Poster #563

Mycoplasma genitalium with Fluoroquinolone Resistance: Design and Development of a Multiplex Real-Time PCR Assay for Detection and Differentiation.

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BACKGROUND

Mycoplasma genitalium is a sexually transmitted bacterium causing urethritis in men and associated with cervicitis and pelvic inflammatory disease in women. Only a few antimicrobial classes have activity against mycoplasmas including tetracyclines, macrolides, fluoroquinolones and streptogramins. The first-line treatment for M. genitalium infections is doxycycline followed by azithromycin. Due to overuse of azithromycin to treat infections, resistance to azithromycin has been rapidly increasing and has been confirmed in multiple studies. Subsequently, the urgency of new and efficient antibiotics has arisen. Moxifloxacin of fluoroquinolone family of antibiotics, was found to be a 100% effective against M. genitalium, but recently the resistance markers for fluoroquinolone treatment have also emerged. Multiple studies attribute fluoroquinolone treatment failure to single nucleotide polymorphisms (SNPs) in parC and gyrA genes. In parC, SNPs corresponding to the amino acid changes S83I, S83R, D87N and D87Y have been associated with fluoroquinolone failure. The contribution of gyrA SNPs alone is unknown; however, the presence of a parC S83I and a concurrent SNP in gyrA may increase the risk of treatment failure.

We aim to demonstrate proof of principle for a Real-Time PCR assay that simultaneously detects M. genitalium and fluoroquinolone resistance.

ASSAY PRINCIPLE

The MGB Alert *M. genitalium* with fluoroquinolone resistance RUO Detection Reagent is a multiplex of real-time PCR reagents that simultaneously detect M. genitalium DNA and distinguish fluoroquinolone resistance-associated mutations and a wild type in *M. genitalium*. The assay targets parC and gyrA genes and includes a set of two primers and three probes specific to *M. genitalium*. The DSQ TaqMan hydrolysis probe is specific to *M. genitalium* and identifies the species DNA. The Pleiades hybridization probes serve to identify and distinguish the wild type genotype and several known resistance-conferring point mutations by melt curve analysis. The internal control primers and probe are included into assay to monitor assay performance and the presence of inhibitors.

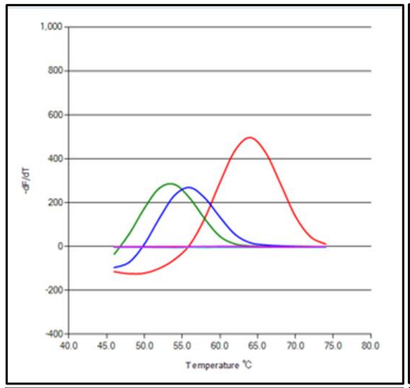
ASSAY COMPOSITION

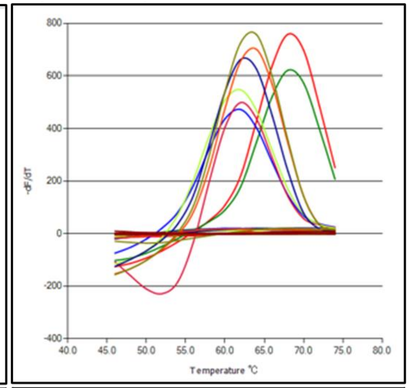
Table 1. MGB Alert M. genitalium with fluoroquinolone resistance RUO Detection Reagent components description. The number in the AP fluorophore name indicates its peak excitation wavelength.

Target template	Probe type and chemistry	Probe fluorophore	Analogous fluorophore (for optical channel selection)
M. genitalium parC gene species-specific region	DSQ hydrolysis	AP639	Cy5, Quasar 670, Alexa Fluor 647
M. genitalium parC wild type, or point mutations A247C, G248A, G248T, T249A, G259T, G259A, A260G	MGB Pleiades hybridization	FAM	FAM
M. genitalium gyrA wild type, or point mutations G285C and G286A	MGB Pleiades hybridization	AP593	ROX, Texas Red
Internal control IC2	DSQ hydrolysis	AP525	VIC, JOE, HEX

ANALYTICAL PERFORMANCE EVALUATION STUDY

Fig 1. Melt curve discrimination of the wild type and parC and gyrA gene mutants. Clean separation of the wild type and mutant melt curve peaks indicate unambiguous differentiation. The results were obtained on the ELITe InGenius® automated sample-to-result platform.





