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Validating the utility of heavy water (Deuterium Oxide) as a potential Raman spectroscopic probe for identification of antibiotic resistance

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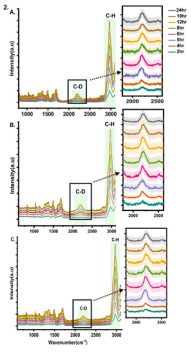
HIGHLIGHTS

- Utility of heavy water in identifying colistin resistant microbes.
- Live tracking of bacterial growth and early detection of antibiotic-resistant by C-D peak.
- Validation of the sensitivity of C-D peak at low biomass bacteria sample in an artificial urine medium.

GRAPHICAL ABSTRACT



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ABSTRACT

The impact of microbial infections is increasing over time, and it is one of the major reasons for death in both developed and developing countries. colistin is considered as the antibiotic of last choice for infections brought by major multidrug-resistant (MDR), gram-negative bacteria such as *Enterobacter species, Acinetobacter species,* and *Pseudomonas aeruginosa.* Existing approaches to diagnose these resistant species are relatively slow and take up to 2 to 3 days. In this work, we propose a novel interdisciplinary method based on Raman spectroscopy and heavy water to identify colistin-resistant microbes. Our hypothesis is based on the fact that resistant bacteria will be metabolically active in the culture medium containing antibiotics and heavy water, and these bacteria will take up deuterium instead of hydrogen to newly synthesized lipids and proteins. This effect will generate a 'C – D' bond-specific Raman spectral marker. Successful identification of this band in the spectral profile can confirm the presence of colistin-resistant bacteria. We have validated the efficacy of this approach in identifying colistin-resistant bacteria spiked in artificial urine and have compared sensitivity at different bacterial concentrations. Overall findings suggest that heavy water can potentially serve as a suitable Raman probe for identifying metabolically active colistin-resistant bacteria *via* urine under clinically implementable time and can be used in clinical settings after validation.

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1. Introduction

Antimicrobial resistance (AMR) is a significant worldwide health concern acknowledged by major global associations for healthcare professionals, namely the Centers for Disease Control and Prevention (CDC), the World Health Organization (WHO), and the Department of Biotechnology (DBT) in India. Currently, AMR is responsible for an estimated annual global death toll of 700,000 individuals, and this alarming figure is expected to rise sharply, potentially reaching 10 million deaths per year by 2050 [1]. Multidrug-resistant (MDR) gram-negative bacteria can cause various infections, such as, Pneumonia, and wound infections [2]. Urinary tract infections (UTI) are one of the most prevalent infections caused by gram-negative bacteria, and approximately 50 % of women experience UTI infection in their lifetime. It can ascend to the kidney and lead to pyelonephritis and life-threatening urosepsis [3]. In the global effort to combat AMR, there is an urgent need to develop new screening methods, particularly against pathogens like *Escherichia coli*, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa,* which are classified under critical priority in the Indian priority pathogen list by WHO and the Department of Biotechnology, India [4].

The increasing prevalence of antibiotic resistance in major multidrug-resistant (MDR) Gram- negative bacteria (MDR-GNB) poses an important global health challenge. This issue is compounded by the fact that several vital antibiotics, such as β-lactams, aminoglycosides, fluoroquinolones, and broad-spectrum penicillin are slowly reducing impact against MDR Gram- negative bacteria. Furthermore, the lack of new antibiotics capable of combating these formidable 'superbugs' exacerbates the problem. In response to this crisis, there has been a re-evaluation of older drugs like polymyxin B, also known as colistin which are commercially available.

Colistin re-appeared as a last-resort treatment option in the mid-1990 s to fight drug- resistant Gram-negative bacteria [5]. The frequent usage of Colistin has triggered the emergence of resistance to this drug as well. The well-known mechanism responsible for colistin resistance is the presence of MCR-1 plasmid, which is involved in the synthesis of phosphoethanolamine transferase that catalyzes the modification of the target site and incorporation of a phosphoethanolamine moiety to lipid A in the bacterial outer membrane (OM), resulting in a decrease in the membrane electronegativity and consequently reducing the binding affinity of colistin [6].The rise and growing prevalence of colistin resistance has led to heightened challenges in managing infections as the availability of treatment choices has dwindled.

