Development and Application of Cas13a-based Diagnostic Assay for *Neisseria Gonorrhoeae* Detection and Identification of Azithromycin Resistance

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Abstract

Gonorrhea caused by *Neisseria gonorrhoeae* has spread world-wide. Antimicrobial-resistant strains have emerged to an alarming level to most antibiotics, including to the ceftriaxone-azithromycin combination, currently recommended as first-line dual therapy. Rapid testing for antimicrobial resistance will contribute to clinical decision-making for rational drug use and will slow this trend. Herein, we developed a Cas13a-based assay for *N. gonorrhoeae* detection (porA target) and azithromycin resistance identification (A2059G and C2611T point mutations). We evaluated the sensitivity and specificity of this method, and 10 copies per reaction can be achieved in porA detection and C2611T identification, with no cross-reactions. Comparison of the Cas13a-based assay (porA target) with Roche Cobas 4800 assay (n=23 urine samples) revealed 100% concordance. Isolated *N. gonorrhoeae* strains were used to validate the identification of A2059G and C2611T resistance mutations. All tested strains (8 A2059G strains, 8 C2611T strains, and 8 wild-type strains) were successfully distinguished by our assay and verified by testing MIC for azithromycin and sequencing the 23S rRNA gene. We adopted lateral flow for the SHERLOCK assay readout, which showed a visible difference between test group and NC group results. To further evaluate the capability of our assay, we tested 27 urethral swabs from patients with urethritis for *N. gonorrhoeae* detection and azithromycin-resistance identification. Of these, 62.96% (17/27) strains

37 were detected with no mutant strains and confirmed by sequencing. In conclusion, the
38 novel Cas13a-based assay for rapid and accurate *N. gonorrhoeae* detection combined
39 with azithromycin drug resistance testing is a promising assay for application in clinical
40 practice.
41
42 **Introduction**

43 Gonorrhea is a common bacterial sexually transmitted infection (STI) in the world,
44 caused by *Neisseria gonorrhoeae* [1]. As estimated by the World Health Organization
45 (WHO)，there were 78 million global cases in 2012 and 86.9 million cases in 2016
46 worldwide [2, 3]. The prevalence of *N. gonorrhoeae* has increased rapidly and remains a
47 public health concern. In the absence of an effective vaccine, antibiotic treatment is
48 critical to cure and slow the spread of *N. gonorrhoeae* infections [4, 5]. However, due to
49 the use and abuse of antibiotics, antimicrobial resistance (AMR) of *N. gonorrhoeae* has
50 emerged to all first-line therapeutic drugs used to date [4-7]. In particular, AMR to
51 azithromycin and ceftriaxone currently used as first-line dual therapy has been reported
52 as a cause of treatment failure in both the United Kingdom and Australia [8, 9], and the
53 resistance has shown a gradual increasing trend according to the gonococcal surveillance
54 program data from Europe and the United States [10, 11]. There is, therefore, a need for
55 clinicians to rapidly acquire resistance data for antibiotics, which could help manage

rational drug use and further slow the development of drug resistance.

Traditional antimicrobial resistance detection methods are mainly culture-based. The quantitative agar dilution method which can determine the minimum inhibitory concentration (MIC) of antimicrobials is recognized as the 'gold standard' method, but complicated protocol steps and long turnaround times hinder its development to satisfy clinical requirements [1, 6]. To achieve this goal, non-culture-based nucleic acid amplification tests (NAATs) have been introduced and are being developed. Sequencing technology has been widely used to identify plasmid-mediated or chromosomally-mediated drug resistance to discover antimicrobial resistance towards penicillin, ciprofloxacin, tetracycline, azithromycin, extended-spectrum cephalosporin, and multidrug resistance [6, 12]. With the discovery of a strong correlation between single nucleotide polymorphisms (SNP) and drug resistance in *N. gonorrhoeae*, more convenient assays have been established [13-17]. These methods generally use PCR to amplify target genes, and combine with specific probes, high resolution melting (HRM) analysis, or mass spectrometry to differentiate specific point mutations. The protocol is time-saving, however, large precision instruments are necessary to ensure accurate temperature control and results also require skilled evaluation.

Cas13a was first described by Zhang et al. and exploits endonuclease activity of target RNA and collateral cleavage activity of the target sequence [18]. Based on this principle, the SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) was developed, which combines recombinase polymerase amplification (RPA) and Cas13a in an isothermal system with single molecule sensitivity, high specificity, single-base resolution, and convenient acquisition of results [19]. Given the advantages of this technology, SHERLOCK is becoming a potential tool for rapid diagnostic testing of emerging infectious diseases, and has been developed for the diagnosis of *Plasmodium*, SARS-CoV-2, Ebola virus, and Lassa virus [20-22]. Thus, this robust Cas13a-based diagnostic tool may satisfy the requirements of *N. gonorrhoeae* detection and antibiotic resistant-SNPs identification in clinical practice.

Azithromycin combined with ceftriaxone is the currently recommended treatment for *N. gonorrhoeae* given the increasing MIC of ceftriaxone [23] and the emergence of high-level azithromycin resistance strains in various geographical regions showing a tendency to spread to other areas [24-30]. The main cause of elevated azithromycin resistance has been highly correlated with A2059G and C2611T mutations in the 23S rRNA subunit of the bacterial ribosome [31-33]. Herein, we attempt to develop and apply this Cas13a-based method to develop a rapid and accurate assay for *N. gonorrhoeae*

detection and azithromycin resistance identification that will contribute to rational drug use by clinicians.

Materials and Methods

Ethics approval

All human samples used for this study were evaluated and approved by the Ethics Review Committee at Dermatology Hospital of Southern Medical University (2020056).

Culture and azithromycin susceptibility testing

The *N. gonorrhoeae* strains used to verify azithromycin resistance were isolated from clinical samples of patients from Guangzhou, China and were identified by Gram Staining, oxidase, catalase, and sugar fermentation tests. Isolates were cultured in Thayer-Martin medium and incubated in 5% CO2 in a 37°C incubator.

Antimicrobial susceptibility to azithromycin was tested using the agar dilution method, according to WHO recommendations [34]. Briefly, all strains were cultured for 18 h and adjusted to a 0.5 McFarland standard suspension, and the cultures were then multipoint inoculated onto antimicrobial agar plates containing different drug concentrations.

Sample preparation

Clinical urine samples were collected and stored at 4°C temporarily before DNA extraction. Urethral swabs were collected and stored in DNA/RNA Shield (Zymo Research; R1100-250) and 4°C for temporary storage before DNA extraction. Genome

DNA extraction was carried out using the HiPure Bacterial DNA Kit (Magen; D3146-02) according to the manufacturer's instructions. Extracted genomic DNA was stored at -20℃ until use.

