Supplementary Material

Organism	Gene	Associated with resistance to	Position targeted	Primer Sequence
N. gonorrhoeae	*gyrA*	Fluoroquinolones	S91, D95	NG-gyrA-A (5'-ATCCGCCACGACCACAAAT-3'; forward) NG-gyrA-B (5'-CGTCCACCGATCCGAAGTT-3'; reverse (35)
M. genitalium	23S *rRNA*	Macrolides	A2058, A2059	Primers (5'–3') Mg 23S-1992F: CCATCTCTTGACTGTCTCGGCTAT. Mg 23S-2138R: CCTACCTATTCTCTACATGGTGGTGTT. (36)
M. genitalium	*gyrA*	Fluoroquinolones	M95, D99	MG_gyrA_F 5'-cctgatgctagagatggacttaaa-3' MG_gyrA_R 5'-aagttctgctgcaagtttagataat-3'(13)
M. genitalium	*parC*	Fluoroquinolones	S83, D87	MG_parC_F 5'-gtgctgttggggagatcat-3' MG_parC_R 5'-ccatggatagaaacagttgttca-3' (13)

Supplementary Material Table 1: Resistance-associated mutations targeted in NG and MG

Laboratory testing: Laboratory testing was completed blind to the clinical NAAT result. Urine samples were immediately stored at 2-8°C, until shipment on ice to the laboratory, in which samples were separated into 1ml aliquots and stored at -80°C within 24 hours of collection. Swabs were stored at room temperature until shipment to the testing laboratory and then processed within 3 weeks or frozen at -20°C until testing. Frozen samples were defrosted at room temperature on the day of testing, and underwent vortexing and brief centrifugation prior to testing. Blank eNAT media

for swabs and nuclease free water (Ambion, USA) for urines were included as controls in each extraction.

All samples identified as MG positive by the FTD Urethritis plus kit (FTDUP) and an equal number of randomly selected (using Excel's RAND function) samples identified as MG negative underwent testing by in-house real-time PCR (qPCR), targeting the MgPa sequence [16] in triplicate using the Quantifast +IC kit (Qiagen, location); in a final reaction volume of 25µl; 5µl Quantifast mastermix 5x, 0.16QM primers and 0.2QM probe, Quantifast IC assay 2.5µl, 11.2µl nuclease free water (Ambion, USA), 2.5µl IC DNA and 2.5µl extracted DNA in each reaction. PCR was carried out on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). A synthetic positive control was generated by the addition of positions 1400 to 1517 in the MgPa operon sequence into a plasmid (pEX-A2) (Eurofins, Luxembourg) and added at a concentration of $1x10^6$ copies/Ql to the reaction.

Discrepant testing: The first reference test for CT/NG was the clinic's routine clinical NAAT (Becton Dickinson (BD) ProbeTec™ CT/GC Qx assay) run on the BD Viper analyser, used at all participating SHCs. For extra-genital samples, two clinics performed confirmatory testing by Xpert® CT/NG (Cepheid, USA) and Aptima Combo 2 test for CT/NG (Hologic, USA) at the other clinic. For samples where test outcome results between the FTDUP and routine clinic NAAT testing differed for either CT or NG, 15Ql of extracted DNA was tested using the Artus® CT/NG QS-RGQ assay (Qiagen) on the Rotor-Gene Q 5plex HRM PCR thermocycler, using the manufacturer's manual set-up instructions for extracted DNA with fluorescence detection carried out in the elongation step of the PCR.

For MG, where results were discordant between FTDUP and qPCR, extracted DNA was tested with the Plexzyme MG Resistance Plus™ assay (research use only kit) (SpeeDx, Australia).

Test positivity, negativity and invalids: For CT/NG, reference test results were reported from the clinic as positive, negative or invalid. Positivity by FTDUP was defined as a signal crossing a threshold of 0.05 normalised fluorescence as per local laboratory practice. Negative and positive controls had to pass for results to be considered valid. In addition, the internal control had to be positive with a

cycle threshold (Ct) value ≤33, or where it was above 33 the value had to be within +/- 3.3 cycles of the extraction control's Ct value. Threshold values for positivity by the Artus CT/NG QS-RGQ assay were based on those recommended in the kit handbook.

For MG, samples tested by qPCR were tested in triplicate and were only considered positive if all replicates produced positive results i.e. above a threshold of 1000 Relative Fluorescent Units (RFU), as per local practice. Negative and positive controls had to pass for results to be considered valid. Samples with no positive replicates were considered negative, samples with 1 or 2 positive replicates were defined as equivocal and were re-tested identically once only. If a sample gave an equivocal result twice, it was tested by the Plexzyme MG Resistance Plus™ assay to resolve its status. Sample status by Plexzyme MG Resistance Plus™ assay was provided by the SpeeDx Analysis Software for CFX96 with results only valid if the negative control was negative.

Resolved Reference Standard: For any sample where discrepant testing was performed, a resolved reference standard was defined as those where 2/3 of the test results were in agreement. Thus for a positive CT/NG resolved result, two of the three tests (clinic NAAT, FTDUP and Artus CT/NG QS-RGQ) had to be positive. For a positive MG result, 2/3 (FTDUP, in-house PCR, Plexzyme MG Resistance Plus™) had to be positive. Likewise, for a negative result, 2/3 results had to be negative.

Sequencing: PCR was performed using the Platinum Supermix kit (Invitrogen, USA) with the following reaction set up: 45µl Mastermix, 0.2nM of each primers, 2µl template, 1µl nuclease free water. References for primers used are listed in table 1.

Thermal cycling was performed using a GS-1 thermocycler (G-Storm, UK). Cycling conditions were as follows: 94ºC for 2 minutes followed by 35 cycles of 30 seconds at 94ºC, 60 seconds at 55 or 56ºC depending on primers used (56°C used for MG parC primers, 55°C used for all other primer pairs) and 2 minutes seconds at 72ºC with a final elongation step of 72°C for 2 minutes. Samples that failed to amplify underwent identical set up with an increase from 35 to 40 cycles and template from 2µl to 3µl.

PCR products were tested using the Bioanalyzer (Agilent, USA) using the Agilent DNA 1000 kit (Agilent, USA) for the presence of product. If product was present, samples were sent to Source Bioscience for sample clean-up and Sanger sequencing. Where sequencing failed, PCR reactions were repeated and analysed using 2% size select gel on the E-gel system (Thermo Fisher, USA). Desired bands were removed and underwent clean-up using Qiagen MinElute Reaction Clean up Kit (Qiagen, Germany). DNA concentrations were assessed using Qubit HS DNA kit on the Qubit 3.0 device (Thermo Fisher, USA) and adjusted to meet the requirements of Source Bioscience for Sanger sequencing. Analysis of sequenced PCR products was carried out using Chromas (Technelysium Pty Ltd) and mutations searched for at the positions listed in Table 1. Genotypic resistance or susceptibility was only defined if a result was available at all relevant positions for that class of antimicrobials, i.e. for MG resistance to fluoroquinolones a valid result was needed for both the *parC* and *gyrA* sequences and at all the positions listed. Samples were defined as susceptible if all resistance associated mutations were absent, and defined as resistant if any of the associated mutations were present.