs and changes in the AMR target genes were identified.

Conclusion. Molecular characterization on non-cultured or culture negative specimens for gonorrhoea can successfully be applied directly to genital and extra-genital samples. Thus permit to identify the presence of the same strain in patients with gonorrhoea infection in multiple anatomic sites and to predict the antimicrobial susceptibility pattern.

Key words

- *Neisseria gonorrhoeae*
- NG-MAST
- typing
- non-cultured specimens

INTRODUCTION

Neisseria gonorrhoeae (NG) infection is nowadays considered a public health issue at international level [1].

The diagnosis of gonorrhoea consists in the cultivation of the pathogen, as a gold standard, and/or the identification of DNA by nucleic acid amplification tests (NAATs) [2]. NAATs are approximately twice as sensitive as culture on oropharyngeal specimens [3, 4], however, it could generate false-positive results due to the presence of other *Neisseria* species in the oropharynx [5].

N. gonorrhoeae multiantigen sequence typing (NG-MAST) is routinely used in gonorrhoea typing system for its discriminatory power and international comparison [6]. In addition, molecular typing permits to monitor the circulation of specific NG clones, as *e.g.* the genogroup (G) 1407, an international multi-drug resistant gonococcal strain with decreased susceptibility or resistance to cefixime, ceftriaxone and azithromycin, and resistance to ciprofloxacin [7, 8].

Moreover, the analysis of antimicrobial resistance (AMR) target genes is crucial for enhancing gonococcal AMR (GONO-AMR) surveillance. Several mechanisms have been extensively described to be associated with resistance in gonorrhoea. From them, changes of *penA* gene (encoding the penicillin-binding protein 2-PBP2) were responsible for decreased susceptibility or resistance to extended-spectrum cephalosporin (ESCs) [9].

Address for correspondence: Paola Stefanelli, Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: paola.stefanelli@iss.it.

Additionally, overexpression of the MtrCDE efflux pump, due to mutations in the promoter and coding region of the *mtrR* gene, together with the decreased membrane permeability caused by *porB1b* gene mutations, contribute to the decreased susceptibility and resistance to ESCs [9]. The role of other genes associated with penicillin resistance, as *e.g.*, *ponA* gene (encoding penicillin-binding protein 1-PBP1), remains unclear.

The object of this study was to identify the same gonococcal strain in patients, mainly men who have sex with men (MSM), in multiple anatomic sites and to type them by NG-MAST and by gene sequencing of *penA, mtrR*, *porB1b* and *ponA* genes known to be involved in the AMR in gonorrhoea, even with the lack of cultivated strain.

MATERIALS AND METHODS

Samples

Seventeen clinical samples (genital and extra-genital), collected, in 2014, from eight patients with symptoms of gonorrhoea (urethritis and/or proctitis or cervicitis; no one had overtly symptomatic pharyngitis) at the STI Clinic, Amedeo di Savoia Hospital, Turin, Italy, resulting negative or positive by culture and samples not cultured at all, were analyzed. For six patients, pharyngeal and rectal paired samples were collected; in one, an urethral sample was also gathered. In two patients pharyngeal swabs were paired with urethral and vaginal samples.

For three samples (gathered at pharyngeal, rectal and urethral sites), collected from the same patient, the culture was not performed at all. All the remaining pharyngeal samples were culture-negative. Among the residual 5 rectal samples, only two were culture-positive and the vaginal together with the remaining urethral samples were culture-negative and culture-positive, respectively.

Patient clinical data were anonymously collected for surveillance purposes, thus no ethical approval was required.

Nucleic acid amplification test (NAAT) for CT/NG

Swabs were collected in Xpert CT/NG (*Chlamydia trachomatis/Neisseria gonorrhoeae*) Swab Transport Reagent tubes and processed with the Xpert CT/NG Assay (Cepheid, SystemsCepheid Europe SAS, Maurens-Scopont France), that is an automated *in vitro* diagnostic test for the qualitative detection and differentiation of DNA from CT and NG. The assay was performed on the Cepheid GeneXpert Instrument that integrates sample purification, nucleic acid extraction, amplification and detection of the target sequences using real-time PCR platform [10].

NG-MAST, penA, mtrR, porB1b, ponA analyses

A total of 17 samples resulting positive by CEPHEID, were then sent to Istituto Superiore di Sanità (ISS) for molecular characterization.

DNA was purified using QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Genes for *penA, mtrR, porB1b, ponA* genes and *porB* and *tbpB* for the NG-MAST analysis, were amplified using primers and amplification parameters, as already described [6, 9, 11].

Thermocycling for PCR was performed using Veriti 96 well instrument, (Applied Biosystems). Samples not successfully amplified in the initial reaction, were subsequently re-tested in a temperature gradient amplification program with the following cycling condition: 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min with decreasing 0.5 °C per cycle, followed by 10 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min; and a final extension step of 72 °C for 2 min.

The *porB* and *tbpB* alleles were analyzed at the NG-MAST website (www.ng-mast.net). Closely related STs were clustered using published definitions as well as those belonging to the same genogroup [8].