Clinical urine specimens for Roche COBAS 4800 NG/CT tests were prepared according to Roche's standard operation protocols.

Serial dilutions of dsDNA (porA, A2059G, C2611T) were prepared by using Q5 High-Fidelity DNA Polymerases (New England Biolabs; M0492S) to amplify the target gene in a total reaction volume of 50 μL (25 μL of 2× Master Mix, 2.5 μL of each 10 μM primer, 2 μL of DNA template, and 18 μL ddH2O). PCR was performed as follows: initial denaturation at 98℃ for 30 s, then 35 cycles at 98℃ for 10 s, 60℃ for 20 s, and 72℃ for 30 s, followed by 72℃ for 2 min. After amplification, PCR products were verified and purified following agarose gel electrophoresis. The Universal DNA purification kit (TIANGEN; DP214) was used according to the manufacturer's protocol to extract target DNA from agarose gel. Purified dsDNA was quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher; Q33230) and stored at -20℃ until use. PCR primers used to produce dsDNA are reported in **Table S2**.

Protein expression and purification of Cas13a

LwCas13a expression and purification were carried out according to the protocols described by Zhang et al. with some modifications [35]. Briefly, LwCas13a expression vectors (NovoPro Bioscience; V010159) were transformed into Rosetta (DE3) Competent

Cells (Tiangen; CB108). Competent cells containing LwCas13a vectors were inoculated into LB Broth media (Sangon Biotech; A507002) containing 50 μg/mL ampicillin (Sangon Biotech; A100339) and grown at 37℃, 220 rpm until the OD600 reached 0.6. Isopropyl-beta-D-thiogalactopyranoside (Sangon Biotech; B541007) was added to the media at a final concentration of 0.5 mM to induce protein expression. Cells were then centrifuged at 4℃, and cell pellets were harvested and stored at -80℃ for further purification.

Protein purification was performed at 4℃. Cell pellets were crushed and purified with His-tag Protein Purification Kit (Beyotime Biotechnology; P2226). Then incubated at 25℃ for 3 h with SUMO protease (Novoprotein; PE007-01A) to digest SUMO tag. Purified Cas13a protein was stored at -80℃ in storage buffer (50 mM Tris, 600 mM NaCl, 5% glycerol, 2 mM DTT, pH 7.5). All purification steps were analyzed and confirmed by SDS-PAGE and Coomassie Blue staining (Sangon Biotech; C510041). The concentration of protein was quantified using the BCA Protein Assay Kit (Beyotime Biotechnology; P0012S).

crRNA preparation

For crRNA preparation, oligonucleotides containing the T7 promoter sequence, spacers (complement to target RNA), and the crRNA core sequence (bind to Cas13a) were designed by SnapGene 4.1.9 and NCBI BLAST, and synthesized by Sangon Biotech. Synthetic ssDNA (100 μM) binds to short T7 primer sequence (100 μM) by gradient

154 annealing from 95℃ to 25℃ with cooling rate of 0.1℃/s. The product was then
155 transcribed to crRNA using HiScribe T7 Quick High Yield RNA Synthesis kit (New
156 England Biolabs; E2050S) incubating at 37℃ overnight. Transcribed crRNA was
157 purified using RNA XP Clean Beads (Beckman; A63987), and the concentration was
158 quantified using the Qubit RNA HS Assay Kit (ThermoFisher; Q32852). crRNA was
159 stored at -20℃ until use. All crRNA used in this study are reported in **Table S2**.

160 **Recombinase Polymerase Amplification**

161 Recombinase Polymerase Amplification (RPA) primers were designed by SnapGene
162 4.1.9 and NCBI BLAST according to the TwistAmp Assay Design Manual instructions,
163 which can be downloaded from the official website
164 (https://www.twistdx.co.uk/en/support/ manuals/twistamp-manuals), with the condition
165 that primers must flank the crRNA target region. RPA primers were synthesized by
166 Sangon Biotech. The forward primer contained the T7 promoter sequence for initiating
167 the transcription. TwistAmp Basic (TwistDx; TABAS03KIT) was used to amplify the
168 target DNA. In a total reaction volume of 25 μL (containing 1.2 μL of each 10 μM primer,
169 14.75 μL of rehydration buffer, 1.25 μL of Magnesium Acetate (MgOAc), 1 μL of input,
170 and 5.6 μL ddH2O). The mixture was run at 37°C for 2 h and then subjected to Cas13a
171 detection assays. All RPA primers used in this study are available in **Table S2**.

172 **LwCas13a collateral detection**

173 Cas13a detection assays were mainly performed according to the protocol described by

Zhang et al. [19]. Briefly, the assay was carried out in a 25 μL reaction volume consisting of 40 mM Tris-HCl (pH7.5), 9 mM $MgCl_2$, 1 mM rNTPs (New England Biolabs; N0466L), 50 U RNase inhibitor (New England Biolabs; M0314L), 37.5 U T7 RNA Polymerase (New England Biolabs; M0251L), 225 nM crRNA, 45nM purified LwCas13a, 125 nM RNA reporter (5'-6FAM-UUUUU-BHQ1-3' as described by Gootenberg et al.[36]), and 1.25 μL RPA reaction solution was added to the above mixture. The reaction mixture was allowed to incubate at 37°C for 3 h in a 96-Well Half-Area Microplate (Corning; CLS3694-100EA). Fluorescence emission (excitation 490 nm/detection 520 nm) was tested every 5 min.

Lateral flow readout

The lateral flow dipstick (Milenia Biotec; MGHD 1) was used to acquire the results of Cas13a collateral cleavage, which was based on the cleavage of the FITC-RNA-Biotin reporter. It basically replaces the RNA reporter used in the system described above, with a new RNA reporter (5'-FITC-UUUUUUUUUUUUU-Biotin-3' described by Myhrvold et al. [37]), and then was subjected to the same process. Subsequently, a 20 μL volume of Cas13a detection solution was added and the reaction mixture was incubated at 37°C for 3h in 80 μL dipstick buffer, with thorough mixing. A lateral flow dipstick was inserted into the mixture to obtain the results.

Sanger sequencing

The 23s rRNA gene of *N. gonorrhoeae*, which contains the A2059G and C2611T point

mutations, was amplified using the Q5 High-Fidelity DNA Polymerases (New England Biolabs; M0492S). In a 25 μL reaction volume, comprised of 12.5 μL of 2x Master Mix, 1.25 μL of each 10 μM primer, 1 μL of input, and 8 μL ddH2O. PCR was performed as follows: initial denaturation at 98℃ for 30 s, then 35 cycles of 98℃ for 10 s, 64℃ for 20 s, and 72℃ for 30 s, followed by 72℃ for 2 min. PCR products were verified by Sanger sequencing (Sangon Biotech). The results of sequencing were blasted in SnapGene 4.1.9. PCR primers are available in **Table S2**.

