Automated and rapid identification of multidrug resistant Escherichia coli against the lead drugs of acylureidopenicillins, cephalosporins, and fluoroquinolones using specific Raman marker bands

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Abstract

A Raman-based, strain-independent, semi-automated method is presented that allows the rapid (<3 hours) determination of antibiotic susceptibility of bacterial pathogens isolated from clinical samples. Applying *a priori* knowledge about the mode of action of the respective antibiotic, we identified characteristic Raman marker bands in the spectrum and calculated batch-wise weighted sum scores from standardized Raman intensity differences between spectra of antibiotic exposed and nonexposed samples of the same strains. The lead substances for three relevant antibiotic classes (fluoroquinolone ciprofloxacin, third-generation cephalosporin cefotaxime, ureido-penicillin piperacillin) against multidrug-resistant Gram-negative bacteria (MRGN) revealed a high sensitivity and specificity for the susceptibility testing of two *Escherichia coli* laboratory strains and 12 clinical isolates. The method benefits from the parallel incubation of control and treated samples, which
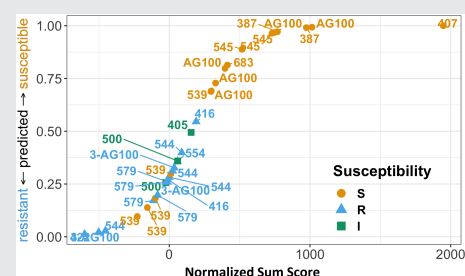
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reduces the variance due to alterations in cultivation conditions and the standardization of differences between batches leading to long-term comparability of Raman measurements.

KEYWORDS

antibiotic susceptibility testing, *Escherichia coli*, multidrug-resistant Gram-negative bacteria, Raman spectroscopy, weighted sum score

1 | INTRODUCTION

The global spread of multidrug resistant pathogens is a major threat for public health [1–3] and requires scientific advances in three critical areas (a) antimicrobial discovery, (b) rapid diagnostics and (c) infection prevention and antimicrobial stewardship [4]. This paper presents a powerful approach for rapid diagnostics (b). Standard clinical microbiological techniques, which include antibiotic susceptibility testing (AST) assays in automated machines after time-consuming bacterial culture, usually yield results only after 1 or 2 days. Thus, new fast, reliable and cost-efficient screening tools for AST are urgently needed. Those tools should be able to comprehensively cover known and emerging resistance mechanisms with both high sensitivity and specificity [5].

Current and emerging AST methods have been reviewed recently [6]. Most of the novel strategies for rapid diagnostics that found their way into clinical practice rely on polymerase chain reaction (PCR)-based techniques, which can detect only known resistance genes [7]. While those can provide the result already within only a few hours, they suffer from several drawbacks, expensive reagents being only one of them [8]. PCR-based methods require a known gene sequence, thus, they are not suitable for the screening of newly emerging resistances [9]. Furthermore, the correlation between resistance genes detected by these methods and the clinically decisive resistance phenotype is limited [10]. This is explained by the level of expression of different resistance genes, that can highly vary and the (particularly in Gram-negatives) complex interplay of many different resistance mechanisms (e.g., mutation of the target protein, efflux pumps, porin loss, different beta-lactamases) [11]. Hence, methods that characterize the phenotypic antibiotic resistance are advantageous and more powerful. One of these promising methods is Raman spectroscopy [12–15]. It has a high potential to change clinical routine as it delivers a full molecular fingerprint in a label-free manner without any toxic immunochemical staining and with only minimal sample preparation times.

Raman spectroscopy can easily be combined with techniques for catching bacteria like microfluidics and dielectrophoresis as it allows analysis of aqueous suspensions [16–18]. Hence, this vibrational spectroscopic method enables the identification of the pathogen and within the same procedure, the characterization of its antimicrobial susceptibility [12], even on the single-cell level [19]. Assmann *et al.* showed that they could detect vancomycin resistance in enterococci after only 30 minutes interaction time [20] and ciprofloxacin resistance in *Escherichia coli* after 60 minutes of interaction time [21], including a simple algorithm to determine the minimal inhibitory concentration (MIC) within 2 hours [22]. The whole procedure from urine sample to pathogen identification and AST result was accomplished within 3.5 hours using a combined dielectrophoresis-Raman setup and a statistical classification model [18]. Other groups reported rapid Raman spectroscopic screening of colistin resistance in *E. coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [23] and characterization of kanamycin resistance in *Acinetobacter baylyi* [24]. Raman spectroscopy was also applied to detect resistances against different antibiotics in a range of Gram-positive and Gram-negative bacterial pathogens isolated from positive blood cultures within 5 hours [25]. The spectroscopic signature of the phenotypic response to the antibiotic is dependent on the mode of action of the drug [26].

