2. SPECIFIC AIMS

Over the past 75 years, *Neisseria gonorrhoeae* (NG) infections have become increasingly resistant to antimicrobial therapy: first to sulfa-based antibiotics, then penicillins, tetracyclines, fluoroquinolones and now extended-spectrum cephalosporins.[1] Currently the only recommended treatment for NG infection is dual antibiotic therapy with injectable ceftriaxone and oral azithromycin or doxycycline.[2] Resistance to extended-spectrum cephalosporins was first identified in Japan and is increasing in the western United States and among men who have sex with men.[3] The mechanisms of antimicrobial resistance have been fairly well-described as mutations in target proteins, alterations in cell wall components or increases in efflux pumps.[1]

Our group has used the modern molecular techniques of polymerase chain reaction (PCR) or genetic sequencing to demonstrate that NG DNA mutations can be easily and reproducibly identified for surveillance.[4-6] Surveillance data critically inform treatment recommendations but cannot be used for real-time clinical management. Along with the growing trends in decreased antimicrobial susceptibility of NG, culture and conventional susceptibility testing in the diagnosis of NG infections have decreased and nucleic acid (DNA or RNA) amplification testing to detect NG in clinical specimens has increased.[7] Nucleic acid amplification testing (NAAT) is more sensitive than culture, but does not readily allow for bacterial isolation and phenotypic antimicrobial susceptibility determination. Newer methods of susceptibility determination based on molecular markers of resistance are urgently needed.

Our team has developed and validated a protocol for the detection of alterations in the gyrase A gene (*gyrA*), the target of fluoroquinolone (FQ) anti-gonococcal activity.[4] By using real-time PCR we identified with 100% sensitivity all 51 ciprofloxacin-susceptible NG isolates (Minimum Inhibitory Concentration (MIC) < 0.125 µg/mL) when compared with susceptibility determination by conventional agar dilution methods. We also found the real-time PCR assay to be 93% sensitive in identifying ciprofloxacin-resistant NG.[4]

In 2002, due to the increasing levels of FQ-resistant NG infections, the California Department of Health Services ceased recommending FQs for the treatment of gonorrhea. Subsequent to that recommendation, the frequency of FQ resistance dramatically declined.[8] In 2007, due to high rates of FQ resistance in NG nationally, the CDC recommended the discontinuation of FQ treatment for gonorrhea, which similarly was followed by a decline in FQ NG resistance nationally.[9] Currently the nationwide frequency of FQ decreased susceptibility (MIC ≥ 0.125 µg/ml) or resistance (MIC ≥ .5 µg/ml) is relatively low at 12%. Those observations, along with the experience from the restriction of erythromycin use in Finland to control an increase in macrolide-resistant group A streptococcal infections,[10] strongly suggest that antibiotic use in humans may modify the ecology of drug-resistance. In this project we propose to verify the performance of RT-PCR to detect FQ-susceptibility in clinical specimens positive for NG by nucleic acid amplification for routine diagnosis and offer susceptibility results to clinicians. *We hypothesize that clinicians receiving results of FQ susceptibility prior to treatment will be more likely to use a FQ for gonorrhea treatment compared with clinicians not receiving such a result.*

Our proposed project has the following three Specific Aims:

Specific Aim 1: To verify the performance of real-time PCR in the detection of ciprofloxacin-susceptible NG infections versus conventional antimicrobial susceptibility testing in clinical specimens used in the diagnosis of gonorrhea.

Specific Aim 2: To develop a molecular antimicrobial susceptibility detection program for clinical use at a large metropolitan public health laboratory STD testing program.

Specific Aim 3: To determine the impact of providing clinicians with molecular antimicrobial susceptibility results on their prescribed treatment of patients diagnosed with NG.

The findings from this project may enable the routine use of molecular FQ-susceptibility testing in large clinical laboratories and drive NG test manufacturers to add FQ-susceptibility determination to current NG assays. Ultimately, those changes may reduce the continued emergence of drug resistance.

3. RESEARCH STRATEGY

A. SIGNIFICANCE

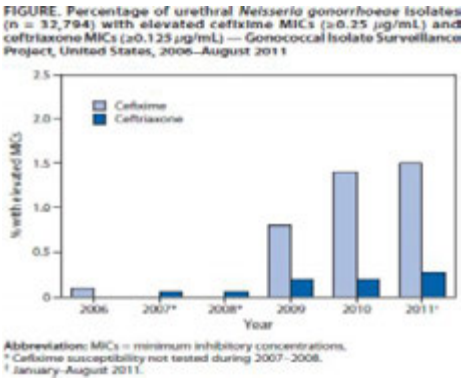


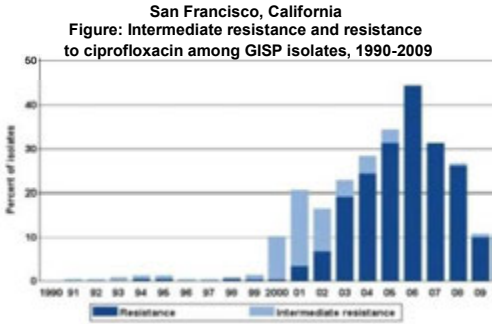
FIGURE. Percentage of urethral *Neisseria gonorrhoeae* isolates (n = 32,794) with elevated cefixime MICs (≥0.25 µg/mL) and ceftriaxone MICs (≥0.125 µg/mL) — Gonococcal Isolate Surveillance Project, United States, 2006–August 2011

Abbreviation: MICs = minimum inhibitory concentrations.
* Cefixime susceptibility not tested during 2007–2008.
† January–August 2011.