Analysis of fluorescence data

Prism 8 software (GraphPad, La Jolla, CA, USA) was used for visualization of results and data analyses. Data are presented as mean ± SEM and were tested for normality with the Shapiro-Wilk test. Differences were considered significant at *P*-values < 0.05.

Results

Schematic of Cas13a based *N. gonorrhoeae* detection and azithromycin resistance identification

The SHERLOCK assay was performed as established by Zhang et al. [19], and combined RPA and Cas13a to create an isothermal detection system. The target sequence was amplified by RPA, and the T7 promoter was appended to the front of the RPA product as the template to be used to initiate subsequent RNA transcription. Synthetic crRNA guided

the Cas13a protein to recognize the specific target and enable its RNA cleavage and collateral cleavage activities (**Fig. 1**) [18]. For *N. gonorrhoeae* detection, we selected porA as the target as was frequently used to identity *N. gonorrhoeae* in other methods [17, 38-40]. Based on the characteristics of its single-base resolution, we constructed two crRNA sequences that could identify A2059G and C2611T separately. Although a single synthetic mismatch of crRNA pairing to the target enabled the assay to identify the A2059G mutation (**Fig. S1A**), this design failed to achieve the identical results for the detection of the C2611T mutation. Thus, we introduced one more synthetic mismatch of crRNA for C2611T mutation testing (**Fig. S1B-D**). Both crRNA designs were successfully utilized for 23s rRNA mutations detection.

Evaluation of the Cas13a-based method with sensitivity and specificity

To determine the sensitivity of the *N. gonorrhoeae* detection and azithromycin resistance identification assay, we prepared serial dilutions of dsDNA template, ranging from 10^0 copy/μL to 10^5 copies/μL (**Table 1**). The RPA step included the addition of 1 μL input of dsDNA template, which was then transferred to the mixture for Cas13a detection. Detection of 10 copies/μL was achieved for porA and C2611T identification (**Fig. S2A, S2E**). For A2059G identification, the detection limit was an order of magnitude lower, at about 10^2 copies/μL (**Fig. S2C**). We further evaluated the specificity of the assay using a panel of urogenital tract pathogenic bacteria (n=12) and *Neisseria meningitides* (**Table 2**).

No cross-reactivity was observed for both *N. gonorrhoeae* detection and azithromycin resistance identification (**Fig. S2B, S2D, S2F**). All RPA primers and crRNA sequences had been confirmed by BLAST before we tested its specificity. The SHERLOCK exhibits robust capability for *N. gonorrhoeae* detection and azithromycin resistance identification.

Validation of *N. gonorrhoeae* detection in clinical urine samples

Twenty-three clinical urine samples with low concentrations previously tested using the standard procedure of the Roche Cobas 4800 NG/CT test were used to validate the performance of SHERLOCK. DNA was extracted from urine samples after centrifugation and a maximum volume of 6.6 μL of DNA was amplified by RPA, followed by Cas13a detection. The method was repeated using 3 technical replicates and the fluorescence signal of each sample was normalized against the negative controls. Using this method, a total of 12 of 23 positive samples were detected, showing a 100% coincidence rate with the Roche assay (**Fig. 2**). The fluorescence signals of 3 samples (samples 3, 6, 10) were weaker than other specimens, but still could be distinguished with the negative control.

Validation of azithromycin resistance identification

A2059G mutant strains (n=8), C2611T mutant strains (n=8), and wild-type strains (n=8) isolated from clinical specimens were used to validate Cas13a-based SNPs detection. We measured the MICs of azithromycin in each strain and sequenced their 23S rRNA gene (**Fig. 3, 4**). Strains containing either the A2059G or C2611T point mutation were more

likely to be a high-level azithromycin-resistant strain. Of the 8 A2059G isolated strains, all 8 strains had MICs of >1 mg/L, and 5 A2059G strains had MICs ≥512 mg/L. Of the 8 C2611T isolated strains, 7 had MICs of >1 mg/L and 3 C2611T strains had MICs ≥512 mg/L. Compared with mutant strains, the wild-type strains possessed lower MICs, corresponding to ≤1 mg/L in 7 strains and the MIC of the remaining strain was 4 mg/L, which was above the average MICs of all mutant strains. We extracted DNA of all mutant and wild-type strains to validate this Cas13a-based assay. Paired with A2059G crRNA or C2611T crRNA, this assay successfully differentiated all 8 A2059G mutant strains and 8 C2611T strains from 8 wild-type strains (**Fig. 3, 4**). Sixteen strains harboring A2059G and C2611T mutations were identified by exhibiting a higher fluorescence intensity than wild-type strains by our assay (**Fig. S3A, S3B**). Thus, we successfully applied Cas13a based assay in azithromycin resistance identification.

Lateral flow for *N. gonorrhoeae* detection and azithromycin resistance identification

We applied lateral flow to provide a more convenient readout tool. The FAM and BHQ1 markers in the RNA reporter were replaced by FITC and Biotin. Compared to the fluorescent intensity detection, the lateral flow is inserted directly into the reaction liquid instead of using a specific device or instrument for the readout of results. The lateral flow contains a control band and a test band. Generally, a positive test will show only one test band or two bands (test band and control band), due to its different cleavage efficiency

which will result in varying amounts of cleaved RNA reporter captured by the antibody in the test band. We tested lateral flow for porA detection, A2059G identification, and C2611T identification separately (**Fig. 5A-C**). Three positive groups generated visual signals in the test bands, while all wild-type groups and the no-input group only showed a single control band.

Applying Cas13a based *N. gonorrhoeae* detection and azithromycin resistance identification in urethritis

To confirm the efficacy of the assay in clinical specimens. We collected 27 urethral swabs from patients with urethritis requiring differential diagnosis for potential gonococcal infection and to determine whether azithromycin was still effective and this information is important for the clinician. We extracted DNA from urethral swabs directly and then tested all 27 samples with the SHERLOCK assay (**Fig. 6**). Overall, 62.96% (17/27) samples showed porA positivity, and the fluorescence intensity of 17 samples was higher than that of the negative samples and the no-input group. We further tested for azithromycin resistance with A2059G crRNA and C2611T crRNA, and no mutant strain was discovered in the 17 porA positive samples. For 27 specimens, we sequenced the 23S rRNA gene and the results showed a 100% coincidence rate with our assay (**Table S1**). Sequencing data demonstrated that 17 porA positive samples were wild-type strains and no signals were detected in 10 porA negative samples.

