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Rapid Antibiotic Susceptibility Testing of Pathogenic Bacteria Using Heavy-Water-Labeled Single-Cell Raman Spectroscopy in Clinical Samples

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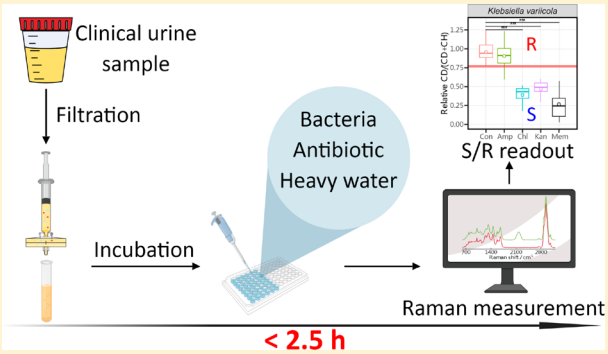
Supporting Information

ABSTRACT: Speeding up antibiotic susceptibility testing (AST) is urgently needed in clincial settings to guide fast and tailored antibiotic prescription before treatment. It remains a big challenge to achieve a sample-to-AST answer within a half working day directly from a clinical sample. Here we develop single-cell Raman spectroscopy coupled with heavy water labeling (Raman-D_2O) as a rapid activity-based AST approach directly applicable for clinical urine samples. By rapidly transferring (15 min) bacteria in clinical urine for AST, the total assay time from receiving urine to binary susceptibility/resistance (S/R) readout was shortened to only 2.5 h. Moreover, by overcoming the nonsynchronous responses between microbial activity and microbial growth, together with setting a new S/R cutoff value based on relative C−D ratios, S/R of both pathogenic isolates and three clinical urines against antibiotics of different action mechanisms determined by Raman-D_2O were all consistent with the slow standard AST assay used in clincial settings. This work promotes clinical practicability and faciliates antibiotic stewardship.



Antibiotic resistance is a growing global health concern.[1,2] More and more pathogenic bacteria have developed resistance to one or multiple antibiotics. Infections with antibiotic-resistant bacteria are predicted to surpass cancer and heart disease to become the leading cause of death in 2050.[1] World Health Organization (WHO) published its first list of antibiotic-resistant "priority pathogens" that pose the greatest threat to human health.[3] This problem is being aggravated by the misuse and abuse of antibiotics.[4] Rapid antibiotic susceptibility testing (AST) of infecting bacteria is urgently needed in clinical settings to timely guide correct antibiotic prescription before treatment.[5,6] However, the current growth-based standard AST assay is very time-consuming. It typically takes 2 d to 1 wk from receiving clinical samples, including 24 h to 5 d for bacteria precultivation and additional 16−24 h for AST.[7,8] Clinicians are often left with the choice of broad-spectrum antibiotics to prevent worsening infection. This practice promotes the occurrence and spread of antibiotic resistance.[4]

To overcome the delay for bacterial growth, rapid AST based on fast phenotypic responses to antibiotics or genotypes of bacteria was developed,[9] such as molecular or metabolic activity responses monitored by Raman, IR, and fluorescence spectroscopy,[10−17] single-cell imaging of bacterial division or morphology in a microfluidic chip,[18−20] alteration of DNA replication by digital PCR,[21,22] etc. A greatly shortened antibiotic exposure time down to even 15 min was reported.[21] However, antibiotic exposure is just one step in the whole AST assay; all the other steps including pathogen isolation, measurement, data analysis, and readout take time before a diagnosis can be made. Notably, most of the reported AST methods were validated with clinical bacterial isolates,[10−12,14−16,21] which take 24 h to 5 d to obtain by precultivating clinical samples (urine, blood, sputum),[7,8] greatly lengthening the whole diagnosis time. An ideal rapid AST should be able to shorten the whole AST time from receipt of clinical samples to antibiotic susceptibility/resistance

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(S/R) readout. Skipping the precultivation by performing AST directly on clinical samples or bacteria rapidly extracted from clinical samples is critical to achieve this rapidness. However, because of the complexity of clinical sample matrixes and low cell loads, it is a challenging task and very few methods can be performed directly on clinical samples. Recently, a nanoliter array coupled with the fluorescence dye resazurin indicating cell viability can allow AST directly on bacteria extracted from urine and provide results within 6 h.[17] In addition, nucleic acid quantification via an ultrafast digital loop-mediated isothermal amplification assay enabled an AST result directly from clinical urine samples within only 30 min.[22]