For the ultimate clinical application of the powerful Raman-based method, an automated data analysis algorithm is needed that is robust against inter-instrument or repetition variability and provides reliable results in real time [5]. To assure transferability between different spectrometers, advanced data treatment algorithms have been developed that include, *inter alia*, corrections for intensity variations due to changes in laser focusing or fluctuations of an autofluorescence background as well as for different instrument response functions [27]. Other approaches include model transfer algorithms to make spectroscopic data from different measurement conditions comparable [28]. Despite all efforts, reliable predictions for spectroscopic data from different measurement conditions remain challenging.

Here, we present a new, mostly data-driven analysis method, which is based on multi-criteria decision analysis using complex Raman spectra. To the best of our knowledge, one new aspect is that our method uses a paired experimental approach in combination with the standardization in the analysis, which can exclude spectral variability. By this experimental design, variations between Raman instruments can be overcome, as effects introduced by the antibiotic treatment are standardized, thus, allowing comparison of results between instruments and paving the way for future clinical application. In addition, the presented approach of effect strength per wavenumber allows reducing the complexity of statistical models by the previous extraction of most informative spectral regions. The identified characteristic Raman marker bands are specific for the mode of action of the tested drug assuring at the same time interpretability of results combined with *a priori* knowledge. The whole approach requires only minimal computation time, so that results can be obtained immediately after spectra acquisition and it even allows for an *in vivo* metabolic analysis. The power of the new method was demonstrated with rapid AST for three of the most relevant classes of antibiotics used to treat moderate to severe infections in hospitalized patients following the definition of the German Commission of Hospital Hygiene and Infection Prevention (KRINKO) [29]: fluoroquinolones (ciprofloxacin), third-generation cephalosporins (cefotaxime) and ureidopenicillins (piperacillin) (3MRGN, see Table S1). We have focused our proof-of-concept study on *Escherichia coli*, which is a major pathogen of human infections world-wide [30–32], the leading pathogen in urinary tract infections as well as in Gram-negative blood stream infections and sepsis [33]. It is a representative of Gram-negative, rod-shaped bacteria, which are known for the emergence and rapid spread of newly evolving resistance genes. Rapid exchange of resistance genes between different Gram-negative species yields multidrug-resistant bacteria. The limited treatment options for multidrug resistant pathogens are associated with increased mortality [34].

2 | METHODS

Fourteen *E. coli* strains (see Table 1 and Supporting information [SI]) were grown as overnight cultures. Optimal antibiotic incubation time points were determined in pretest to 90 minutes for ciprofloxacin and after 120 minutes for cefotaxime and piperacillin (see SI for details). For each batch, two aliquots were prepared equally treated in parallel from the same overnight culture, one aliquot with antibiotics (treated) and one without antibiotics (untreated). Antibiotic concentrations were selected according to the EUCAST breakpoint table [35] for Enterobacterales and

chosen to be the first or second concentration above the breakpoint. As no further cultivation steps were necessary, results can be obtained within 2 hours.

Prior to Raman measurements, bacterial suspensions (treated and untreated) were washed twice in 0.5× phosphate-buffered saline (PBS Dulbecco, Merck Biochrom GmbH, Germany) and resuspended in 0.5× PBS. Raman measurements were performed with a commercial Raman microscope (CRM 300, WITec, Ulm) using Raman excitation wavelength of 532 nm in combination with a dielectrophoresis (DEP) setup as described earlier [17, 21] (see SI for more details). In total, 30 487 spectra were recorded in 54 different experiments to characterize laboratory strains as well as real-world patient's isolates (Table 1). Data were recorded within a time span of 3 years, where data acquired within the first 1.5 years (data set 1) were used for the establishment of the analysis procedure and data acquired in the last year served as independently cultured validation data set (data set 2, Table 1).

Before data analysis, Raman spectra were preprocessed including wavenumber axis calibration, baseline correction as well as truncating spectra to regions of interest, spike removal, smoothing and normalization (see SI for details). Spectral data were finally truncated to the spectral fingerprint region from 600 to 1800 cm^{-1}, where most of the biologically relevant vibrational bands are found.

