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Emergence and Spread of Neisseria Gonorrhoeae Antimicrobial Resistance in California

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Emergence and Spread of *Neisseria gonorrhoeae* Antimicrobial Resistance in California

by

Severin Oyloe Gose

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctorate of Public Health

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor George Sensabaugh, Chair
Professor Gertrude Buehring
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Fall 2012
Emergence and Spread of *Neisseria gonorrhoeae* Antimicrobial Resistance in California

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by

Severin Oyloe Gose
Abstract

Emergence and Spread of *Neisseria gonorrhoeae* Antimicrobial Resistance in California

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University of California, Berkeley

Professor George Sensabaugh, Chair

The development of antimicrobial resistance in *Neisseria gonorrhoeae* has severely limited the treatment options available for uncomplicated urogenital infection. As of August 2012, dual therapy with an injectable third generation cephalosporin and a macrolide is the only Centers for Disease Control and Prevention recommended treatment regimen for empirical therapy in the United States. Verified treatment failures with oral cephalosporins used for urethral infections and with injectable cephalosporins used for pharyngeal infections have been reported in Asia, Europe and Australia. At this time, there have been no verified treatment failures with injectable cephalosporins in a urethral infection. Isolates with reduced susceptibility to the both oral and injectable cephalosporins have been described in the U.S., but no treatment failures have been described. This dissertation is formatted as three self-contained research papers. The first paper compares Etest to agar dilution for the determination of *N. gonorrhoeae* minimum inhibitory concentrations. The second paper describes the genotypic and phenotypic surveillance of *N. gonorrhoeae* resistance to third generation cephalosporins and macrolides in California in 2011. The third paper details the development of a new molecular assay for the detection of genes associated with reduced susceptibility to cephalosporins.
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Chapter 1

Introduction

Infection with *Neisseria gonorrhoeae* is one of the oldest infections of humans. Descriptions of the clinical presentation of gonorrhea can be found in the historical record dating back over 5,000 years in ancient Chinese writings, the book of Leviticus and the writings of Hippocrates in 4th and 5th century Greece [32]. The causative organism of this ancient disease was discovered in 1879 by the German physician Albert Neisser, for whom the genus *Neisseria* is named. After its discovery, *N. gonorrhoeae*, also known as the “gonoccoccus”, could not be cultured until Leistikow and Löffler successfully grew the bacterium in 1882 and facilitated the detailed study of its biology [21].

*N. gonorrhoeae* is a fastidious Gram-negative coffee bean-shaped bacteria usually observed as diplococci and a strict human pathogen. Of the eleven *Neisseria* spp. that colonize humans, only *N. gonorrhoeae* and *N. meningitidis* are pathogenic. *N. gonorrhoeae* cultures exhibit growth on chocolate agar at 37°C with 5% CO₂. *N. gonorrhoeae* can be differentiated from other *Neisseria* spp. by nucleic acid amplification testing (NAAT) or metabolic tests for the production of oxidase and the utilization of glucose, but not maltose or sucrose. The organism’s primary sites of infection are the mucous membranes of the urethra, endocervix, anal crypts, pharynx and conjunctiva [42].

*N. gonorrhoeae* has a variety of adaptations and virulence factors that allow the bacterium to colonize host sites, evade immune detection and prevent its physical removal from mucosal surfaces. The bacterium utilizes twitching motility for movement and type IV pili to adhere to host mucosal surfaces. This bacterial pili-host receptor interaction is incredibly strong and has been calculated to exert a force of one nanonewton or 100,000 times the bacterium’s own weight, making it the most powerful biological motor described [5]. The bacterium is also capable of evading the host’s immune response through antigenic variation of the Opa protein repertoire that forms a protective coating on its surface. This variation in surface protein expression
leads to an inefficient immune response and prevents the acquisition of immunological memory in the host, allowing repeated infections [26]. *N. gonorrhoeae* has been shown to be a particularly promiscuous bacterium which explains its ability to rapidly acquire antimicrobial resistance genes through horizontal gene transfer [3]. *N. gonorrhoeae* is naturally competent for DNA transformation, expresses plasmid-encoded genes and is capable of exchanging DNA through bacterial conjugation. These properties allow the organism to easily exchange DNA with other *N. gonorrhoeae* strains during mixed infections and unrelated bacteria that colonize the same host sites.

In the pre-antibiotic era, complications from gonococcal infections were common. With the advent of modern medicine and chemotherapy, effective treatment options became available. Although not a large cause of mortality in the United States, gonococcal infection causes a significant amount of morbidity amongst adults. In men, urethral infection can result in severe dysuria, epididymitis, prostatitis and urethritis. In women the infection is often initially asymptomatic, but may lead to severe sequelae such as pelvic inflammatory disease (PID), ectopic pregnancy and infertility [29]. Pharyngeal or anal exposure to *N. gonorrhoeae* can cause pharyngitis or proctitis, but often results in asymptomatic colonization which further facilitates transmission. Those infected with *N. gonorrhoeae* are commonly co-infected with *Chlamydia trachomatis* and like many other sexually transmitted infections, gonococcal infection increases the risk of human immunodeficiency virus (HIV) infection and transmission.

Currently, gonorrhea is the second most common sexually transmitted infection in the United States with over 300,000 cases reported annually to the Centers for Disease Control and Prevention (CDC) and an estimated 700,000 cases occurring each year [12]. Gonococcal infection is much more common among young persons, especially the 15-24 year old age group. Rates are also higher among persons of lower socio-economic status, men who have sex with men (MSM), illicit drug users, commercial sex workers, persons held in correctional facilities, and racial or ethnic minority groups. Globally, gonococcal infection is a major cause of morbidity with an estimated 62.4 million infections annually and the highest rates occurring in Asia, Southeast Asia and Sub-Saharan Africa [4].

### 1.1 Treatment options

Beginning with the introduction of the sulfonamide class of antimicrobials in the late 1930s, a variety of chemotherapeutic agents have been used to treat gonococcal infection. Unfortunately, the bacterium has proven to be incredibly adept at developing antimicrobial resistance through bacterial conjugation, chromosomal mutation, and the acquisition of plasmids and naked DNA from its environment. Over the last 70 years, the bacterium developed resistance to a stunning array of antimicrobial
classes including sulfonimides, penicillins, $\beta$-lactams, tetracyclines, macrolides, and fluoroquinolones. With these antimicrobial classes rendered ineffective for the empirical treatment of uncomplicated gonococcal infection, clinicians have come to rely heavily upon the third generation cephalosporins. Resistance to these antimicrobials has recently started spreading and relatively few alternative treatment options exist. Spectinomycin has been used in the past to treat gonococcal infection, but is no longer approved for use in the United States. Resistance to spectinomycin has also been documented to arise rapidly through a single point mutation [27]. Other antimicrobials such as the carbapenems, tigecycline, and fourth-generation cephalosporins have been investigated, but their clinical utility in the context of multi-drug resistant infections remains unclear.

The 2011 CDC treatment recommendations for the treatment of uncomplicated gonococcal infection in order of preference were:

1. Ceftriaxone 250 mg single intramuscular dose
2. Cefixime 400 mg single oral dose
3. Single dose injectable cephalosporin regimen plus azithromycin 1 g single oral dose
4. Single dose injectable cephalosporin regimen plus doxycycline 100 mg orally twice a day for 7 days

Due to the increasing number of isolates detected in the United States with reduced susceptibility to oral cephalosporins, the CDC updated the treatment recommendations for uncomplicated gonococcal infection in August 2012 to:

1. Ceftriaxone 250 mg single intramuscular dose plus azithromycin 1 g single oral dose

1.1.1 Ceftriaxone

Ceftriaxone (shown in Figure 1.1) is an injectable third generation cephalosporin that has broad spectrum activity against both Gram-positive and Gram-negative bacteria. Its mechanism of action involves the inhibition of bacterial wall synthesis and maintenance by binding to the bacterial penicillin binding proteins. The drug is typically administered as a single intramuscular injection for the treatment of uncomplicated gonococcal infection and has been shown to be well tolerated at a dose of 250 mg.
1.1.2 Cefixime

Cefixime (shown in Figure 1.2) is an oral third generation cephalosporin that has broad spectrum activity against both Gram-positive and Gram-negative bacteria. Its mechanism of action involves the inhibition of bacterial wall synthesis and maintenance by binding to the bacterial penicillin binding proteins. The drug is typically administered as a single oral dose for the treatment of uncomplicated gonococcal infection and has been shown to be well tolerated at a dose of 400 mg. Its safety profile and effectiveness allow its use in patient-delivered partner therapy (PDPT).

1.1.3 Cefpodoxime

Cefpodoxime (shown in Figure 1.3) is an oral third generation cephalosporin that has broad spectrum activity against both Gram-positive and Gram-negative bacteria. Its mechanism of action involves the inhibition of bacterial wall synthesis and maintenance by binding to the bacterial penicillin binding proteins. The drug is typically administered as a single oral dose for the treatment of uncomplicated gonococcal infection and has been shown to be well tolerated at a dose of 400 mg. Its safety profile and effectiveness allow its use in PDPT.
1.1.4 Azithromycin

Azithromycin (shown in Figure 1.4) is a member of the azilide subclass of macrolides. It is effective against both Gram-negative and Gram-positive bacteria, but has a more limited spectrum of activity against Gram-negative infections than the third generation cephalosporins. Its mechanism of action involves inhibition of bacterial mRNA translation and protein expression by binding to the 50S ribosomal subunit. The drug is typically administered as a single oral dose for the treatment of uncomplicated gonococcal infection and has been shown to be well tolerated at a dose of 1 g. 2 g doses have been suggested as an option to overcome increasing bacterial minimum inhibitory concentrations (MICs), but this dosage is not well tolerated due to gastrointestinal side effects. Newer time-release formulations have been developed to prevent these side effects at higher doses but their effectiveness has not yet been demonstrated.

1.2 Classification of antimicrobial resistance

Antimicrobial resistance is defined in the United States by the Clinical and Laboratory Standards Institute (CLSI). CLSI defines the breakpoints at which a bacterial MIC is
considered susceptible or non-susceptible. Determining clinically relevant breakpoints for cephalosporins has been difficult since relatively few treatment failures have been described. Breakpoints for these antimicrobials have not been defined for resistance and instead have only been defined for sensitive isolates [10]. The CLSI standards issued in 2008 defined resistance as $\text{MIC} \geq 2.0 \mu g/mL$ for azithromycin and sensitivity as $\text{MIC} \leq 0.25 \mu g/mL$ for third generation cephalosporins. The CDC Gonococcal Isolate Surveillance Program (GISP) also defines alert value breakpoints for isolates with reduced susceptibilities. The CDC GISP 2010 protocol update defined alert value MICs as $\geq 2.0 \mu g/mL$ for azithromycin, $\geq 0.25 \mu g/mL$ for cefixime and cefpodoxime and $\geq 0.125 \mu g/mL$ for ceftriaxone.