Current methods for identifying antibiotic-resistantance are inherently slow, such as culturing microorganisms relying on the visible growth of cells, which takes around 16 to 18 h and another 18 to 24 h to conduct antibiotic susceptibility tests. This extended waiting period often leads to the prescription of broad-spectrum antibiotics based solely on the patient's symptoms. Other methods such as PCR are prone to background contamination from external DNA, and immunoassay requires a certain level of sensitivity and a sufficient amount of antigen [7,8]. To tackle these challenges, the development of a rapid diagnostic tool capable of providing colistin resistance or sensitivity results within a few hours is warranted. It will enable the optimal utilization of this last- resort antibiotic and help mitigate the spread of colistin resistance.

Raman spectroscopy is widely known as a non-invasive and non-destructive technique with the capability to furnish insights about the chemical bonds within molecules. A novel approach that has emerged recently involves employing Raman spectroscopy to track the integration of deuterium into newly synthesized biomolecules such as in proteins and lipids. This integration allows the identification of metabolically active bacteria within a culture medium in a short and clinically implementable time [9–11]. The carbon-hydrogen (C-H) bonds in a Raman spectrum of a biological system are typically identified through peaks in the 2800–3100 cm^{-1} region. However, in the presence of deuterium the hydrogens are replaced, and the bonds become heavier as deuterium is about twice as heavy as hydrogen. This increase in mass causes a reduction in the vibration frequency of the bonds and produces distinct Raman signals around 2040—2300 cm^{-1}, where normally no Raman signals are observed and also known as Raman silent region. The shift in vibrational frequency (υ) from C-H stretching to C-D stretching in Raman spectroscopy can be calculated using the reduced mass (μ) and the initial and final vibrational frequencies. The reduced or decreased mass can be estimated by $\mu = m1m2/m1 + m2$; where m1 is the mass of the carbon atom, m2 is the mass of the hydrogen (for C-H) or deuterium (for C-D atom) [12]. This unique Raman band shift can serve as a semi-quantitative measure of bacterial metabolic activity.

A previous study demonstrated the feasibility of monitoring incorporation of deuterium oxide (D_2O) in a single cell utilizing stimulated Raman scattering (SRS) imaging [13,14]. Another study showed that time for antibiotic susceptibility test could be reduced to 2–3 hrs using deuterium labelling at Raman single cell spectroscopy [15,16]. Single-cell Raman spectroscopy along with utilizing heavy water labelling was employed to observe and analyze the metabolic reactions of native bacteria in chicken feces when subjected to different antibiotics [17]. In this study, our overarching goal is to demonstrate the feasibility of heavy water labelling and Raman spectroscopy to identify colistin resistant *E. coli, A. baumannii* and *P. aeruginosa*, which belongs to the critical category in the priority pathogen list. The effectiveness of the proposed method has been assessed through spectral measurements in spiked artificial urine samples. Futuristic utilization of this method in clinical applications has been validated through important parameters such as sensitivity and time consumption.

2. Materials and methods

2.1. Chemicals, bacterial strain, and growth conditions

Growth medium Luria Bertani (LB) broth, Luria Bertani Agar were purchased from SRL Pvt. Ltd. Deuterium oxide (D_2O) and colistin sulphate salt were procured from Sigma-Aldrich. Three priority pathogens *E. coli* (MCC 3019), *Acinetobacter baumannii* (MCC 3114), *Pseudomonas aeruginosa* (MCC 2082) were obtained from National Centre for Microbial Resource (NCMR), India. Bacterial cultures were initially grown in LB-Agar incubated at 37 °C for 18-24 h. A single isolated colonies were picked and inoculated in Luria Bertani broth and kept in an incubator at 37 °C overnight. All strains were stored in glycerol solution (50 %) for further use at −80 °C.

