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# Real-Time PCR and Melt Curve Analysis Targeting *gyrA* Gene for Prediction of Ciprofloxacin Resistance in Clinical *Neisseria gonorrhoeae* Isolates

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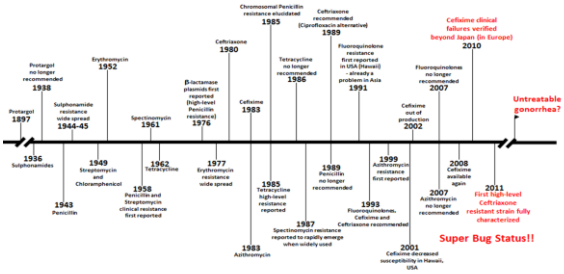
## History of Antimicrobial Resistance in *Neisseria gonorrhoeae*

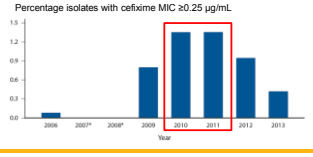



CDC, Antibiotic Resistance Threats in the US 2013
Unemo M, Shafer WM. Ann N Y Acad Sci. 2011;1230:E19-28



## Epidemiology of Resistance in US

- Resistance constantly shifting: changes to treatment guideline
- U.S. CDC's Gonococcal Isolate Surveillance Project (GISP) 2012
  - Significant increase in prevalence of isolates with elevated MICs (≥0.25 µg/mL) to cefixime: unsuitable for empirical treatment
- Current CDC treatment guideline for uncomplicated cervical/urethral infection in adults: ceftriaxone 250 mg IM+ azithromycin 1 g PO




CDC 2012. MMWR 61:590-594.



## Fitness Cost of Mutations Conferring Resistance

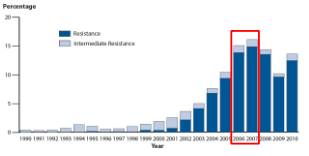
- Organisms carrying mutations: less fit than wild type in absence of selective pressure[1]
- Theoretical models predict decrease in resistance among microbial population when antimicrobial use is reduced[2]
- Evidence of decreased prevalence of resistance in several microorganism
  - Finland: macrolide resistance in *S. pyogenes*[3]
  - Iceland: penicillin resistance in *S. pneumoniae*[4]
  - Israel: fluoroquinolone resistance among urine isolates of *E. coli*[5]

[1]Melnyk AH, Wong A, Kassen R. 2015. Evolutionary applications 8:273-283.
[2]Andersson DI, Hughes D. 2011. FEMS microbiology reviews 35:901-911.
[3]Seppala H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P. 1997. NEJM 337:441-446.
[4]Austin DJ, Kristinsson KG, Anderson RM. 1999. PNAS 96:1152-1156.
[5]Gottesman BS, Carmeli Y, Shitrit P, Chowers M. 2009 CID 49:869-875



## Resistance to Ciprofloxacin in *N. gonorrhoeae* in US

- Ciprofloxacin resistance peaked in 2007: CDC stopped recommending FQ as an empirical treatment
- Prevalence of FQ resistance declined shortly thereafter
- In 2013, only 16.1% of isolates were resistant to CIP (MIC≥1.0 µg/mL)
  - CIP may be a viable treat option for susceptible strains
- Resistance is conferred by Y91S mutation of *gyrA* in >99% of R isolates

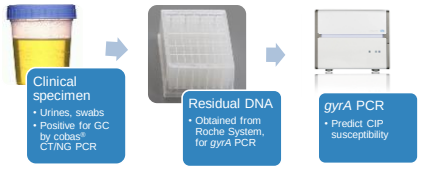



Providing physicians with information about potential resistance may be beneficial.

CDC 2013 Sexually Transmitted Diseases Surveillance
Siedner MJ et al. 2007. JCM 45:1250-1254.