### 1.3 Cephalosporin resistance

The first treatment failures with an oral third generation cephalosporin were described in two Japanese patients treated with cefdinir in 2001 [1]. Treatment failure isolates spread rapidly from a single clone to many contacts in Japan and were resistant to multiple drug classes including $\beta$-lactams, penicillins and oral third generation cephalosporins [25,35]. Molecular characterization of the multi-drug resistant isolates from Japan in 2000 and 2001 showed that their reduced susceptibilities to oral third generation cephalosporins were caused by alterations of the $\text{penA}$ gene, which encodes the penicillin binding protein 2 (PBP-2) and is targeted by penicillins, $\beta$-lactams and cephalosporins. The new $\text{penA}$ alleles were termed “mosaic-like” because they contained portions of sequence homology with other Neisseria spp. including Neisseria flavescens, Neisseria cinerea, Neisseria perflava and Neisseria meningitidis. It was assumed that these homologous regions were acquired through multiple recombination events with the other commensal and pathogenic Neisseria spp. These mosaic strains were capable of producing MICs at or above the susceptibility breakpoint for oral third generation cephalosporins and were associated with cefixime treatment failures in Japan [2]. In 2003, multi-drug resistant strains with reduced susceptibilities to oral and injectable cephalosporins and resistance to penicillin, tetracycline, and ciprofloxacin were described in Hawaii. These strains were associated with a potential treatment failure after oral administration of 400 mg cefixime [53]. Further molecular characterization of the Japanese isolates from 2000 and 2001 showed that a variety of closely related mosaic alleles were circulating in Japan and that certain alleles were associated with reduced susceptibility to oral cephalosporins, specifically pattern X [24]. Investigation of other drug resistance determinants within the $N. \text{gonorrhoeae}$ genome demonstrated that although the mosaic alterations of the $\text{penA}$ gene were the primary determinant of reduced oral cephalosporin susceptibility, multiple other resistance determinants were capable of affecting drug susceptibilities through
mechanisms like efflux pumps and alterations of the bacterial surface porins [45]. In 2006, specific point mutations common to multiple mosaic penA alleles (G545S, I312M, V316T) were shown to be associated with reduced susceptibility to cefixime and other oral third generation cephalosporins [44]. In 2007 four more cefixime treatment failures were reported in Japan with cefixime MICs of 0.5-1.0 µg/mL [58]. In 2008, homology modeling studies of strains with reduced susceptibilities to both oral and injectable cephalosporins demonstrated that the mosaic penA alleles and alleles showing certain point mutations resulted in conformational changes to the β-lactam binding pocket where cephalosporins bind [40]. In late 2008 and 2009, further treatment failures with oral third generation cephalosporins and the first ceftriaxone treatment failure of a pharyngeal gonococcal infection were described [31, 46]. Between 2009 and 2012, mosaic penA alleles were found in multiple countries including the United States [14,16,18,22,28,33,39,41,48,56] and treatment failures with both oral and injectable cephalosporins were described in multiple countries [23, 49–52]. No verified treatment failure with either an oral or injectable cephalosporin have been documented in the United States.

1.4 Macrolide resistance

Azithromycin treatment failures have been reported in the literature dating back to the early 1990s in the United States and abroad [43,55,59]. Unlike the third generation cephalosporins, azithromycin MICs are not strongly associated with clinical outcomes. CLSI defines resistance as MIC $\geq$ 2.0 µg/mL for azithromycin, but some isolates associated with treatment failures have displayed MICs of 0.125-0.5 µg/mL. In a treatment failure reported in 1997, comparison of the pre-treatment isolate and post-treatment isolate clearly showed selection for a higher azithromycin MIC with an increase from 0.125 µg/mL to 3.0 µg/mL [59]. These contradictory results were explained as being due to other complex pharmacokinetic variables such as differences in the rates of absorption and distribution of the drug in tissue fluids [47]. Another difficulty in assessing potential azithromycin treatment failures is that azithromycin is rarely used as a monotherapy. It is typically prescribed as part of a dual-therapy regimen because patients with gonococcal infection are commonly co-infected with Chlamydia trachomatis, against which it is highly effective [7].

Alert value MICs for azithromycin are associated with mutations to both the drug target and a bacterial efflux pump. Azithromycin targets the 50S ribosome and a single mutation of the peptidyltransferase loop in domain V of the 23S subunit (C2611T) leads to an alteration of the ribosome structure that prevents macrolides from binding [34]. The N. gonorrhoeae genome contains four copies of the 23S ribosomal subunit gene and the number of resistant copies is associated with higher azithromycin MICs.
Chapter 1. Introduction

In 2010, a novel 23S ribosomal subunit mutation (A2143G) was described in an Argentinean isolate that resulted in an azithromycin MIC of 2,048 µg/mL. Interestingly, this same mutation has been associated with high-level macrolide resistance in *Helicobacter pylori*, several Mycobacterium spp., *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, Propionibacterium spp., and *Treponema pallidum* [17].

Mutations in the *mtrR* gene are also associated with elevated MICs for azithromycin and a number of other antimicrobials. *MtrR* encodes a repressor of the bacterial MtrC-MtrD-MtrE multi-drug resistance efflux pump and a single base-pair deletion in the 13-bp inverted repeat results in increased expression of the efflux pump on the bacterial surface and lower levels of intracellular azithromycin [15]. Mutations in the coding region of the *mtrCDE* operon itself are also associated with reduced susceptibility to azithromycin (G115A,G131A), and can produce MICs between 4.0-16.0 µg/mL when combined with the *mtrR* promoter mutation [54].

1.5 Research Papers

This dissertation follows the three-paper format with a unifying introduction and conclusion focusing on antimicrobial resistance in *N. gonorrhoeae*. The first paper is a methodological comparison of *N. gonorrhoeae* MICs obtained by Etest and agar dilution. The second paper describes the genotypic and phenotypic surveillance of isolates with reduced susceptibilities to third generation cephalosporins and macrolides in California in 2011. The third paper presents a new TaqMan real time polymerase chain reaction (RTPCR) assay for the detection of isolates with reduced susceptibility to cephalosporins.

1.6 Funding source

This research was funded through a collaboration between the San Francisco Department of Public Health (SFDPH), CDC and California Department of Public Health (CDPH). Student support was provided through the Lab Aspire program which was administered by CDPH and distributed through the University of California, Berkeley (UCB).

1.7 IRB and human subjects

The research involved in this dissertation was not considered human subjects research and did not require institutional review board approval. This status was confirmed through communications with both the UCB Committee for the Protection of Human
Chapter 1. Introduction

Subjects and the University of California, San Francisco (UCSF) Committee on Human Research. The process of isolate collection was not considered human subjects research because the isolates were initially collected for public health surveillance purposes and were deidentified prior to their use in this research.

1.8 Study sites and collaborators

Isolate collection and initial selective plating occurred at public health clinics in four California counties - San Francisco, Los Angeles, Orange County and San Diego. Species confirmation and isolate purification were done at each county’s respective public health laboratories. Collaborators at the CDC Gonococcal Isolate Surveillance Project (GISP) regional laboratory at the University of Washington (Seattle, WA) and CDPH (Richmond, CA) were responsible for collection of the agar dilution MIC data. DNA sequencing was done by MCLAB Inc. (South San Francisco, CA) and Elim Biopharmaceuticals Inc. (Hayward, CA). All other work was done by the author at the San Francisco Department of Public Health Laboratory (San Francisco, CA).
Chapter 2

Comparison of *N. gonorrhoeae* MICs obtained by Etest and agar dilution

2.1 Introduction

*N. gonorrhoeae* is currently the second most common sexually transmitted infection in the United States with an estimated 700,000 cases occurring annually [12]. As of August 2012, dual therapy with an injectable third generation cephalosporin and a macrolide is the only CDC recommended treatment regimen for empirical therapy. In the last ten years, treatment failures caused by isolates with reduced susceptibility to third generation cephalosporins have been reported in Europe and Asia. Recently, it was reported that that emerging *N. gonorrhoeae* strains may soon be capable of causing untreatable infections [38].

In an era of increasing *N. gonorrhoeae* antimicrobial resistance, the ability of laboratories to conduct antimicrobial susceptibility testing (AST) is crucial to both treatment and surveillance efforts. Etest is an alternative method to agar dilution that requires fewer resources and is capable of rapidly producing quantitative MIC results. Previous reports have evaluated the Etest method by using agar dilution as a reference standard for ceftriaxone and azithromycin [6,57]. This study presents the first data comparing Etest with agar dilution for cefixime and cefpodoxime.

2.2 Materials and methods

2.2.1 Isolate collection

During 2011, urethral isolates were collected from men visiting public health clinics in Los Angeles, San Francisco, San Diego and Orange Counties in California.
presumptive identification of *N. gonorrhoeae* was based on the following criteria: growth of typical appearing colonies on a selective medium such as Thayer-Martin at 35° C to 36.5° C in 5% CO₂, a positive oxidase test, and the observation of gram-negative, diplococci in stained smears. A single *N. gonorrhoeae* was picked and subcultured from the selective primary medium on a Chocolate II Agar (GC II Agar with Hemoglobin and 1% IsoVitaleX, Beckton-Dickenson, USA) plate. After 18 to 20 hours incubation, growth from the pure culture was suspended in TSB containing 20% (v/v) glycerol, frozen at -80° C and sent overnight on dry ice to the SFDPH laboratory for AST by Etest and the CDC GISP regional laboratory for AST by agar dilution.

### 2.2.2 Etest

Frozen isolates were thawed in a biosafety cabinet at room temperature, inoculated on a Chocolate II Agar plate and incubated at 37°C with 5% CO₂ for 20-25 hrs. A sterile swab was then used to collect colonies off the plate and suspend them in TSB with 15% glycerol to a turbidity of 0.5-1 MacFarland units. Using the sterile swab, the liquid culture was plated out onto three Chocolate II Agar plates in three 90 degree turnings and an Etest strip was placed in the center of each plate using the manual applicator. After incubation for 20-25 hrs at 37°C with 5% CO₂, the plates were visualized and the MIC was recorded according to the manufacturer’s instructions (bioMerieux, France). Examples of Etest plates from a wildtype *N. gonorrhoeae* isolate and an isolate with reduced susceptibility to cephalosporins are shown in Figure 2.1 and Figure 2.2.

### 2.2.3 Agar dilution

Agar dilution was completed as described previously [11]. Briefly, Difco GC medium base (Becton Dickenson, Cockeysville, MD) supplemented with 1% IsoVitaleX was used to prepare plates containing a range of antimicrobial concentrations from 0.008 to 8.0 µg/mL. Frozen isolates were thawed in a biosafety cabinet at room temperature, inoculated on a chocolate agar plate and incubated at 37°C with 5% CO₂ for 16-18 hours. The isolate was then subcultured a second time on chocolate agar. Isolate growth was collected, diluted to 0.5 MacFarland units using Mueller-Hinton broth and inoculated onto the plates containing the set of antimicrobial concentrations. Growth in the presence of antimicrobials was examined after 24 hours incubation at 37°C with 5% CO₂. An isolate’s MIC was defined as the lowest concentration of antimicrobial that completely inhibited visible growth.
Chapter 2. Comparison of \textit{N. gonorrhoeae} MICs obtained by Etest and agar dilution

Figure 2.1: Example cefixime and cefpodoxime Etest plates of an isolate with reduced susceptibility. Black arrows indicate the limit of growth and the isolate’s MIC is 0.125 \( \mu \text{g/mL} \) for cefixime and 0.75 \( \mu \text{g/mL} \) for cefpodoxime.