The data analysis algorithm involved two main steps: (a) the identification of Raman marker bands that are characteristic for the mode of action of the antibiotic drug in data set 1 and (b) the calculation of weighted sum scores based on Raman intensity differences between spectra of treated and untreated pathogens for both data sets. All data analysis was performed according to the cellular target of the drug: while ciprofloxacin targets the topoisomerases, cefotaxime and piperacillin both target peptidoglycan biosynthesis and thus were analyzed together. For the identification of drug-specific Raman marker bands, effect strength values per wavenumber were calculated using the difference of mean spectra of treated and untreated bacteria divided by the square root for their mean-variance (see SI for details). Effect strength is a scale-free measure and defined in standard deviations. Since there exist no published values with spectroscopic content so far, we determined own cutoff values. A Raman marker band was identified when the effect strength exceeded three standard deviations, or when the effect strength of a specific wavenumber was at least twice as high as the absolute effect strength from other wavenumbers within this batch (Figure 1).

The selected wavenumbers were used for calculating the weighted sum scores as following: an exhaustive bootstrap of every possible combination of band intensities of the beforehand-specified wavenumbers of treated and untreated
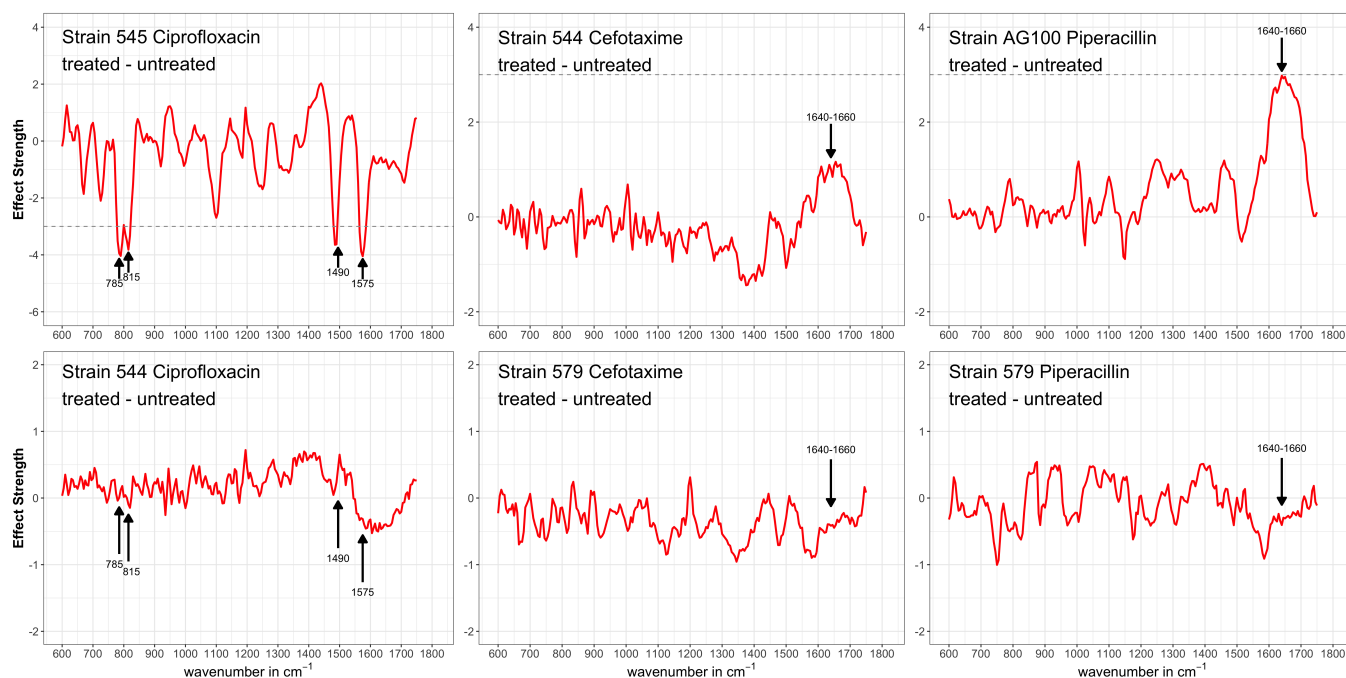
FIGURE 1 Top row: effect strengths for *E. coli* strains 545, 544 and AG100, which are susceptible to the three tested antibiotic classes: the fluoroquinolone ciprofloxacin, the cephalosporin cefotaxime and the acylureidopenicillin piperacillin. Bottom row: effect strengths for *E. coli* strains 544, 579 and 579 which are resistant to the three tested antibiotic classes, respectively. Identified wavenumbers are indicated with black arrows. See the supplementary material for further information

pathogens in each batch was drawn and calculated the differences between spectra of treated and untreated separately for each batch (paired experiment approach). Difference values were ordered ascendingly, separated into equally sized bins and summed up weighted by their bin number (see details in SI). The result is a weighted sum score. As the number of bins used differs between batches, the weighted sum score was normalized by dividing by the number of counts used resulting in the normalized sum score (NSS).