Single-cell Raman spectroscopy is also very promising for measuring bacteria directly from clinical samples. Its ability to measure down to one single cell exerts limited demands on the low cell counts in clinical samples. In addition, Raman spectroscopy can provide rich chemical profiles of bacterial cells and their biochemical responses to antibiotics.[10,23] When further combined with stable isotope probing (SIP) such as with ^{13}C, ^{2}D, and ^{15}N, intracellular assimilation of isotope-labeled substrates can generate characteristic Raman shifts induced by the replacement of a light atom with a heavy isotope in the newly synthesized biomolecules.[24−30] Because this process is governed by cellular metabolic activity,[27] isotope-induced Raman band shifts therefore provide a simple and even quantitative manner to monitor the microbial activity and the associated responses of activity to antibiotics.[14,15,27] Recently, Raman combined with D_2O or stimulated Raman imaging with ^{2}D-glucose was employed to assess the metabolic activity-inhibiting effect of antibiotics on bacteria.[14,15] Incorporation of D into the newly synthesized lipid or protein generated a new C−D bond in a silent spectral region,[27] enabling its sensitive and specific detection. Comparison of the C−D band allows resistant and susceptible cells to be distinguished due to their different activities in response to antibiotics after a short 30 min incubation.[14,15]

Despite this progress, Raman-^{2}D SIP has not been established as a reliable and rapid AST for clinical applications, especially for directly testing clinical samples without precultivation. There are three major technical gaps. First, how to overcome the nonsynchronous responses of microbial activity and growth to achieve AST results from Raman-^{2}D SIP consistent with that of standard growth-based AST assay. Growth-arrested cells have been widely found to be still metabolically active.[14,31,32] For instance, ampicillin at a concentration as high as 60 × MIC was not enough to inhibit microbial activities measured as the C−D band.[14] The MIC of vancomycin determined by the C−D band was two times the MIC determined by the conventional growth-based method.[16] To deploy clinically, it is a prerequisite for the new AST to conform to growth-based clinical interpretation criteria, such as inhibition zone diameter or MIC breakpoints produced by the organizations of the Clinical & Laboratory Standards Institute (CLSI).[33] Second, intrinsic D incorporation rates or microbial activities vary dramatically among different bacteria species, as demonstrated by the varying C−D ratios (CD/(CD+CH)) among different bacteria.[27] It is therefore not feasible to use absolute C−D ratios to determine S/R. A more robust S/R criterion has to be developed for Raman-^{2}D SIP. Third and more importantly, despite its potential, the single-cell Raman-^{2}D SIP AST method has never been applied directly on clinical samples. To skip the lengthy precultivation and shorten the whole assay time from clinical sample receipt to S/R readout, a workflow enabling rapid transfer of bacteria directly from clinical sample for antibiotic exposure and Raman measurement has to be developed.

Herein we aim to develop single-cell Raman combined with D_2O (Raman-D_2O) as a sufficiently rapid and accurate AST toward clinical implementations, especially on urinary tract infections (UTIs) which are the most prevalent infections and have exhibited widespread antibiotic resistance. To accomplish this goal, we established a proper antibiotic treatment condition for Raman-D_2O that ensured S/R readout consistent with that of standard AST assay used in clinical settings. A criterion based on relative C−D ratios with a reliable S/R threshold value overcoming intrinsic differences of metabolic activities were established. Finally, a simple workflow enabling directly extracting clinical urine bacteria for AST without the lengthy urine precultivation was developed. The total assay time from receiving the urine to S/R readout was shortened to only 2.5 h.

MATERIALS AND METHODS

Bacterial Strains and Antibiotics. Bacterial strains used here included five *E. coli* strains with different resistance profiles, one quality control strain recommended by CLSI (*E. coli* 25922), three pathogens within the WHO "priority pathogens" list (i.e., *Salmonella enterica, Shigella flexneri, Proteus vulgaris*), and five UTI isolates (i.e., *Klebsiella variicola, Escherichia fergusonii, Providencia rettgeri, Klebsiella singaporensis, Klebsiella pneumoniae*). More information about their identification and origins are summarized in Table S1. Antibiotics used in this study included ampicillin, kanamycin, chloramphenicol (Solarbio, Beijing, China), nitrofurantoin, norfloxacin, and fosfomycin (Macklin, Shanghai, China), and meropenem (Target Molecule Corp., Boston, MA). Information about the preparation and concentrations of stock solution can be found in Table S2. All antibiotic solutions were filtered through a 0.22-μm sterile syringe filter (Millipore Millex, Burlington, MA) and stored in the dark at −20 °C before use.

AST via a Disk Diffusion Assay. AST was determined following AST performance standards published by CLSI.[33] Briefly, the inhibition zone diameter was measured by the disk diffusion method as follows: (i) Inoculate one single colony of each bacteria into 10 mL of fresh LB broth and culture at 37 °C overnight. After harvesting, adjust the turbidity of bacterial solution to 0.5 McFarland standard (OD_{600} ∼ 0.08−0.135) with sterile fresh LB broth. (ii) Dip a swab in the as-prepared suspension, express excess liquid, and streak the swab evenly over the entire surface of a Muller−Hinton agar (MHA) plate. (iii) Place antibiotic disks (Solarbio, Beijing, China) evenly (no smaller than 24 mm from center to center) on the MHA plate and incubate at 37 °C for 16−18 h. (iv) Measure the diameter of the complete inhibition zone using a ruler. AST was determined using clinical zone diameter interpretive criteria from CLSI.[33] All experiments were carried out in triplicates.