2.2. Generation of Colistin resistant strains by cloning of MCR-1 gene

To generate colistin resistant clones, MCR-1 plasmid was procured from Addgene, New Delhi, India. The transformation of the MCR-1 plasmid to WT (wild type) *Escherichia coli*, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* was performed as per the manufacturer's instructions by employing heat-shock method. Initially bacterial cells were made competent using calcium chloride treatment for 2 hr followed by continuous thawing the cells in ice. One (1) μl of plasmid was added to the competent cells (50 μl) and incubated over ice for 30 mins. This was followed by heat shock in a water bath set at 42 °C for 30 sec. The screening of positive colonies weas performed by kanamycin screening after 18-hour incubation, positive colonies were chosen for plasmid isolation and verification. To isolate tMCR-1 plasmid, miniprep kits from QIAGEN India Pvt Ltd. was utilized. The purity of the isolated plasmid was determined through spectrophotometry, and the size of the plasmid was verified *via* agarose gel electrophoresis, Fig. S1. The stable colonies of higher colistin resistant strains were obtained by exposing the transformed clones to continuous colistin treatment at higher concentrations. The 16S rRNA gene sequencing was performed for species identification to check the purity of the strains after the transformation. NCBI database was used to compare the dataset obtained from

sequencing to confirm the species.

2.3. Identification of optimal heavy water concentration

Cell viability in presence of heavy water was evaluated by growing bacterial cells at different concentrations of D_2O. Single colony from the overnight culture was inoculated in different concentrations of D_2O ranging from 0 %, 25 %, 50 %, 75 %, 100 % and incubated at 37 °C overnight. The positive control samples were grown under normal conditions (without heavy water) in LB broth. Optical density at 600 nm from the growing cells was measured at different time intervals 2, 4, 6, 8, 12, 18, and 24 h after incubation. The experiments were repeated thrice. The growth kinetics at different heavy water concentrations were achieved by plotting the optical density(absorbance) at 600 nm against time.

2.4. Microbial growth in artificial urine

Artificial urine (AU) was purchased from Nanochemazone, India. This closely resembles human urine and has a similar composition. In the first step, the microbial growth kinetics study was performed to rule-out any negative effects of the artificial urine. The AU was autoclaved for 15 mins at 121 °C and pH was adjusted to 7 prior to use. Final culture medium was developed with 50 % D_2O with 2 × LB-broth and 50 % AU. Bacterial strains were inoculated in presence of colistin for resistant strains and without colistin for sensitive strains. The amount of LB broth media was adjusted to double so that there would not be any changes in nutrients with the addition of equal amount of artificial urine. The culture was incubated at 37 °C, and the optical density was recorded at different time points ranging from 2, 4, 6, 8, 12, 18, and up to 24 hrs.

2.5. Raman spectra acquisition

The Raman spectra were obtained using WITec alpha 300R Raman micro spectrometer coupled with 532 nm wavelength laser (spot size: 2.96 μm), 50X objective (Zeiss, NA = 0.55; WD 9.1 mm), 600 groove/mm grating and thermo cooled charged coupled detector (CCD). Five (5) μl of the resuspended bacterial pellets were put on the top of the calcium fluoride (CaF_2) window and spectra were collected with an exposure time of 10 sec and 15 accumulations for each point. Multiple spectra were collected at different locations on the sample to covering the entire pellets. The CaF_2 windows were cleaned after each use to remove the residues. The acquired spectra were pre-processed using the MATLAB based in-house codes. The spectra were recorded in 352–4016 cm^{-1} range which was interpolated to 1800-2800 cm^{-1} range to analyze the C-D peak followed by preprocessing steps such as filtering, baseline removal and unit normalization.

