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Using Infrared Spectroscopy and Multivariate Analysis to Detect Antibiotics' Resistant *Escherichia coli* Bacteria

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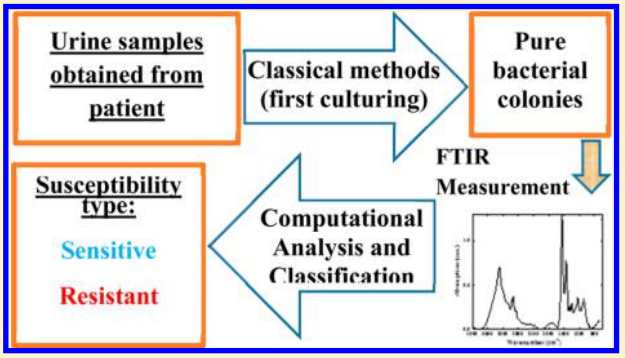
Supporting Information

ABSTRACT: Bacterial pathogens are one of the primary causes of human morbidity worldwide. Historically, antibiotics have been highly effective against most bacterial pathogens; however, the increasing resistance of bacteria to a broad spectrum of commonly used antibiotics has become a global health-care problem. Early and rapid determination of bacterial susceptibility to antibiotics has become essential in many clinical settings and, sometimes, can save lives. Currently classical procedures require at least 48 h for determining bacterial susceptibility, which can constitute a life-threatening delay for effective treatment. Infrared (IR) microscopy is a rapid and inexpensive technique, which has been used successfully for the detection and identification of various biological samples; nonetheless, its true potential in routine clinical diagnosis has not yet been established. In this study, we evaluated the potential of this technique for rapid identification of bacterial susceptibility to specific antibiotics based on the IR spectra of the bacteria. IR spectroscopy was conducted on bacterial colonies, obtained after 24 h culture from patients' samples. An IR microscope was utilized, and a computational classification method was developed to analyze the IR spectra by novel pattern-recognition and statistical tools, to determine *E. coli* susceptibility within a few minutes to different antibiotics, gentamicin, ceftazidime, nitrofurantoin, nalidixic acid, ofloxacin. Our results show that it was possible to classify the tested bacteria into sensitive and resistant types, with success rates as high as 85% for a number of examined antibiotics. These promising results open the potential of this technique for faster determination of bacterial susceptibility to certain antibiotics.



Since the midtwentieth century, antibiotics have constituted the dominant method for control and treatment of bacterial human diseases. However, the uncontrolled and excessive use of antibiotics has promoted the emergence of bacterial mutants that are resistant to traditional antibiotics. In a modern clinical setting, effective treatment of a bacterial infection with an antibiotic depends mainly on a rapid identification of the bacterium susceptibility to antibiotics. Early identification of the optimal antibiotic to be used is important for successful treatment of infections; and, in some cases, early determination of susceptibility can be lifesaving.

The intent of this study was to demonstrate novel and cost-effective laboratory method, based on infrared spectroscopy, which can significantly reduce the time it takes to identify bacterial susceptibility to antibiotics and to determine the optimal choice for effective treatment.

A bacterial strain is considered to be resistant to a specific antibiotic when it can no longer be controlled by this antibiotic and continues to multiply in the presence of the antibiotic, similarly to untreated bacteria. The bacteria develop resistivity to antibiotic as a result of repeated and uncontrolled use of antibiotics.

In fact, in many countries, it is possible to purchase and use antibiotics without physician prescription, even though in most

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cases there is no need for or benefit from antibiotic treatment. In many clinical settings, patients are advised by physicians to start blind treatment with uncertain antibiotics while awaiting for laboratory results, although many of the infections are later determined to be viral in origin.[1−3]

Bacteria can acquire resistance to antibiotics by different mechanisms such as genetic mutations,[4] acquiring plasmids and transposons[5,6] with resistant genes from other bacteria by the process of conjugation. Resistance to antibiotics is considered as a serious global health problem, since a large number of different bacteria have already acquired resistance to various antibiotics, and a few have become resistant to all antibiotics.[7] Therefore, the success of many surgeries performed today is compromised by the use of ineffective antibiotics, and the costs of health care, including longer stays at hospitals, has significantly increased.[5,8]