AST via Single-Cell Raman-D_2O. Antibiotic treatment and D_2O incubation were carried out in a 96-well plate which allows multiple strain and antibiotic treatments at the same time. Overnight-cultured bacterial stains were inoculated into the LB media containing 100% D_2O (99.8 atom % D, Aldrich, Milwaukee, WI) and different concentrations of antibiotics and incubated at 37 °C, 400 rpm for 0, 30, 60, 90, and 120 min, respectively. Typically, an incubation time of 30 min and antibiotics at 10 × CLSI MIC were applied for rapid AST by

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Raman-D_2O. Bacteria were then harvested and washed twice with sterilized DI water by centrifuging at 5000 rpm for 3 min. A 2-μL aliquot of sample was spotted on aluminum (Al) foil with a low and featureless background signal[34] and dried in air at room temperature prior to single-cell Raman spectra acquisition.

Single-cell Raman spectra were acquired using a LabRAM Aramis (HORIBA Jobin-Yvon, Japan) confocal micro-Raman system equipped with a 532 nm Nd:YAG excitation laser and a 300 grooves/mm diffraction grating.[24,25] A 100× dry objective with a numerical aperture of 0.9 (Olympus, Japan) was employed for bacterial observation and Raman signal acquisition. The acquisition time for each spectrum was 25 s, and 20 single-cell spectra were acquired from each sample. After preprocessing Raman spectra via baseline correction, batch calculations of the C−D ratio (CD/(CD+CH)) from a large amount of single-cell Raman spectra were performed. To do this, "Spectral Profile" operation in LabSpec 5 software (HORIBA Jobin-Yvon, Japan) was used to create a mapping file from multiple spectra. "Map Analysis" operation was then used to generate a map based on the signal ratio of CD/CH in specified spectral regions of 2040−2300 cm^{-1} (C−D) and 2800−3100 cm^{-1} (C−H), respectively. A simple mathematical transformation was used to get CD/(CD+CH) from CD/CH.

AST of Patient Urine Samples via Disk Diffusion and Single-Cell Raman-D_2O Assays. Three anonymous discarded fresh clinical urine samples (~50 mL) confirmed to have urinary tract infections (UTIs) by physicians were obtained from The Second Affiliated Hospital of Xiamen Medical College (Xiamen, China) with the consent from the institutional review board protocol of this hospital. For the disk diffusion assay, pathogenic bacteria were cultivated and isolated from clinical urine samples by plating urine on LB agar plates and incubating at 37 °C for 24 h to form colonies. Bacterial colonies were then subjected to disk diffusion AST following the same procedure as described above. These colonies were identified via 16S rRNA gene sequencing (Table S1).[24]

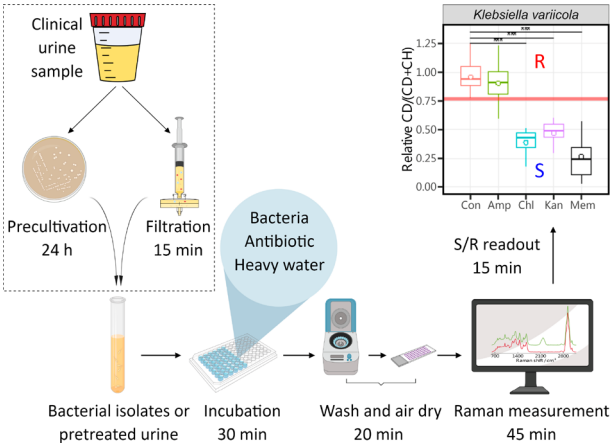
For Raman-D_2O assay, the urine samples were filtered through a 5-μm filter (Millipore Millex) to remove big particles such as leukocytes or epithelial cells (Scheme 1). Subsequently, the filtered urine was centrifuged at 5000 rpm for 3 min. After removal of supernatant, 2 μL of the as-prepared urine was inoculated into 200 μL of LB media containing 100% D_2O and 10 × CLSI MIC antibiotic for 30 min. Subsequent steps were the same as that for pure bacterial isolates described above.

Statistical Analysis. All data analyses were performed by R (version 3.4.3). Statistical comparisons were performed using the Wilcox test via package "ggsignif" with customized scripts.[35] All statistical tests were considered significant at $P < 0.01$. Diameters of the disk diffusion inhibition zone were presented as the mean ± sd from biological triplicates. The relative C−D ratios from Raman spectra of 20 individual cells were denoted by points, and quartile distribution was displayed as box plots.