***Neisseria gonorrhoeae* (NG) is increasingly resistant to common and easily available antibiotics.** In August 2012, due to a steady increase in elevated minimum inhibitory concentrations (MICs) of NG to the oral cephalosporin antibiotic cefixime (see figure at right), the CDC recommended to discontinue oral cephalosporin use in addition to not using ciprofloxacin, penicillins or tetracyclines for the treatment of NG infection.[2] The only currently recommended treatment is dual therapy with injectable cefriaxone plus oral azithromycin or doxycycline. The continued emergence of drug resistance in NG has many experts stating that NG infection might become an untreatable "superbug".[3,11]

Current efforts to control the continued emergence of drug-resistant NG are inadequate. Already in 2013, a major report described 9 extended-spectrum cephalosporin treatment failures among NG-infected patients in Canada.[12] Other recent reports documented increasing extended-spectrum cephalosporin resistance in Hong Kong,[13] France[14] and Japan.[15] As predicted, the emergence of multi-drug resistant, extensively-drug resistant and untreatable gonorrhea marches on.[11] Yet, the response to this serious public health problem continues to rely on ensuring the use of recommended empiric treatment, clinical vigilance for treatment failure, partner treatment, maintenance of culture-based surveillance, risk-reduction counseling, condom use, repeat screening and calls for novel antimicrobial drug development.[2,3,16] Clearly those recommendations are insufficient. *The control of antimicrobial resistance requires a more tailored approach to treatment that may be provided by the use of real-time antimicrobial susceptibility determination.* In fact, the National Coalition of STD Directors (NCSD) recently called on industry partners to "develop testing technologies for gonorrhea to bring to market rapid molecular antimicrobial susceptibility testing…to help health care providers more immediately identify resistance [and] potentially provide tailored and appropriate treatment regimens" (see Appendix B for more information). Susceptibility results including those from molecular tests form the foundation for infectious disease practice. Similar to the Xpert MTB-RIF assay for multi-drug resistant *M. tuberculosis* infection, the use of real-time molecular assays for antimicrobial susceptibility determination in NG infection could be a "game changer".[17]

Selective reduction in the use of antibiotics may impact the population-level ecology of drug-resistant infections. In the early 1990s there was a major increase in erythromycin resistance among group A streptococci in Finland.[10] In response, national policies were instituted to reduce the use of macrolide antibiotics in outpatients. From 1991 to 1992, there was a marked 50% reduction in erythromycin use followed by a subsequent large decrease in erythromycin resistance (19.0% to 8.6%) (see figure at right). Similar changes in the frequency of ciprofloxacin resistance have been observed with the treatment of gonorrhea. The figure at left shows the rapid increase in ciprofloxacin resistance in San Francisco and subsequent decrease starting in 2007 following California recommendations to cease the use of ciprofloxacin for NG.[8] Nationally the picture was similar, with an increase followed by a decrease after national treatment recommendations were changed in 2007.[18] A recent study also found that in one year following the cessation of cefixime in England and Wales, cefixime resistance decreased from 17.1% to 10.8%.[19] Importantly, the study editorial stated: "What is more encouraging is the return of antibiotic sensitivity with the disuse of an agent: thus, abandoned drugs can again be useful."[20] Those observations form the basis for our strategy to reduce the continued emergence of extended-spectrum cephalosporin-resistant NG infection through decreasing cephalosporin use. *The underlying hypothesis for this project is that real-time antimicrobial susceptibility testing will result in a decreased use of extended-spectrum cephalosporin antibiotics for the treatment of NG infection.*



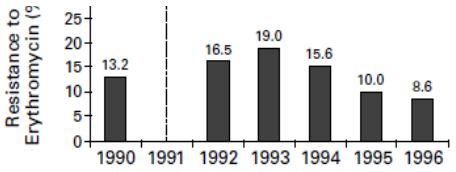


Figure 2. Frequency of Resistance to Erythromycin among Group A Streptococcal Isolates from Throat-Swab and Pus Samples in Finland in 1990 and in 1992 through 1996.

Real-time PCR will provide timely, actionable drug susceptibility results to clinicians. In 2011 the Los Angeles (LA) County Public Health Laboratory (PHL) diagnosed 4329 cases of gonococcal infection.[21] Of those cases, 56% were treated at the time of specimen collection, resulting in 40% (n=1918) of cases treated

7-30 days later (4% had no treatment documentation). Importantly, among those at most risk for drug-resistant gonorrhea (men who have sex with men), only 35% were treated at the time of specimen collection, resulting in 61% treated 7-30 days later (4% untreated). The pie chart to the right shows that in 2011 28.1% of NG surveillance isolates in LA were resistant to ciprofloxacin (any Quinolone Resistant NG, or QRNG). Thus, about 72% of NG cases not receiving treatment the same day as collection may receive ciprofloxacin and not an extended-spectrum cephalosporin. Based on those estimates, the provision of susceptibility results could reduce the use of extended-spectrum cephalosporins by over 40%. Such a reduction in antibiotic use was sufficient to have a marked decrease in on the ecology of drug-resistant streptococcus in Finland.[10]

Clinician prescribing practices are readily measured. The LA County Department of Public Health (DPH) routinely collects treatment information for all gonorrhea cases countywide. In 2011 96% of treated gonorrhea cases in LA County received ceftriaxone (95%) or another extended-spectrum cephalosporin (0.6%).[22] The existing collaboration between UCLA and LA County DPH will allow us to use routinely-collected treatment data to measure the impact of providing NG ciprofloxacin susceptibility information to gonorrhea treating clinicians.

Demonstrating the impact of providing drug-susceptibility results will change clinical practice and thwart the emergence of untreatable drug resistant infections. A 2010 study found that rapid methicillin-susceptibility results of *Staphylococcus aureus* had a dramatic effect on clinical practice, reducing unnecessary antibacterial antibiotic exposure by more than 75%.[23] Susceptibility data are routinely used in clinical practice, and molecular markers of resistance have been used in the management of tuberculosis[24] and *S. aureus* infection[23] but not yet with gonorrhea. Importantly, once we have demonstrated proof of concept and effect size, STD control officials may recommend the use of rapid assays for susceptibility determination, leading diagnostic test manufacturers to add molecular markers of antimicrobial susceptibility to current NAATs for NG infection (see NCSD Press Releases, September 16, 2013, Appendix B).

