

Mosaic structure of the *penA* gene in the oropharynx of men who have sex with men negative for gonorrhoea

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Abstract

The oropharynx represents a crucial site for the emergence of multi-drug resistance in *Neisseria gonorrhoeae*. The mosaic *penA* alleles, associated with decreased susceptibility to cephalosporins, have emerged by DNA recombination with partial *penA* genes, particularly those from commensal pharyngeal *Neisseria* species. Here, we investigated the prevalence of the mosaic structure of the *penA* gene in the oropharynx of men who have sex with men testing negative for pharyngeal gonorrhoea. From January 2016 to June 2018, 351 gonorrhoea-negative men who have sex with men attending a sexually transmitted infection clinic in Italy were enrolled. Pharyngeal swabs underwent a real-time polymerase chain reaction (PCR) for the detection of the mosaic *penA* gene. In case of positivity, PCR products were sequenced and searched against several sequences of *Neisseria* strains. Overall, 31 patients (8.8%) were found positive for the presence of the mosaic *penA* gene. The positivity was significantly associated with previous cases of pharyngeal gonorrhoea (relative risk [RR]: 3.56, 95% confidence interval 1.44–8.80) and with recent exposure to beta-lactams (RR: 4.29, 95% confidence interval 2.20–8.38). All *penA*-positive samples showed a high relatedness (90–99%) with mosaic-positive *Neisseria* strains. Our data underline that commensal *Neisseria* species of the oropharynx may be a significant reservoir for genetic material conferring antimicrobial resistance in *N. gonorrhoeae*.

Keywords

Gonorrhoea (*Neisseria gonorrhoeae*), antibiotic, epidemiology, homosexual, prevention

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Introduction

Sexually transmitted pharyngeal infections due to *Neisseria gonorrhoeae* are very common among men who have sex with men (MSM).^{1,2}

The oropharynx represents a crucial site for the emergence of multi-drug resistance in *N. gonorrhoeae*.³ Indeed, it has been reported that the mosaic *penA* alleles, associated with decreased susceptibility and resistance to extended-spectrum cephalosporins, have emerged by DNA transformation and recombination with partial *penA* genes, particularly those from *N. meningitidis* or commensal oropharyngeal *Neisseria* species, such as *N. perflava*, *N. cinerea*, *N. flavescens*, *N. polysaccharea*.⁴

Several studies have described the detection of molecular markers associated with cephalosporin

resistance directly from *N. gonorrhoeae* NAAT-positive samples, including pharyngeal specimens.^{5–7} Nevertheless, no exhaustive information is available regarding the presence of mosaic *penA* genes in gonorrhoea-negative pharyngeal samples. Since MSM are at higher risk for pharyngeal gonorrhoea transmission, the presence of the *penA* genes at the pharyngeal

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site in *N. gonorrhoeae*-negative subjects could be considered a useful predictor and a potential precursor for a future acquisition of cephalosporin resistance in case of gonorrhoea infection.

In this context, the aim of this study was to investigate the prevalence of *penA* gene's mosaic structure in the oropharynx of MSM reporting condomless oral intercourse, who were negative for pharyngeal gonorrhoea.

Methods

Study population and sample collection

From January 2016 to June 2018, 351 consecutive MSM attending the sexually transmitted infection (STI) Outpatients Clinic of St Orsola-Malpighi Hospital in Bologna (Italy) and reporting condomless oral intercourse were enrolled for the study. Exclusion criteria were the following: being younger than 18 years and testing positive for pharyngeal *N. gonorrhoeae*.

After a preliminary interview, a clinical visit was carried out for each patient. Information about oropharyngeal symptoms, previous STIs and HIV status were collected, following the regular STI evaluation at the Clinic.⁸

Afterwards, a pharyngeal swab (E-Swab, Copan, Italy) for the molecular detection of *Chlamydia trachomatis* and *N. gonorrhoeae* was collected. To avoid biases related to antimicrobial treatment, only samples collected at the first visit were considered, excluding specimens obtained during the follow-up period.

Written consent was obtained from all the patients and the study protocol was reviewed and approved by the Ethics Committee of St Orsola-Malpighi Hospital in Bologna (Italy) (78/2017/U/Tess).

Diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections and detection of the *penA* gene

Pharyngeal swabs were processed by the Versant CT/GC DNA 1.0 Assay (Siemens Healthcare Diagnostics, Tarrytown, USA), a commercial NAAT simultaneously detecting the presence of *C. trachomatis* and/or *N. gonorrhoeae* DNA.⁹

This molecular assay has proved to be extremely sensitive in the detection of extra-genital *N. gonorrhoeae* infections (limit of detection: 1.0 copies/ml), with an excellent specificity (no false positive results due to the presence of non-gonococcal *Neisseria* species).⁹

Starting from the remaining DNA eluate of the Versant polymerase chain reaction (PCR) plate, gonorrhoea-negative samples were processed for the detection of the mosaic structure of the *penA* gene by

a TaqMan real-time PCR, as previously described in detail by Ochiai et al.¹⁰ Specific amplification of the *penA* gene was performed with the primers NG89-F2 (5'-GTTGGATGCCCGTACTGGG-3') and NG89-R1 (5'-ACCGATTTTGTAAGGCAGGG-3') and with the probe NG89-P1 (5'-56-FAM-CGGCAAAGTGGATGCAACCGA-3IABkFQ-3'). The components of PCR in a final volume of 50 µl included Platinum Quantitative PCR Super Mix-UDG with ROX (Invitrogen), 1 µM of the primers, 250 nM of the probe and 5 mM of MgCl₂. Finally, 5 µl of template was added to each reaction mixture. An ABI PRISM 7300 real-time PCR system (Applied Biosystems, Japan) was used for amplification and detection, using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C.

penA amplicon sequencing

In case of a *penA*-positive sample, the 220 bp fragment PCR products were sequenced using forward and reverse primers. The *penA* amplicons were sequenced by the dye terminator method with an automatic sequencer. First, to confirm the specificity for Neisseriaceae, *penA* amplicon sequences were searched against the entire nucleotide database on the National Center for Biotechnology Information (NCBI) website, using online BLAST analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Second, *penA* sequence data were compared using the genome comparator tool, implemented within the PubMLST.org/neisseria website. As previously described, in this website defined loci are allocated a value free nomenclature using the prefix NEIS followed by four digits.¹¹ Penicillin binding protein 2 was defined as NEIS1753 and was associated with the locus tag NGO1542 (from the reference *N. gonorrhoeae* isolate FA1090) and the gene name, *penA*.¹¹ Through this comparator tool, the allelic variant of *penA* gene (2131 available alleles) is therefore obtained.

Finally, to confirm the presence of *penA* mosaic structure, obtained electropherograms were BLAST searched against several *Neisseria* strains, including sequences from both mosaic-positive (accession numbers: AY294556; AB071984; AB546858) and -negative strains (CP003909; CP001050).

Statistical analysis

All statistical analyses were performed by using GraphPad Prism software (GraphPad Software, San Diego, California, USA, www.graphpad.com). To evaluate statistically significant differences, t-test was used to compare quantitative data, whereas categorical data were analysed through the calculation of

relative risk (RR) and odds ratio (OR), considering a 95% confidence interval (CI). A *P* value <0.05 was considered significant.

Results

Study population

The mean age of the 351 subjects enrolled was 33.7 ± 10.3 years (± standard deviation) (min–max 19–77 years). Out of the 351 patients, 56 (15.9%) complained about various symptoms, including pharyngeal pain, hoarseness and painful cervical adenopathy. Globally in the cohort of subjects enrolled, 71 patients (71/351; 20.2%) were HIV-positive and 20 (20/351; 5.7%) were positive for pharyngeal *C. trachomatis* infection.

Prevalence of *penA* gene at the pharyngeal site

Overall, 31 patients (31/351; 8.8%) were found positive for the presence of the mosaic structure of the *penA* gene. The detection of *penA* gene was not associated with the age of patients (mean age of positive versus negative subjects: 33.6 ± 10.8 versus 33.7 ± 10.2 years; *P* = 0.96) nor with the presence of pharyngeal symptoms (RR: 0.70 with 95% CI of 0.28–1.78; OR: 0.68 with 95% CI of 0.25–1.85), nor with the HIV status (7.0% of HIV-positive subjects versus 11.8% of HIV-negative patients; RR: 1.58 with 95% CI of 0.64–3.88; OR: 1.67 with 95% CI of 0.60–4.65).