RESULTS AND DISCUSSION

Principle and Workflow of Raman-D_2O-Based AST. Scheme 1 shows the workflow of AST via single-cell Raman-D_2O. Pure bacterial isolates from lengthy precultivation on an agar plate or clinical urine samples after a rapid filtration were incubated with LB culture medium containing 100% D_2O and antibiotics in a 96-well plate at 37 °C for different time

Scheme 1. Workflow for Antibiotic Susceptibility Testing via Single-Cell Raman-D_2O from Clinical Sample Collection to Susceptibility/Resistance (S/R) Readout[a]



[a]Pretreatment of urine via rapid filtration or a lengthy precultivation on an agar plate is outlined by a dotted rectangle.

(typically 30 min). After harvesting, the bacteria were washed twice with deionized water and then dropped on Al foil for air-drying, followed by single-cell Raman spectra acquisition and batch calculations of C−D ratios (CD/(CD+CH)) to determine S/R. Figure 1 shows incubation time-dependent

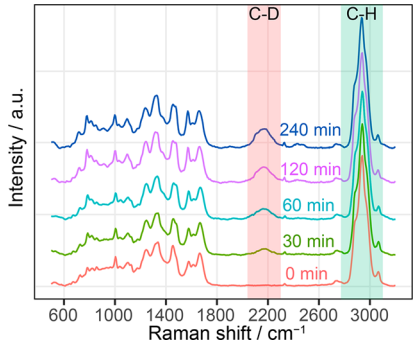


Figure 1. Time-dependent single-cell Raman spectra of *E. coli* DH5α (n = 20) incubated with LB culture medium amended with heavy water without antibiotic treatment.

Raman spectra of single *E. coli* cells. The intensity of the C−D band at 2040−2300 cm^{-1} was clearly observed to increase with time due to the increasing formation of C−D bonds on newly synthesized biomolecules.[27] The C−D band can be observed at as early as 30 min in the silent spectral region, enabling great potential for rapid AST.

Establishing Raman-D_2O as a Rapid and Accurate Method for AST. One of key issues in applying Raman-D_2O for rapid AST is the inconsistency with the growth-based S/R clinical interpretive criteria, because susceptible bacteria can still be metabolically active at growth-based MIC.[14,15] To seek a proper antibiotic treatment condition to ensure a rapid and reliable AST by Raman-D_2O, one susceptible (DH5α) and three resistant *E. coli* strains against ampicillin (Amp^R), chloramphenicol (Chl^R), and kanamycin (Kan^R), respectively, were incubated with 0, 1 × , 10 × , and 100 × CLSI MIC of antibiotics in the D_2O-amended media for different time. These antibiotics have different mechanisms of action(Table 1). Amp and Kan are bactericidal by inhibiting the synthesis of

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Table 1. Mechanisms and Susceptible Breakpoints of MIC and Zone Diameter of Antibiotics for *Enterobacteriaceae* Recommended by CLSI[33]

antibiotics	mechanism of action	CLSI MIC (μg/mL) S ≤	CLSI zone diameter (mm) S ≥
ampicillin (Amp)	cell wall (bactericidal)	8	17
meropenem (Mem)	cell wall (bactericidal)	1	23
kanamycin (Kan)	protein (bactericidal)	16	18
chloramphenicol (Chl)	protein (bacteriostatic)	8	18
nitrofurantoin (Nit)	multiple mechanisms (bactericidal)	32	17
norfloxacin (Nor)	DNA (bactericidal)	4	17
fosfomycin (Fos)	cell wall (bactericidal)	64	16

the bacterial cell wall and proteins, respectively. Chl is bacteriostatic by inhibiting protein synthesis. Susceptible MIC breakpoints produced by CLSI was used here and displayed as CLSI MIC throughout this paper (Table 1). Time- and dose-dependent C−D ratios from single-cell Raman spectra are shown in Figure 2A. With the increase of antibiotic dose, C−D ratios of susceptible *E. coli* in response to all three antibiotics decreased (Figure 2A, left panel), while that of the three resistant strains remained unchanged except at 100 × CLSI MIC (Figure 2A, right panel), indicating that the activities of susceptible cells were more easily inhibited by antibiotics than resistant cells. However, for susceptible cells after the shortest 30 min incubation (Figure 2A, red dotted line), the C−D ratios under 1 × CLSI MIC Amp or Kan treatment cannot separate well from control (0, no antibiotic treatment). Clear separation at 30 min was observed under 10 × CLSI MIC treatment for all three antibiotics and even better at a higher antibiotic dose and a longer incubation time. However, 100 × CLSI MIC or a higher dose was not recommended, because the C−D ratio even in the resistant strains of Chl^R and Kan^R decreased, reducing S/R discrimination sensitivity. Therefore, 10 × CLSI MIC was more proper for a rapid Raman-D_2O AST when taking incubation time (30 min), discrimination sensitivity, and applicability to different antibiotics into account. Considering that clinical MIC breakpoints are continually updated due to the increasing microbial resistance to antibiotics over time,[33] this quantitative association (i.e., 10 × CLSI MIC) also provides a means to update the antibiotic dose for Raman-D_2O AST.