Multiple sequence and amino acid alignments were performed using CromasPro version 1.15 and Clustal Omega web site (www.ebi.ac.uk/Tools/msa/clustalo/).

Antimicrobial susceptibility

A total of three strains, isolated from three patients, were collected from rectal (n = 2) and urethral (n = 1) sites. After growth on Thayer-Martin medium (Oxoid Ltd, Italy) with 1% IsoVitalex (Oxoid, Ltd) at 37 °C in a 5% CO_2 atmosphere, antimicrobial susceptibility tests have been performed following the European Gonococcal Antimicrobial Surveillance Programme (EURO-GASP) [12].

In particular, antimicrobial susceptibility to cefixime, ceftriaxone, ciprofloxacin and azithromycin was assessed by Etest (bioMérieux, Sweden), carried out in agreement with the manufacturer's instructions. World Health Organization (WHO) *N. gonorrhoeae* G, K, M, O, and P control strains were added in the assay [13].

RESULTS

Samples and patients

A total of 17 samples, from eight patients, were lab-confirmed by CEPHEID. All of them were symptomatic with urethritis and/or proctitis or cervicitis but none had overtly symptomatic pharyngitis.

The patient's nationality was as following: six Italians, one Rumanian and one Brazilian and the median age was of 26.6 years (years range 19-42). Seven were MSM and one was women who have sex with men (WSM). All the patients denied sexual contacts outside the city of Turin; three of them had a previous gonococcal infection and none was HIV positive. The WSM patient was co-infected with *Trichomonas vaginalis* (data not shown). The patients were treated with a combined therapy of 0.5 g of intramuscular ceftriaxone plus 1 g of oral azithromycin.

Molecular analyses

For all samples, except for one collected from pharynx, it was possible to assign the *porB* and *tbpB* alleles (*Table 1*). The same Sequence Type (ST) was found in all paired samples except for one couple (ID8a, pharyngeal, and 8b, urethral) showing different *porB* and *tbpB* alleles (*Table 1*). In particular, as showed in *Table 1*, the ST292,

Table 1
Site of sampling, sex, sexual orientation and age of patients and molecular characterization by NG-MAST, *mtrR, penA, ponA and porB1b* gene sequence analyses

ID	Specimen site	Sex	Sexual orientation	Patient age, years	NG-MAST		ST	*penA*		*mtrR*		*ponA*	*porB1b*
					porB	*tbpB*		*penA* pattern	GAC insertion	promoter	aa substitution	aa substitution	aa substitution
1a	Oropharynx	M	MSM	21	28	4	ST292	IV	D345a	wt	T86A; H105Y	wt	A121S
1b	Rectum				28	4	ST292	IV	D345a	wt	T86A; H105Y	wt	A121S
2a	Oropharynx	F	WSM	26	301	4	ST1352	NA	NA	NA	NA	NA	A121S
2b	Vaginal				301	4	ST1352	NA	NA	NA	NA	NA	A121S
3a	Oropharynx	M	MSM	28	90	953	ST5624	IV	D345a	wt	A insertion**	wt	G120D; A121G
3b	Rectum				90	953	ST5624	IV	D345a	wt	A insertion**	wt	G120D; A121G
4a	Oropharynx	M	MSM	21	6816	33	ST11632	IV	D345a	A deletion°	G45D	L421P	A121S
4b	Rectum				6816	33	ST11632	IV	D345a	A deletion°	G45D	L421P	A121S
5a	Oropharynx	M	MSM	31	1489	563	ST2400	IV-A501T/ P551L*	D345a	A deletion°	D79N; T86A; H105Y	L421P	G120K; A121D
5b	Rectum				1489	563	ST2400	IV-A501T/ P551L*	D345a	A deletion°	D79N; T86A; H105Y	L421P	G120K; A121D
6a	Oropharynx	M	MSM	42	1489	4	ST10259	NA	NA	NA	NA	L421P	G120K; A121D
6b	Rectum				1489	4	ST10259	NA	NA	NA	NA	NA	G120K; A121D
7a	Oropharynx	M	MSM	19	5853	241	ST9909	IV	D345a	wt	A39T	wt	wt
7b	Rectum				5853	241	ST9909	IV	D345a	wt	A39T	wt	wt
7c	Urethra				5853	241	ST9909	IV	D345a	wt	A39T	wt	wt
8a	Oropharynx	M	MSM	25	870	1005^	-	NA	-	A deletion°	D79N; T86A	wt	A121D
8b	Urethra				182	4	ST1935	V-A501T	D345a	A deletion°	D79N; T86A	L421P	G120K; A121D

* = new *penA* sequence pattern, accession number KP677512 [15]
** = adenine (A) insertion at nt 17 from ATG of *mtrR* gene
° = adenine (A) deletion in the *mtrR* promoter
^ = 96% of similarity with the *tbpB* allele 1005
Wt = wild-type
MSM = men who have sex with men
WSM = women who have sex with men
NA = not applicable

ST1352, ST5624 (belonging to G5624), ST11632 (belonging to G4995), ST2400 (belonging to G2400), ST10259, ST9909 and ST1935 have been assigned.

The *penA* sequences were compared with *N. gonorrhoeae* LM306 (Accession number: M32091) and *N. gonorrhoeae* NG-3 (Accession number AB071984).