For instance, *E. coli* bacteria, which is a common cause of urinary tract infections and is responsible for more than 85% of urine tract infections, has developed resistance to fluoroquinolone antibiotic, which is commonly used by physicians to treat these infections; consequently, in many countries, this antibiotic has become ineffective for more than 50% of the patients.[9]

Therefore, the key for effective treatment, and for reducing the rate of development of new bacterial resistance to antibiotics, is an early identification of the cause of the infection and, if bacterial, its susceptibility to antibiotics.[10−12] Reports have shown that 10 to 30% of patients suffering from bloodstream infections in intensive care units do not initially receive the correct antimicrobial therapy. The death rate among this group has been reported to be 30 to 60% higher than in patients that are treated with an effective antibiotic.[13,14] Thus, if the time of bacterial identification and diagnosis of antibiotic resistance can be shortened, the morbidity, mortality, and costs associated with infectious diseases can also be significantly reduced.[15]

Currently the methods used routinely in hospitals for identification of bacterial susceptibility, phenotypic and genotypic methods, are time-consuming and/or expensive.[16−21] Genotypic methods, like PCR[22,23] and in situ hybridization, are used for detection of bacterial infection by targeting their conserved 16S rRNA.[22−24] These genotypic methods could be developed as effective methods for microbe detection and probably for bacterial susceptibility. However, since the molecular methods are expensive, they are not commonly used in laboratories, even though they are accurate; they are, however, used widely in academic settings.[25] Traditional phenotypic tests are preferable over molecular tests for detection of resistance, because molecular tests are based on specific primers that are not available for many bacteria,[25] rendering phenotypic tests as the "gold standard" in medical centers, for comparison against newer technologies, based on performance, cost, and ease of use.[25] Today, in some of the medical centers, matrix assisted laser desorption/ionization (MALDI) time-of-flight-mass spectrometry (TOF-MS) is used for species identification of the isolated bacterial colonies from patients specimens.[26]

In qualitative and quantitative analytical applications, the potential of IR spectroscopy[27] to identify chemical components by analysis of their vibrational spectra fingerprints can be of great value. IR spectroscopy is commonly used in the fields of biochemistry, biomedical science, materials science, and medicine, where its value has been demonstrated.[27−38] The mid-infrared spectral region is rich with structural and functional information regarding the cell's molecular components,[27,39,40] which enables the technique to detect molecular changes related to early stages of the development of cancer, for instance refs 48 and 55. Moreover, earlier studies showed that IR spectroscopy was able to detect cancer cells at very early stages of the cancer, even when the histological appearance of the cells still looks normal.[37,38,41] Despite the completely different biochemistry of cancer development in eukaryotic cells vs resistivity to antibiotics in prokaryotes, both transformations are the result of complicated and subtle biochemistry changes, and we submit that successful discrimination by FT-IR of such changes in eukaryotes may give reasonable expectations for a similar success in prokaryotes.

Although FT-IR was used for the identification of microorganisms as early as the 1950s,[42−44] it has benefitted from the more recent development of modern IR spectrometers, high-performance computers, and powerful new algorithms that enable the use of multivariate statistical analysis and pattern recognition methodologies.[45] In 1988, Naumann et al. reported on the potential of this technique to identify bacteria at the strain level,[46,47] a finding that has been replicated by other researchers in the following decades.[29,46,48−65]

In our previous study, we successfully classified resistant and sensitive *Phytophthora infestans* isolates to mefenoxam with high success rate using IR spectroscopy.[66] Moreover, in a different study, we used FT-IR spectroscopy in tandem with principal component analysis (PCA) and linear discriminant analysis (LDA) for taxonomic classification of 35 isolates of *Colletotrichum coccodes* at the isolate level and to classify these isolates into eight vegetative compatibility groups (VCGs) with high accuracy.[67] A VCG consists of isolates (belonging to the same species) that can transfer genetic material by contact[68] to produce new stable heterokaryons (cells that contain multiple, genetically different nuclei), thereby forming subpopulations that tend to be similar due to a common genetic pool and which exhibit similar pathogenic aggressiveness.[68]

We also showed in previous studies that FT-IR can monitor abnormality at a stage when the cells show normal morphology.[37,38,41,69] These characters render the infrared microscopy technique for the detection of molecular changes associated with developing the resistance to a specific antibiotic.