Figure 2.2: Example cefixime and cefpodoxime Etest plates of a wildtype isolate. Black lines indicate the limit of growth and the isolate’s MIC is \(<0.016 \mu \text{g/mL} \) for cefixime and \(<0.016 \mu \text{g/mL} \) for cefpodoxime.
2.2.4 Quality Control

For every Etest batch, two *N. gonorrhoeae* isolates with predetermined MICs were included as quality control strains. One isolate was susceptible to all four antimicrobials and one isolate displayed alert value MICs to the cephalosporins. For agar dilution, *N. gonorrhoeae* strains ATCC 49266 (CLSI control strain), SPJ-15 (azithromycin control strain), SPL-4 (cephalosporin control strain), and four other control strains were included with each agar dilution AST batch. For both Etest and agar dilution AST, control strains were considered accurate if their MICs were within $\pm 1\log_2$ of the predetermined MIC. MICs for agar dilution control strains were determined by the CDC. MICs for Etest control strains were determined through repeat testing by the SFDPH laboratory.

2.2.5 Data analysis

Etest has a finer gradation of MIC values and a larger range than agar dilution. To facilitate comparison of the two methods, all Etest values were rounded up to the nearest $\log_2$ agar dilution MIC value. Concordance was defined categorically using the 2010 CDC GISP alert value breakpoints, which were 0.125, 0.25, 0.25, and 2.0 $\mu g/mL$ for ceftriaxone, cefpodoxime, cefixime and azithromycin respectively. Agreement was defined as an Etest MIC within $\pm 1\log_2$ of the reference agar dilution MIC following the CDC GISP quality assurance guidelines. Correlations were calculated using Pearson’s product-moment correlation coefficient.

2.2.6 Human subjects

Isolates used in this study were collected for public health surveillance and de-identified prior to AST, thus this work was considered public health practice and was exempt from human subjects regulations. This category of research is not considered human subjects research and is not subject to Institutional Review Board oversight.

2.3 Results

2.3.1 AST results obtained by the reference agar dilution method

The results of AST using the reference agar dilution and Etest methods are shown in Table 2.1 and Table 2.2. AST was completed by both Etest and agar dilution for 664 isolates with ceftriaxone and azithromycin, 351 isolates with cefpodoxime and 315 isolates with cefixime. 0.5%, 7%, 2.5% and 0.5% of isolates had MICs above the alert value breakpoint for ceftriaxone, cefpodoxime, cefixime and azithromycin.
respectively. Two isolates had alert value MICs to both ceftriaxone and cefpodoxime and one isolate had alert value MICs to both ceftriaxone and cefixime. Twenty-three isolates had alert value MICs to cefpodoxime only and seven isolates had alert value MICs to cefixime only. Three isolates had alert value MICs to azithromycin, but none of these isolates had alert value MICs to any of the cephalosporins.

Table 2.1: Susceptibility of *N. gonorrhoeae* isolates by agar dilution

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
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<td>66</td>
<td>43</td>
<td>3</td>
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<td>0</td>
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<td>0</td>
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<td>164</td>
<td>236</td>
<td>151</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Shaded cells indicate alert value MICs.

Table 2.2: Susceptibility of *N. gonorrhoeae* isolates by Etest

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriaxone</td>
<td>378</td>
<td>132</td>
<td>75</td>
<td>76</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>112</td>
<td>103</td>
<td>54</td>
<td>58</td>
<td>6</td>
<td>5</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefixime</td>
<td>189</td>
<td>84</td>
<td>28</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>55</td>
<td>259</td>
<td>233</td>
<td>96</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Shaded cells indicate alert value MICs.

### 2.3.2 Comparison of MICs obtained by Etest and agar dilution

The results of the comparison between the reference agar dilution method and Etest are summarized in Figure 2.3 and Table 2.3. In Figure 2.3, dark grey shading indicates isolates that had an Etest value which matched the corresponding agar dilution value, medium grey shading indicates isolates that had an Etest value within \( \pm 1 \log_2 \) of the corresponding agar dilution value, light grey shading indicates isolates that had an Etest value within \( \pm 2 \log_2 \) of the corresponding agar dilution value and thick black lines indicate the alert value breakpoints for each antimicrobial. Unshaded numbers indicates isolates that had an Etest value \( > 2 \log_2 \) or \( < 2 \log_2 \) of the corresponding agar dilution value.
Chapter 2. Comparison of *N. gonorrhoeae* MICs obtained by Etest and agar dilution

Cefpodoxime, cefixime and azithromycin showed high levels of agreement. Ceftriaxone Etest MICs displayed a trend towards higher values relative to agar dilution. From Table 2.3, one hundred and twenty-two (18%) isolates had ceftriaxone MICs that were $2 \log_2$ higher than their corresponding agar dilution values and 28 (4.2%) isolates had MICs that were $>2 \log_2$ higher than their corresponding agar dilution values. This lack of agreement at the higher end of the ceftriaxone MIC range has been reported previously and may be related to the use of chocolate agar for AST instead of GC agar [30].

**Table 2.3: Comparison of *N. gonorrhoeae* MICs obtained by Etest relative to agar dilution.**

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>&lt;2</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
<th>&gt;2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriaxone</td>
<td>0</td>
<td>4</td>
<td>46</td>
<td>343</td>
<td>121</td>
<td>122</td>
<td>28</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>3</td>
<td>36</td>
<td>108</td>
<td>119</td>
<td>76</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Cefixime</td>
<td>6</td>
<td>37</td>
<td>165</td>
<td>69</td>
<td>36</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0</td>
<td>22</td>
<td>192</td>
<td>303</td>
<td>128</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

*Indicated by counts of isolates with Etest MICs log$_2$ above or below the reference agar dilution MIC value. Shaded areas indicate isolates in agreement.*

### 2.3.3 Statistical measures of agreement between Etest and agar dilution

Table 2.4 shows statistical measures of agreement between Etest and agar dilution. Agreement was high for all antimicrobials tested with the exception of ceftriaxone. Ceftriaxone showed the lowest level of agreement (77%), which was caused by the trend towards higher Etest MICs as explained in Table 2.3. Concordance between the two methods was high for all antimicrobials with 89% to 100% of MICs correctly established as above or below the alert value breakpoints by both methods. For cefpodoxime, cefixime and azithromycin, all discordant Etest MICs were within $\pm1 \log_2$ of the reference agar dilution MIC. Discordant ceftriaxone MICs showed much larger MIC differences. Correlations between the two methods were high for all antimicrobials (0.75-0.88).
Chapter 2. Comparison of *N. gonorrhoeae* MICs obtained by Etest and agar dilution

<table>
<thead>
<tr>
<th>Etest ceftriaxone MIC (µg/mL)</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar dilution ceftriaxone MIC (µg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.125</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0.06</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>35</td>
<td>1</td>
<td>43</td>
</tr>
<tr>
<td>0.03</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>24</td>
<td>31</td>
<td>0</td>
<td>66</td>
</tr>
<tr>
<td>0.015</td>
<td>328</td>
<td>0</td>
<td>65</td>
<td>19</td>
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<tr>
<td>Total</td>
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<td>0</td>
<td>132</td>
<td>75</td>
<td>76</td>
<td>3</td>
<td>664</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Etest cefpodoxime MIC (µg/mL)</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar dilution cefpodoxime MIC (µg/mL)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>0.06</td>
<td>3</td>
<td>0</td>
<td>22</td>
<td>26</td>
<td>20</td>
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<td>0.03</td>
<td>34</td>
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<td>4</td>
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<td>107</td>
</tr>
<tr>
<td>0.015</td>
<td>75</td>
<td>0</td>
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<td>3</td>
<td>2</td>
<td>0</td>
<td>111</td>
</tr>
<tr>
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<tr>
<td>Total</td>
<td>112</td>
<td>0</td>
<td>103</td>
<td>54</td>
<td>58</td>
<td>6</td>
<td>351</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Etest cefixime MIC (µg/mL)</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar dilution cefixime MIC (µg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>0.125</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>0.06</td>
<td>6</td>
<td>0</td>
<td>12</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>0.03</td>
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</tr>
<tr>
<td>0.015</td>
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<td>177</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>189</td>
<td>0</td>
<td>84</td>
<td>28</td>
<td>11</td>
<td>3</td>
<td>315</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Etest azithromycin MIC (µg/mL)</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar dilution azithromycin MIC (µg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>0.125</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>0.06</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>0.03</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>55</td>
<td>259</td>
<td>233</td>
<td>96</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>664</td>
</tr>
</tbody>
</table>

Figure 2.3: Heat maps of MICs obtained by Etest and agar dilution.
Table 2.4: Agreement between MICs obtained by Etest and agar dilution

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>n</th>
<th>agreement(%)^a</th>
<th>concordance(%)^b</th>
<th>r^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriaxone</td>
<td>664</td>
<td>77</td>
<td>89</td>
<td>0.79</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>351</td>
<td>86</td>
<td>100</td>
<td>0.88</td>
</tr>
<tr>
<td>Cefixime</td>
<td>315</td>
<td>86</td>
<td>98</td>
<td>0.82</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>664</td>
<td>94</td>
<td>100</td>
<td>0.75</td>
</tr>
</tbody>
</table>

^a Defined as ± 1 log₂ of the reference agar dilution MIC value.
^b Defined categorically as having an MIC above or below the alert value breakpoint.
^c Calculated using Pearson’s correlation coefficient.

2.4 Discussion

With fewer treatment options available and *N. gonorrhoeae* antimicrobial resistance increasing, the ability of laboratories to conduct AST is crucial. Compared with the reference agar dilution method, Etest is faster, requires fewer supplies and requires less labor. The data presented in this study confirm that Etest is a valid alternative to agar dilution for azithromycin and presents the first data showing the same for oral third generation cephalosporins. Ceftriaxone, however, showed lower levels of agreement with a trend towards higher Etest MICs relative to agar dilution. It may, therefore, be appropriate to set method specific alert value breakpoints for ceftriaxone. For example, if the Etest ceftriaxone alert value breakpoint were raised to 0.25 µg/mL and the agar dilution ceftriaxone alert value breakpoint remained at 0.125 µg/mL, the concordance between the two methods would improve from 89% to 100%. This study also demonstrates that chocolate agar can successfully be used in place of the much more expensive GC agar for cefpodoxime, cefixime and azithromycin AST, but that for ceftriaxone, it may produce higher MICs as described previously [30].

Limitations of this study include the fact that isolates could not be retested by either method due to time and cost constraints. Agar dilution was also completed at the GISP regional laboratory in Seattle, while Etest AST was completed at the SFDPH laboratory in San Francisco. Finally, an isolate with an alert value MIC to azithromycin was not available for use in Etest quality control and the majority of isolates displayed MICs below the alert value breakpoint. These limitations may increase the variability in MICs seen between the two methods, but also give a closer approximation of their agreement in a real world situation where repeat testing is typically not possible.
Chapter 3

Genotypic and phenotypic surveillance of \textit{N. gonorrhoeae} antimicrobial resistance in California

3.1 Introduction

\textit{N. gonorrhoeae} is currently the second most common sexually transmitted infection in the United States with an estimated 700,000 cases occurring annually [12]. As of August 2012, dual therapy with an injectable third generation cephalosporin and a macrolide is the only CDC recommended treatment regimen for empirical therapy. In the last ten years, treatment failures caused by isolates with reduced susceptibility to third generation cephalosporins have been reported in Europe and Asia. Recently, it was reported that emerging \textit{N. gonorrhoeae} strains may soon be capable of causing untreatable infections [38].

