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Detection of *gyrA* mutations associated with ciprofloxacin resistance in *Neisseria gonorrhoeae* by rapid and reliable pre-programmed short DNA sequencing

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Abstract

Quinolone resistance is rapidly increasing in *Neisseria gonorrhoeae* and is posing a significant public health threat that requires constant surveillance. A rapid and reliable mutation detection assay has been developed. The assay is based on pre-programmed short DNA sequencing and is designed to detect point mutations in the *gyrA* gene that are highly related to ciprofloxacin resistance, i.e. in codons 91 and 95. By developing an assay based on pyrosequencing and exploiting the pre-programmed nucleotide dispensation capability of this technology, the sequence comprising the mutations will be analysed and promptly reveal whether the *N. gonorrhoeae* pathogen carries resistance to ciprofloxacin. A panel of 40 *N. gonorrhoeae* clinical isolates, of which 27 phenotypically displayed decreased susceptibility or resistance to ciprofloxacin, was used in the present study. All point mutations in the short stretch of the *N. gonorrhoeae gyrA* gene were easily discriminated, and the genotypic results obtained by pre-programmed sequencing were mainly in agreement with the phenotypically identified decreased susceptibility or resistance to ciprofloxacin. The new method used in the present study has the potential for rapid and reliable identification of known as well as previously unknown drug resistance mutations.

Keywords: DNA sequencing; Ciprofloxacin resistance; *Neisseria gonorrhoeae*; Pre-programmed DNA sequencing; Pyrosequencing technology

1. Introduction

The ability of pathogenic microorganisms to develop resistance to antimicrobial drug treatments has been recognised for decades [1] and continues to increase. This increasing antimicrobial resistance poses a serious threat to public health; thus, as the effects of antimicrobial therapeutic agents decrease, morbidity, mortality and healthcare expenditure increase [2,3].

Neisseria gonorrhoeae is the aetiological agent of gonorrhoea, one of the most common sexually transmitted diseases globally. Quinolone resistance has increased rapidly in *N. gonorrhoeae*, mainly as a result of point mutations in the bacterial genes *gyrA* and *parC* that code for the target enzymes DNA gyrase and topoisomerase IV, respectively [4]. The level of resistance appears to be correlated with the location and number of mutations in these genes. Treatment failures have been shown to be associated with quinolone resistance due to mutations primarily in the *gyrA* gene [5–7]. Moreover, resistance to ciprofloxacin in many isolates of *N. gonorrhoeae* is emerging, and genotyping these mutations is of great importance for resistance surveillance.

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DNA sequencing is the gold standard method for genotyping and mutation detection. It produces the highest resolution for nucleic acid-based diagnosis of mutant microorganisms and, moreover, provides de novo mutation information of the region being sequenced. In the present study, we describe a rapid, reliable and cost-effective pre-programmed DNA sequencing approach that uses the pyrosequencing technique [8,9] for point mutation sequencing and analysis of the *gyrA* gene in a single sequencing reaction. The new approach is time efficient (two times faster than conventional pyrosequencing) and the results and sequence read-outs are significantly improved. Furthermore, we have reduced the cost and labour of amplification by using a new pyrosequencing system (PSQ HS 96A), originally intended for single nucleotide polymorphism (SNP) analysis, that requires significantly lower (10-fold) polymerase chain reaction (PCR) volumes. By modifying the SNP software of the PSQ HS 96A system, we have included a pre-programmed sequencing protocol. We have also utilised a rapid and time-efficient Sepharose bead sample preparation system. Moreover, interpretation of the sequence data of point mutations is simple and user friendly, and de novo mutations are easily detected.