2.6. Sensitivity evaluation with respect to the gold standard methods

Resistant MCR-1 positive *E. coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* were inoculated into a 10 mL culture medium consisting of 5 mL LB-broth with 50 % heavy water (D_2O) and 5 mL artificial urine along with 5 μg/mL colistin. Each bacterial strain was grown to OD_{600} of 0.4. Then, bacterial cells were diluted to yield end concentration of 10^3,10^4,10^5, 10^7 and 10^8 colony forming unit (CFU/mL). The number of cells present in each dilution was confirmed by the OD_{600} . Bacterial cells from each dilution were then collected by centrifugation at 17500xg for 10 mins followed by washing twice in 0.8 % saline for complete removal of any culture media traces. The laser exposure time was 10 sec and spectra were averaged over 10 accumulations for cell pellets corresponding to 10^7 and 10^8 CFU/mL dilutions. The exposure time was increased to 15 sec and averaged over 15 accumulations for pellets of 10^5 CFU/mL due to reduced cell numbers. In order to prepare cells for gold standard culture testing the colistin resistant (5μg/mL) bacterial cells were inoculated in equal volume of 50 % D_2O-LB and artificial urine, maintained at 37 °C in a incubator at 180 RPM for 10–18 hrs. Once cells reached log phase (OD_{600}), samples were prepared for plating. The samples were serially diluted 10-fold in the range of 10^1 to 10^8 CFU/mL. 100 μl of cells were collected from each dilutions and plated directly on the LB agar culture plates and evaluated for growth. Plates were kept at 37 °C and growth on the plates were monitored up to 24 hrs.

3. Results and discussion

3.1. Generation of Colistin resistant clones

The minimal inhibitory concentration (MIC) for all three bacterial strains was determined using the broth microdilution and colistin strip testing methods under the EUCAST guidelines [18]. MIC for all three wild type strains was found to be 1.9 μg/mL. At this concentration no visible growth was seen in the presence of colistin. Therefore, microbial clones if they could grow to concentration more than 1.9 μg/mL were considered as resistant. In order to generate colistin resistant clones, transformation of MCR-1 plasmid in the wild type strains was performed. The plasmid incorporation was confirmed by agarose electrophoresis, Fig. S1 Band around 5081 bp was observed for resistant strains. In order to generate stable colistin resistant clones, after the transformation microbes were screened under increasing colistin pressure. Bacterial cells were collected when it reached to the log phase (0.6–0.8 OD600), then added to LB broth containing a higher colistin concentration just above the MIC value starting from 3.9 μg/mL and incubated for 18–24 hrs. After visible growth, cells were again transferred to a fresh LB-medium with a 3.9 μg/mL of colistin and kept overnight for growing at 37 °C. This step was carried out around 4–5 times until stable colonies were obtained at a certain concentration. Same procedure was repeated with the higher concentrations, *i.e.*, 5.0, 6.5, 7.8 μg/mL, until stable colonies were obtained for all the desired colistin concentrations [19]. After resistance induction MIC values were reconfirmed by colistin strip test.

Genomic validation of the transformed clones was performed using 16 s rRNA sequencing. Nucleotide sequences from16S rRNA gene from all three strains were submitted to basic local alignment search tool (BLAST) available in NCBI and strains were identified as *E. coli* Strain (NR_114042.1), *Acinetobacter baumannii* (NR_117620.1), and *Pseudomonas aeruginosa* (CP080511.1). Supporting table 1 has the summary of the topmost hits on the query sequences.

3.2. Identifying optimal heavy water concentration

Heavy water is known to suppress cellular activities in animals at higher concentrations. Therefore heavy water or deuterium oxide (D_2O) is often utilized in low amounts for physiological investigations, different responses of deuterated water on growth ranging from no effect to complete inhibition have been reported [20] Therefore in the first step, the optimal heavy water concentration for all three colistin resistant bacteria was standardized. Bacterial growth is defined by the increase in the number of bacterial cell mass at different growth phases of bacteria. The typical growth curves were produced in low-salt LB-broth with different concentrations of heavy water ranging from 0 %, 25 %, 50 %, 75 %, 100 %. Based on optical density (OD_{600}) values growth curve was generated, different growth phases of bacteria were observed such as 0–4 h for the lag phase, 6–12 h represents the exponential phase and 18–48 h stationary phase. As shown in Fig. 1, D_2O concentration over 50 % causes reduction in the OD_{600} value for all three microbes. However, the heavy water concentration below 50 % had no significant influence (p-value more than 0.05) on the growth of all three microbes. Therefore, further experiments involving D_2O were performed at 50 % D_2O concentration.