B. INNOVATION

This project is novel in three main ways: 1) The verification for clinical use of real-time ciprofloxacin PCR susceptibility testing has not been done in any clinical laboratory. Verifying a clinical laboratory test is critical for the use of that test in accordance with Clinical Laboratory Improvement Amendment (CLIA) regulations.[25] Once a test has been verified in a large clinical laboratory like the LA County PHL, additional laboratories can conduct their own verification studies and provide that test to clinicians; 2) Despite successful use with other diseases, molecular markers for antimicrobial susceptibility have not yet been used to target antimicrobial therapy in the clinical management of gonorrhea, despite this infection being the second most common reportable infectious disease in the U.S. and on the verge of becoming untreatable with currently available antimicrobials. The use of real-time molecular markers of susceptibility is transformational in the management of gonorrhea; and 3) The use of targeted rather than empiric "one size fits all" treatment of gonorrhea is novel and will change the practice of sexually transmitted disease medicine. The findings from this project may demonstrate that timely FQ-susceptibility results can decrease the prescribing of extended-spectrum cephalosporins and cause policy makers to recommend larger commercial NG-testing laboratories to add FQ-susceptibility testing by RT-PCR. Furthermore, the findings may drive NG test manufacturers to add molecular FQ-susceptibility determination to current nucleic acid amplification assays. Scaled-up FQ-susceptibility determination with resultant declines in the use of extended-spectrum cephalosporins could have a major impact mitigating the continued emergence of extended-spectrum cephalosporin resistance.

C. APPROACH

C.1. **Overview and Timeline.** This study encompasses 3 phases, as detailed in Table 1 below:

- Phase I involves start-up, approvals, and assay verification with known isolates in accordance with CLIA.
- Phase II involves the development of the antimicrobial susceptibility testing program including (a) a laboratory component; (b) information management and communication; and (c) education and training of clinicians in test result interpretation through a laboratory generated letter and face-to-face site trainings.
- Phase III involves the evaluation of the new assay among clinicians treating STD patients with gonorrhea, including: (a) monitoring of time to delivery of drug-susceptibility information relative to time of treatment, (6 mo); (b) treatment selection (6 mo) (c) a follow-up assessment of the usefulness of the molecular susceptibility results (1 mo); and (d) data analysis and dissemination (4 mo).

Table 1: Study Timeline

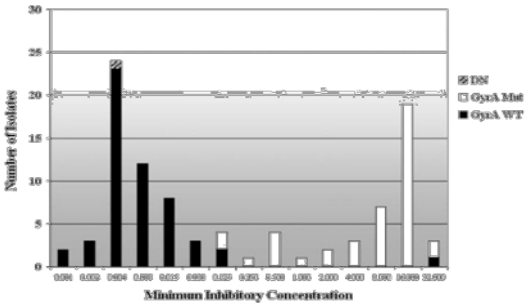
Component and Task Name	Start	Finish	Duration	Year 1				Year 2			
				Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
I. Start-up, preparation; protocol verification with known isolates	7/1/14	12/31/14	6 mo.								
II. Development of RT-PCR testing program	1/1/15	6/30/15	6 mo.								
III. Pilot implementation, evaluation and analysis	7/1/15	7/31/16	12 mo.								

C.2. Preliminary Studies.

Epidemiology of NG drug resistance, The Philippines. Years ago in The Philippines we found the proportion of NG highly resistant to ciprofloxacin (MIC ≥ 4 µg/mL) increased from 9% to 49% among female sex workers and resistance was highly associated with self-prescribed antimicrobial use.[26] In 1996, we conducted a randomized clinical trial of oral ciprofloxacin vs. cefixime for the treatment of gonorrhea and found a high rate of treatment failure in those treated with ciprofloxacin leading to updated treatment recommendations.

Molecular markers of drug resistance in syphilis. After identifying an azithromycin treatment failure in syphilis in 2002-2003,[27] we were the first to describe the molecular epidemiology of azithromycin-resistant *T. pallidum* in the United States and Europe[28] and the clinical response to treatment.[29 - 32] We then developed a real-time PCR assay for *T. pallidum* that was eventually adopted by the US CDC for surveillance.[33]

Molecular markers of drug resistance in gonorrhea. In 2007, we developed and tested a real-time PCR assay for detecting mutations in the ser91 codon of the *gyrA* gene of NG.[4] We compared wild type and mutated *gyrA* gene products with ciprofloxacin susceptibility results from agar dilution. With an MIC threshold of 0.125 µg of ciprofloxacin per mL, we found that 51 of 51 susceptible specimens were correctly identified (100% sensitivity) and 41 of 44 specimens with intermediate resistance or greater were correctly identified (93% sensitivity). The figure on the next page shows the distribution of MIC results and *gyrA* gene findings.



(DN indicates non-amplified specimens.) Our laboratory[34] then investigated the mosaic *penA* allele associated with extended-spectrum cephalosporin resistance[33-34] on 54 NG isolates in San Francisco. Our assay correctly identified all 5 isolates with the mutant *penA* gene and elevated MICs for extended-spectrum cephalosporins.

Performance of molecular markers of susceptibility in clinical specimens. The current proposal relies on the detection of antimicrobial susceptibility markers in remnant clinical swab or urine specimens collected in APTIMA transport tubes. Our prior work demonstrated adequate DNA extraction and PCR product detection in 95 (99%) of 96 remnant urine specimens collected in male patients with positive urethral swabs.[4] Among 100 female urine specimens with positive NG nucleic acid detection, 72 (72%) had amplifiable *gyrA* gene product. ***No (0%) NG negative clinical specimens had detectable molecular markers of resistance demonstrating the specificity of our gyrA test for NG in clinical specimens potentially contaminated with other bacteria.***

C.3. Methodology and Study Aims.