Discussion

Antimicrobial resistance is the largest concern in the treatment of gonorrhea, with its decreasing susceptibility to antibiotics used in previous or current treatment approaches [5]. Treatment failures have been reported to the current first-line drug combination of azithromycin and ceftriaxone [8, 9], and represent a significant treatment challenge to clinicians. Rapid information regarding antimicrobial resistance would be beneficial to rational drug use in the clinic and would slow this growing trend. Traditional culture-based drug resistance methods have been widely used in the clinical laboratory and are of great importance in antimicrobial resistance surveillance, although the testing is time-consuming and is unable to meet clinical requirements rapidly [41]. Newly NAATs-based assays may overcome some of the disadvantages of culture-based methods and can be applied to identify antimicrobial resistance associated mutations simultaneously. Current molecular methods to identify mutations are mainly based on specially designed probes, HRM analysis, and mass spectrometry [13, 16, 17]. Compared with culture-based antimicrobial resistance detection methods, these methods effectively shorten assay times, but still present several limitations. If the alteration in gene has little impact on Tm values or GC content, for example, C to G variations, the method would be invalidated, moreover, short insertions or deletions may make the method unreliable [17,

42]. Furthermore, these assays are essentially PCR-based and require PCR amplification instruments coupled with other detection equipment, which limits the convenience and flexibility of the assay. Thus, the development of an assay with ultra-high resolution is desired for distinguishing mutant from the wild-type.

CRISPR/Cas molecular diagnostics have been developed and applied for testing various organisms, including SARS-CoV-2, HPV, Zika virus, Dengue virus, Ebola virus, and plasmodium [20, 21, 37, 43, 44]. Benefiting from its high specificity, sensitivity, and ability to identify SNP with the isothermal process, we have adopted a Cas13a-based strategy for *N. gonorrhoeae* detection and antimicrobial resistance identification in this study. The SHERLOCK contains two nucleic acid amplification steps: DNA amplification by recombinase polymerase amplification and RNA amplification by T7 transcription. With double signal amplification cycles, this strategy allowed to detect low levels of *N. gonorrhoeae*. Cross-reactivity is the major concern of currently developed diagnostic methods [12]. Attempts have been made to introduce two targets to uniquely identify a species, but this approach complicates the assay [13, 15-17]. The combination of specially-designed RPA primers and crRNA makes the whole reaction extremely specific. As expected, our assay exhibited high specificity in testing a panel of non-gonococcal bacteria. In addition, our Cas13a-based assay showed an excellent

concordance rate with the Roche Cobas assay currently used for clinical urine samples. With regard to SNPs detection, assay has been developed that exploits CRISPR/Cas13a technology to recognize single point mutations [19, 36, 45]. Our Cas13a-based assay achieved a sensitivity of 10 copies per reaction, which is more sensitive than previous HRM-based assays [46]. The diagnostic capability of the Cas13a-based assay has also been examined by testing clinical isolates harboring the SNP mutation. This isothermal assay which relies on a reaction temperature of 37℃ over the entire process without complex equipment has a great potential to be applied as a POCT device.

Azithromycin is a widely used macrolide antimicrobial agent and primarily acts on domain V of the 23S rRNA gene. Previous studies and our observation have revealed that *N. gonorrhoeae* strains harboring A2059G and C2611T mutations in the 23s rRNA gene are strongly associated with high-level azithromycin resistance [28, 32, 33, 47-49]. Sixteen *N. gonorrhoeae* isolates containing 23s rRNA mutations were utilized to evaluate the performance of our assay. The results showed that our Cas13a-based assay could provide drug resistance information in real-time. We also tested a small number of urethral swabs collected from the clinic in Guangzhou, China. In porA-positive(17/27) urethral swabs from patients with urethritis, no 23s rRNA mutant was identified, which is consistent with previous reports, indicating that the high-level azithromycin-resistant *N.*

346 *gonorrhoeae* has not wildly spread in Guangzhou, China [14, 16, 17]. Because of the
347 ongoing use of ceftriaxone and azithromycin dual therapy, the surveillance of 23s rRNA
348 mutation is still a requirement.

349

350 There are several limitations to our study. We only tested a small number of samples and
351 limited sources of clinical specimen. Cervical, anus, and pharynx specimens should be
352 considered in further study. Moreover, the cost of SHERLOCK is higher than
353 HRM-based method, though lower than most reported assays to date. In summary, we
354 developed a CRISPR/Cas13a-based assay for *N. gonorrhoeae* detection and azithromycin
355 resistance identification with great potential for providing drug resistance information to
356 assist clinical diagnosis and treatment.

357

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Author contributions

Conceived and designed the study: HL and HPZ. Collected samples: ZDM, XML, JJY, JLO, QQX, and ZQF. Isolation of clinical strains: LHZ, YYP, QHX. Tested Susceptibility to azithromycin: XML, XXL, and YWL. Performed the laboratory work: HL. Analyzed the data: HL and WC. Wrote the initial draft of the paper: HL and WC. Funding supported the study: XLQ, XML, and HPZ. All authors viewed and contributed to the final paper.

Competing interests

The authors declare no interest of conflicts.

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Figure legends

535 **Fig. 1** Schematic overview of the SHERLOCK assay. Illustration of LwCas13a combined
536 with RPA for detection of *N. gonorrhoeae*. The target gene was amplified by RPA and
537 appended with the T7 promoter. The unpurified RPA product is added to the Cas13a
538 system. Once crRNA was matched the transcribed RNA target, Cas13a cleaves the RNA
539 reporter and the reporters emit a fluorescent signal.

540 **Fig. 2** Validation of the SHERLOCK assay for urine samples**.** The performance of
541 SHERLOCK assay in clinical urine samples compared to verification using the Roche
542 Cobas 4800 (NG/CT test).

543 **Fig. 3** Identification of the SHERLOCK assay for the identification of the A2059G point
544 mutation**.** Sixteen *N. gonorrhoeae* strains were tested to measure their MICs for
545 azithromycin and then assayed for 23S rRNA for 2059 and 2611 point mutations.
546 Extracted DNA was tested by SHERLOCK assay directly.

547 **Fig. 4** Evaluation of the SHERLOCK assay for C2611T point mutation detection. Sixteen
548 *N. gonorrhoeae* strains were evaluated to determine MICs of azithromycin and were then
549 assayed for 23S rRNA 2059 and 2611 point mutations. Extracted DNA was tested by
550 SHERLOCK assay directly.