Interestingly, analysing clinical data of patients within six months prior to enrolment, we found that the detection of *penA* was significantly associated with previous cases of pharyngeal gonorrhoea (12.8% versus 3%; RR: 3.56 with 95% CI of 1.44–8.80; OR: 4.59 with 95% CI of 1.34–15.63), as well as with a recent exposure to beta-lactam antibiotics (e.g. beta-lactams for genital and/or extra-genital gonorrhoea, active syphilis, pharyngeal infection due to *Streptococcus pyogenes*) (33.3% versus 7.7%; RR: 4.29 with 95% CI of 2.20–8.38; OR: 5.61 with 95% CI of 2.38–13.24).

Sequencing of *penA* amplicons

When searched against the entire nucleotide database on the NCBI website, all *penA* amplicon sequences always align only with *Neisseria* species, confirming the specificity of PCR products. Through the genome comparator tool implemented within the PubMLST.org/*neisseria* website, we found that most of *penA* sequences showed the closest match with *penA* allele 2028 (18/31; 58%), followed by allele 879 (11/31; 35.5%) (Table 1).

All the alignments between *penA* amplicon sequences and the closest *penA* allelic variant are shown in the Supplementary material.

As shown in Table 1, all *penA*-positive samples showed a high relatedness (ranging from 90 to 99%) with several mosaic-positive *Neisseria* strains. At the same time, *penA* sequences showed no match or low relatedness (<81%) with mosaic-negative strains.

Discussion

The possibility of acquiring cephalosporin resistance from pharyngeal commensal *Neisseria* species underlines the crucial role of the pharynx in the creation of 'multi-drug resistant' gonorrhoea.^{12–15} The detection of molecular markers associated with cephalosporin resistance in *N. gonorrhoeae*-negative pharyngeal samples in patients at higher risk for gonorrhoea (e.g. MSM) could open new perspectives for the prevention of antimicrobial resistance.

In this study, we assessed the prevalence of the mosaic structure of the *penA* gene in the oropharynx of 351 MSM reporting condomless oral intercourse but negative for pharyngeal gonorrhoea. For this purpose, we used a previously described real-time PCR employed both on *Neisseria* isolates and directly on *N. gonorrhoeae*-positive clinical samples.^{6,10}

In this context, it should be taken into account that *penA* genes could be potentially fragmented by enzymes present in the pharyngeal environment, thus leading to an underestimation of *penA*-positive samples.

First, we found that more than 8% of the subjects enrolled were positive for the presence of the *penA* gene and we confirmed that *penA* amplicons were highly related with *Neisseria* strains harbouring the mosaic structure of the *penA* gene.

It has been shown that commensal *Neisseria* spp. are a source of altered *penA* alleles and that the horizontal gene transfer is a common mechanism for beta-lactam resistance in *Neisseria* species.¹⁶ As example, *N. subflava* and *N. cinerea* may be involved in the emergence of *N. gonorrhoeae* strains with either intermediate or total resistance to penicillin or cephalosporin by the horizontal genetic exchange of the *penA* gene.^{14,17}

Second, we found that *penA* positivity was associated with previous cases of pharyngeal gonorrhoea and with the recent exposure to beta-lactams (e.g. cephalosporins for genital and extra-genital gonorrhoea, penicillin for syphilis).

Recently, Dong et al.,¹⁸ by means of a culture-based approach, showed that MSM harbour in the oropharynx a high rate of commensal *Neisseria* species with reduced susceptibility to cephalosporins. Moreover, they demonstrated that the recent use of antibiotics is strongly associated with the presence of antimicrobial-resistant *Neisseria* strains.¹⁸

These results emphasise how the use of antibiotics to treat common STIs could contribute to the onset of

Table 1. Characteristics of *penA* amplicon sequences. For each sequence, the closest match with *penA* alleles, as well as the relatedness with several mosaic-positive (AY294556, AB071984, AB546858) and -negative *Neisseria* strains (CP003909, CP001050), are reported.