Isolates with reduced susceptibility to cephalosporins have been linked to altered penicillin-binding protein 2, encoded by the \textit{penA} gene. These \textit{penA} alleles were termed “mosaic” because their sequence appears to have been formed through homologous recombination events with other \textit{Neisseria} spp. that are naturally resistant to cephalosporins. In 2009, isolates with mosaic \textit{penA} alleles were first described in the United States and were associated with reduced susceptibility to oral cephalosporins in San Francisco isolates [41].

To investigate the link between mosaic \textit{penA} alleles and cephalosporin resistance, isolates were collected from men with symptomatic urethral infections at four sites in California in 2011 and their MICs to ceftriaxone, cefpodoxime, cefixime and azithromycin were determined by Etest. Next, RTPCR was used to screen for isolates with mosaic \textit{penA} alleles and mosaic \textit{penA} alleles were subjected to DNA sequencing. Finally, \textit{N. gonorrhoeae} multi-antigen sequence typing (NG-MAST), which assesses
the genetic similarity of \textit{N. gonorrhoeae} strains, was used to examine the population structure of mosaic and non-mosaic isolates.

3.2 Materials and methods

3.2.1 Isolate collection

During 2011, urethral isolates were collected from men visiting public health clinics in Los Angeles, San Francisco, San Diego and Orange Counties in California. A presumptive identification of \textit{N. gonorrhoeae} was based on the following criteria: growth of typical appearing colonies on a selective medium such as Thayer-Martin at 35$^\circ$C to 36.5$^\circ$C in 5\% CO$_2$, a positive oxidase test, and the observation of gram-negative, diplococci in stained smears. A single \textit{N. gonorrhoeae} was picked and subcultured from the selective primary medium on a Chocolate II Agar (GC II Agar with Hemoglobin and 1\% IsoVitaleX, Beckton-Dickenson, USA) plate. After 18 to 20 hours incubation, growth from the pure culture was suspended in TSB containing 20\% (v/v) glycerol, frozen at -80$^\circ$C and sent overnight on dry ice to the SFDPH laboratory for AST by Etest.

3.2.2 Human subjects

Isolates used in this study were collected for public health surveillance and de-identified prior to AST, thus this work was considered public health practice and was exempt from human subjects regulations. [20]. This category of research is not considered human subjects research and is not subject to Institutional Review Board oversight.

3.2.3 Etest

Frozen isolates were thawed in a biosafety cabinet at room temperature, inoculated onto a Chocolate II Agar plate and incubated at 37$^\circ$C with 5\% CO$_2$ for 20-25 hrs. A sterile swab was then used to collect colonies off the plate and suspend them in TSB with 15\% glycerol to a turbidity of 0.5-1 MacFarland units. Using the sterile swab, the liquid culture was plated out onto three Chocolate II Agar plates in three 90 degree turnings and an Etest strip was placed in the center of each plate using the manual applicator. After incubation for 20-25 hrs at 37$^\circ$C with 5\% CO$_2$, the plates were examined and the MIC was recorded according to the manufacturer’s instructions (bioMerieux, France). Examples of Etest plates from a wildtype \textit{N. gonorrhoeae} isolate and an isolate with reduced susceptibility to cephalosporins are shown in Figure 3.1 and Figure 3.2.
Chapter 3. Genotypic and phenotypic surveillance of *N. gonorrhoeae* antimicrobial resistance in California

Figure 3.1: Example cefixime and cefpodoxime Etest plates of an isolate with reduced susceptibility. Black arrows indicate the limit of growth and the isolate’s MIC is 0.125 $\mu$g/mL for cefixime and 0.75 $\mu$g/mL for cefpodoxime.

Figure 3.2: Example cefixime and cefpodoxime Etest plates of a wildtype isolate. Black lines indicate the limit of growth and the isolate’s MIC is $<0.016$ $\mu$g/mL for cefixime and $<0.016$ $\mu$g/mL for cefpodoxime.
3.2.4 RTPCR

A TaqMan RTPCR was used to detect penA alleles with the mosaic structure as described previously [37]. Primers and probe used are shown in Table 3.1. Briefly, samples were prepared by diluting 5 µL of the liquid culture into 245 µL of distilled water. 5 µL of this diluted liquid culture was used as the template in the 20 µL reaction mix below. Liquid N. gonorrhoeae cultures rapidly autolyse so it is not necessary to extract the bacterial DNA prior to amplification. The samples were run on a Roche LightCycler 2.0 using LightCycler FastStart DNA Master HybProbe master mix (Roche Applied Science, Switzerland) and were declared positive if their curves rose above 1.0 fluorescence before cycle 35 after absolute quantification. A previously characterized mosaic isolate that was prepared following the same protocol was included in every run as a positive control along with a negative water control. The following amplification conditions were used: 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.

Table 3.1: DNA sequences of primers used in RTPCR and amplification of the por, tbpB and penA genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTPCR forward</td>
<td>5'-GTTGGATGCCCCTACTGGG-3'</td>
</tr>
<tr>
<td>RTPCR reverse</td>
<td>5'-ACCGATTTTCTAAGGCCAGGG-3'</td>
</tr>
<tr>
<td>RTPCR probeb</td>
<td>5'-CGGCAAGGTGCAACCCGA-3'</td>
</tr>
<tr>
<td>por forward</td>
<td>5'-CAAGAAGACCTCGGCAA-3'</td>
</tr>
<tr>
<td>por reverse</td>
<td>5'-CCGACAAACCACTTGGT-3'</td>
</tr>
<tr>
<td>tbpB forward</td>
<td>5'-CGTTGTGCAGCGGCAAAC-3'</td>
</tr>
<tr>
<td>tbpB reverse</td>
<td>5'-TTCATCGGCTCGCTGCTGTTG-3'</td>
</tr>
<tr>
<td>penA forward 1st half</td>
<td>5'-GATCAAGTGATAATAAAGAAG-3'</td>
</tr>
<tr>
<td>penA reverse 1st half</td>
<td>5'-CTTAAGGCAAGGTATTTGAAT-3'</td>
</tr>
<tr>
<td>penA forward 2nd half</td>
<td>5'-GTTGGATGCCCGTACTG-3'</td>
</tr>
<tr>
<td>penA reverse 2nd half</td>
<td>5'-CAGCCAAAGGGGTTACTGTGAC-3'</td>
</tr>
</tbody>
</table>

a Reverse primers are presented in reverse and complimentary order to the genome.
b 5-6-carboxyfluorescein-3-6-carboxytetramethylrhodamine

3.2.5 Quality Control

For every Etest batch, two N. gonorrhoeae isolates with predetermined MICs were included as quality control strains. One isolate was susceptible to all four antimicrobials.
crobiolals and one isolate displayed alert value MICs to the cephalosporins. Control strains were considered accurate if their MICs were within ± 1 log₂ of the predetermined MIC. MICs for Etest control strains were determined through repeat testing by the SFDPH laboratory. For every RTPCR batch, a well characterized N. gonorrhoeae isolate with the SF-A mosaic penA allele was used as a positive control and a negative water control was included.

3.2.6 DNA sequencing

Isolates positive by RTPCR were subjected to penA sequencing as described previously [36]. Primers used in this study are shown in Table 3.1. Briefly, samples were prepared by diluting 5 µL of the liquid culture into 245 µL of distilled water. Liquid N. gonorrhoeae cultures rapidly autolyse so it is not necessary to extract the bacterial DNA prior to amplification. 5 µL of this diluted liquid culture was used as the template in the 20 µL reaction mix below. The samples were run on a Roche LightCycler 2.0 using LightCycler FastStart DNA Master HybProbe master mix and the amplification products were purified using the QiaQuick PCR Purification Kit (Qiagen, Netherlands) on the automated Qiacube platform. Sequencing was completed by MCLAB (South San Francisco, CA) and Elim Biopharmaceuticals (Hayward, CA) using the same primers that were used for amplification.

3.2.7 NG-MAST

The por and tbpB genes from isolates positive by RTPCR and a random sample of 50 RTPCR negative isolates from each county were amplified and sequenced as described previously [34]. Primers used in this study are shown in Table 3.1. Briefly, samples were prepared by extracting bacterial DNA from 200 µL of liquid culture using the QIAamp DNA Mini Kit on the automated Qiacube platform. DNA extraction was done because amplification efficiencies were low when using diluted liquid culture. 5 µL of this purified bacterial DNA was used as the template in a 20 µL reaction mix. The samples were run on a Roche LightCycler 2.0 using LightCycler FastStart DNA Master HybProbe master mix and the amplification products were purified using the QiaQuick PCR Purification Kit on the automated Qiacube platform. Sequencing was completed by MCLAB (South San Francisco, CA) and Elim Biopharmaceuticals (Hayward, CA) using the same primers that were used for amplification.

3.2.8 Data analysis

Isolates with alert value MICs were classified according to the 2010 CDC GISP alert value breakpoints, which were 0.125, 0.25, 0.25, and 2.0 µg/mL for ceftriaxone, cef-
podoxime, cefixime and azithromycin respectively. DNA sequence alignments were produced using MEGA 5.05 and the TeXshade package in \LaTeX. Boxplots and tables were produced using STATA 11. All sequences presented in this study are publicly available through Genbank (http://www.ncbi.nlm.nih.gov/genbank) and accession numbers are presented in Table 3.3.

3.3 Results

3.3.1 Phenotypic surveillance of isolates with alert value MICs

AST was completed on 678 isolates for ceftriaxone, 351 isolates for cefpodoxime, 332 isolates for cefixime and 682 isolates of azithromycin. Proportions of isolates with alert value MICs for each antimicrobial for San Francisco, Los Angeles, Orange County and San Diego are shown in Table 3.2. Isolates with alert value MICs were present at all four sites for cefpodoxime, three sites for ceftriaxone and two sites for azithromycin. No isolates with alert value MICs to cefixime were observed. Cefpodoxime showed the highest proportion of isolates with alert value MICs with San Francisco and San Diego above 5%. Ceftriaxone showed lower proportions of isolates with alert value MICs with San Francisco, San Diego and Orange County above 2%. Isolates with azithromycin alert value MICs were rare with only a single alert value isolate found in San Francisco and a single alert value isolate found in San Diego.