551 **Fig. 5** The SHERLOCK assay using lateral flow readout for the detection of *N.*
552 *gonorrhoeae*. (A) Lateral flow readout system applied to the SHERLOCK assay for the
553 detection of the porA gene. (B) Lateral flow result for the detection of the A2059G point

554 mutation. (C) Lateral flow result for the detection of the C2611T point mutation.

555 **Fig. 6** Fluorescence intensity of *N. gonorrhoeae* SHERLOCK assay for the detection of
556 gonococcal urethritis. The performance of the CRISPR/Cas13a-based assay for using
557 urethral swab samples from patients with urethritis for identification of porA, A2059G,
558 and C2611T.

559 **Fig. S1** Schematic diagram illustrating the crRNA design for the detection A2059G and
560 C2611T. (A) crRNA for the identification of A2059G and one synthetic mismatches are
561 highlighted. (B) crRNA for the identification of C2611T; one synthetic mismatches are
562 highlighted in crRNA1 and two synthetic mismatches are highlighted in crRNA2. (C)
563 Fluorescence intensity of crRNA1 and crRNA2 for the detection of C2611T and
564 wild-type template (n=3 technical replicates). (D) The ratio of fluorescence intensity of
565 C2611T to WT.

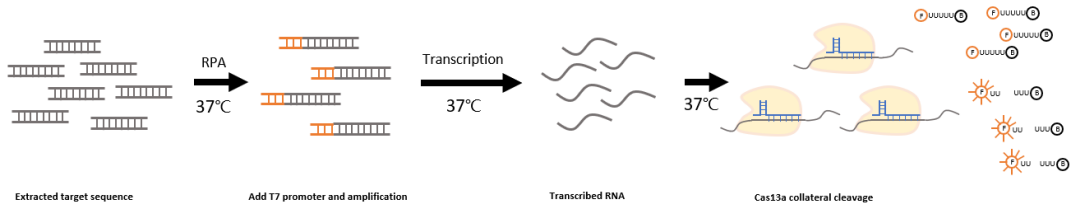
566 **Fig. S2** Sensitivity and specificity of the SHERLOCK assay for the detection of porA,
567 A2059G, and C2611T. (A) Sensitivity of porA gene detection (n=3 technical replicates,
568 two-tailed Student t-test; ns, not significant; **, $p < 0.01$; ****, $p < 0.0001$; bars
569 represent mean ± SEM). (B) Specificity of porA detection (n=3 technical replicates). (C)
570 Sensitivity of A2059G detection (n=3 technical replicates, two-tailed Student t-test; ns,
571 not significant; ****, $p < 0.0001$; bars represent mean ± SEM). (D) Specificity of
572 A2059G detection (n=3 technical replicates). (E) Sensitivity of C2611T detection (n=3

technical replicates, two-tailed Student t-test; ns, not significant; ***, $p < 0.001$; ****, $p < 0.0001$; bars represent mean ± SEM). (F) Specificity of C2611T detection (n=3 technical replicates).

Fig. S3 Fluorescence intensity relative to azithromycin resistance mutation strain detection. (A) Fluorescence intensity of the SHERLOCK assay for the detection of 16 *Neisseria gonorrhoeae* strains (8 A2059G mutant strains and 8 wild-type strains) for A2059G detection. (n=3 technical replicates, two-tailed Student t-test; ns, not significant; *, $p < 0.05$, ***, $p < 0.001$; ****, $p < 0.0001$; bars represent mean ± SEM). (B) Fluorescence intensity of 16 *N. gonorrhoeae* strains (8 C2611T mutant strains and 8 wild-type strains) for C2611T detection. (n=3 technical replicates, two-tailed Student t-test; ns, not significant; ****, $p < 0.0001$; bars represent mean ± SEM)

Figure

Fig. 1 Schematic overview of the SHERLOCK assay.



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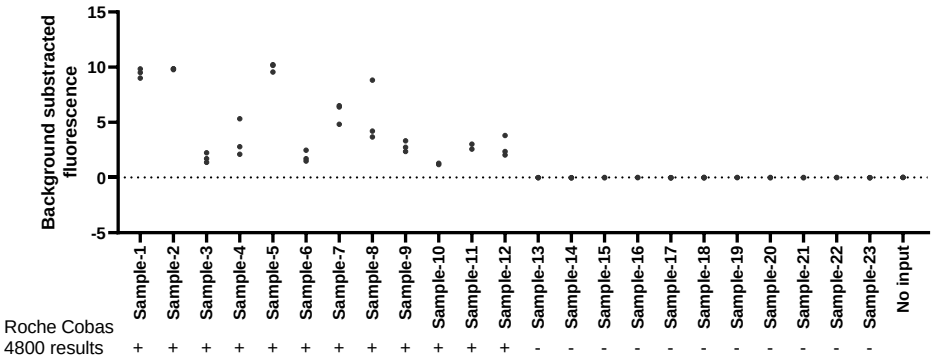
590 **Fig. 2** Validation of the SHERLOCK assay for urine samples.



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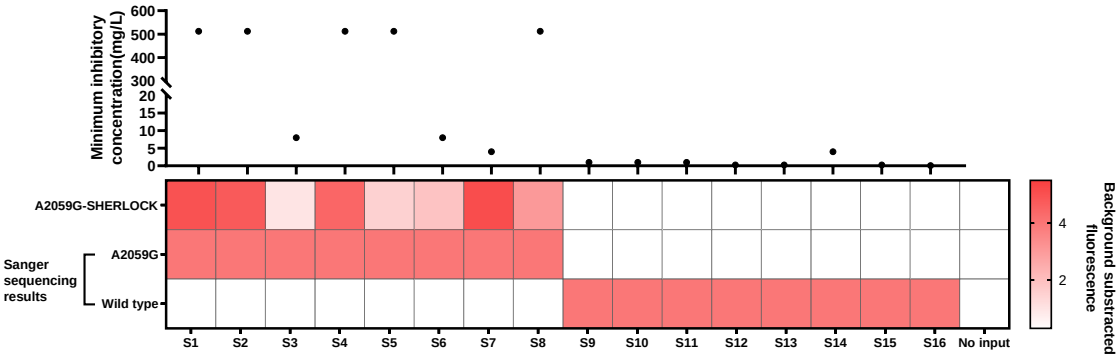
597 **Fig. 3** Identification of the SHERLOCK assay for the identification of the A2059G point

598 mutation.