Data-specific NSS cutoffs to discriminate between strains susceptible and resistant to antibiotics were obtained using the Youden method [36]. The 95% confidence interval for classifier sensitivity and classifier specificity was calculated according to Agresti-Coull using the number of batches as sample size [37]. A common cutoff was determined together for all ciprofloxacin wavenumbers and a common cutoff was determined for cefotaxime and piperacillin.

For statistical substantiation, we performed a logistic regression with the dependent binary variable "susceptibility" and the independent variable "NSS" for each identified wavenumber and resistance mechanism. Probabilities were calculated for each batch for being classified as susceptible (ciprofloxacin) or resistant (cefotaxime/piperacillin) to antibiotics (see SI for details). In addition, a permutation test was performed using the Mann-Whitney-Wilcoxon test (exact and two-sided). Tests were performed separately for each wavenumber. Results were considered as significant when $P \leq .05$ (see SI for details).

3 | RESULTS AND DISCUSSION

3.1 | Identification of different Raman marker bands according to the antibiotic effect mechanism

To extract relevant biochemical changes induced by the treatment with antibiotics for a reliable computational approach, effect strength values per wavenumber have been calculated by comparing Raman spectra of treated susceptible bacteria and untreated controls (Figure 1). Using *a priori* knowledge is advantageous for the band selection and leads to an immense dimension reduction, which is of particular interest in case of a small number of samples. Furthermore, statistical models rely on estimates calculated from input variables. When decreasing the number of input variables, the overall (unnecessary) variation of the data set is reduced leading to a more reliable and robust model. In addition, the road is paved for nonlinear analysis methods, which require often extensive computation times.

Four wavenumbers were identified where spectral changes are observed upon efficient interaction of ciprofloxacin with the *E. coli* bacteria: 785, 815, 1490 and 1575 cm^{-1}. For cefotaxime and piperacillin, the wavenumber range from 1640 to 1660 cm^{-1} was identified as being associated with successful β-lactam action (Figure 1). Those wavenumbers were the same in all

TABLE 1 Measured strains, resistance profile (including minimal inhibitory concentration, MIC), number of batches (independent biological replicates) and total number of spectra per strain, antibiotic treatment and assignment to data set

Treatment condition	*E. coli* strain	Antibiotic susceptibility[a]	MIC[b] (in mg/L)	# Batches	# Spectra Data set 1 (2014-2016)	Data set 2 (2016-2017)
Ciprofloxacin (1 mg/L), 90 min after treatment	AG100	S	≤0.25 (0.032)	5	1800	1200
	3-AG100	R	1 (1)	3		1800
	387	S	≤0.25 (0.032)	2		1300
	405	I	0.5 (0.5)	1		513
	407	S	≤0.25 (0.016)	1		600
	416	R	1 (1)	2		1200
	422	R	1 (1)	1		600
	500[b]	I	0.5 (0.5)	2		1200
	539	S	≤0.25 (0.25)	5	1900	1200
	544	R	≥4 (128)	4	1800	600
	545	S	≤0.25 (0.125)	3	1900	
	554	R	≥4 (≥32)	1		600
	579[b]	R	≥4 (256)	4	2000	600
	683	S	≤0.25 (0.032)	1		600
Cefotaxime (2 mg/L), 120 min after treatment	AG100	S	≤1 (0.125)	3	1800	
	539	R	≥64 (256)	3	1800	
	544	S	≤1 (0.25)	3	1800	
	545	S	≤1 (0.032)	3	1774	
	579[c]	R	≥64 (≥256)	3	1900	
Piperacillin (16 mg/L), 120 min after treatment	AG100	S	≤4 (2)	2	1200	
	579[c]	R	≥256 (≥256)	2	1200	
	Σ			**54**	**32 887**	