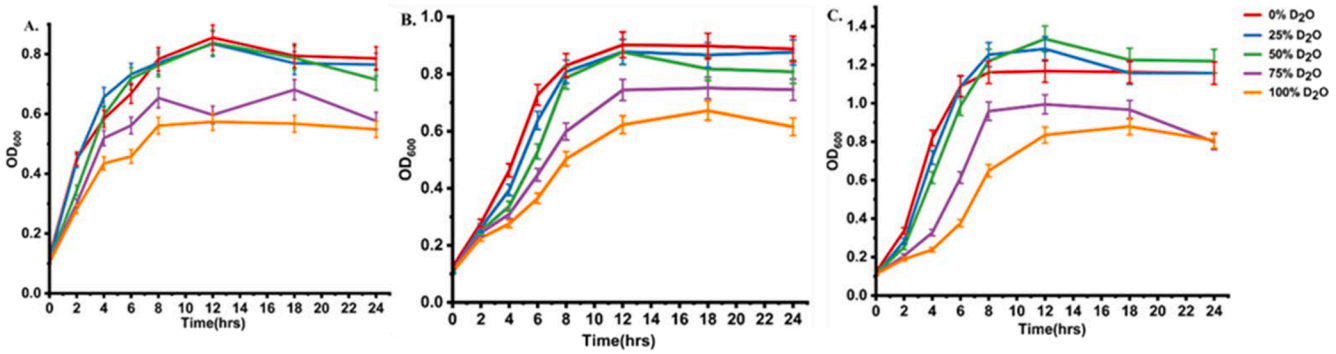

Fig. 1. Identification of optimal heavy water concentration for labeling **A**. *E. coli*, **B.** *A. baumannii*, and **C.** *P. aeruginosa*. The error bar represents the standard deviation across three different experiments.

3.3. Identification of C-D spectroscopic signature from metabolically active microbes

Microorganisms that are metabolically active takes deuterium through the NADH/NADPH electron transport chain. As a result, a characteristic Raman carbon-deuterium (C-D) band appears in the spectra [21,22]. To confirm, if the proposed method can sense the metabolically active microbes in a short time (less than 6 h), we also acquired spectra at different intervals before the initiation of the log phase. As shown in Fig. 2A-C, in addition to the spectral bands in the fingerprint region and carbon hydrogen in 2800–3100 cm^{-1} band a broad peak attributable to C-D vibration was also observed in 2040–2300 cm^{-1} region. This suggests successful incorporation of heavy water in the metabolome of all three microbes used in this study. However, the C-D band had varying intensity during the growth from 2 to 24 h. Further we observed that the C-D band-associated spectral features were visible as early as 2 h post incubation (inset). As the population of the resistance cells increases, changes in the C-D peak are expected. Due to high cell density and number of microbes in the log or exponential phase, the C-D band intensity was found to be highest at 6 h post incubation, Fig. 2(inset). The band intensity closely follows the microbial growth kinetics *i.e.,* strong intensity in the early phases which slowly stabilizes in the later phases of growth. The spectral characteristics exhibited a gradual increase during the logarithmic growth phase from 5 to 12 h. This is followed by a constant peak from 12 to 24 h, indicating that the microbes had reached the stationary phase because of decrease of nutrients from the culture medium. Further we also observed that spectra acquired before 4 h were very noisy. This is expected as microbial cells during the lag phase are known to adjust to their environment, synthesizing essential biomolecules and preparing for rapid growth, typically marked by low rate of metabolic activity. Therefore, they do not incorporate much deuterium in the metabolome.