Table 3.2: Proportions of isolates with alert value MICs by county

<table>
<thead>
<tr>
<th>County</th>
<th>Ceftriaxone</th>
<th>Cefpodoxime</th>
<th>Cefixime</th>
<th>Azithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% alert</td>
<td>n</td>
<td>% alert</td>
</tr>
<tr>
<td>San Francisco</td>
<td>197</td>
<td>2.5</td>
<td>85</td>
<td>7.1</td>
</tr>
<tr>
<td>Los Angeles</td>
<td>172</td>
<td>2.3</td>
<td>108</td>
<td>4.6</td>
</tr>
<tr>
<td>Orange County</td>
<td>107</td>
<td>0</td>
<td>51</td>
<td>3.9</td>
</tr>
<tr>
<td>San Diego</td>
<td>202</td>
<td>3</td>
<td>107</td>
<td>7.5</td>
</tr>
</tbody>
</table>

3.3.2 Genotypic surveillance of mosaics penA alleles

Unfortunately, mosaic penA alleles have been described using multiple nomenclature systems that have yet to be standardized. To provide some clarity on the topic, Table 3.3 provides any alternate names, accession numbers, references and notable antimicrobial susceptibilities for all mosaic penA alleles described in this study.
Proportions of \textit{penA} genotypes by county are shown in Table 3.4. For this study, \textit{penA} genotypes were defined by 100% identity at the nucleotide sequence level. Overall, 59 (8.6%) isolates were positive by RTPCR, indicating the presence of a mosaic \textit{penA} allele. DNA sequencing revealed that 39 (5.7%) isolates had the SF-A \textit{penA} allele and four (0.6%) isolates had the SF-B \textit{penA} allele. The SF-A and SF-B alleles were both previously discovered in San Francisco in 2009 [41]. The SF-B mosaic \textit{penA} allele was initially characterized as a “partial mosaic” with slightly elevated, but not alert value MICs. The SF-A \textit{penA} allele is a well characterized mosaic \textit{penA} allele that has previously been associated with reduced susceptibility to cephalosporins. A pharyngeal isolate with the SF-A allele recently caused a treatment failure in Slovenia [51] with ceftriaxone and closely related mosaic \textit{penA} alleles have caused treatment failures in France (\textit{penA}-CI) [50], Austria (SF-A with T534A amino acid alteration) [49] and Hong Kong (Mosaic X) [44]. Sixteen (1.8%) isolates had a novel mosaic \textit{penA} allele that will be referred to as LA-A. SF-A was the most common mosaic \textit{penA} allele found in San Francisco, San Diego and Orange County. SF-B was rare and was only found in San Francisco and San Diego. LA-A was the most common mosaic \textit{penA} allele in Los Angeles and was also found in small numbers in Orange County and San Diego. It should be noted that it is possible that other mosaic \textit{penA} alleles are circulating in these counties that were not detected in this study. The RTPCR used to screen for the presence of a mosaic \textit{penA} allele was designed to detect a central region of homology shared between many mosaic \textit{penA} alleles, but it is possible that mosaic \textit{penA} alleles exist that are not detected by this assay [37].

Figure 3.3 shows a full length (1746 bp) multiple alignment of the mosaic \textit{penA} alleles found in this study, closely related \textit{penA} alleles that have been associated with treatment failures and the \textit{penA} allele from a wildtype reference strain (LM306). Using the wildtype \textit{penA} allele as a reference, only variable positions are shown with nucleotide positions displayed vertically above the alignment. Between bp 294-889, the SF-A, Mosaic X and penA-CI alleles all share sequence identity, while the SF-B...
Table 3.4: Proportions of *penA* alleles by county

<table>
<thead>
<tr>
<th><em>penA</em> genotype</th>
<th>San Francisco (n=199)</th>
<th>Los Angeles (n=172)</th>
<th>Orange County (n=107)</th>
<th>San Diego (n=206)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-A</td>
<td>6%</td>
<td>4.1%</td>
<td>3.7%</td>
<td>7.8%</td>
</tr>
<tr>
<td>SF-B</td>
<td>1.6%</td>
<td>0%</td>
<td>0%</td>
<td>0.5%</td>
</tr>
<tr>
<td>LA-A</td>
<td>0%</td>
<td>7.6%</td>
<td>1.9%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Wildtype</td>
<td>92.4%</td>
<td>88.3%</td>
<td>94.4%</td>
<td>91.2%</td>
</tr>
</tbody>
</table>

and LA-A sequences more closely resemble the wildtype sequence with a number of point mutations. Between bp 930-1182, all the mosaic alleles share high sequence homology with only four point mutations differentiating them. Previous studies have shown that this central region of homology is shared between many different mosaic *penA* alleles. For this reason, the RTPCR used to differentiate mosaic from non-mosaic alleles was designed to detect this region. From bp 1191 to the 3’ end of the gene, the SF-B sequence reverts to the wildtype sequence with a number of point mutations. From bp 1191-458, the SF-A, Mosaic X, *penA*-CI, and LA-A alleles continue to share high sequence homology. From bp 1464-1638, the SF-A, Mosaic X and *penA*-CI alleles continue to share high sequence homology while the LA-A allele diverges, but does not revert to the wildtype sequence. From bp 1645 to the 3’ end of the gene, the SF-A, *penA*-CI and SF-B alleles revert to the wildtype sequence while the Mosaic X and LA-A alleles show sequence differences until bp 1725 after which all five mosaic alleles share identity with the wildtype sequence.

Figure 3.4 shows a partial multiple alignment of the mosaic *penA* alleles found in this study, closely related *penA* alleles that have been associated with treatment failures and the *penA* allele from a wildtype reference strain (LM306). The SF-A allele has all three amino acid changes (I312M, V316T, and G545S) previously associated with reduced susceptibility to oral cephalosporins [44]. Homology modeling studies have shown that these amino acid changes result in conformational alterations of the β-lactam-binding pocket and affect the ability of cephalosporins to bind, particularly those with large R groups [40]. SF-B and LA-A both have two of the three amino acid changes previously associated with reduced susceptibility to oral cephalosporins (I312M, V316T). None of the mosaic *penA* alleles found in this study have the recently described amino acid change (A501P) found in the *penA*-CI allele that has been associated with high level resistance to both cefixime (MIC = 4.0) and ceftriaxone (MIC = 2.0). [9,50].
Chapter 3. Genotypic and phenotypic surveillance of *N. gonorrhoeae* antimicrobial resistance in California

Figure 3.3: Multiple alignment of full length mosaic penA alleles. Using the wildtype penA sequence as a reference, only variable positions are shown with nucleotide position presented vertically.
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Figure 3.4: Alignment of mosaic *penA* alleles (bp 930-955, 1495-1510, 1625-1640). Lower case letters indicate sequence differences from the wildtype allele (LM306). Shaded regions indicate codons that have been associated with reduced susceptibility to cephalosporins.

### 3.3.3 Association of the SF-A mosaic *penA* allele with alert value MICs

Table 3.5 shows proportions of isolates with alert value MICs by *penA* genotype. Every isolate with an alert value MIC for a cephalosporin had the SF-A *penA* allele, but not every SF-A *penA* allele resulted in an alert value MIC. The association was strongest for cefpodoxime where 88% of SF-A isolates displayed an alert value MIC. For ceftriaxone, 38% of SF-A isolates displayed an alert value MIC. These data closely match previously reported cephalosporin MICs from isolates with the SF-A *penA* allele and present additional evidence supporting the hypothesis that the G545S amino acid change is crucial to producing elevated cephalosporin MICs. The I312M and V316T amino acid changes seen in the SF-B and LA-A alleles may be necessary, but not sufficient to produce alert value MICs to the cephalosporins. No isolates with alert value MICs were observed for cefixime and both isolates that displayed alert value MICs for azithromycin had wildtype *penA* alleles. Azithromycin’s mechanism of action targets the 50s ribosomal subunit so alterations to the *penA* gene should not alter its MICs.

Table 3.5: Proportions of isolates with alert value MICs by *penA* genotype

<table>
<thead>
<tr>
<th><em>penA</em> genotype</th>
<th>Ceftriaxone</th>
<th>Cefpodoxime</th>
<th>Cefixime</th>
<th>Azithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% alert</td>
<td>n</td>
<td>% alert</td>
</tr>
<tr>
<td>SF-A</td>
<td>39</td>
<td>38</td>
<td>24</td>
<td>88</td>
</tr>
<tr>
<td>SF-B</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>LA-A</td>
<td>16</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Wildtype</td>
<td>619</td>
<td>0</td>
<td>314</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3.5 shows box plots of MIC distributions by *penA* genotype. The SF-A
penA allele was clearly associated with elevated MICs for all three cephalosporins, although for ceftriaxone and cefixime, the majority of MICs remained below the alert value breakpoints. The MIC distributions of isolates with the SF-A allele also covered a wide range, indicating that other determinants of resistance are likely important in producing alert value MICs. Three wildtype isolates showed slightly elevated MICs for the cephalosporins and were considered outliers of the distributions.

Figure 3.5: Boxplots of MIC distributions by penA genotype. N = 678, 351, 332 and 682 for ceftriaxone, cefpodoxime, cefixime and azithromycin respectively. Boxes indicate the 25th, 50th, and 75th percentiles. Whiskers indicate the lowest datum within 1.5 IQR of the lower quartile, and the highest datum within 1.5 IQR of the upper quartile. Outliers are shown as gray circles. Gray horizontal bars indicate the alert value breakpoint for each antimicrobial.
3.3.4 Population structure of isolates with mosaic penA alleles

Table 3.6 shows the por alleles, tbpB alleles and NG-MAST sequence types (STs) for isolates with a mosaic penA allele. In 2009, the SF-A and SF-B penA alleles were only found in ST1407 and a very closely related strain (ST1513, tbpB 110, por 971 - 1 bp difference from por 908) in San Francisco and appeared to be spreading in a clonal fashion. In this study, ST1407 (tbpB allele 110, por allele 908) was the most common ST of isolates with a mosaic penA allele. Every ST1407 isolate had either the SF-A or SF-B penA allele and this sequence type was not seen in any isolate with a wildtype penA allele. The SF-B penA allele has remained clonal within ST1407. In SF-A isolates with STs other than ST1407, the tbpB allele was the same as ST1407 (tbpB allele 110) or was a closely related tbpB allele (tbpB allele 1431, 2 bp difference). Por alleles from isolates with the SF-A penA allele, however, varied widely from por 908 and are discussed below.

ST7268 (tbpB allele 18, por allele 4340) was the most common NG-MAST sequence type of isolates with the LA-A penA allele. This ST, however, was also commonly seen in isolates with a wildtype penA allele in the same counties where the LA-A penA allele was found. In LA-A isolates with STs other than ST7268, the tbpB allele was similar to tbpB allele 18 in one case (tbpB allele 1429, 1 bp difference) and quite different in two others (tbpB allele 81, 27 bp different and tbpB allele 1430, 21 bp different).

Figure 3.6 shows a multiple alignment of the 490 bp por sequences used for NG-MAST of isolates with a mosaic penA allele. Por allele 908 was the dominant por allele associated with the SF-A and SF-B penA alleles, but the SF-A penA allele was also found in isolates with unrelated por alleles. From bp 225 to the end of the sequence, the set of closely related por alleles found in isolates with the SF-A penA allele (1903, 1900, 3669, 2684, 4599, 2622, 3424, 1388) are nearly identical with fewer then four mutations seperating them. Por 4544 is also closely related to this set, but appears to have a codon insertion at bp 333. The SF-A allele was also found in isolates with por 2700, which is very closely related to por 4547 and por 4340 from bp 1-216. The LA-A allele was restricted to isolates with por 4340, 4545, 4546 and 4547. Por 4545 and 4546 appear to have a codon insertion at bp 347, while por 4340 and 4547 are closely related to por 908 from bp 1-437 after which they share homology with por 2700, but diverge from por 908.