SPECIFIC AIM 1: To verify a real-time PCR assay in the detection of ciprofloxacin susceptible NG infections in accordance with CLIA regulations.

Rationale. While we have demonstrated the excellent performance of the real-time PCR assay in the SF PHL and have successfully transferred and tested the technology with the US Navy Medical Research Laboratory Detachment in Lima, Peru,[35] we have not verified the real-time PCR assay for clinical use in the LA County PHL. According to CLIA regulations,[36] laboratories that introduce a new test system must demonstrate adequate performance for accuracy, precision, reportable range, and normal values in order to provide a novel test to clinicians for clinical management.[37] The LA County PHL has verified novel molecular tests in the past, and has the technical capacity to conduct DNA extraction and real-time PCR testing, as well as the capacity to conduct gene sequencing for further confirmation.[38] The LA County PHL has extensive experience with test verification and serves as a reference center for other clinical laboratories. No potential challenges are expected with this Aim, as the project team includes nationally recognized experts in molecular biology including Drs. Janda Green and Pandori and both the LA County PHL and SF PHL already have large numbers (>1000) of well-characterized clinical NG specimens.

Methods and Procedures.
(a) Performance testing: Using a panel of 100 well-characterized NG isolates from the 2011 and 2012 GISP, we will determine presence or absence of mutations in the *gyrA* gene (S91P) by real-time PCR and compare molecular typing results of bacterial isolates with ciprofloxacin phenotypic susceptibility testing results. Agar dilution will be used for conventional susceptibility testing comparison. For conventional susceptibility testing, Clinical Laboratory Standards Institute clinical MIC breakpoints of S = <0.06µg ciprofloxacin / µl and R = ≥1µg ciprofloxacin / µL will be used. Sequence analysis for *gyrA* will be performed on samples with discrepant results.
(b) Clinical specimens: Remnant APTIMA NG positive specimens from urethral swabs, vaginal swabs, and urine previously tested at the LA County PHL will be used in a pilot study to determine the best method for nucleic acid extraction (manual or automated) and parameters needed for PCR amplification. The limit of detection for APTIMA CT/NG testing may be lower than real-time PCR because transcription mediated amplification uses abundant ribosomal RNA as the amplification target rather than a single gene target and has an excellent analytical sensitivity (low limit of detection approximately 250-500 cells/mL specimen). It will be necessary to determine cut-off APTIMA Relative Light Unit (RLU) values for positive specimens to ensure adequate amplification of *gyrA* real-time PCR. Once PCR parameters are optimized, the assay limit of detection (analytical sensitivity) will be determined using serial ten-fold dilutions of spiked NG negative remnant specimens compared to colony forming units/mL and RLU value. Analytical specificity will be determined by cross-reactivity studies using specimens spiked with bacteria, fungi, yeast, and viruses representing normal and abnormal genitourinary tract flora. Interference studies will be performed to determine the effect of amplification inhibition due to substances which may be found in urine or genital tract. Internal amplification control representing wild type NG *gyrA* target or other chromosomal gene will be used for all specimens.

Data Collection. Data will be collected by the laboratory study team and recorded in a secure electronic database by specimen identification number. The specimen identification number is only linked to the specimen source (anatomic site), specimen collection date, and specimen NG test result and not with any patient personal identifiers. The data will be maintained in aggregate.

Data Analysis. Using the agar dilution results as the gold standard for ciprofloxacin susceptibility determination, we will use Cohen's kappa statistic[39] as well as its 95% confidence interval (CI) to measure the inter-method agreement of the molecular results summing both susceptible and decreased susceptible results. The lower bound of 95% CI of Cohen's kappa is found to be 0.86 when 100 specimens are tested by both methods and yield an estimated kappa of 0.95. Our prior results demonstrated a percent agreement of 96% (95% CI 92%-100%) with 100% (93%-100%) detection of susceptible specimens.[4]

SPECIFIC AIM 2: To develop a molecular antimicrobial susceptibility detection program for clinical use at a large metropolitan public health laboratory STD testing program.

Methods and Procedures.
(a) Specimen testing program: Currently, clinical specimens are tested for NG with the APTIMA Combo2 CT/NG test on the Hologic Inc. (formerly Gen-Probe) TIGRIS DTS platform at the LA County PHL. Over 8,000 APTIMA tests are done each month. Newly-identified NG positive clinical specimens will undergo real-time PCR for ciprofloxacin susceptibility within 24 hours of NG detection. The NG ciprofloxacin-susceptibility result will be reported back to clinicians within 2 business days of the original NG test result.
(b) Real-time PCR testing protocol: We will follow the testing protocol as previously described[4] with modifications. For real-time PCR analysis of the *gyrA* gene, remnant APTIMA Combo2 specimens will be extracted by using either manual spin column or automated nucleic acid extraction techniques. Extracted samples will be use for real-time PCR. Amplification and melting-curve analysis of specimens will be performed by using a Roche LightCycler 2.0 (Roche Diagnostics). Amplified regions of the *gyrA* gene will be probed for mutations in the Ser 91 codon through use of melting-curve analysis with probes specific to that region of the *gyrA* gene. Primer and probes will be selected from prior published reports of similar assays, and confirmed by using online BLAST analysis from the National Center for Biotechnology Information at the National Institutes of Health. The probes for the Ser91 amplicon (*gyrA*-ser-Flu and *gyrA*-ser-LC) are a paired set of FRET probes, including a LightCycler Red640 probe and a fluorescein-labeled probe, separated by 1 bp. The Red640 oligonucleotide has a maximal absorption and emissions of 622 and 638 nm, respectively. Amplification of PCR product will be followed by melt-curve analysis to confirm specificity of amplification and genotype. Melting-curve analysis will be performed by using LightCycler software (v.4.0). We will plot the negative value of the first derivative of fluorescence per unit time to distinguish peak melting temperatures from melting-curve plots.
(c) Education and training of clinicians: The laboratory will circulate a letter upon initiation of the additional testing program describing the epidemiology of drug-resistance in NG, the methodology of the new RT-PCR test, and test performance including association with phenotypic cipro-resistance. Additionally, Dr. Klausner will