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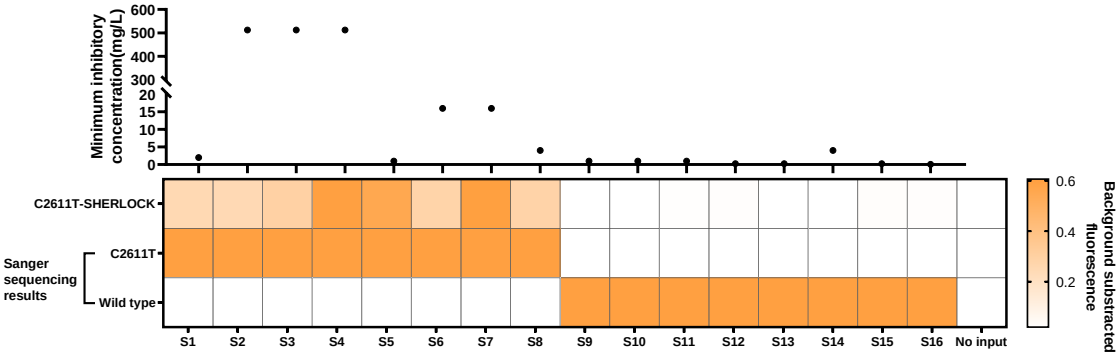
601 **Fig. 4** Evaluation of the SHERLOCK assay for C2611T point mutation detection.



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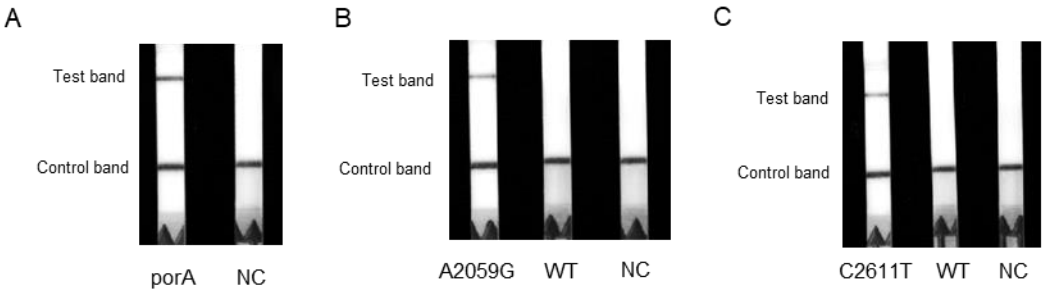
607 **Fig. 5** The SHERLOCK assay using lateral flow readout for the detection of *N.*

608 *gonorrhoeae*.



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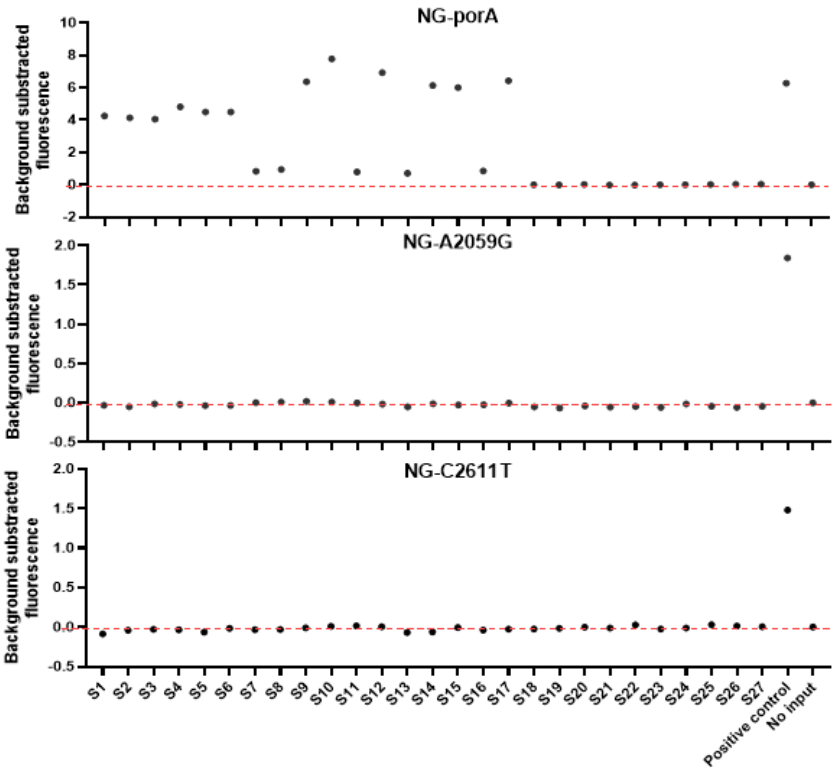
611 **Fig. 6** Fluorescence intensity of *N. gonorrhoeae* SHERLOCK assay for the detection of

612 gonococcal urethritis.



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614 **Table**

615 **TABLE 1** LODs of porA detection and 23S rRNA point mutation identification (copies

per reaction)

Assay	10^4	10^3	10^2	10^1	10^0	Wild type[a]
porA	3/3	3/3	3/3	3/3	0/3	N/A
A2059G	3/3	3/3	3/3	0/3	0/3	0/3
C2611T	3/3	3/3	3/3	3/3	0/3	0/3

[a]N/A, not applicable

TABLE 2 Specificity of porA detection and 23S rRNA point mutation identification

Organism	Assay[a]		
	porA	A2059G	C2611T
porA	Pos	N/A	N/A
A2059G template	N/A	Pos	Neg
C2611T template	N/A	Neg	Pos
Neisseria Meningitidis	Neg	Neg	Neg
Treponema pallidum	Neg	Neg	Neg
Herpes Simplex Virus-1	Neg	Neg	Neg
Herpes Simplex Virus-2	Neg	Neg	Neg
Human Papilloma Virus-2	Neg	Neg	Neg
Human Papilloma Virus-7	Neg	Neg	Neg
Candida Albicans	Neg	Neg	Neg
Trichomonas Vaginalis	Neg	Neg	Neg
Chlamydia Trachomatis	Neg	Neg	Neg
Ureaplasma Urealyticum	Neg	Neg	Neg
Mycoplasma Humanum	Neg	Neg	Neg
Escherichia Coli	Neg	Neg	Neg
Enterococcus Faecalis	Neg	Neg	Neg