Figure 2B shows the C−D ratios under 10 × CLSI MIC antibiotic treatment after only 30 min incubation. It was clear that the C−D ratios of susceptible *E. coli* were significantly lower than that of control for all three antibiotics, while no significant decreases were observed on three resistant *E. coli* strains. In addition, from the corresponding C−D Raman bands (Figure 2C), the susceptible strain still held a clear C−D band even under 10 × CLSI MIC treatment, consistent with previous work showing that growth-arrested bacteria can still be metabolically active and keep incorporating D.[14,15] The effect of D_2O content on microbial growth and D incorporation and the justification of using 100% D_2O are discussed in Supporting Information (Figures S1 and S2).

Rapid AST of Different *E. coli* Strains against Different Antibiotics. Antibiotics at 10 × CLSI MIC and incubation of 30 min were then applied to perform AST on five *E. coli* strains against three types of antibiotics by single-cell Raman-D_2O (Figure 3), a total of 16 bacteria−antibiotic combinations including a triple treatment. To provide a solid basis to validate Raman-D_2O AST results, the standard growth-based disk diffusion assay was also performed to determine antibiotic susceptibility. The disk diffusion assay was performed by plating overnight cultured bacteria onto a Muller−Hinton agar plate. After incubation at 37 °C for 16−18 h, diameters of clear area indicative of the bacterial inhibition zone were measured.

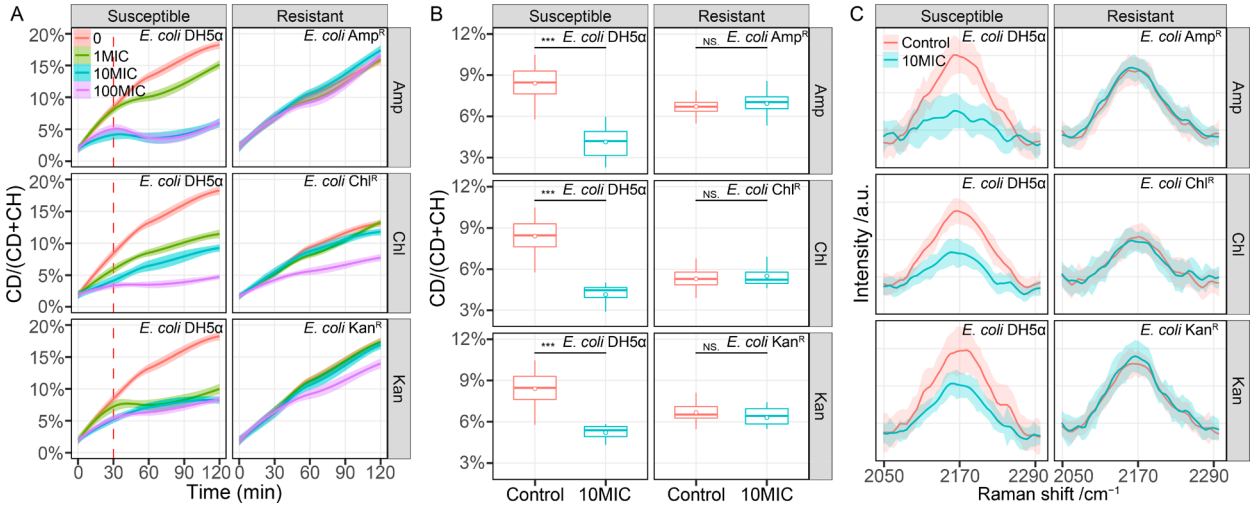

Figure 2. Incorporation of D from D_2O into susceptible and resistant *E. coli* as detected by single-cell Raman spectroscopy. (A) Time- and dose-dependent C−D ratios of one susceptible (DH5α) and three resistant *E. coli* strains (Amp^R, Chl^R, and Kan^R) in response to 0, 1 × , 10 × , 100 × CLSI MIC antibiotic treatment with ampicillin (Amp), chloramphenicol (Chl), and kanamycin (Kan), respectively. Mean (solid lines) and standard error (shaded regions) from 20 individual cells are depicted with 99.9% confidence interval. (B) C−D ratios and (C) C−D bands of susceptible and resistant strains after 30 min incubation with and without 10 × CLSI MIC of antibiotic treatments (Wilcox test, ***$P < 0.001$; NS, not significant.).