conduct face-to-face site clinician trainings in the epidemiology and clinical management of drug-resistant gonorrhea in each of the treatment settings, to answer any questions and concerns that arise.
(d) Delivery of results**:** Providers submitting specimens for NG testing will be informed electronically or by fascsimile of the molecular NG ciprofloxacin susceptibility test results along with a statement on the test performance from Aim 1 (accuracy, sensitivity, specificity) within 2 business days of the original NG test results.

Information management. Ciprofloxacin susceptibility results will be reported to providers ordering NG testing through the current LA County PHL information management system electronically and through facsimile, in accordance with federal regulations.[25] We will use the current ~~antibiotic~~ NG treatment reporting system to monitor the outcome of antibiotic selection for NG cases. As part of routine disease surveillance activities, type, dose and duration of the prescribed antibiotic for NG cases is regularly reported to the LA County DPH.

Potential Challenges. There could be delays in program implementation due to the time required to complete the assay verification (Aim 1); however, verification for molecular assays usually requires weeks not months. There will be changes made to the current LA County laboratory information management system that will require additional computer programming. The changes are modest and such changes are commonplace.

SPECIFIC AIM 3: To determine the impact of providing clinicians with molecular antimicrobial susceptibility results on their prescribed treatment of patients diagnosed with NG.

Methods and Procedures.

(a) Data Monitoring: We will monitor monthly the number of NG tests, NG test results and NG ciprofloxacin susceptibility results from the period of study inception through the period of intervention development and implementation. We will assess the time to ciprofloxacin susceptibility result, the frequency of ciprofloxacin susceptibility among tested specimens, and will describe routinely collected basic demographic/behavioral correlates (patient age, sex, reported residence zip code, treatment location, and gender of sex partners) of cases of NG positivity and NG ciprofloxacin susceptibility. We will monitor the proportion of NG cases treated with extended spectrum cephalosporin and evaluate any changes over time, specifically testing whether the provision of NG ciprofloxacin susceptibility results is associated with any changes in antibiotic treatment type.
(b) Treatment selection: Most (>95%) treating providers follow LA County DPH recommendations for NG treatment. We will update those NG treatment recommendations to recommend the use of ciprofloxacin 500 mg orally once based on a molecular NG test result demonstrating susceptibility.
(c) Clinician survey: Following 6 months of intervention implementation, in order to understand better the use of the NG ciprofloxacin susceptibility results we will use provider data from the 3 largest NG treating sites sending clinical specimens to LAC DPH laboratory (LAC DPH CHS clinics, LAGLC and AHF) to generate a list of all NG-treating providers at those testing sites and select a simple random sample of 50 providers. We will send up to 3 e-mails over 2 weeks to recruit survey participants. Any provider who is non-responsive to the SurveyMonkey® email link after 2 weeks will be replaced by the next provider on the list. Invitations will proceed until we have 50 completed survey responses. The survey wil measure provider characteristics (demographics, practice location, frequency of NG treatment); their knowledge, attitudes, and practices regarding the epidemiology of drug-resistance; the use of the NG susceptibility test result; and the treatment of NG infection (see Appendix C).

Quality Assurance. We will conduct ongoing quality assurance of the NG ciprofloxacin susceptibility testing and reporting program using periodic control specimens (susceptible and decreased susceptible isolates) provided by the SF PHL. Ten specimens every month will be tested in LA for the presence of *gyrA* wild-type and compared with the known antimicrobial susceptibility test results from the SF PHL.

Potential Challenges. Clinicians might not understand the new test results and not know how to use these results in clinical practice; however, the LA County PHL routinely introduces new clinical tests and educates clinicians on the interpretation and use of those tests. The project team will educate all sites and providers submitting specimens for gonorrhea testing about the new susceptibility test and how to interpret the results.

C.4. Sample Size Estimations and Statistical Analyses

Sample Size. During the first six months of 2012, the LA County PHL identified 1231 positive NG specimens from providers (~200 per month). Expecting that 40% of cases are treated after time of specimen collection (80 per month) and up to 20% of cases might be dual infections at several anatomic sites (rectal/pharyngeal/ urethral), it will take about 6 months to observe 400 cases where treatment decisions may be based on the molecular NG ciprofloxacin susceptibility result.

Primary analysis. The primary analysis is to compare the difference in the proportion of NG cases treated with an extended spectrum cephalosporin before and after the provision of NG susceptibility results. Based on the Finland experience, we hypothesize a 50% reduction in extended spectrum cephalosporin use from 95% to

50%. A sample size of 400 cases achieves >99% power to detect a 50% reduction (from 95% to 50%) in NG cases treated with extended spectrum cephalosporin when using a two-sided binomial test for proportion, at a 0.05 significance level.

Secondary analyses. In addition to the primary analysis, we will conduct analyses to explore the association of non-extended spectrum cephalosporin use (e.g. use of ciprofloxacin) based on both provider and case-patient characteristics. Those characteristics may include provider location and duration of practice, as well as patient age, race/ethnicity, sex, and gender of sex partners. Unpaired t tests (for continuous variables) and chi-square tests/Fisher's exact tests (for categorical variables) will be used when deemed appropriate.