A recent study carried out by Lechowics et al. has reported 95% success in classification of 109 *E. coli* isolates into sensitive and resistant to Cephalothin, using FT-IR-ATR[70] and classification by an artificial neural network. A few other studies used mass spectrometry (MS)[71−73] for detecting resistance of bacteria to antibiotics.

Thus, given its potential to monitor subtle chemical and molecular changes in cells,[66] FT-IR microscopy may be applicable for clinical laboratory diagnosis of bacterial resistance because it is noninvasive, rapid, safe, and low-cost. Insofar as it allows the microorganisms to be studied in their intact states and reflects the overall molecular composition of a sample, IR spectroscopy is also a promising method to study microbial metabolism and other factors related to bacterial susceptibility.[74]

EXPERIMENTAL SECTION

Bacterial Isolation and Identification from Patients' Samples. Deidentified urine samples from patients were provided by physicians in the Bacteriological Laboratory, Soroka Medical Center (SUMC at Beer-Sheva, Israel), after

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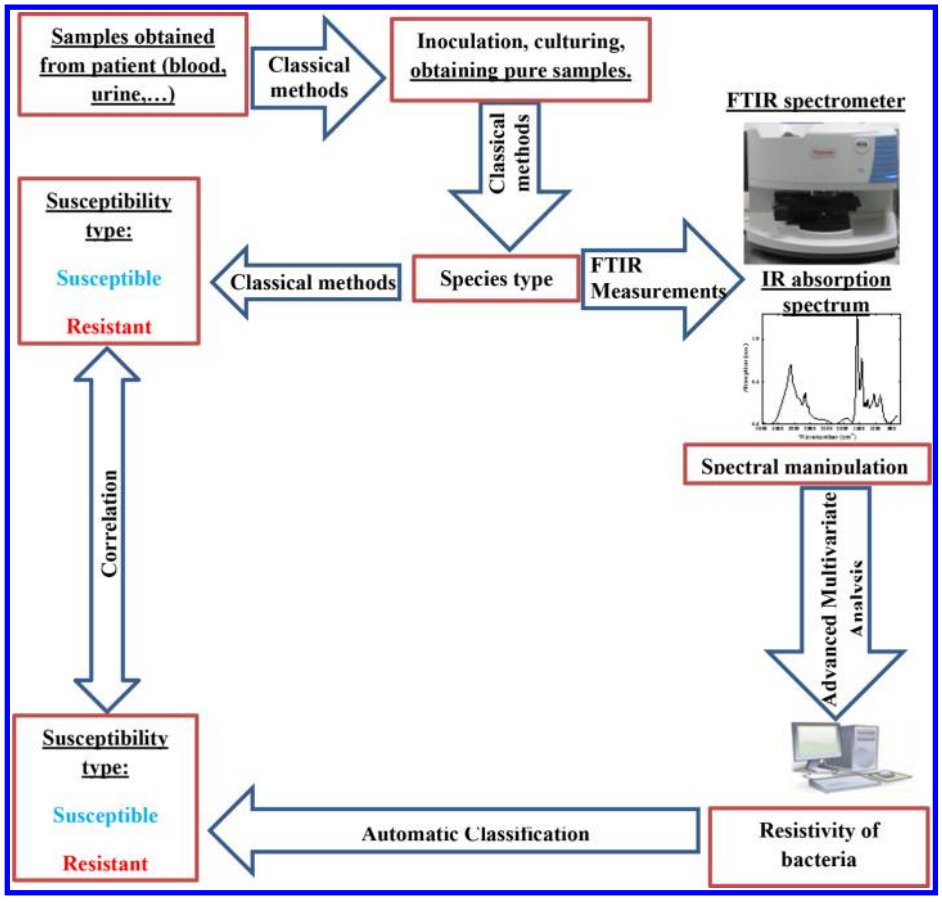