3.3.5 Population structure of isolates with wildtype penA alleles

Table 3.7 shows the most common NG-MAST sequence types of isolates with a wildtype penA allele. Except for ST7268, none of the STs from isolates with a mosaic penA allele were found in wildtype penA isolates. The most common wildtype penA
Table 3.6: NG-MAST sequence types of isolates with a mosaic penA allele

<table>
<thead>
<tr>
<th>count</th>
<th>penA genotype</th>
<th>tbpB allele</th>
<th>por allele</th>
<th>NG-MAST ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>SF-A</td>
<td>110</td>
<td>908</td>
<td>1407</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>110</td>
<td>1388</td>
<td>2212</td>
</tr>
<tr>
<td>2</td>
<td>SF-A</td>
<td>110</td>
<td>1903</td>
<td>3149</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>110</td>
<td>2622</td>
<td>4275</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>110</td>
<td>2684</td>
<td>4378</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>110</td>
<td>3424</td>
<td>5643</td>
</tr>
<tr>
<td>4</td>
<td>SF-A</td>
<td>110</td>
<td>2700</td>
<td>5895</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>110</td>
<td>3669</td>
<td>6200</td>
</tr>
<tr>
<td>2</td>
<td>SF-A</td>
<td>110</td>
<td>4544</td>
<td>7560</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>110</td>
<td>4599</td>
<td>7647</td>
</tr>
<tr>
<td>1</td>
<td>SF-A</td>
<td>1431(^a)</td>
<td>1900</td>
<td>7566</td>
</tr>
<tr>
<td>4</td>
<td>SF-B</td>
<td>110</td>
<td>908</td>
<td>1407</td>
</tr>
<tr>
<td>10</td>
<td>LA-A</td>
<td>18</td>
<td>4340</td>
<td>7268</td>
</tr>
<tr>
<td>2</td>
<td>LA-A</td>
<td>18</td>
<td>4546</td>
<td>7564</td>
</tr>
<tr>
<td>1</td>
<td>LA-A</td>
<td>18</td>
<td>4545</td>
<td>7646</td>
</tr>
<tr>
<td>1</td>
<td>LA-A</td>
<td>81(^b)</td>
<td>4340</td>
<td>7561</td>
</tr>
<tr>
<td>1</td>
<td>LA-A</td>
<td>1429(^c)</td>
<td>4340</td>
<td>7562</td>
</tr>
<tr>
<td>1</td>
<td>LA-A</td>
<td>1430(^d)</td>
<td>4547</td>
<td>7565</td>
</tr>
</tbody>
</table>

\(^a\) tbpB allele 1431 is 2 bp different from \(tbpB\) allele 110.
\(^b\) tbpB allele 81 is 27 bp different from \(tbpB\) allele 18.
\(^c\) \(tbpB\) allele 1429 is 1 bp different from \(tbpB\) allele 18.
\(^d\) \(tbpB\) allele 1430 is 21 bp different from \(tbpB\) allele 18.
Figure 3.6: Multiple alignment of NG-MAST por sequences. Using the por 908 sequence as a reference, only variable positions are shown with nucleotide position presented vertically.
STs from isolates with wildtype penA alleles were found in similar numbers at each study site and no geographic pattern was observed. Por 908 was a common por allele in isolates with wildtype penA alleles, but the tbpB alleles from these isolates (tbpB 29, tbpB 563) were not closely related to tbpB 110, which was found in ST1407.

Table 3.7: Most common NG-MAST sequence types of isolates with a wildtype penA allele

<table>
<thead>
<tr>
<th>count</th>
<th>penA genotype</th>
<th>tbpB allele</th>
<th>por allele</th>
<th>NG-MAST ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Wildtype</td>
<td>29</td>
<td>1808</td>
<td>2992</td>
</tr>
<tr>
<td>10</td>
<td>Wildtype</td>
<td>29</td>
<td>908</td>
<td>3935</td>
</tr>
<tr>
<td>7</td>
<td>Wildtype</td>
<td>743</td>
<td>30</td>
<td>3307</td>
</tr>
<tr>
<td>7</td>
<td>Wildtype</td>
<td>18</td>
<td>4340</td>
<td>7268</td>
</tr>
<tr>
<td>7</td>
<td>Wildtype</td>
<td>563</td>
<td>1489</td>
<td>2400</td>
</tr>
<tr>
<td>5</td>
<td>Wildtype</td>
<td>4</td>
<td>19</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>Wildtype</td>
<td>563</td>
<td>908</td>
<td>7574</td>
</tr>
</tbody>
</table>

*a Only NG-MAST STs found in 5 or more copies are shown.

### 3.4 Discussion

_N. gonorrhoeae_ has an extensive record of drug resistance and has demonstrated that it is capable of utilizing a variety of mechanisms to escape antimicrobial pressure. Third generation cephalosporins have been the CDC recommended treatment only since 2007, but it appears that their usefulness is already on the decline. Cephalosporin resistance caused by strains with mosaic penA alleles continues to spread. In 2012, the CDC again updated its recommendations and abandoned oral cephalosporins in favor of dual therapy with an injectable third generation cephalosporin and a macrolide. In California in 2011, proportions of isolates with alert value MICs to cefpodoxime rose above 5%, which is traditionally the level at which a new class of antimicrobials is recommended. In San Francisco and San Diego, the proportions were above 7% for cefpodoxime and three counties had proportions above 2% for ceftriaxone. These proportions indicate that the useful lifespan of third generation cephalosporins will likely be limited for the treatment of _N. gonorrhoeae_ in this setting.

The SF-A mosaic penA allele found in this study is of most concern because it resulted in cephalosporin MICs capable of causing treatment failures. Although the SF-B and LA-A penA alleles both have two of the three amino acid changes associated with reduced susceptibility to oral cephalosporins, the distributions of cephalosporin
MICs for isolates with these alleles were similar to those with wildtype penA alleles. These data agree with previously published reports that the I312M and V316T amino acid changes act synergistically with the G545S amino acid change, but that they are incapable of producing alert value cephalosporin MICs without it.

Since the SF-A penA allele was first detected in 2009 in San Francisco, it has been described as an internationally successful clone within ST1407 (por 908, tbpB 110) and closely related STs. Unlike previous reports, however, this study found the SF-A allele in isolates with por alleles not closely related to por allele 908. This indicates that the new por alleles associated with the SF-A penA allele were not created by genetic drift of por 908, but instead represent new strain lineages. The multiple alignments of mosaic penA alleles in Figure 3.3 and por alleles in Figure 3.4 both show recombination as the major driver of diversity. N. gonorrhoeae is known to be a highly recombinogenic organism and the sequence block diversity seen in both penA and por appear to have been created through this mechanism.

ST1407 remains the dominant ST associated with cephalosporin resistance in California, but strains with unrelated STs now carry the SF-A penA allele. Whole genome sequencing of an ST5895 isolate from San Francisco recently detailed the transfer of the SF-A penA allele into a completely different genetic background [19]. In this study, four ST5895 isolates with the SF-A allele were found in Los Angeles, Orange County and San Diego indicating that this strain is likely circulating throughout California. Unfortunately, the horizontal spread of the SF-A penA allele into new STs makes tracking the spread of cephalosporin resistance with NG-MAST problematic.

Of note is the fact that the SF-A penA allele was only found in isolates with tbpB allele 110 or a very closely related allele (tbpB 1431, 2 bp different from tbpB allele 110). In the bacterial genome, penA is located 50 kb downstream of tbpB. The linkage between these two loci likely explains the high level of clonality of tbpB alleles in isolates with the SF-A penA allele. Interestingly, the LA-A penA allele shares many of the mutations in the 3’ half of the SF-A penA allele, but is not associated with tbpB allele 110. This result would be explained by a model where the recombination event that formed the LA-A penA allele occurred in the middle of the SF-A penA allele, but did not include the additional sequence containing tbpB allele 110. The linkage between penA and tbpB is also seen in isolates with the LA-A penA allele where 81% have tbpB 18.

Another concern with the SF-A penA allele is its potential to produce isolates with high level resistance to injectable cephalosporins. The SF-A penA allele is only a single point mutation different from the penA-CI penA allele described in France that exhibited high-level resistance to both oral and injectable cephalosporins [50]. This additional mutation might be capable of increasing ceftraxone MICs from the elevated levels seen in this study (0.094-0.25 µg/mL) to levels at which ceftriaxone treatment failures have occurred even with much higher 1 g doses (MIC = 1.0-2.0
The penA-CI allele was also found in an ST1407 strain indicating a genetically similar strain, but it is possible that determinants of resistance other than penA also affect the cephalosporin MICs observed.

Dual therapy with an injectable cephalosporin and a macrolide is now recommended by the CDC in an attempt to contain the spread of resistant strains and prevent the emergence of new ones. The data here support this recommendation since no isolates were found with alert value MICs to both azithromycin and a cephalosporin. Two isolates with alert value MICs to azithromycin were detected in San Francisco and San Diego, however, so the emergence of dual-resistant strains in California remains a possibility. Unfortunately, N. gonorrhoeae isolates resistant to both azithromycin and cephalosporins have already been reported in Japan and France [38,50].

Detecting strains with reduced susceptibility to cephalosporins is a challenge in the U.S. The vast majority of N. gonorrhoeae infections are diagnosed using NAAT methods where no culture is available. Without a culture and AST, molecular methods must be developed that are capable of detecting isolates with reduced susceptibility to cephalosporins. The data presented here demonstrate that NG-MAST is no longer a suitable method for this task. Assays capable of detecting the specific penA alleles associated with reduced susceptibility to cephalosporins will provide clinical and public health laboratories with the information necessary to track the spread of resistant strains and detect potential treatment failures.

The main limitation of this study is the sampling strategy used to collect N. gonorrhoeae isolates. The samples were collected in parallel with the CDC GISP which collects isolates only from men. Additionally, the samples were collected from public health clinics whose populations may not be representative of the general population and are known to oversample MSM. Since MSM populations have historically been associated with higher rates of antimicrobial resistance, it is possible that these data overestimate the prevalence of alert value isolates in the general population.

In conclusion, N. gonorrhoeae isolates with reduced susceptibility to cephalosporins are currently circulating in California. Isolates with the same NG-MAST ST, penA allele and cephalosporin MICs have caused verified treatment failures elsewhere. Enhanced surveillance efforts such as the use of molecular assays to detect resistance determinants and the implementation of test of cure protocols will be necessary to ensure that empirical treatment remains effective.
Chapter 4

Improved RTPCR for the detection of *N. gonorrhoeae* mosaic penA alleles associated with elevated cephalosporin MICs

4.1 Introduction

*N. gonorrhoeae* isolates with reduced susceptibility to cephalosporins are currently circulating worldwide and have been linked to treatment failures with both oral and injectable cephalosporins [8]. These treatment failures have been caused by isolates with mosaic penA alleles, resulting in altered penicillin-binding protein 2 (PBP-2) and elevated cephalosporin MICs. In 2008, Ochiai et al. published a TaqMan RTPCR assay capable of detecting mosaic penA alleles that share a region of homology between bp 970 and bp 990 [37]. In 2011, a study in California detected three different mosaic penA alleles using this assay, but only one of these penA alleles (SF-A) was associated with elevated cephalosporin MICs. This penA allele was also recently found in an isolate that caused a verified treatment failure to ceftriaxone in Slovenia [51]. Since isolates with elevated MICs are of the greatest interest to public health, the RTPCR primers were modified to improve the assay by detecting only the SF-A mosaic penA allele and closely related mosaic penA alleles that have caused treatment failures in France (penA-CI) [50], Austria (SF-A with T534A amino acid alteration) [49], and Hong Kong (mosaic X) [44]. This was accomplished by designing forward and reverse primers with terminal 3’ mismatches to prevent the amplification of mosaic penA alleles that are not associated with elevated cephalosporin MICs and treatment failures. This study presents data on the validation of this improved RTPCR for use
on both *N. gonorrhoeae* isolates and clinical specimens.