2. Materials and methods

2.1. Bacterial isolates and phenotypic antibiotic susceptibility testing

A total of 40 *N. gonorrhoeae* clinical isolates cultured from different patients in Sweden between 2001 and 2005 were included to evaluate the pre-programmed *gyrA* gene pyrosequencing technique (Table 1). Selection of the isolates was based on their minimum inhibitory concentration (MIC) to ciprofloxacin, which was phenotypically determined by the Etest method (see below). Consequently, 12 ciprofloxacin-susceptible (MIC $\leq$ 0.064 mg/L) *N. gonorrhoeae* isolates, 9 isolates showing decreased susceptibility to ciprofloxacin and 19 ciprofloxacin-resistant (MIC > 0.25 mg/L) isolates were included (Table 1). Nearly all MIC values within the range detectable by the Etest strip, i.e. from <0.002 to >32 mg/L, were represented by at least one isolate. All isolates were subsequently analysed in a blinded fashion to establish the existence of polymorphisms in the short stretch of the *gyrA* gene.

Phenotypic susceptibility to ciprofloxacin was analysed using the Etest method (AB Biodisk, Solna, Sweden) on GC II agar (BBL, Becton Dickinson and Company, Cockeysville, MD) supplemented with 1% haemoglobin and 1% IsoVitaleX enrichment (BBL, Becton Dickinson and Company) as previously described [10].

2.2. DNA extraction and PCR

Isolation of bacterial DNA was performed using magnetic silica particles in a robotized system (MagNA Pure LC; Roche Molecular Biochemicals, Mannheim, Germany) according to the instructions of the manufacturer. In brief, bacterial suspensions (ca. 3×10^8 cells/mL) were prepared in sterile 0.15 M NaCl. A total of 1 mL from each suspension was pelleted and re-suspended in 20 μL of sterile distilled water. Then, 130 μL of Bacteria Lysis Buffer (Roche Diagnostics GmbH, Mannheim, Germany) and 20 μL of Proteinase K solution (Roche Diagnostics GmbH) were added to each sample and the suspensions were incubated at 65 °C for 10 min followed by 95 °C for 10 min. DNA was isolated from 100 μL of these final suspensions with the MagNA Pure LC DNA Isolation Kit III (Roche Diagnostics GmbH) and eluted in 100 μL of elution buffer (Roche Diagnostics GmbH) according to the manufacturer's instructions.

The PCR primers GYRA2-1 and GYRA2-2, used to amplify a stretch of the *gyrA* gene [11] and optimised for use with the pyrosequencing method, were originally designed using the OLIGO 4.0 software (National Biosciences, Inc., Plymouth, MN) from a known sequence available in GenBank for the *gyrA* gene (strain MUG116; accession number U08817). The sequencing primer GYRA2-3 [11], used to sequence the amplified *gyrA* region, was designed in the same way. One of the amplification primers in the primer pair was biotin-labelled for single-strand separation. The primers were synthesised by ThermoHybaid (Ulm, Germany). PCR amplification of the segment of the *gyrA* gene was performed in a 50 μL mixture containing 1 × PCR gold buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 10 pmol of each primer, 0.2 mM mix of deoxynucleoside triphosphates (Sigma Aldrich, St Louis, MO), 1.5 U AmpliTaq Gold™ (Applied Biosystems, Stockholm, Sweden) and 5 μL of the DNA template. The target region was amplified by a PCR protocol using the following temperature profile: initial heating at 95 °C for 10 min; a cycle of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s and elongation at 72 °C for

Table 1
Mutations in the sequence encoding amino acid positions 90–96 of the *gyrA* gene, and the minimum inhibitory concentration (MIC) and phenotypic susceptibility to ciprofloxacin of *Neisseria gonorrhoeae* isolates ($N = 40$)

gyrA gene sequence	No. of isolates	Ciprofloxacin MIC (mg/L)	Phenotypic ciprofloxacin susceptibility
Wild-type	9	0.002–0.047	Susceptible
S91P and D95A alterations	11	0.125–32	Decreased susceptibility to high resistance
S91P and D95G alterations	10	0.125–32	Decreased susceptibility to high resistance
S91P alteration	8	0.047–0.38	Susceptible
D95N alteration	2	0.032–0.064	Susceptible