The ratio of C-D against the entire hydrogen pool (depicted by C-H band) can provide vital clues about the amount of deuterium incorporated in the bacterial metabolome. Therefore, we calculated the amount of deuterium incorporated in the bacterial cells by computing ratio of C-D intensity against the total of C-D and C-H, Fig. 3A. This ratio can be utilized as a semi-quantitative measure of the overall metabolic activity of cells. Our findings are suggestive of the fact that %CD incorporated in the metabolome was almost linearly associated with the growth of bacteria at different phases of growth. In the next step, to evaluate any possible negative influence of artificial urine on the overall growth of the bacteria we also performed growth curve analysis in presence of urine and heavy water. As shown in Fig. 3B, the growth pattern of all three colistin-resistant microbes in a solution consisting of LB broth, 50 % D_2O-LB, artificial urine, and antibiotic (colistin) show no significant differences in the overall growth kinetics. The full range spectrum of artificial urine is presented in Fig. S2. The presence of artificial urine does not lead to appearance of any additional band as no bands in the

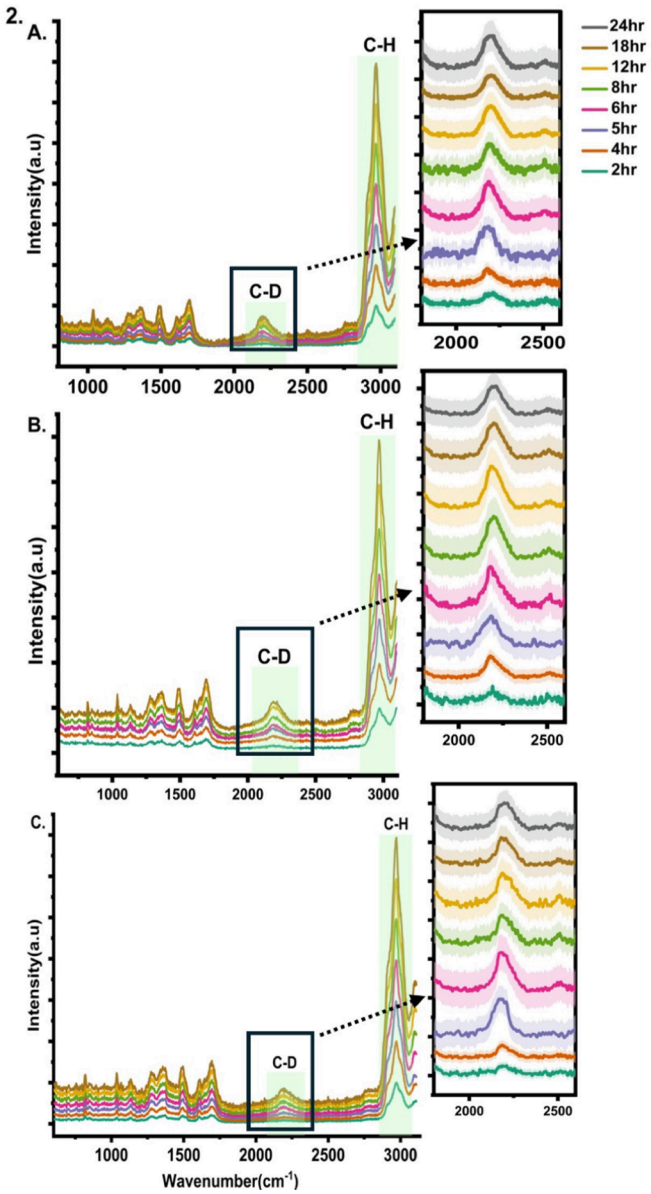

Fig. 2. Evaluation of minimum time required for the identification of deuterium oxide specific Raman spectral signature **A.** E. coli, **B.** A. baumannii, **C.** P. aeruginosa. Both C-D and C-H peaks are highlighted. The inset picture demonstrates changes in C-D band intensity with respect to the microbial growth.