[a]The assessment is based on European Committee on Antibiotic Susceptibility Testing (EUCAST) clinical breakpoints (version 8.0, 2018): I, susceptible, increased exposure, R, resistant; S, susceptible; mostly ~600 spectra per batch (300 treated and 300 untreated).
[b]Determined by automated VITEK2 analysis in clinical routine diagnostics (first value) as well as by broth microdilution analysis or E-test (Liofilchem MIC Test Strip) (values in brackets). Breakpoints to discriminate resistant from susceptible phenotypes were used according to the EUCAST [38].
[c]Strains, which are resistant against three lead substances of the KRINKO definition (3MRGN, see Table S1). Spectra are divided into two independent data sets according to time of measurement.

analyzed strains. Furthermore, all selected wavenumbers are in accordance with the known mode of action of the drugs: Fluoroquinolones, such as ciprofloxacin, bind to and inhibit the topoisomerase, that is, the gyrase-DNA complex (gyrA/B) and the topoisomerase IV (parC/E), which leads to impaired DNA supercoiling, inhibition of DNA synthesis and ultimately, promoting breakage of double-stranded DNA [39]. All four wavenumbers identified to be associated with efficient ciprofloxacin action can be assigned to DNA moieties: the band around 785 cm^{-1} originates from O-P-O symmetric stretching vibration of the DNA backbone [40], the band at 815 cm^{-1} can be assigned to ordered ribonucleic acid structures [41], the band at 1490 cm^{-1} is a Raman marker band of DNA-protein interactions [42] and the band at 1575 cm^{-1} reflects purine vibrations [40]. Ciprofloxacin reduces intact DNA, thus, all significant ciprofloxacin effect strengths in Figure 1 are negative, indicating that Raman intensities correlating with DNA content are higher in the untreated controls. Our results are in agreement with previous work [22] as well as with studies analyzing the effect of ciprofloxacin treatment to *Bacillus pumilis* [43], indicating that the identified Raman marker bands are independent from species but unique for a specific mode of action.

For the cephalosporin cefotaxime as well as for the ureidopenicillin piperacillin, only one wavenumber region was identified as being significantly affected by
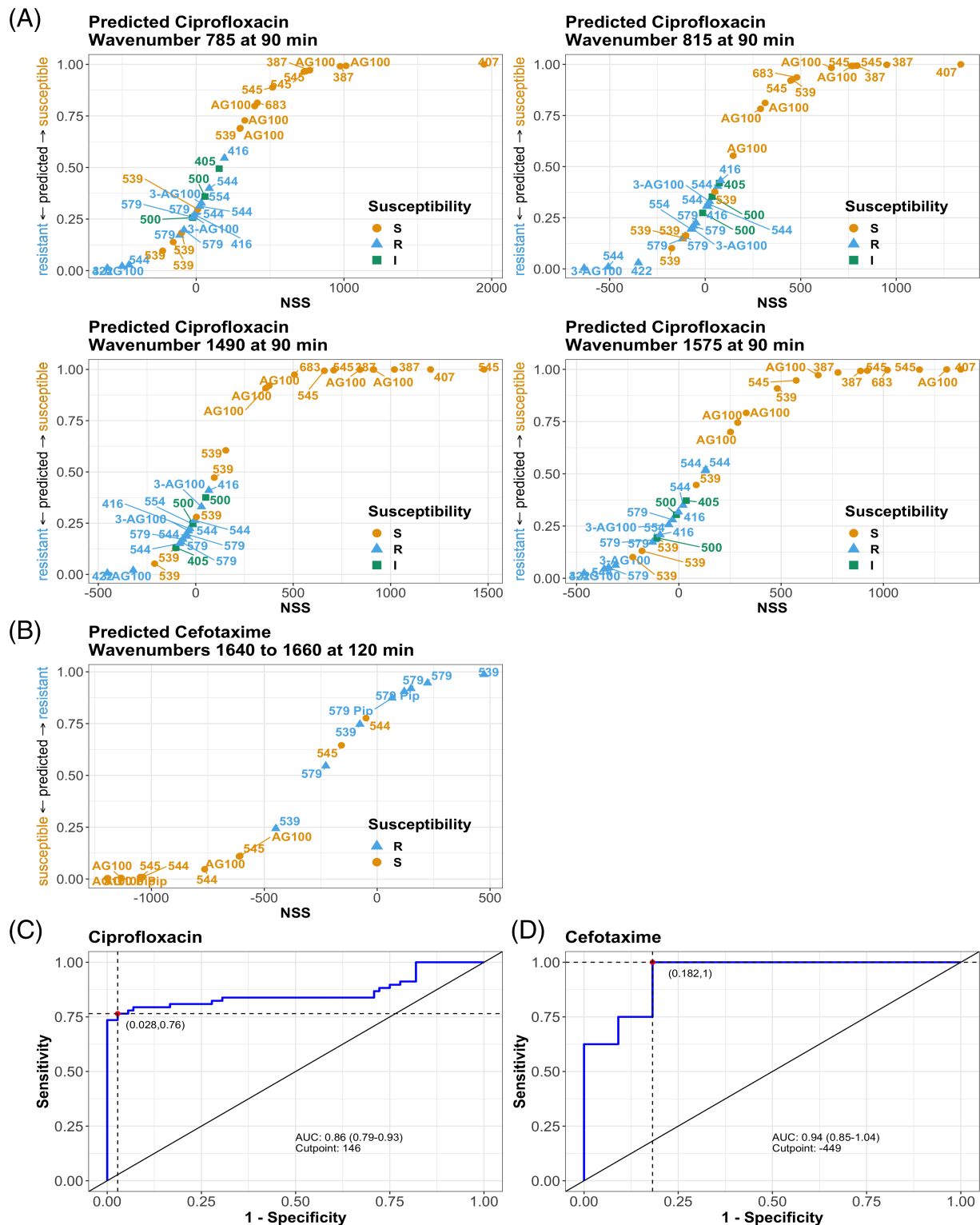
FIGURE 2 A, B, Scatter plots showing the normalized sum score (NSS) plotted against the probabilities of the logistic model: A, for ciprofloxacin at wavenumbers 785, 815, 1490 and 1575 cm^{-1} and B, for cefotaxime and piperacillin treatment for the wavenumber region 1640-1660 cm^{-1}. Piperacillin batches are marked with "Pip." Susceptible (S, orange), resistant strains (R, blue) and strains requiring increased drug concentrations (I, green squares) are labeled in the figure with the strain name. C,D, receiver operating characteristic (ROC) curves for C, ciprofloxacin and D, cefotaxime and piperacillin. Further ROC curves computed with different methods can be found in Figure S2 and S3