Feasibility and acceptability. The provider survey (n=50) will be analyzed to describe survey participant knowledge, attitudes and practice frequencies and correlates of those frequencies based on basic provider characteristics (age, sex, race/ethnicity, practice location and duration).

Statistical considerations and data management. Descriptive statistics including mean, standard deviation, median, inter-quartile range and frequency distribution will be generated for outcome variables as well as provider and case-patient characteristics. Graphics such as bar charts, box-plots, and histograms will be used to present the data and check for skewness and normality. Transformations of the outcome variables will be explored and performed if needed. For all statistical investigations, tests for significance are two-tailed. All analyses will be conducted with Stata 9.0 (Stata Corporation, College Station, TX, 2006).

6. PROTECTION OF HUMAN SUBJECTS

Introduction

The present proposal will verify a new real-time PCR assay for ciprofloxacin-susceptibility in *Neisseria gonorrhoeae* specimens in a clinical laboratory and evaluate the provision of the assay result to clinicians treating patients diagnosed with *Neisseria gonorrhoeae* (NG). We will be seeking an exemption from human subjects (IRB) approval, as real-time PCR assays are approved for marketing by the FDA and the use of real-time PCR assays for laboratory specific diagnoses after verification are allowable and consistent with US CLIA regulations. There will be no contact between study staff and patients testing for NG. Testing to complete the verification protocol will only occur on remnant laboratory specimens, and the only potential impact of the new PCR assay human subjects is better-targeted treatment selection from their clinician. The only human subjects who will be recruited for participation in this study are NG treating providers (n=50) who will be asked to take a simple anonymous survey via an online program such as Survey Monkey to give us a better understanding of clinician knowledge, attitudes and practices regarding the use of NG ciprofloxacin susceptibility results to guide treatment selection.

Protocol Status

The research protocol for this project is under development but will be consistent with the procedures outlined in this grant application (see Research Strategy).

1) Research Activities for Human Subjects

Aim 1 of this study involves the verification of real-time PCR for the detection of ciprofloxacin-susceptible NG infections, as required by CLIA regulations.[24] Only remnant de-identified but antimicrobial-susceptibility characterized laboratory specimens will be used for this verification, performed according to standard laboratory practice. No human subjects will be involved in this Aim.

Aims 2 and 3 involve clinical gonorrhea specimens collected in LA County and sent to the LA County Public Health Laboratory (PHL) for testing. As Aim 2 will not begin until the verification of real-time-PCR for this purpose has been completed, the molecular antimicrobial susceptibility results provided to clinicians as part of this Aim will be provided according to CLIA regulations and after verification is standard practice for clinical laboratories. There is no human subjects involvement in the study for this Aim, therefore, because all patients receiving results (>50,000 per year in LA County) will be receiving results according to US regulations and interpreted individually by their clinician. The impact evaluation in Aim 3 is a retrospective evaluation of the impact of a new laboratory test on clinical practice, and involves no personal identifiers of patients, only routinely reported data regarding the treatment type selected by the clinician for each case of NG infection. Aim 3 *will* involve the recruitment of 50 clinicians who provide NG testing and treatment; these providers will be asked to complete an anonymous survey about their knowledge, attitudes, and practices regarding the use of NG susceptibility testing results, via an online data collection system such as SurveyMonkey®.

Human Subjects Involvement and Characteristics. All patients in LA County presenting to an STD testing location that uses the LA County PHL for NG testing will potentially be affected by this study. In LA

County that includes people over the age of 12 who are able to consent to their own testing according to standard of care and California State law. More than 100,000 test are performed for NG infection through the LA County PHL each year, and approximately 200 specimens per month are identified as positive for NG. It would be these ~200 patients per month whose clinicians would receive additional antimicrobial susceptibility information as a result of our project. No personal data or other identifying information will ever be transmitted to study staff. Any publications resulting from this project will contain no personal identifiers. This study does not involve any risks of treatment, and no direct contact or data collection will occur between study staff and NG patients at any time.

Up to 50 clinicians will be randomly selected from a list of NG-treating providers at the 3 largest LA County DPH-supported NG testing sites in 2013, and recruited via email for anonymous completion of a brief survey using a system such as SurveyMonkey®. No personally identifying information will be collected about those providers during the survey.

Sources of Materials/ Information. All research information used in this study will be retrospectively gathered through already routinely collected data as part of standard clinical, public health and laboratory practice in LA County. Research material will include laboratory results and provider and case-patient data for NG cases, reported to the LA County Department of Public Health as required by law; as well as results of the online clinician survey, which will include only anonymously-collected data.

Potential Risks of Study Participation. There is zero risk to patients whose specimens are tested for ciprofloxacin susceptibility through this study, as their clinician is responsible for interpreting any laboratory results and selecting the best course of treatment based on available information. There is a very small risk of emotional discomfort to clinicians when being asked to respond to survey questions about their knowledge, attitudes, and practices regarding the use of NG susceptibility testing, but this is unlikely; their involvement will be anonymous, completely voluntary, and they will be told that they can discontinue the survey at any time if desired.

2) Adequacy of Protection against Risks

Informed Consent. It is not feasible nor necessary to collect informed consent for each patient presenting for NG testing in LA County during the implementation period of the study. Since no patients will be classified as human subjects for the duration of the project, no informed consent will be sought by our team. Informed consent for testing and treatment will be obtained by the clinical site offering NG testing as current clinical practice, according to CA state regulations, but this process is completely independent from our study. IRB review and request for waiver of informed consent will be pursued for the clinician survey, as the survey is anonymous and poses very minimal risk.

Protections Against Risks. No extra effort will be needed to protect patients against risks, as there is no risk to patients as a result of this study. Providers who are completing the clinician survey will be told that their involvement is completely anonymous and that they can discontinue the survey at any time should they grow uncomfortable with the questions asked.