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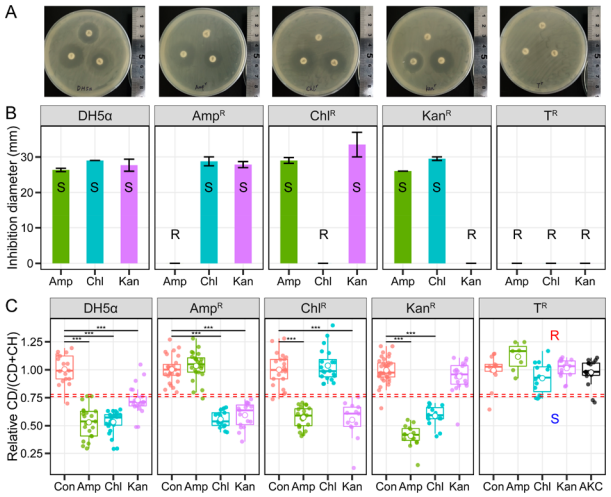

Figure 3. Antibiotic susceptibility testing of five different *E. coli* strains by standard disk diffusion and single-cell Raman-D_2O assays. (A) Image of disk diffusion inhibition zone. (B) Diameters of disk diffusion inhibition zone presented as the mean ± sd from biological triplicates. (C) Relative C−D ratios measured from Raman spectra of 20 individual cells and displayed as a quartile distribution using box plots. Con: control (without antibiotic), Amp: ampicillin, Chl: chloramphenicol, Kan: kanamycin, AKC: Amp+Kan+Chl. All comparisons denoted by a black bar are statistically significantly different (Wilcox test, $***P < 0.001$). The red dotted lines (0.76−0.78) are the criteria to determine antibiotic susceptibility.

Figure 3, parts A and B, shows the image and diameter of inhibition zones displayed as a clear area around each white antimicrobial disk. Bacteria with an inhibition zone larger than the CLSI zone diameter interpretive criteria (Table 1) were reported as susceptible (S) or otherwise resistant (R). For instance, the inhibition diameter of *E. coli* Amp^R in response to Amp was almost zero, but obviously larger than the zone diameter criteria of 18 mm to Chl and Kan, indicating that it was R to Amp but S to Chl and Kan. For *E. coli* T^R, the inhibition diameters were almost zero to all three antibiotics, indicating that it was a multiresistant strain. In the same way, susceptibility profiles of all *E. coli* strains were determined and indicated as S or R in Figure 3B.

Figure 3C displays the corresponding AST results from around 20 individual cells randomly selected for Raman measurement. C−D ratios of control (Con, without antibiotic) from different *E. coli* strains were found to vary slightly from ∼5% to ∼9% (Figure S3). This is because of the intrinsic difference in metabolic activity among the different bacterial strains. To overcome this problem and set a common criterion irrespective of different bacterial species or strains, all C−D ratios were normalized with that of control. The resulting relative C−D ratios were used to analyze antibiotic susceptibility. For *E. coli* Amp^R, the relative C−D ratios from Amp were as high as that of control but significantly lower than control (= 1) for Chl and Kan. For multiresistant *E. coli* T^R, all the relative C−D ratios from either single or triple antibiotic (AKC) antibiotic treatment were at a high level close to that of control. The same case was observed in *E. coli* Chl^R and Kan^R. These results demonstrated that antibiotic susceptibility of *E. coli* profiled by Raman-D_2O agreed well with the standard disk diffusion assay but with a much reduced incubation time of 30 min in comparison with the conventional 16−18 h.

Rapid AST of Diverse Pathogenic Isolates against Different Antibiotics. In addition to *E. coli*, Raman-D_2O-based AST was also examined for other pathogenic bacteria, and a quality control (QC) strain of *E. coli* 25922 recommended by CLSI to exclude factors affecting S/R readout accuracy, such as antibiotic efficiency, culture medium, and personnel competency, was also included.[33] We first tested three bacteria within the WHO "priority pathogen" list that poses the greatest threat to human health (Table S1),[3] i.e., *Proteus vulgaris* (causing wound infection) and *Salmonella enterica* and *Shigella flexneri* (causing diarrhea, fever, and stomach cramps). An additional last-resort antibiotic of meropenem (Mem) was also included to test its efficacy against pathogenic bacteria. AST was then performed on these 16 pairs (4 bacteria × 4 antibiotics) by both standard disk diffusion and Raman-D_2O assays (Figure 4). S/R determined

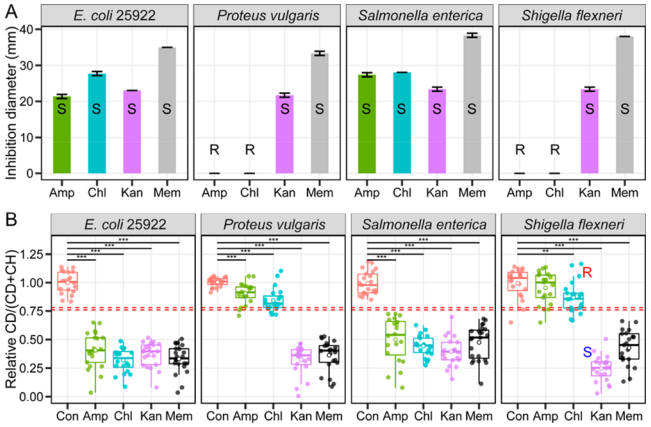

Figure 4. Antibiotic susceptibility testing of one quality control strain (*E. coli* 25922) and three WHO pathogens by disk diffusion and single-cell Raman-D_2O assays. (A) Diameters of the disk diffusion inhibition zone presented as the mean ± sd from biological triplicates. (B) Relative C−D ratios measured from Raman spectra of 20 individual cells and shown as a quartile distribution using box plots. Con: control (without antibiotic), Amp: ampicillin, Chl: chloramphenicol, Kan: kanamycin, Mem: meropenem. All comparisons denoted by a black bar are statistically significantly different (Wilcox test, $**P < 0.01$, $***P < 0.001$). The red dotted lines (0.76−0.78) are the criteria to determine antibiotic susceptibility.