<i>penA</i> amplicon sequence	<i>penA</i> allele ^a (NEIS1753)	Per cent identity after alignment with mosaic-positive <i>Neisseria</i> strains (accession numbers)			Per cent identity after alignment with mosaic-negative <i>Neisseria</i> strains (accession numbers)	
		AY294556	AB071984	AB546858	CP003909	CP001050
01	2068	95.1	95.1	90.6	80.5	80.5
02	2068	96.1	96.1	91.4	80.5	80.5
03	2068	95.5	95.5	90.8	80.0	80.0
04	2068	94.9	94.9	90.1	80.6	80.6
05	2068	95.5	95.5	90.8	80.0	80.0
06	879	96.7	98.9	94.5	0.0	0.0
07	879	96.2	98.3	93.9	0.0	0.0
08	879	97.2	99.4	94.9	0.0	0.0
09	879	96.2	98.3	93.9	0.0	0.0
10	931	93.2	95.2	93.8	0.0	0.0
11	879	96.3	98.4	97.2	0.0	0.0
12	2068	94.7	94.7	93.8	80.6	80.6
13	2068	96.1	96.1	93.8	80.6	80.6
14	2068	95.2	95.2	93.4	80.7	80.7
15	2068	94.9	94.9	90.3	79.4	79.4
16	2068	94.3	94.3	90.7	80.0	80.0
17	879	96.2	98.3	93.9	0.0	0.0
18	2068	95.1	95.1	90.6	80.5	80.5
19	879	96.2	98.3	96.6	0.0	0.0
20	2068	95.4	95.4	90.8	79.9	79.9
21	2068	94.9	94.9	90.3	79.8	79.8
22	2068	95.1	95.1	90.6	80.5	80.5
23	879	96.1	98.3	93.9	0.0	0.0
24	2068	95.4	95.4	90.8	79.8	79.8
25	2068	95.1	95.1	90.6	80.5	80.5
26	2068	94.2	94.2	90.0	79.7	79.7
27	931	92.6	94.7	93.3	0.0	0.0
28	879	96.7	98.9	94.5	0.0	0.0
29	2068	96.1	96.1	91.4	80.5	80.5
30	879	95.8	97.9	96.6	0.0	0.0
31	879	96.2	98.3	93.9	0.0	0.0

^a*penA* sequences were compared using the genome comparator tool, implemented within the PubMLST.org/neisseria website. Penicillin binding protein 2 locus is defined as NEIS1753 and is associated with the locus tag NGO1542 (from the reference *N. gonorrhoeae* isolate FA1090).

antimicrobial resistance in commensal *Neisseria* species of the oropharynx. The prevention of bacterial STIs is therefore a crucial component to fight the hard challenge of drug resistance.

In this context, modelling work by Fingerhuth et al.¹⁹ demonstrated that higher treatment rates result in faster spread of antibiotic resistance in *N. gonorrhoeae*. We should probably revise the current notion that more screening and treatment will limit the spread of *N. gonorrhoeae*, perhaps considering not to screen and treat asymptomatic pharyngeal gonorrhoea. Treatment recommendations for *N. gonorrhoeae*

should therefore carefully balance prevention of infection and avoidance of resistance spread.

Our molecular-based approach does not allow us to identify the particular *Neisseria* species harbouring the *penA* mosaic structure. In addition, no detailed information about the susceptibility/resistance to the various cephalosporins can be obtained. However, unlike culture, our approach is easier and faster and it can be successfully combined with a molecular assay for STI screening of the oropharynx.

We are fully aware of a significant limitation of this study: the entire *penA* gene should be sequenced to

obtain a detailed molecular analysis of the mosaic structure. In this way, it would be possible to evaluate the association with specific bacterial strains, as well as the presence of clusters within the population enrolled.

In conclusion, even though preliminary, our data underline that commensal *Neisseria* species of the oropharynx may be a significant reservoir for genetic material conferring antimicrobial resistance in *N. gonorrhoeae*. For this reason, to prevent the spread of antimicrobial resistance in *N. gonorrhoeae*, individuals should be advised on correct and consistent use of the condom even during oral intercourse to minimise the potential transmission of resistance genes.

Longitudinal studies, including also the heterosexual population, will be needed to investigate the relationship between the previous pharyngeal detection of *penA*-positive *Neisseria* species and the future acquisition of mosaic *penA*-positive *N. gonorrhoeae*.

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