the mode of action of the two drugs with the current data set. This wavenumber region was the same for both antibiotic classes (1640-1660 cm^{-1}; Figure 1), which is in agreement with other studies of the action of penicillin on *E. coli* [44]. Cephalosporins and ureidopenicillins are both β-lactam antibiotics, which inhibit synthesis of the bacterial cell wall by binding to one or more penicillin-binding proteins (PBPs), which inhibit the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls: cephalosporins preferentially bind to PBP 1a while ureidopenicillins bind preferentially to PBP 3. Despite different interactions on the molecular level, the two different β-lactam antibiotics lead to an inhibition of bacterial cell wall synthesis, which is visible with the same effect in the Raman spectra. Therefore, we decided to combine the two β-lactam groups (cephalosporins and ureidopenicillins) for subsequent statistical analysis. The effect strength value is positive indicating a stronger intensity in treated bacteria in comparison to the control.

3.2 | NSS and logistic model for prediction of antibiotic susceptibility

For the selected wavenumbers (Figure 1) an exhaustive bootstrap of every possible combination of difference spectra from treated and untreated bacteria was computed. The difference values were organized in a histogram comprising 20 binned values. To selectively enhance large difference values, a weighted sum score was calculated by summing up the counts multiplied by the bin number. In order to make different batches with a different number of spectra comparable, the sum score was normalized to the overall number of counts, yielding the NSS. In line with this, it is possible to down weight or exclude two main sources of spectral variability in Raman-based antibiotic resistance detection: (a) the paired experiment with the parallel incubation of control and treated sample is fully exploited, reducing the variance due to alterations in cultivation conditions and (b) the standardization of differences between the batches facilitates long-term (and potentially also inter-instrument) comparability of Raman measurements. The latter is of particular importance if the presented Raman-based method will be implemented in clinical routine in the future as each site will have their own Raman devices, but results should be the same and reproducible. The NSS was used to predict the susceptibility of unknown bacteria as visualized in the scatter plots for ciprofloxacin (Figure 2A) as well as for cefotaxime and piperacillin (Figure 2B).