by the disk diffusion assay is indicated in Figure 4A. As expected, QC strain was determined to be susceptible to all four antibiotics, indicating that all operations went properly. More importantly, after a short 30 min incubation with antibiotics, completely consistent susceptibility profiles were determined by Raman-D_2O (Figure 4B). For instance, relative C−D ratios of QC strain and *S. enterica* were all significantly lower than that of control and thus reported as S, while that of *P. vulgaris* and *S. flexner* were only significantly lower than that of control to Kan and Mem, indicating that they were S to Kan and Mem but R to Amp and Chl. Readout consistency is highly important to enable the new Raman-D_2O AST assay for accurate clinical interpretation.

We have now tested totally 32 bacteria−antibiotic combinations (Figures 3 and 4) including different bacterial species and antibiotics with different mechanisms of action. The obtained relative C−D ratios provided a database to set a binary S/R cutoff value for Raman-D_2O AST. A deterministic algorithm based on the quartile distribution of relative C−D ratios were employed here, i.e., relative C−D ratios between

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the lower quartile (25% of values) of resistant strains and the upper quartile (75% of value) of sensitive strains in the box plots of Figures 3B and 4B were compared. The common range of 0.76−0.78 in the 32 bacteria−antibiotic combinations was set as the S/R cutoff value for Raman-D_2O (red dotted line in Figures 3B and 4B), i.e., S: ≤0.76, R: ≥0.78. To test its robustness and also applicability on other bacterial isolates and antibiotics, this cutoff value was further employed to determine S/R of five bacteria isolates from urine against three antibiotics typically used for UTI (Table 1), i.e., nitrofurantoin (Nit), norfloxacin (Nor), and fosfomycin (Fos) (Figure S4). It is interesting to find that AST results from the new 15 combinations together with the previous 32 ones were all correctly determined by Raman-D_2O (100% categorical agreement with the standard disk diffusion assay). Because no UTI isolates tested here were resistant to Nit and Fos, the diagnostic sensitivity for Nit and Fos resistance remained to be validated for additional isolates. Compared with direct spectral analysis that requires a chemometric method to analyze subtle spectral changes and build a classification model before differentiating S and R,[10−12,16] such a simple criterion based on relative C−D ratios greatly simplifies and accelerates S/R readout.

Rapid and Direct AST of Clinical Urine Samples by Single-Cell Raman-D_2O. For clinical samples, the current clinical protocol normally takes 24 h to 5 d to obtain bacterial isolates via precultivation before AST (Scheme 1).[7,8] Skipping this cultivation and directly transferring clinical samples for AST can greatly reduce the diagnosis time. Although bacterial counts in clinical samples are normally very low, single-cell Raman-D_2O that can work on bacteria at the single-cell level provides a good means to perform AST directly on the limited number of bacteria in clinical samples. More importantly, it has been demonstrated in the above 32 bacteria−antibiotic combinations that 20 bacteria were enough to deliver reliable AST results by Raman-D_2O.

Urinary tract infections (UTIs) are one of the most prevalent infections affecting almost half of the population at least once in their whole life and has exhibited widespread antibiotic resistance.[7] Here we examined the applicability of single-cell Raman-D_2O for rapid AST directly on clinical urine samples without the precultivation step. Three anonymous fresh urine samples confirmed to have positive UTIs by physicians were obtained from a local hospital. After simply filtering urine through a 5-μm membrane to remove large debris, leukocytes, and epithelial cells (Scheme 1), the urine was centrifuged to remove supernatants and then inoculated into the culture medium amended with D_2O and 10 × CLSI MIC antibiotic in a 96-well plate. The total pretreatment time was only 15 min, much shorter than the 24 h required for urine preculturing. The resulting bacterial counts were around 5 × 10^5 cells/mL, similarly low to that in fresh urine samples. Figure S5 shows a photograph of lab consumables used for AST of urine via Raman-D_2O, mainly including a syringe filter and a 96-well plate. All of them are easily accessible in the lab. After incubation for 30 min, urine bacteria were harvested for single-cell Raman measurements. Subsequent procedures for AST were the same as that on pure bacteria isolates. To validate Raman-D_2O results, AST was also performed by the standard assay following the lengthy clinical protocols, i.e., urine was cultured on an agar plate for 24 h for bacteria isolation and identification, followed by AST via a disk diffusion assay. Pathogenic bacteria in urine samples were identified as *Klebsiella variicola*, *Escherichia fergusonii*, and *Providencia rettgeri* via 16S rRNA gene sequencing, respectively (Table S1). All of them are pathogens commonly found in UTI patients.[36,37] Inhibition diameters of all three pathogens (Figure 5A) to Amp were found to be less than 17 mm of zone