### 4.2 Materials and methods

#### 4.2.1 Isolate and specimen collection

*N. gonorrhoeae* isolates with the SF-A, SF-B, LA-A and non-mosaic *penA* alleles were collected as part of a state-wide phenotypic and genotypic surveillance study examining the link between mosaic *penA* alleles and cephalosporin MICs in California in 2011. Briefly, urethral isolates were collected from men visiting public health clinics in Los Angeles, San Francisco, San Diego and Orange Counties in California. A presumptive identification of *N. gonorrhoeae* was based on the following criteria: growth of typical appearing colonies on a selective medium such as Thayer-Martin at 35 $^\circ$C to 36.5 $^\circ$C in 5% CO$_2$, a positive oxidase test, and the observation of gram-negative, diplococci in stained smears. A single *N. gonorrhoeae* was picked and subcultured from the selective primary medium on a Chocolate II Agar (GC II Agar with Hemoglobin and 1% IsoVitaleX, Beckton-Dickenson, USA) plate. After 18 to 20 hours incubation, growth from the pure culture was suspended in TSB containing 20% (v/v) glycerol, frozen at -80 $^\circ$C and sent overnight on dry ice to the SFDPH laboratory. Isolates with the penA-CI, mosaic X and Austrian *penA* alleles were not available for testing, but sequences were obtained from their corresponding publications for alignment. Clinical specimens were obtained from the San Francisco Department of Public Health Laboratory in 2012.

#### 4.2.2 Human subjects

Isolates used in this study were collected for public health surveillance and de-identified prior to AST, thus this work was considered public health practice and was exempt from human subjects regulations. [20]. This category of research is not considered human subjects research and is not subject to Institutional Review Board oversight.

#### 4.2.3 NAAT

All clinical specimens were tested using the APTIMA Combo 2 (Gen-Probe, San Diego, CA) to determine whether *N. gonorrhoeae* was present prior to testing by RTPCR. For three of the urine specimens, matched urethral isolates with the SF-A mosaic *penA* allele and elevated MICs to cephalosporins were available for testing.
4.2.4 RTPCR

Table 4.1 shows the primers and probe used in this study. From isolates, samples were prepared by diluting 5 µL of liquid culture into 245 µL of distilled water. 5 µL of this diluted liquid culture was used as the template in a 20 µL RTPCR reaction. Liquid *N. gonorrhoeae* cultures rapidly autolyse so it is not necessary to extract the bacterial DNA prior to amplification. From clinical specimens, samples were prepared by extracting bacterial DNA from 200 µL of the APTIMA specimen buffer using the QIAamp DNA Mini Kit on the automated Qiacube platform. 5 µL of this purified genomic DNA was used as the template in a 20 µL RTPCR reaction. The samples were run on a Roche LightCycler 2.0 using LightCycler FastStart DNA Master HybProbe master mix (Roche Applied Science, Switzerland) and were declared positive if their curves rose above 1.0 fluorescence before cycle 35 after absolute quantification. The following amplification conditions were used: 2 min at 50°C, 10 min at 95°C, and 35 cycles of 15 s at 95°C and 60 s at 65°C.

Table 4.1: DNA sequences of primers used in the improved RTPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improved RTPCR forward</td>
<td>5'-TCAATACGCCTGCCTATGAG-3'</td>
</tr>
<tr>
<td>Improved RTPCR reverse</td>
<td>5'-GCACATCCAAAGTAGGATAAACG-3'</td>
</tr>
<tr>
<td>RTPCR probeb</td>
<td>5'-CGGCAAAGTGATGCAACCGA-3'</td>
</tr>
</tbody>
</table>

a Reverse primers are presented in reverse and complimentary order to the genome.

b 5-6-carboxyfluorescein-3-6-carboxytetramethylrhodamine

Figure 4.1 shows a multiple sequence alignment of mosaic penA alleles found in California in 2011 and the improved RTPCR primers and probe. Sequences of the primers and probe match the SF-A penA allele’s sequence. PenA-CI, mosaic X, and the penA allele described in Austria in 2011 (SF-A with T534A amino acid alteration) all have 100% sequence identity with SF-A in this region (bp 797-1116).

4.2.5 Data Analysis

Statistical measures of assay performance were calculated using a dataset of 684 isolates collected for a previous study in California in 2011. The dataset included MICs obtained by Etest (ceftriaxone, cefpodoxime, cefixime and azithromycin), RTPCR results using the Ochiai et al. (2008) assay and penA genotypes. For this study, penA genotypes were defined by 100% identity at the nucleotide sequence level. MICs were classified according to the 2010 CDC GISP alert value breakpoints, which were 0.125,
Chapter 4. Improved RTPCR for the detection of \textit{N. gonorrhoeae} mosaic \textit{penA} alleles associated with elevated cephalosporin MICs

Figure 4.1: Alignment of mosaic \textit{penA} alleles (bp 797-1116), a wildtype strain (LM306) and improved RTPCR primers and probe. Lower case letters indicate sequence differences from the wildtype allele (LM306). Ochiai et al. (2008) primers are shown in gray and the improved primers are shown in black.
Chapter 4. Improved RTPCR for the detection of *N. gonorrhoeae* mosaic *penA* alleles associated with elevated cephalosporin MICs

0.25, 0.25, and 2.0 µg/mL for ceftriaxone, cefpodoxime, cefixime and azithromycin respectively. DNA sequence alignments were produced using MEGA 5.05 and the TeXshade package in \LaTeX. Boxplots and tables were produced using STATA 11. RTPCR crossing points (CP), the cycle at which the sample’s fluorescence rises above background, were automatically calculated using the Roche LightCycler 2.0 software package and the absolute quantitation setting.

### 4.3 Results

#### 4.3.1 Validation of the improved RTPCR using urethral isolates

Table 4.2 shows urethral isolates tested by the improved RTPCR. The assay successfully detected isolates with the SF-A mosaic *penA* allele and showed no amplification of the SF-B or LA-A *penA* alleles. Six isolates with each mosaic *penA* allele and 20 wildtype isolates were tested to validate the assay. CP values for positive urethral isolates were between 16-22 cycles.

Table 4.2: Isolates tested with the improved RTPCR

<table>
<thead>
<tr>
<th>Count</th>
<th>Source</th>
<th><em>penA</em> genotype</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Urethral</td>
<td>SF-A</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Urethral</td>
<td>SF-B</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Urethral</td>
<td>LA-A</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Urethral</td>
<td>wildtype</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 4.3.2 Validation of the improved RTPCR using clinical specimens

Table 4.3 shows clinical specimens tested by the improved RTPCR. Again, the assay successfully detected the three urine specimens matched to urethral isolates with the SF-A allele. No amplification was seen in pharyngeal specimens without *N. gonorrhoeae* indicating that the assay does not cross react with *penA* alleles from other commensal *Neisseria* spp. or unrelated bacteria in the pharynx. Matched pharyngeal *N. gonorrhoeae* positive specimens were not available for testing. CP values for positive urine specimens were between 24-28 cycles.

#### 4.3.3 Statistical and graphical measures of RTPCR performance

Table 4.4 shows statistical measures of assay performance for the improved RTPCR and the Ochiai et al. (2008) assay. Results of the Ochiai et al. (2008) assay and
Chapter 4. Improved RTPCR for the detection of *N. gonorrhoeae* mosaic *penA* alleles associated with elevated cephalosporin MICs

Table 4.3: Clinical specimens tested with the improved RTPCR

<table>
<thead>
<tr>
<th>Count</th>
<th>Source</th>
<th><em>penA</em> genotype</th>
<th>NAAT result$^a$</th>
<th>RTPCR Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>urine</td>
<td>SF-A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>urine</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>pharyngeal swab</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Tested by APTIMA Combo 2.

Etest MICs from 684 *N. gonorrhoeae* collected in California in 2011 were used to compare the assays. The data for the improved RTPCR was generated *in silico* using the same dataset with the assumption that only isolates with the SF-A mosaic *penA* allele would be reactive as was observed during the validation presented in Table 4.2. The improved RTPCR showed better performance in predicting all three alert value MIC categories. Positive predictive values (PPV) were high for the cefpodoxime (PPV = 88%) and the any cephalosporin (PPV = 74%) alert value categories. The assay was less able to predict ceftriaxone alert values (PPV = 38%). No alert value MICs were seen in RTPCR negative isolates, resulting in 100% sensitivity and 100% negative predictive value (NPV) for all alert value categories. Few false negatives were seen and specificity was high for all alert value categories (>96%). Data for cefixime is not shown because no alert value MICs were observed for this antimicrobial. Data for azithromycin is not shown because mosaic *penA* alleles are not associated with elevated MICs to this antimicrobial.

Table 4.4: Statistical measures of RTPCR performance in predicting cephalosporin alert value MICs

<table>
<thead>
<tr>
<th>Assay</th>
<th>n</th>
<th>Alert value category$^a$</th>
<th>PPV$^b$</th>
<th>NPV$^c$</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ochiai</td>
<td>350</td>
<td>any cephalosporin</td>
<td>49%</td>
<td>100%</td>
<td>100%</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>678</td>
<td>ceftriaxone</td>
<td>25%</td>
<td>100%</td>
<td>100%</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>351</td>
<td>cefpodoxime</td>
<td>57%</td>
<td>100%</td>
<td>100%</td>
<td>95%</td>
</tr>
<tr>
<td>Improved</td>
<td>350</td>
<td>any cephalosporin</td>
<td>74%</td>
<td>100%</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>678</td>
<td>ceftriaxone</td>
<td>38%</td>
<td>100%</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>351</td>
<td>cefpodoxime</td>
<td>88%</td>
<td>100%</td>
<td>100%</td>
<td>99%</td>
</tr>
</tbody>
</table>

$^a$ Alert value breakpoint were 0.125 µg/mL, 0.25 µg/mL, 0.25 µg/mL, and 2.0 µg/mL for ceftriaxone, cefpodoxime, cefixime and azithromycin respectively.

$^b$ Positive predictive value.

$^c$ Negative predictive value.

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Chapter 4. Improved RTPCR for the detection of *N. gonorrhoeae* mosaic *penA* alleles associated with elevated cephalosporin MICs

Figure 4.2 shows boxplots of MIC distributions by improved RTPCR positivity. Isolates positive by the improved RTPCR clearly showed elevated MICs to ceftriaxone, cefpodoxime and cefixime compared with RTPCR negative isolates. No association between azithromycin MICs and improved RTPCR positivity was observed.

### 4.4 Discussion

The RTPCR developed in this study successfully detected isolates with the SF-A mosaic *penA* allele without amplifying other mosaic *penA* alleles that are not associated with elevated MICs to cephalosporins (SF-B, LA-A). It also successfully detected the SF-A *penA* allele in urine specimens and produced no false positives from commensal *Neisseria* spp. or other flora in *N. gonorrhoeae* negative urine and pharyngeal specimens. In a retrospective *in silico* analysis, the assay showed increased ability to detect isolates with alert value MICs to cephalosporins compared with the Ochiai et al. (2008) assay. These data indicate that the improved RTPCR may be useful as a surveillance tool to track the spread of isolates with elevated MICs to cephalosporins.