Privacy and Confidentiality. No personally identifiable information will be collected about patients or providers. Information privacy is already severely regulated by federal and CA rules and regulations and these standards would be in effect.

3) Potential Benefits of the Proposed Research to the Subjects and Others

Individuals whose clinicians receive ciprofloxacin-susceptibility information as a result of our study may be treated with a more optimal antibiotic than would have otherwise been selected by their provider. All individuals at risk for NG infection might benefit from decreased drug resistance of NG overall, as a result of decreased use of extended-spectrum cephalosporin antibiotics after provision of susceptibility information at the time of test result reporting.

4) Importance of the Knowledge to Be Gained

The study will help to assess the potential impact of a strategy to address the critical problem of emerging drug resistance in NG. Specifically, the information gained from this project can be used to influence national STD treatment policies and therefore result in a marked decrease in extended-spectrum cephalosporin resistant NG infection, improving the availability of treatment options for people infected with NG.

INCLUSION OF WOMEN AND MINORITIES

No individuals or human subjects are recruited or participating AIMS 1 or 2 of this project. AIM 3 includes an anonymous survey of up to 50 clinicians. The anticipated gender, race/ethnicity and age distribution of the participating clinicians reflect the current practicing clinicians who use the LA County PHL for gonorrhea testing. Women and minorities will be included in this research project to the extent that they are currently clinicians submitting patient-specimens for gonorrhea to the LA County PHL. Data from LA County in 2012 suggest that women and minorities will be represented in the clinician sample in the following proportions: Women (40%) and Racial/Ethnic Minorities (36%) of all clinicians who submit NG samples to the LA County PHL.

Data shall be analyzed by gender and by racial/ethnic minority status as described in the Research Approach.

BIBLIOGRAPHY AND REFERENCES CITED

[1] Barry PM, Klausner JD. The use of cephalosporins for gonorrhea: the impending problem of resistance. *Expert Opinion on Pharmacotherapy*. 2009 Mar;10(4): 555-577. [PMID: 19284360]

[2] Centers for Disease Control and Prevention (CDC). Update to CDC's Sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer a recommended treatment for gonococcal infections. *Morbidity and Mortality Weekly Report*. 2012 Aug 10; 61(31): 590-594. [PMID: 22874837]

[3] Bolan GA, Sparling PF, Wasserheit JN. The emerging threat of untreatable gonococcal infection. *New England Journal of Medicine*. 2012 Feb 9; 366(6): 485-487. [PMID: 22316442]

[4] Siedner MJ, Pandori M, Castro L, Barry P, Whittington WL, Liska S, Klausner JD. Real-time PCR assay for detection of quinolone-resistant Neisseria gonorrhoeae in urine samples. *Journal of Clinical Microbiology.* 2007 Apr; 45(4): 1250-1254. [PMID: 17267635]

[5] Pandori M, Barry PM, Wu A, Ren A, Whittington WL, Liska S, Klausner JD. Mosaic penicillin-binding protein 2 in Neisseria gonorrhoeae isolates collected in 2008 in San Francisco, California. *Antimicrobial Agents and Chemotherapy*. 2009 Sept; 53(9): 4032-4034. [PMID: 19546370]

[6] Buono S, Wu A, Hess DC, Carlson JS, Rauch L, Philip SS, *et al.* Using the Neisseria gonorrhoeae multiantigen sequence-typing method to assess strain diversity and antibiotic resistance in San Francisco, California. *Microbial Drug Resistance*. 2012 Oct; 18(5): 510-517. [PMID: 22686196]

[7] Dicker LW, Mosure DJ, Steece R, Stone KM. Testing for sexually transmitted diseases in U.S. Public health laboratories in 2004. *Sexually Transmitted Diseases*. 2007 Jan; 34(1): 41-46. [PMID: 16735955]

[8] CDC. Increases in fluoroquinolone-resistance Neisseria gonorrhoeae—Hawaii and California, 2001. *Morbidity and Mortality Weekly Report*. 2004 Nov 22; 51(46): 1041-1044. [PMID: 15123985]

[9] CDC. Cephalosporin susceptibility among Neisseria gonorrhoeae isolates—United States, 2000-2010. *Morbidity and Mortality Weekly Report*. 2011 Jul 8; 60(26): 873-877. [PMID: 21734634]

[10] Seppälä H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. *New England Journal of Medicine*. 1997 Aug 14; 337(7): 441-446. [PMID: 9250845]

[11] Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future Microbiology*. 2012 Dec; 7: 1401 – 1422. [PMID: 23231489]

[12] Allen VG, Mitterni L, Seah C, Rebbapragada A, Martin IE, Lee C, *et al.* Neisseria gonorrhoeae treatment

failure and susceptibility to cefixime in Toronto, Canada. *Journal of the American Medical Association*. 2013 Jan 9; 309(2): 163-170. [PMID: 23299608]

[13] Lo JY, Ho KM, Lo AC. Surveillance of gonococcal antimicrobial susceptibility resulting in early detection of emerging resistance. *Journal of Antimicrobial Chemotherapy*. 2012 June; 67(6): 1422-1426. [PMID: 22334602]

[14] Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. High-level cefixime- and ceftriaxone-resistant Neisseria gonorrhoeae in France: novel penA mosaic allele in a successful international clone causes treatment failure. *Antimicrobial Agents and Chemotherapy*. 2012 Mar; 56(3): 1273-1280. [PMID: 22155830]

[15] Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, *et al.* Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhea?: *detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrobial* Agents and Chemotherapy. 2011 July; 55(7): 3538-3545. [PMID: 21576437]

[16] Kirkcaldy RD, Bolan GA, Wasserheit JN. Cephalosporin-resistant gonorrhea in North America. *Journal of the American Medical Association*. 2013 Jan 9; 309(2): 185-197. [PMID: 23299612]