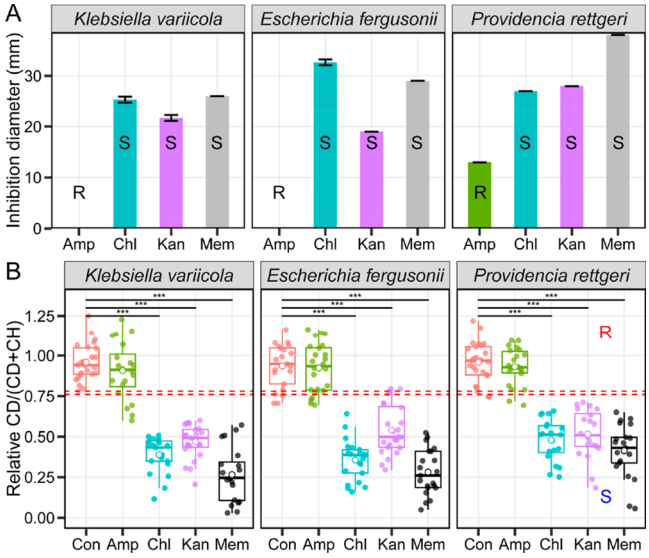

Figure 5. Antibiotic susceptibility testing of three clinical urine samples with positive urinary tract infections by disk diffusion after 24 h precultivation and single-cell Raman-D_2O after 15 min filtration of urine. (A) Diameter of disk diffusion inhibition zone presented as the mean ± sd from biological triplicates. (B) Relative C−D ratios measured from Raman spectra of 20 individual cells and shown as quartile distribution using box plots. Con: control (without antibiotic), Amp: ampicillin, Chl: chloramphenicol, Kan: kanamycin, Mem: meropenem. All comparisons denoted by a black bar are statistically significantly different (Wilcox test, ***$P < 0.001$). The red dotted lines (0.76−0.78) are the criteria to determine antibiotic susceptibility.

diameter criteria (Table 1), indicating they all had developed resistance to Amp. Fortunately, they were all susceptible to Chl, Kan, and Mem as indicated by the larger inhibition diameter compared with zone diameter criteria.

Figure 5B shows AST results from single-cell Raman-D_2O based on the measurement of around 20 individual cells. By using the relative C−D ratio criteria set above (i.e., S: ≤0.76, R: ≥0.78), three pathogens can be clearly reported as R against Amp, but S against Chl, Kan, and Mem. Obviously, susceptibility profiles of clinical urine samples determined by single-cell Raman-D_2O totally agreed with the standard disk diffusion assay. More importantly, the whole assay time from the receipt of urine to S/R readout, including urine pretreatment (15 min), incubation (30 min), wash and dry (20 min), Raman measurement (45 min per urine sample, 9 min per antibiotic), and C−D ratio calculations (15 min), was shortened to only within 2.5 h (Scheme 1), demonstrating the sufficient rapidness of single-cell Raman-D_2O in determining antibiotic susceptibility of clinical samples.

CONCLUSION

We developed single-cell Raman-D_2O as a sufficiently rapid and accurate activity-based AST method directly applicable for clinical urine samples. The total time from receiving urine to antibiotic susceptibility readout was shortened to 2.5 h,

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Anal. Chem. 2019, 91, 6296−6303

enabling a rapid diagnosis and timely guidance for antibiotic selection by the clinician. A proper antibiotic treatment condition of 10 × CLSI MIC breakpoint was established for Raman-D_2O, well offsetting the effect of nonsynchronous microbial responses (activity vs growth) on susceptibility determination. A simple S/R cutoff value based on relative C−D ratios was established, not only overcoming the interference of intrinsic activity variation of bacteria on S/R determination but also greatly facilitating S/R readout. In the test of 14 pathogenic bacterial strains including 3 in clinical urine samples in response to antibiotics with different mechanisms of action, susceptibility profiles were all correctly determined by Raman-D_2O with a 100% categorical agreement with the slow standard disk diffusion assay. Single-cell level detection enabled the direct performance of AST on a low number of bacteria in clinical samples. The whole workflow is easy to handle, is cost-effective, and involves only easily accessible lab consumables. This work represents a significant contribution to promote a new rapid AST assay of single-cell Raman-D_2O toward clinical practicality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b01064.

Results on the effect of heavy water on bacteria. AST of urine isolates against UTI-typical antibiotics. Photograph of lab consumables for direct AST of urine. Summary of bacterial strains and antibiotics (PDF)

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Notes

The authors declare no competing financial interest.

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