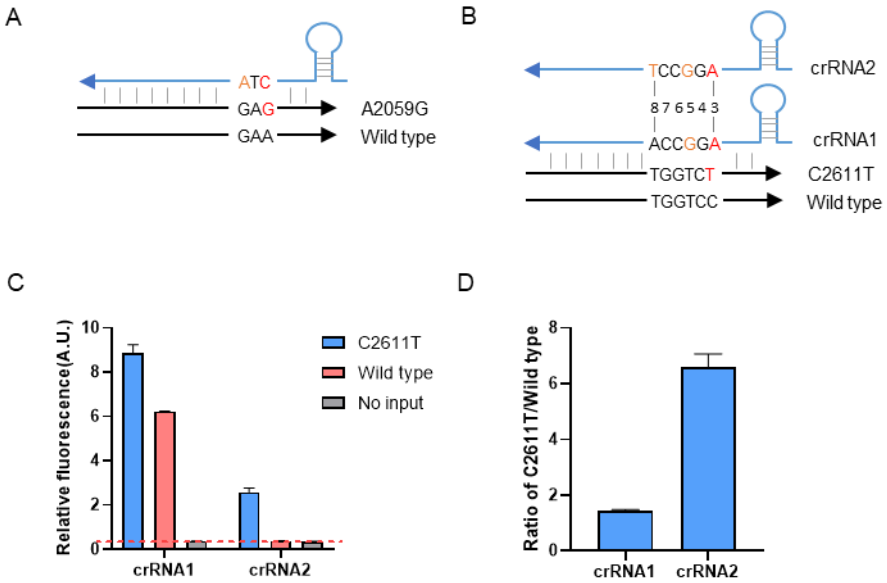
[a]Pos, positive; Neg, negative; N/A, not applicable.

622 **Supplementary Data**

623 **Figure**

624 **Fig. S1** Schematic diagram illustrating the crRNA design for the detection A2059G and

625 C2611T.



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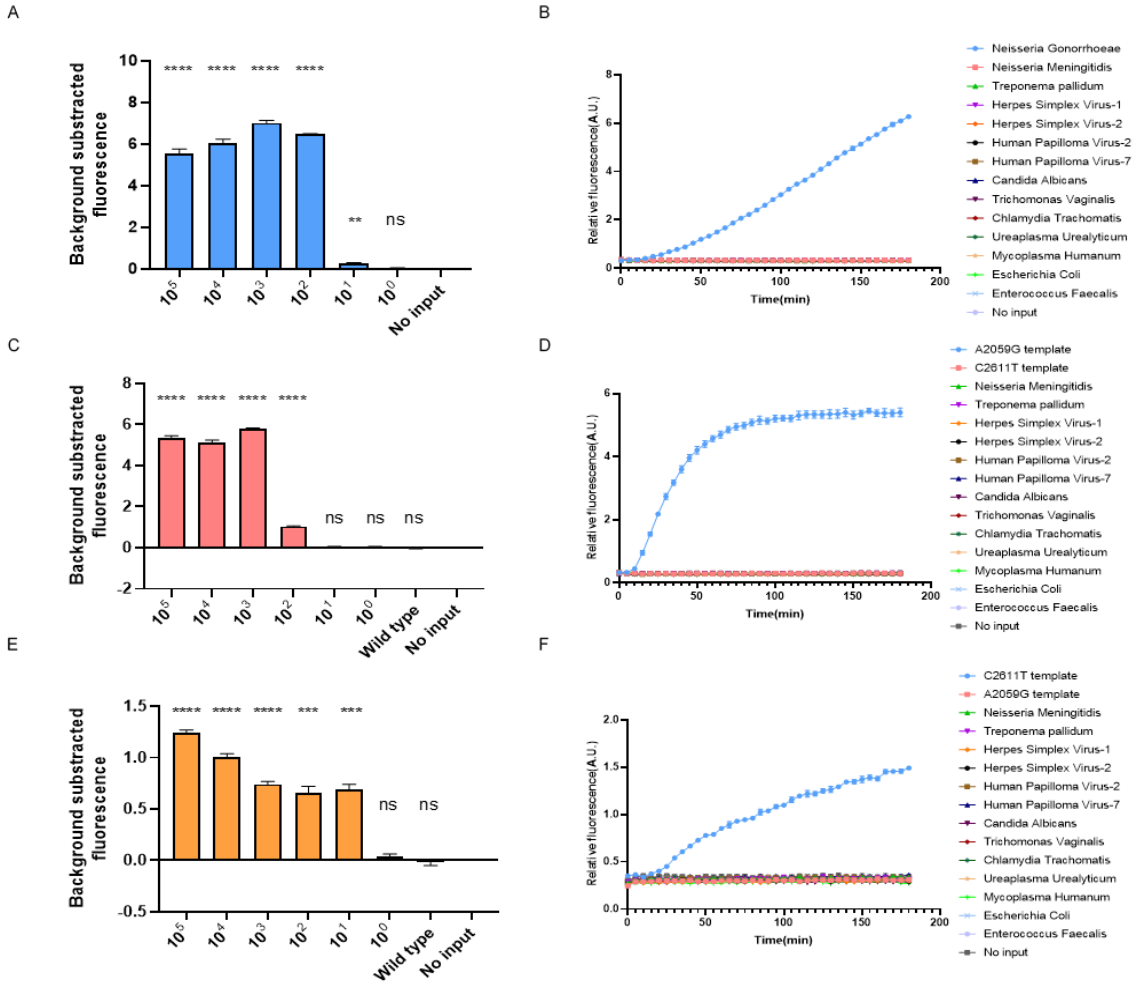
631 **Fig. S2** Sensitivity and specificity of the SHERLOCK assay for the detection of porA,