## Objective of Study

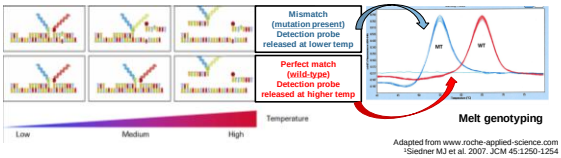
- Develop and verify a molecular assay and workflow to predict CIP susceptibility immediately after a specimen is tested positive for *N. gonorrhoeae* by NAAT
  - Timely, actionable drug susceptibility results to clinician






## Real-time PCR with FRET Probes to Detect Predicted Y91S Mutation in *gyrA*

- Anchor (donor) probe labeled with fluorescein **(green dot)**
  - Sequence homologous to area next to region of interest on *gyrA*
- Detection (acceptor) probe labeled with LC640 **(red dot)**
  - Sequence homologous to wild-type *gyrA*
- Fluorescein excitation➔Energy transfer➔LC640 emission




Adapted from www.roche-applied-science.com
¹Siedner MJ et al. 2007. JCM 45:1250-1254



## Assay Verification: Accuracy

- DNA extracted from 100 clinical isolates from GISP San Francisco Regional Laboratory with known CIP susceptibility
  - 23 S isolates, 77 R isolates
  - Accuracy = 100%
    - *gyrA* PCR able to predict CIP susceptibility via melt genotyping

| Susceptibility to ciprofloxacin | Melt curve genotype from *gyrA* PCR | |
|---|---|---|
| | Wild-type | Mutant |
| Susceptible (MIC <0.12 µg/mL) | 23 | 0 |
| Resistant (MIC >= 1 µg/mL) | 0 | 77 |



## Assay Verification: Limit of Detection

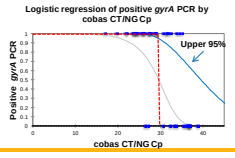
- 32 clinically positive and 16 negative patient urines (n=36) and urethral swabs (n=12)
- 24 additional urines seeded at varying concentrations with a WT and MT *N. gonorrhoeae*
- Performed cobas® CT/NG assay and retrieved residual DNA from cobas® 4800 deep well plate for *gyrA* PCR
- Compared positive crossing points (Cp) of *gyrA* PCR against Cp of cobas ® CT/NG assay



## Assay Verification: Limit of Detection

- Sensitivity and specificity of *gyrA* PCR: 75% and 100%
- False-negative *gyrA* PCR: cobas® CT/NG Cp of > 30
- Probit analysis: CT/NG assay Cp of ≤ 29.45 as having a 95% detection rate on *gyrA* assay
  - About 75% of positive specimens at UCLA have Cp of ≤29.45

| Cobas 4800 CT/NG assay | *gyrA* PCR | |
|---|---|---|
| | Positive (WT/MT) | Negative (no amplification) |
| Positive | 42 (75%) | 14 (25%) |
| Negative | 0 | 16 |






## Assay Verification

- Precision: triplicates of urines seeded with WT or mutant *gyrA* isolate at concentration of 1000 CFU/mL
  - No significant difference between triplicates
- Cross-reactivity study: *N. meningitidis*, *N. sicca*, *N. subflava*, *N. mucosa*, *N. cinerea*, and *N. elongata* from UCLA collection
  - No fluorescence signal could be genotyped

## Conclusion

- *gyrA* real-time PCR was 100% accurate in predicting ciprofloxacin susceptibility in clinical isolates
- No cross-reactivity was observed with other *Neisseria* species tested
- Residual DNA from cobas® 4800 deep well plate that were positive for *N. gonorrhoeae* in cobas® CT/NG assay could be used as template for *gyrA* PCR
  - CT/NG assay Cp ≤ 29.45: 95% detection rate on *gyrA* PCR



## Future Directions

- A study initiated at UCLA to determine impact of providing clinicians with susceptibility prediction on their prescribed treatment
- Include prediction of CIP susceptibility in CT/NG results and determine:
  - Time to ciprofloxacin susceptibility result
  - Frequency of CIP susceptibility
  - Demographic of cases of NG positivity and CIP susceptibility
  - Proportion of cases treated with an extended-spectrum cephalosporin
  - Whether provision of ciprofloxacin susceptibility results is associated with any changes in antibiotic treatment type.



## Future Directions: Cefixime Susceptibility Prediction

- Many mutations could contribute to increased MICs: Which pattern to look for?
  - Surveillance of extended-spectrum cephalosporin susceptibility among 684 *N. gonorrhoeae* in California
  - All 29 isolates with an alert value for extended-spectrum cephalosporin MICs possessed mosaic XXXIV *penA* allele[1]
- Designing melt genotyping PCR using FRET probes to detect *penA* mutations compatible with XXXIV mosaic pattern
- Multiplex with *gyrA* assay to predict CIP and CFM susceptibility simultaneously

[1]Gose, Severin, et al. *BMC infectious diseases* 13.1 (2013): 570.



## Acknowledgements


- UCLA Microbiology Laboratory
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