Irrespective of the analyzed data set, a clear separation in strains susceptible and resistant to ciprofloxacin is

shown for all four selected wavenumbers (Figure 2A and Figure S1). The optimal cutoff value of the NSS to distinguish sensitive and resistant strains is 146.4 with an AUC of 0.86 (confidence interval: 0.79-0.93). The corresponding receiver operating characteristic (ROC) curve for all four wavenumbers is shown in Figure 2C. The ROC curves illustrate the diagnostic capability of a binary classifier (susceptible: yes or no) when the discrimination threshold of the NSS is varied. The area under the curve (AUC) is a measure of how well the NSS can distinguish between susceptible or resistant strains. Note that without *E. coli* 539, the AUC (Figure 2C) would be 0.99 (confidence interval: 0.98-1, supporting information Figure S2). Strains exhibiting a high positive NSS show high differences in the Raman spectra between untreated and treated bacteria, indicating efficient action of ciprofloxacin. Strains exhibiting a low or negative NSS show low or small negative differences indicating resistance to the drug. These predictions (Figure 2A) are in agreement with the resistance profiles determined by gold standard methods (Table 1). For statistical analysis, strains that require increased drug concentrations according to the current EUCAST definition (http://www.eucast.org/newsiandr/) (I), were conservatively classified as resistant by the method (*E. coli* 405 and *E. coli* 416); although, not always for all Raman marker bands (*E. coli* 405 at 785 cm^{-1} is predicted as susceptible, Figure S1). For two ciprofloxacin-resistant strains (*E. coli* 416 and *E. coli* 544) one marker band indicated susceptibility while the other three clearly indicated resistance (Figure 2A, Figure S1). Thus, the analysis of multiple Raman marker bands can make the method very robust and avoid wrong results when the method will be applied later in clinical routine for the identification of suitable antibiotics from a single patient's sample for personalized treatment. Gold standard methods which have all longer interaction times characterized strain *E. coli* 539 as ciprofloxacin susceptible; however, it was classified as resistant by our algorithm with read-out times of only 110 minutes, which are composed of 90 minutes interaction and 20 minutes sample preparation and spectral acquisition time (Figure 2A, Figure S1). This patient isolate was found to have a chromosomal point mutation at the *gyrA* gene (S83L). Since proper function of both topoisomerases (i.e., DNA gyrase and topoisomerase IV) is essential for survival of the bacterial cell, mutations in both enzymes are required for complete fluoroquinolone resistance. However, so-called "first step mutants" like *E. coli* 539 have a by several magnitudes increased risk to acquire the second mutation referring complete resistance. Therefore, it is of medical importance to identify first step mutants which is only possible by sequencing until now, since resistance and treatment failure can

TABLE 2 Results of the logistic bias-reduced general linear model to determine antibiotic susceptibility against ciprofloxacin (top rows) and cefotaxime and piperacillin (bottom two rows)

Treatment	Coefficient	Estimate (SE)	*P*-value	Chance[a] (per 100 points increase in NSS)
Ciprofloxacin	β_{0_785}	0.83 (0.48)	.08	
	β_{785}	0.005 (0.002)	.007	100.5
	β_{0_815}	0.8 (0.47)	.09	
	β_{815}	0.006 (0.002)	.0077	100.6
	β_{0_1490}	0.91 (0.49)	.059	
	β_{1490}	0.007 (0.003)	.0127	100.7
	β_{0_1575}	0.7 (0.47)	.13	
	β_{1575}	0.005 (0.002)	.0065	100.5
Cefotaxime or piperacillin	β_{0_1660}	1.19 (0.8)	.15	
	β_{1660}	0.004 (0.002)	.019	100.44

Note: β_0, intercept for the respective wavenumber, β_x, the influence of the normalized sum score (NSS) value and SE, standard error (note that due to the small number of batches, SEs are relatively high).

[a]For being classified as susceptible to ciprofloxacin or resistant to cefotaxime and piperacillin, respectively.

evolve rapidly during treatment from those first step mutants [45]. It has to be evaluated in future work if the Raman-based method can be used to phenotypically identify point mutations in the *gyrA* gene. Furthermore, strain *E. coli* 539 holds an extended-spectrum ß-lactamase (ESBL) plasmid, which can also be responsible for further resistance mechanisms, like porin loss or increased efflux [46]. This could be confirmed for strain *E. coli* 539 (data not shown). Thus, although gold standard microbiological analysis (VITEK2 analysis, read out after 8 hours) classified this strain as susceptible, the NSS results indicate that the treatment with ciprofloxacin might not be the first choice for this specific patient.