This assay, however, has several limitations. The positive predictive value of this RTPCR was improved when compared with the Ochiai et al. (2008) RTPCR, but isolates were still detected that had MICs below the alert value breakpoints as defined by CDC GISP. CLSI has yet to define resistance categories for cephalosporin MICs in *N. gonorrhoeae*, so the exact MIC breakpoint capable of causing a treatment failure remains unclear. Isolates with MICs at the CDC GISP alert value breakpoints have caused verified treatment failures (ceftriaxone MIC = 0.125 µg/mL), but some of these were from pharyngeal infections where cephalosporins are known to be less effective. The data in Figure 4.2 clearly shows that RTPCR positive isolates had elevated MIC distributions, but the exact relationship between cephalosporin MICs and clinical outcomes at different sites will require further study to fully elucidate. Therefore, the improved assay’s ability to predict treatment failures is not well defined.

Another potential limitation of the assay is its use in different geographic areas. The *penA* genotypes which the assay was designed to discriminate between were detected in California in 2011. It is possible that other mosaic *penA* alleles are circulating elsewhere that produce elevated MICs, but do not share the region of homology that this RTPCR detects. For example, the H041 isolate recently described in Japan [38] produced very high MICs to cephalosporins (ceftriaxone MIC = 4µg/mL) and had a number of mutations in the RTPCR probe and primer sites. Fortunately, this isolate was only found in a single patient and has not been detected elsewhere. An isolate or genetic material from the H041 mosaic *penA* allele was not available for testing so it is unknown whether it would be detected by this RTPCR.

Finally, this RTPCR should be used with caution on clinical specimens because of
Chapter 4. Improved RTPCR for the detection of *N. gonorrhoeae* mosaic *penA* alleles associated with elevated cephalosporin MICs

![Boxplots of MIC distributions by improved RTPCR positivity.](image)

Figure 4.2: Boxplots of MIC distributions by improved RTPCR positivity. Boxes indicate the 25th, 50th, and 75th percentiles. Whiskers indicate the lowest datum within 1.5 IQR of the lower quartile, and the highest datum within 1.5 IQR of the upper quartile. Outliers are shown as gray circles. Gray horizontal bars indicate the alert value breakpoint for each antimicrobial.
Chapter 4. Improved RTPCR for the detection of *N. gonorrhoeae* mosaic *penA* alleles associated with elevated cephalosporin MICs

the potential for cross-reactivity with *penA* alleles from other *Neisseria* spp. Mosaic *penA* alleles are composed of pieces of genetic material obtained through homologous recombination with other *Neisseria* spp. so cross-reactivity is of particular concern with mixed clinical specimens. The primers and probe showed a number of common hits by NCBI BLAST (http://blast.ncbi.nlm.nih.gov) in other *Neisseria* spp. such as *N. meningitidis*, *N. cinerea* and *N. perflava*. In the *N. gonorrhoeae* negative pharyngeal specimens tested no false positives were seen, but ideally the assay should be validated at each laboratory due to geographical differences in the commensal *Neisseria* spp. present. The *N. gonorrhoeae* negative urethral specimens also produced no false positives. False positives should be less of a concern in urine specimens, since, with the exception of rare *N. meningitidis* colonization, *Neisseria* commensals are not present at that site.

In conclusion, the assay presented here is a simple and rapid screening tool for the detection of isolates with mosaic *penA* alleles associated with elevated cephalosporin MICs. It may also be useful for the detection of these alleles in clinical specimens and the identification of potential treatment failures.
Chapter 5

Conclusion

*N. gonorrhoeae* has proven its ability to evolve or acquire the adaptations necessary to escape antimicrobial pressure multiple times. In the last 20 years, the medical community has lost three of the most potent and effective tools in the treatment of *N. gonorrhoeae* - tetracycline, fluoroquinolones (ciprofloxacin) and oral third generation cephalosporins (cefixime). In August 2012, the CDC updated their recommendations for the treatment of uncomplicated gonococcal infection to only include dual therapy with an injectable third generation cephalosporin and a macrolide. The data presented in Chapter 3 demonstrates that during 2011, antimicrobial resistance to oral cephalosporins in California rose above the 5% level at which treatment recommendations are traditionally changed. Interestingly, the pattern of the spread of resistance to third generation cephalosporins caused by mosaic *penA* alleles closely mirrors the patterns seen with tetracyclines and fluoroquinolones. Resistance to these antimicrobials initially emerged in Asia, then spread to Hawaii and the Pacific Islands, then to the western coast of the United States and finally spread from west to east across the United States [13]. Resistance to third generation cephalosporins has followed this pattern exactly with the initial detection of resistance in Japan in 2001, rising MICs and a potential treatment failure in Hawaii in 2003, mosaic *penA* alleles detected in San Francisco in 2007 and finally elevated MICs in isolates with the SF-A *penA* allele in both Northern and Southern California in 2011.

The loss of oral cephalosporins at a treatment option was a major setback for public health efforts since they were the last line of oral antimicrobials available to treat *N. gonorrhoeae*. Many public health agencies lack the capacity to deliver intravenous medications and PDPT is not possible without oral antimicrobials. With such a narrow spectrum of antimicrobials available for the treatment of uncomplicated gonococcal infection, it is crucial that public health agencies increase surveillance efforts to detect resistant isolates and support efforts to prevent their spread.

One major barrier to increasing surveillance efforts is the labor, supplies, and slow
Chapter 5. Conclusion

turn around time involved in traditional AST methods like agar dilution. CDC funded regional laboratories are some of the few public health laboratories in the United States with the capacity to complete this type of testing and the turnaround time for results can range from 1-4 months. The data presented in Chapter 2 showed that Etest is a valid AST method that requires a fraction of the labor and supplies necessary for agar dilution. It should be noted that for ceftriaxone, Etest showed a bias toward higher MICs compared with agar dilution. Ceftriaxone results should therefore be interpreted with caution and the media used for AST with this antimicrobial should be chosen carefully. With Etest, a laboratory can easily determine \(N. \text{gonorrhoeae}\) MICs in less than a week. This greatly increases the chance of detecting treatment failures in the field and allows physicians to quickly follow-up with patients that may need additional treatment. In populations that are transient or that don’t have consistent access to health care, rapid AST can result in effective public health interventions instead of passive surveillance.

Another major barrier to increasing antimicrobial resistance surveillance efforts is the lack of clinical laboratories which are capable of conducting AST on \(N. \text{gonorrhoeae}\). Currently, the vast majority of urethral gonococcal infections are diagnosed by NAAT. The progression from culture-based laboratory methods to NAAT over the last 30 years enabled the development of automated high-throughput testing platforms, but also resulted in a rapid decline in the number of clinical laboratories with the capacity to culture \(N. \text{gonorrhoeae}\). NAAT does not require a live specimen and it is impossible to conduct AST on non-living specimens with culture-based methods like Etest or agar dilution. The RTPCR presented in Chapter 4 offers a potential solution to the problem of detecting potentially resistant strains. Molecular assays capable of predicting phenotypic antimicrobial resistance are already in use for other infectious agents including HIV and tuberculosis. The likelihood that culture capacity in the United States will return is low so identifying and detecting the molecular determinants of resistance directly represents a new model for the future of AST.

Finally, it should be noted that although the rates of isolates with elevated MICs to injectable third generation cephalosporins are low in the United States, they have been increasing steadily over the last five years. Dual therapy with azithromycin and ceftriaxone may delay the spread of these isolates, but using history as a guide, resistance will inevitably increase to the point where injectable third generation cephalosporins will no longer work for empirical therapy. Table 5.1 shows the current antimicrobials used to treat \(N. \text{gonorrhoeae}\) infection and potential replacements if resistance to third generation cephalosporins becomes widespread. Newer classes of antimicrobials such as carbapenems, aminoglycosides, aminocyclitols and glycylcyclines may provide solutions to the growing problem of antimicrobial resistance in \(N. \text{gonorrhoeae}\), but some have argued that these antimicrobials should be reserved for the future or only used for pan-resistant infections that are untreatable with current antimicro-
bials. The other option would be a paradigm shift in the way antimicrobials are used in clinical practice. Rather than using antimicrobials in empirical therapy, targeted therapy would utilize the best antimicrobial based upon the susceptibility profile of each specific infection. This would allow infections that are still susceptible to older antimicrobials to be effectively treated with antimicrobials such as tetracyclines or fluoroquinolones. It would also reserve the newest classes of antimicrobials for infections resistant to all other treatments. At this time, the technology and laboratory capacity to initiate such a paradigm shift does not exist, but the shift from culture-based laboratory methods to molecular methods has laid the foundation for targeted therapy and the intelligent use of antimicrobials to become a reality in the future.

Table 5.1: Current and potential future antimicrobials used in the treatment of *N. gonorrhoeae* infection

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Class</th>
<th>Route</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftriaxone</td>
<td>Cephalosporin</td>
<td>Intramuscular</td>
<td>250 mg</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Macrolide</td>
<td>Oral</td>
<td>1 g</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Aminoglycoside</td>
<td>Oral</td>
<td>2 g</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Rifamycin</td>
<td>Oral</td>
<td>1-2 g</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Aminocyclitol</td>
<td>Intramuscular</td>
<td>2 g</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>Carbapenem</td>
<td>Intramuscular</td>
<td>1 g</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>Glycylcycline</td>
<td>Intravenous</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Glossary

AST  Antimicrobial Susceptibility Testing.
ATCC  American Type Culture Collection.
AZI  Azithromycin.
CDC  U.S. Centers for Disease Control and Prevention.
CDPH  California Department of Public Health.
CFM  Cefixime.
CLSI  Clinical and Laboratory Standards Institute.
CP  Crossing point.
CPD  Cefpodoxime.
CRO  Ceftriaxone.
DNA  Deoxyribonucleic Acid.
GISP  Gonococcal Isolate Surveillance Program.
MIC  Minimum Inhibitory Concentration.
mRNA  Messenger ribonucleic acid.
MSM  Men who have sex with men.

*N. gonorrhoeae*  *Neisseria gonorrhoeae*.

NAAT  Nucleic Acid Amplification Testing.

NG-MAST  *Neisseria gonorrhoeae* Multi-Antigen Sequence Type.
**Glossary**

**NPV** Negative Predictive Value.

*por* *N. gonorrhoeae* gene encoding outer membrane porin.

**PBP-2** Penicillin Binding Protein 2.

**PCR** Polymerase Chain Reaction.

**PDPT** Patient-Delivered Partner Therapy.

**PID** Pelvic Inflammatory Disorder.

**PPV** Positive Predictive Value.

**RNA** Ribonucleic acid.

**RTPCR** Real-Time Polymerase Chain Reaction.

**SFDPH** San Francisco Department of Public Health.

**ST** Sequence Type.

*tbpB* *N. gonorrhoeae* gene encoding the β subunit of transferrin-binding protein.

**TSB** Tryptone Soy Broth.

**UCB** University of California Berkeley.

**UCSF** University of California San Francisco.
Bibliography


[27] M. Kojima, K. Masuda, Y. Yada, Y. Hayase, T. Muratani, and T. Matsumoto. Single-dose treatment of male patients with gonococcal urethritis using 2g specti-