632 A2059G, and C2611T.



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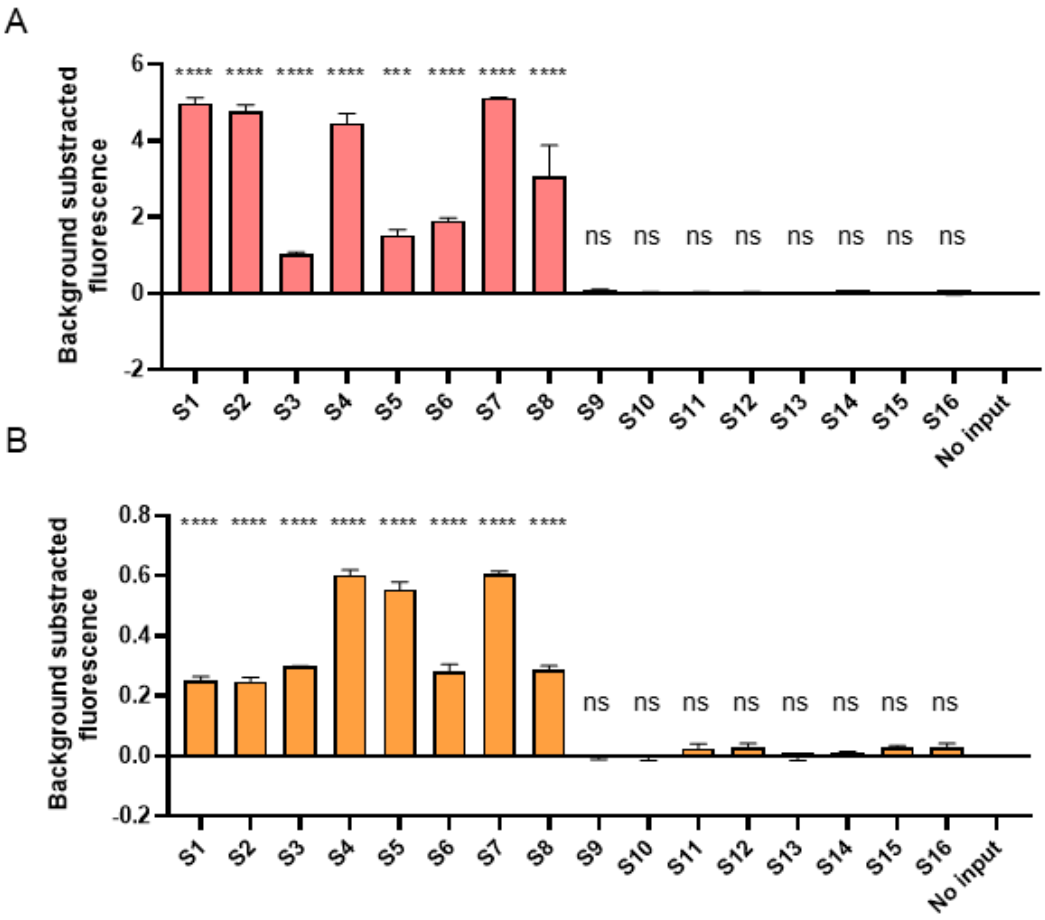
638 **Fig. S3** Fluorescence intensity relative to azithromycin resistance mutation strain

639 detection.



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647 **Table**

Table S1 Applying Neisseria gonorrhoeae SHERLOCK for detection of urethritis

Sample ID	Clinical diagnosis	Assay[a]			Sanger sequencing[a,b]
		poA	A2059G	C2611T	
S1	urethritis	Pos	Neg	Neg	WT
S2	urethritis	Pos	Neg	Neg	WT
S3	urethritis	Pos	Neg	Neg	WT
S4	urethritis	Pos	Neg	Neg	WT
S5	urethritis	Pos	Neg	Neg	WT
S6	urethritis	Pos	Neg	Neg	WT
S7	urethritis	Pos	Neg	Neg	WT
S8	urethritis	Pos	Neg	Neg	WT
S9	urethritis	Pos	Neg	Neg	WT
S10	urethritis	Pos	Neg	Neg	WT
S11	urethritis	Pos	Neg	Neg	WT
S12	urethritis	Pos	Neg	Neg	WT
S13	urethritis	Pos	Neg	Neg	WT
S14	urethritis	Pos	Neg	Neg	WT
S15	urethritis	Pos	Neg	Neg	WT
S16	urethritis	Pos	Neg	Neg	WT
S17	urethritis	Pos	Neg	Neg	WT
S18	urethritis	Neg	Neg	Neg	N/A
S19	urethritis	Neg	Neg	Neg	N/A
S20	urethritis	Neg	Neg	Neg	N/A
S21	urethritis	Neg	Neg	Neg	N/A
S22	urethritis	Neg	Neg	Neg	N/A
S23	urethritis	Neg	Neg	Neg	N/A
S24	urethritis	Neg	Neg	Neg	N/A
S25	urethritis	Neg	Neg	Neg	N/A
S26	urethritis	Neg	Neg	Neg	N/A
S27	urethritis	Neg	Neg	Neg	N/A

[a]Pos, positive; Neg, negative; N/A, not applicable.

[b]WT, 23S rRNA wild type strain

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652 **Table S2** Primer and crRNA sequences

Sequence ID	Target gene	Sequences (5'-3')	Application in this study
porA-RPA-F	porA	GAAATTAATACGACTCACTATAGGGCCGGAACTGGTTTCATCTGATT	RPA
porA-RPA-R	porA	GATTTTCCGGTTTCAGCGGCAGCATTCAAT	RPA
porA-crRNA	porA	GATTTAGACTACCCCAAAAACGAAGGGGACTAAAACCCGAGTCAAAACAGCAAGTCCGCCTATA	CRISPR/Cas 13a
A2059G-RPA-F	A2059G	GAAATTAATACGACTCACTATAGGGTCAGCGAAGTTGAAGTGGTTGTGAAGATGC	RPA
A2059G-RPA-R	A2059G	TACACAAGTGACTTCAAAGTCCAATGCAAAG	RPA
A2059G-crRNA	A2059G	GATTTAGACTACCCCAAAAACGAAGGGGACTAAAACCTCTACGTCTAGCAGCGGGTAGATTG	CRISPR/Cas 13a
C2611T-RPA-F	C2611T	GAAATTAATACGACTCACTATAGGGCCCAAGGGTATGGCTGTTCGCCATTTAAAG	RPA
C2611T-RPA-R	C2611T	TTACAACCGGTACACCAGAGGTTCGTCCAC	RPA
C2611T-crRNA-1	C2611T	GATTTAGACTACCCCAAAAACGAAGGGGACTAAAACAGAGGCCAAACTGTCTCACGACGTTT	CRISPR/Cas 13a
C2611T-crRNA-2	C2611T	GATTTAGACTACCCCAAAAACGAAGGGGACTAAAACAGAGGCCTAACTGTCTCACGACGTTT	CRISPR/Cas 13a
porA-F	porA	ATTCAGACCGGCATAATACACATCC	PCR
porA-R	porA	TAATGTGGCTTCGCAATTGGGT	PCR
A2059G-F	A2059G	GTGCCGGAAGGTTAATTGAA	PCR
A2059G-R	A2059G	CAGGGTGGTATTTCAAGGAC	PCR
C2611T-F	C2611T	CTGCGAGACCGACAAGTC	PCR
C2611T-R	C2611T	GTCTCGAACGACCCTTTAG	PCR
23SrRNA-F	23SrRNA	ACGAATGGCGTAACGATGGCCACA	Sequencing[1]
23SrRNA-R	23SrRNA	TTCGTCCACTCCGGTCCTCTCGTA	Sequencing[1]

653 **1.** Ng LK, Martin I, Liu G, Bryden L. Mutation in 23S rRNA associated with macrolide
654 resistance in Neisseria gonorrhoeae. Antimicrob Agents Chemother. 2002
655 Sep;46(9):3020-5

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