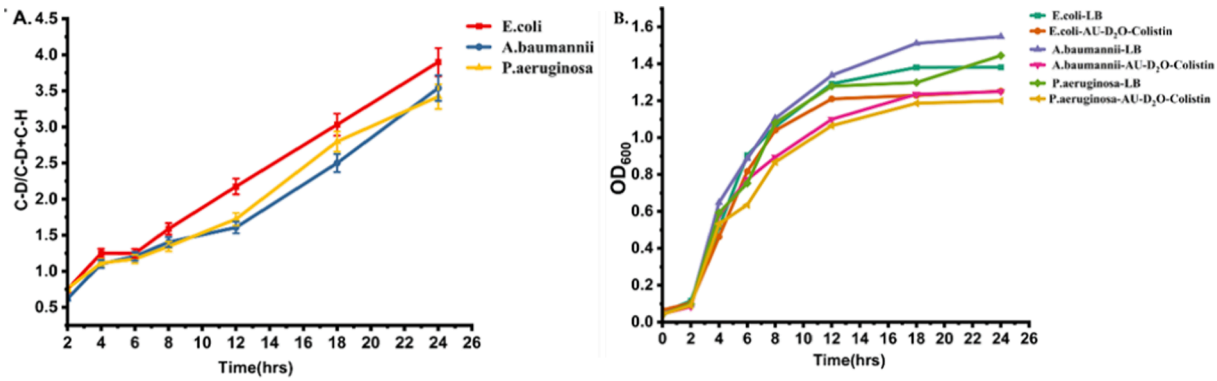

Fig. 3. **A**. Quantification of Deuterium incorporated in the metabolome via C-D/(C-D + C-H) ratio for *E. coli*, *A. baumannii*, *P. aeruginosa* **B.** Evaluating the presence of any negative effects on the microbial growth due to artificial urine or heavy water.

Raman silent region were observed, Fig. S2. During the log phase the OD_{600} value was almost the same for all three microbes indicating the generalized suitability of this approach for identifying colistin resistance microbes during early phases of bacterial growth.

3.4. Sensitivity evaluation of heavy water labeling in detecting Colistin resistant microbes in urine

After demonstrating the less time consumption associated with this approach for identifying resistant species, next we validated the minimum number of microbes required for generating a visible signal of deuterium oxide. Traditionally the diagnosis of bacterial infections via urine is based on the count of bacteria per mL. It is a widely known fact that if the bacterial count is $> 10^5$ CFU/mL the samples are considered as positive for UTI [23–25]. This diagnosis is often accompanied by microscopic examination to rule out any vaginal contamination. Therefore, we evaluated the sensitivity of this approach in successfully identifying C-D Raman band in at least 10^5 CFU/mL bacteria in the urine samples. Different dilutions ranging from 10^4,10^5, 10^7 and 10^8 CFU/mL were created with resistant microbes in presence of colistin, heavy water (50 %) and artificial urine. Average spectra obtained from the bacterial cells at different dilutions are shown in Fig. 4. The shaded area indicates standard deviation across three experiments. A clear C-D peak well above the baseline level at the concentration corresponding to 10^5 CFU/mL was observed. These findings were further compared against the gold standard plating method Fig. S3. Bacteria growing under similar conditions were prepared by 10-fold dilution ranging from 10^1 to 10^8 CFU/mL and were allowed to grow in agar plates for 18–24 hrs at 37 °C. After 18hrs of incubation visible colonies were observed on cells at 10^8 –10^6 CFU/mL concentration. At concentration below this no colonies were observed even after 24–48 hrs of incubation indicating that culturing methods are not only slow but also inefficient to find antibiotics resistance if cells are too few in the sample. This observation further supports the prospective applicability of heavy water coupled Raman spectroscopic approach as an alternate/adjunct mode of identifying colistin resistant uro- pathogens.