As shown in *Table 1*, all samples showed *penA* nonmosaic allele containing an aspartic acid insertion at position 345 (D345a). In particular, 3 paired samples, ID1a and 1b, 3a and 3b, 4a and 4b, and samples ID7a, 7b and 7c showed the same nonmosaic type IV allele.

The nonmosaic type IV allele with two additional amino acid substitutions, A501T and P551L (Accession number: KP677512, [14]) was identified in ID5a and 5b paired samples. The sample with ID8b showed *penA* nonmosaic allele V with the additional substitution V501T. *penA* allele was not determined for ID8a (*Table 1*). No amplification of *penA* gene was obtained in two paired samples ID2a and 2b, 6a and 6b, respectively.

mtrR gene harbored the single adenine deletion in the promoter region and the T86A, H105Y, D79N amino acid changes in the MtrR efflux pump in ID4a and 4b, 5a and 5b, 8a and 8b. ID4a and 4b showed the G45D amino acid change in the MtrR DNA-binding motif; ID7a, 7b, 7c showed the A39T amino acid change and ID3a and 3b samples harboured an insertion of adenine at position 17 from the ATG of the gene (*Table 1*). Two paired samples, ID2a and 2b, 6a and 6b, were negative for *mtrR* amplification reaction.

The L421P amino acid substitution in the *ponA* allele was detected in six couple of samples and in the urethra sample ID8b (*Table 1*). Moreover, samples with ID2a and 2b and ID6b were negatives for *ponA* amplification reaction.

The amino acid substitution A121S was found in PorB1b for three paired samples (ID1a and 1b, 2a and 2b, 4a and 4b); G120D and A121G was detected in one couple of samples (ID3a and 3b) and G120K and A121D in two paired samples (ID5a and 5b, 6a and 6b) (*Table 1*). ID8a and 8b showed different amino acid substitutions: A121D and G120K together with A121D, respectively.

Antimicrobial susceptibility assay results on three cultivated strains were in agreement with the mutation identify in the target genes analyzed in samples collected from the same patients. They were strains cultivated from the samples ID4b, 6b and 8b. The results were as follows: susceptible to cefixime (MIC range values 0.016-0.023 mg/L) and ceftriaxone (MIC range values 0.002-0.023 mg/L); resistant to ciprofloxacin (MIC range values 4-12 mg/L). ID4b and 6b showed a decreased susceptibility to azithromycin with MIC = 0.38 mg/L and 0.25 mg/L and respectively. Moreover, ID8b was resistant to azithromycin with MIC = 1.5 mg/L.

DISCUSSION

Gonococcal infection is nowadays a worrisome reality due to the circulation of AMR and of multi-drug resistant (MDR) strains [15]. In this context, it is crucial to define the patterns of antimicrobial susceptibility and to genotype those samples resulting negative by culture or samples not cultured at all [16]. Since the spread in the use of molecular techniques to identify the presence of gonorrhoea without a culture confirmation, it is essential to be able to predict at least the antimicrobial susceptibility. In order to answer to this request we analyzed 17 samples collected from several anatomic sites from 8 patients.

The analysis of *penA* gene showed the presence of nonmosaic type IV allele. It has been already described to be associated with ESC susceptible profile in NG [9, 11] and recovered in Italy in cefixime susceptible NG [14]. Sustaining this point the T86A, H105Y, D79N, A39T in the MtrR efflux pump and the A121S, A121G, A121D, G120D, G120K amino acid changes in PorB1b were found [15, 17].

Moreover, STs belonging to G2400, found in the samples ID5a and 5b, showed *penA* nonmosaic allele IV variant with two additional amino acid substitutions (A501T and P551L), the substitutions D79N/T86A/H105Y in MtrR, the L421P in PBP1 and G120K/A121D in PorB1b, as already described [14]. For a MSM patient, two *N. gonorrhoeae* strains, one in the pharynx and one in the urethra, with different *porB* and *tbpB* alleles and two molecular profiles for antimicrobial resistant determinants were found. From the urethra it was possible to cultivate the strain confirming the resistance to ciprofloxacin and the susceptibility to cefixime as observed by the molecular analysis on sample.

CONCLUSION

In summary, we display the molecular characterization of gonorrhoea non-cultured or culture-negative.

In the panel of samples analyzed it was possible to characterize the presence of DNA associated to the same gonococcal strain and to predict the susceptibility to the antimicrobials in use for gonorrhoea therapy.

Since the large use of molecular methods for laboratory diagnosis of gonorrhoea the possibility to detect antimicrobial resistance genes provide an alternative to culture-based antimicrobial susceptibility testing especially in the era of global spreading of gonococci resistant or multidrug resistant to antimicrobials.

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Authors' contributions

AC, performed NG-MAST and resistance target genes analyses and drafted the manuscript. VG, IDC, GG, MLS and SDR, collected the samples together with the clinical data of patients and performed the NAATs analysis. PV, performed the DNA extraction and sequence analysis. PS, designed the study and drafted the manuscript. All authors approved the final version of the manuscript.

Conflict of interest statement

None to declare.

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