[17] Small PM, Pai M. Tuberculosis diagnosis—time for a game change. *New England Journal of Medicine*. 2010 Sept 9; 363(11): 1070-1071. [PMID: 20825320]

[18] CDC. 2009 Sexually Transmitted Diseases Surveillance: Gonorrhea. Available online at: http://www.cdc.gov/std/stats09/gonorrhea.htm. Last Accessed 31 January 2013. [No PMID]

[19] Ison CA, Town K, Obi C, Chisholm S, Hughes G, Livermore DM, Lowndes CM. Decreased susceptibility to cephalosporins among gonococci: data from the Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) in England and Wales, 2007–2011. *Lancet Infectious Diseases.* 2013 Sept; 13(9): 762-768. [PMID: 23764300]

[20] Wright DJ, Azadian B. Cephalosporin resistance in gonorrhea. *Lancet Infectious Diseases.* 2013 Sept; 13(9): 728-730. [PMID: 23764301]

[21] LA County Public Health Department, unpublished data. [No PMID]

[22] CDC, Gonococcal Isolate Surveillance Project (GISP). GISP Profiles, 2011. Available online at: http://www.cdc.gov/std/gisp2011/default.htm. Last Accessed 29 Jan 2013. [No PMID]

[23] Parta M, Goebel M, Thomas J, Matioobi M, Stager C, Musher DM. Impact of an assay that enables rapid determination of Staphylococcus species and their drug susceptibility on the treatment of patients with positive blood culture results. *Infection Control and Hospital Epidemiology*. 2010 Oct; 31(10): 1043-1048. [PMID: 20731594]

[24] Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, *et al*. Rapid molecular detection of tuberculosis and rifampin resistance. *New England Journal of Medicine*. 2010 Sept 9; 363(11): 1005-1015. [PMID: 20825313]

[25] CDC. Current CLIA Regulations. Available online at: http://wwwn.cdc.gov/clia/regs/toc.aspx. Last Accessed 29 Jan 2013. [No PMID]

[26] Aplasca de los Reyes MR, Pato-Mesola V, Klausner JD, Manalastas R, Wi T, Tuazon CU, *et al.* A randomized trial of Ciprofloxacin versus Cefixime for treatment of gonorrhea after rapid emergence of gonococcal Ciprofloxacin resistance in The Philippines. *Clinical Infectious Diseases.* 2001; 32(9): 1313-1318. [PMID: 11303266]

[27] CDC. Azithromycin treatment failures in syphilis infections—San Francisco, California, 2002-2003. *Morbidity and Mortality Weekly Report*. 2004 Mar 12; 53(9): 197-198. [PMID: 15017376]

[28] Lukehart SA, Godornes C, Molini BJ, Sonnett P, Hopkins S, Mulcahy F, *et al.* Macrolide resistance in Treponema pallidum in the United States and Ireland. *New England Journal of Medicine*. 2004 Jul 8; 351(2): 154-158. [PMID: 15247355]

[29] Klausner JD, Kohn RP, Kent CK. Azithromycin versus penicillin for early syphilis. *New England Journal of Medicine*. 2006 Jan 12; 354(2): 203-205. [PMID: 16411292]

[30] Mitchell SJ, Engelman J, Kent CK, Lukehart SA, Gordones C, Klausner JD. Azithromycin-resistant syphilis infection: San Francisco, California, 2000-2004. *Clinical Infectious Diseases*. 2006 Feb 1; 42(3): 337-345. [PMID: 16392078]

[31] Katz KA, Klausner JD. Azithromycin resistance in Treponema pallidum. *Current Opinion in Infectious Diseases*. 2008 Feb; 21(1): 83-91. [PMID: 18192791]

[32] Katz KA, Pillay A, Ahrens K, Kohn RP, Hermanstyne K, Bernstein KT, *et al*. Molecular epidemiology of syphilis—San Francisco, 2004-2007. *Sexually Transmitted Diseases*. 2010 Oct; 37(10): 660-663. [PMID: 20601928]

[33] Pandori MW, Gordones C, Castro L, Engelman J, Siedner M, Lukehart S, Klausner J. Detection of azithromycin resistance in Treponema pallidum by real-time PCR. *Antimicrobial Agents and Chemotherapy*. 2007 Sept; 51(9): 3425-3430. [PMID: 17620374]

[34] Ochiai S, Ishiko H, Yasuda M, Deguchi T. Rapid detection of the mosaic structure of the Neisseria gonorrhoeae penA Gene, which is associated with decreased susceptibilities to oral cephalosporins. *Journal of Clinical Microbiology*. 2008 May; 46(5): 1804-1810. [PMID: 18367575]

[35] Siedner MJ, Pandori M, Leon SR, Barry PM, Espinosa BJ, Hall ER, *et al*. Detection of quinolone-resistant Neisseria gonorrhoeae in urogenital specimens with the use of real-time polymerase chain reaction. *International Journal of STD and AIDS*. 2008 Jan; 19(1): 69-71. [PMID: 18275657]

[36] United States Code of Federal Regulations: 42 CFR §493.1253. Standard: Establishment and verification of performance specifications. Available online at http://www.gpo.gov/fdsys/pkg/CFR-2011-title42-vol5/pdf/CFR-2011-title42-vol5-sec493-1254.pdf. Last Accessed 6 February 2013. [No PMID]

[37] Sloan LM. Real-Time PCR in Clinical Microbiology: Verification, Validation, and Contamination Control. *Clinical Microbiology Newsletter*. 2007 June 15; 29(12): 87-95. [No PMID]

[38] Elder BL. Verification and Validation of Procedures in the Clinical Microbiology Laboratory. *Clinical Microbiology Newsletter.* 1997 Oct 15; 19(20): 153-156. [No PMID]

[39] Cohen, J. A coefficient of agreement for nominal scales. *Educational and Psychological Measurement*. 1960; 20: 37-46. [No PMID]