gyrA		
Mutation	Melt Tm (C°)	ΔTm (C°)
WT	63.9	0.00
G285C (M95→I)	53.1	10.80
G286A (A96→T)	55.8	8.10

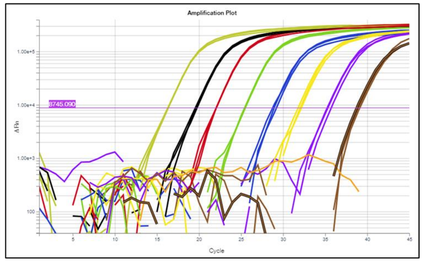
parC		
Mutation	Melt Tm (C°)	ΔTm (C°)
WT	68.4	0.00
A247C (S83→R)	61.6	6.80
G248A (S83→N)	62.3	6.10
G248T (S83→I)	61.4	7.00
G259T (D87→Y)	62.7	5.70
G259A (D87→N)	63.2	5.20
A260G (D87→G)	63.2	5.20

LIMIT OF DETECTION DETERMINATION

The limit of detection was determined by testing serial 3-fold dilutions of M.genitalium genomic DNA and calculated at 1.42 genome copies per PCR using Probit analysis approach. The calculated LoD was verified by testing 20 replicates of M.genitalium genomic DNA at LoD level.

LINEAR RANGE DETERMINATION STUDY

Fig 2. Linear range of M. genitalium RUO reagents 1e7 – 1e0 genome copies per PCR



Target	Detection channel	Slope	R2	Intercept
parC	FAM	-3.18	0.998	37.738
gyrA	AP593	-3.28	0.996	38.509
MG	AP639	-3.21	0.998	38.632

CROSS-REACTIVITY EVALUATION STUDY

The assay was tested for cross-reactivity with the organisms that might be present in normal vaginal swabs and urine specimens by *in silico* analysis and *in vitro*. All the bacteria were tested at concentration of 1e6 genome copies per PCR, and viruses were tested at 1e5 genome copies per PCR, in triplicates.

- Actinomyces israelii
- Bacteroides fragilis
- Candida albicans
- Chlamydia trachomatis
- Corynebacterium genitalium
- Escherichia coli
- Enterococcus faecalis
- Gardnerella vaginalis
- HSV1
- HSV2
- Lactobacillus crispatus
- Micrococcus luteus
- Mycobacterium smegmatis
- Mycoplasma hominis
- Mycoplasma pneumoniae
- Neisseria gonorrhoeae
- Proteus vulgaris
- Staphylococcus aureus
- Staphylococcus epidermidis
- Ureaplasma urealyticum

No cross-reactivity with non-*M.genitalium* species was observed.

MATERIALS AND METHODS

The RUO amplification reagents were used in combination with the ELITe InGenius, a fully automated sample-to-result system with six optical channels for detection and melt curve capabilities (ELITechGroup). The assay was also tested on QuantStudio Dx and QuantStudio 7 Pro (ThermoFisher). A linear range and a limit of detection were established using serial dilutions of M.genitalium gDNA. Wild type and mutant differentiation was carried out using M.genitalium gBlocks. The gBlocks were ordered from Integrated DNA Technologies (IDT).
Cross-reactivity panel was obtained from ATCC or Zeptometrix, with their nucleic acids extracted on ELITe InGenius platform and tested on QuantStudio 7 Pro real-time PCR instrument with M. genitalium RUO reagents.

RESULTS

- **Analytical sensitivity was determined and verified at 1.42 viral genome copy/rxn.**
- **A linear range was established from 1e7 to 1 genome copy/rxn.**
- **Test results showed no cross-reactivity with organisms that might be present in normal vaginal swabs and urine specimens.**
- **Melt curve analysis has shown a reliable differentiation between M. genitalium mutant (conferring resistance to fluoroquinolones) and wild type genotypes.**

CONCLUSION

We have developed a robust real-time PCR assay for detection and differentiation of the wild-type *M. genitalium* and its genotypic mutants conferring resistance to fluoroquinolones. The assay was proven to be sensitive, specific, and easy-to-use. Analytical evaluation of the MGB Alert M. genitalium with fluoroquinolone resistance RUO detection reagent indicates potential as a valuable tool in the diagnosis of *M. genitalium* and resistance guided treatment.

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