The significant separation between susceptible (probability < .5) and resistant (probability ≥ .5) strains was confirmed by a permutation test (for the different marker wavenumbers 785 cm^{-1}: Z = 3.4, *P* < .001; 815 cm^{-1}: Z = 3.56, *P* < .001; 1490 cm^{-1}: Z = 3.9, *P* < .001; 1575 cm^{-1}: Z = 3.5, *P* < .001). Furthermore, the significance of the NSS as a reliable marker for the susceptibility to ciprofloxacin treatment was proven with a bias-reduced logistic model (Table 2). According to the single models (Table 2), the chances for being classified as susceptible to ciprofloxacin increase by about 100.5 times per 100 units increase in NSS. One out of 14 strains was misclassified as resistant while being sensitive (*E. coli* 539), leading to 4 out of 17 batches being mislabeled as resistant, i.e. in a classifier sensitivity of 76% (65% to 85%) for predicting strains susceptible to ciprofloxacin (also in SI). Strains not susceptible (resistant) to ciprofloxacin were recognized with a classifier specificity

of 97% (90% to 99%). Thus, resistant strains exhibit a high detection probability, which reduces the possibility for a wrong treatment.

For the two other drugs cefotaxime and piperacillin, the NSS also proved to be a powerful measure to reliably discriminate between susceptible and resistant strains. Due to the positive effect strength band (Figure 1), susceptible strains exhibit high negative NSS, resistant strains are found at a lower negative or positive NSS (Figure 2B). The optimal cutoff was at 449.3 with an AUC 0.94 (0.85-1, see ROC curve Figure 2D). Susceptibility predictions are in good agreement with gold standard results (Table 1), except for one misclassified batch of the strains 544 and 545. The classifier sensitivity (strain is susceptible to antibiotic) was 100% (67.5%-100%) and the specificity (strain is resistant to antibiotic) was 81% (52%-94%). The permutation test confirmed significant results for the wavenumber region 1640 to 1660 cm^{-1} (Z = 3.22, *P* < .001). According to the logistic model (Table 2), the chances for being classified as resistant to cefotaxime- or piperacillin treatment increase by about 100.4 times per 100 units increase in NSS.

4 | CONCLUSIONS

In this paper, we present a proof-of-principle study, which uses a weighted sum score fed with *a priori* knowledge to detect phenotypic antibiotic resistance in *E. coli* against the lead substances of the 3MRGN with a reliable sensitivity and specificity. Principally, one batch with a

small number of spectra can be sufficient for susceptibility testing, as far as a) there is a (significant) difference between negative control and treatment spectra at an identified Raman marker band in the majority of the single spectra and (b) the mode of action of the antibiotic is known. This was demonstrated with "one-batch" strains like *E. coli* 407 and *E. coli* 422 which were correctly classified as susceptible and resistant, respectively (Table 1, Figure 2). Using clinically relevant drug concentrations, we obtained results in well-characterized laboratory strains but also in clinical isolates from patients with blood stream infections (sepsis patients). The method was optimized to equally work for different antibiotic effect mechanisms and to be fully independent from the investigated strain and the measurement device. Our results might have a significant impact on clinical routine because incubation and analysis times are much faster (less than 2.5 hours, depending mostly on interaction time of bacteria with antibiotic drug) in comparison to the classical clinical setting (>8 hours). In real world, patient's samples (e.g. urine samples) sometimes also mixed infections are encountered. It is suggested to deal with this complexity with a modified sample preparation approach which is beyond the scope of the manuscript.

The presented algorithm can principally be transferred to other antibiotics with different effect mechanisms. As demonstrated here, the presented effect strength values of the paired experimental setting give insight into drug's mode of action, thus, enabling interpretation of results (or even identification of mode of action for unknown drugs). The newly developed weighted sum score can be computed from any specific Raman marker band which contains the characteristic spectral differences for treated and nontreated bacterial samples.

AUTHOR CONTRIBUTIONS
Ute Neugebauer, Peter Schlattmann, Jürgen Popp developed the hypothesis, Johanna Kirchhoff, Uwe Glaser performed experiments, Theresa Götz, Marcel Dahms, Claudia Beleites performed the statistical analysis, Johanna Kirchhoff, Ute Neugebauer, Claudia Beleites, Marcel Dahms, Theresa Götz, Peter Schlattmann interpreted data. Jürgen A. Bohnert and Mathias W. Pletz provided material and clinical advice. All coauthors edited and approved the manuscript.

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