Identification of antibiotic resistant microbes in urine is a multi-step process involving bacterial culturing, identification, quantification, and testing the susceptibility to different antibiotics [26,27]. The outcome of the testing is generally presented as 'antibiogram' with details of the microbial sensitivity to different antibiotics. The timeframe for the completion of the entire diagnostic procedure (even in advanced labs) is often more than 72–96 h [26,27]. The steady but alarming rise in colistin resistance infections indicates a pressing need for a faster, more precise, automated, and relatively inexpensive method of urine-based diagnosis. Such methods will provide immediate benefits especially in terms of screening at the first stage and will have long term benefits for public health [28]. Most of the available diagnostic approaches are limited by complex operating procedures, high time consumption and subjectivity. Novel methods based on Raman spectroscopy have been applied for microbial phenotyping, gene expression analysis, studying physiological states, identifying cellular secretions and metabolic activities [29–33]. These methods offer high sensitivity, objectivity and involve minimal sample preparation. A recently developed approach involves Raman spectroscopy tracking of metabolic activity by incorporating heavy isotopes into the metabolome. The metabolic pathway involves uptake of deuterium(D) into newly formed lipids and proteins instead of hydrogen (H) when bacteria are metabolically active under antibiotic stress. This leads to the appearance of a distinctive signature characterized by a C-D bond. The successful identification of this band in the spectral profile serves as confirmation of the presence of antibiotic resistant microbes in urine, eliminating the need for tedious culturing steps.

The overall findings of this work are suggestive of prospective utility of this approach for identifying antibiotic resistance. Taking colistin as an example we successfully demonstrated that spectra acquired after 4 h

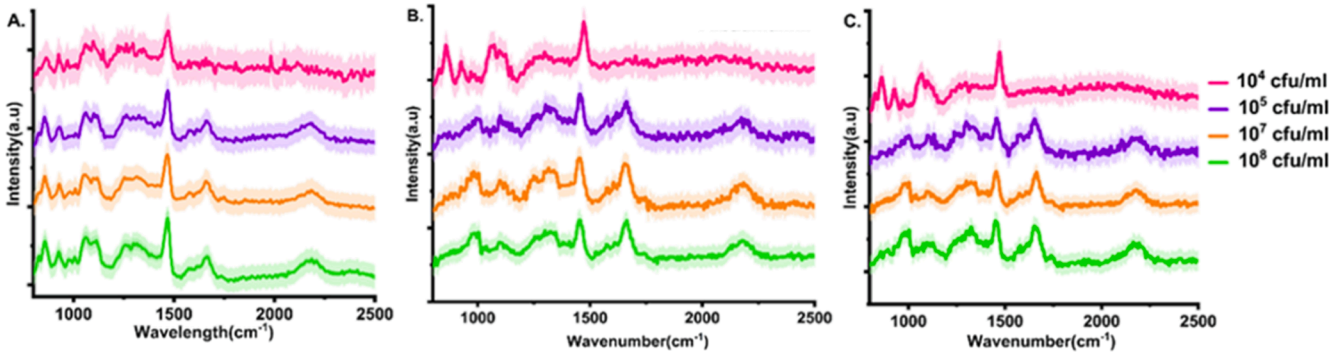

Fig. 4. Evaluating the sensitivity of heavy water labeling approach for identification of colistin resistance **A.** *E. coli*, **B.** *A. Baumannii*, **C.** *P. aeruginosa.*

show a very clear visible C-D band well above the noise level, Fig. 2. We also demonstrated that the proposed method has higher sensitivity (in terms of minimum number of microbes) with respect to the existing gold standard culture-based methods. These are important parameters, especially with respect to the future translation of this approach. A visible differentiation of C-D signal above the noise level coming from minimum number of cells will facilitate the operation and interpretation of this approach in the hands of non-expert users also. The intensity of this band can be tied up with a specific concentration of antibiotic to generate 'metabolic fingerprint' of microbes.

4. Conclusion

Overall findings support the prospective utility of heavy water labelling and Raman spectroscopy as an alternate/adjunct approach for identifying metabolically active colistin resistant bacteria *via* urine in a clinically implementable time.

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CRediT authorship contribution statement

Dimple Saikia: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Arunsree Vijay:** Writing – review & editing, Visualization, Software, Methodology. **Tanan Cebajel Bhanwarlal:** Writing – review & editing, Visualization, Software. **S.P. Singh:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2024.124723.

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