Figure 1. Main steps that were carried out in all stages of classification in our study. Disk diffusion was used as the gold standard phenotypic method for resistivity classification.

determining the types and susceptibilities. The isolates were cultured on selective agar plates (at 24 °C for about 24 h) for bacterial colonies isolation (first culture). Pure bacterial colonies were identified for their species by MALDI-TOF and for determining their susceptibility to antibiotics, by the routine disc method, which takes another 24 h (second culture). The Bacteriological Laboratory in SUMC uses the standard concentrations of the tested antibiotics, which are determined according to the protocols of the companies. Small samples of these colonies were also picked up by the bacteriological needle, placed on a zinc−selenium slide, dried at room temperature for about 15 min, and examined by FT-IR microscopy.

FT-IR Measurements. We used a Nicolet i10 spectrometer that is attached to a MCT detector to perform our measurements, using transmission mode in the range of 600−4000 cm^{-1} with 4 cm^{-1} spectral resolution and 128 coadded scans.

Spectral Manipulation. We manipulated the spectra using OPUS 7 (Bruker Germany) to manipulate our spectra, smoothing (Savitzky−Golay algorithm with 13 points), baseline correction (Concave Rubber Band method with 64 points and five iterations), and normalization (vector and offset normalization) as described in our previous paper.[75]

Multivariate Analysis. In this study, we used linear support vector machines (SVM) as a binary classifier to differentiate between the resistant and sensitive *E. coli* isolates for individual specific antibiotics. The spectra were normalized setting the area under the curve to one for shape enhancement and down-sampled by a factor of 2 for signal smoothing. SVM is a supervised machine learning method that must be trained before the validation process. The classifier was trained on all the data for each specific antibiotic (for the *E. coli*), and then the leave-one-out (LOO) method was used to validate the performance of the resulting classifier output values. The LOO method is run a number of times equal to the number of samples in the set.

The dimensionality of the data was reduced (features selection)[76] before the classification procedure, using various methods for feature selection, including sequential forward feature selection. We then employed ensemble classifiers based SVM[77,78] as a multidimensional classifier.

The computerized classifiers and machine learning algorithms were developed in our laboratories, based on methodologies developed for classification of optical spectroscopic data at visible wavelengths.[79] The developed classifier was used for the classification between the *E. coli* spectra according to their susceptibility as sensitive and resistant for each specific antibiotic. LOO[80,81] variant of k-fold cross-validation approach was used to estimate the performance of the classifier. LOO is commonly used in pattern recognition, when the size of the data set is small. Of course, it is preferable to have independent training and testing data sets, so that classifier performance can be tested on a naïve data, but when the available data set is small and only retrospective statistical analysis is possible, the LOO method is among the more reliable tests.

The performance of the classifier was determined in statistical terms. For each antibiotic, specificity (SP), sensitivity

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(SE), positive-predictive value (PPV), negative-predictive value (NPV) and area under the ROC curve (AUC) were used as metrics of the performance of the preliminary tests of the developed classifier. SE is defined as the probability for identifying sensitive isolate (classical methods) as sensitive (infrared method), while SP is defined as the probability of identifying resistant isolate as resistant for specific antibiotics. PPV is defined as the probability that the classifier is correct, if it predicts drug sensitivity to a specific antibiotic, and the classical method confirms the sensitivity. NPV is defined as the probability that the classical method will confirm an isolate as resistant, if it was identified as resistant by the classifier.

Additionally, a new analysis has been employed, with the intent to provide doctors with recommendations for antibiotics that are likely to work with a high confidence level. This is an ensemble analysis, which relies on the combined results of a few antibiotics with best performance, enabling one or more choices with a high confidence level to treat the bacteria. Using the ensemble analysis, the definitions of the sensitivity and specificity were modified to estimate the performance of the classifier according to the ensemble. SE is defined as the probability of the ensemble to identify correctly one effective antibiotic, when one or more effective antibiotics exist, based on the gold standard. SP is identified as the probability of identifying all antibiotics (of a test group) to which the pathogen is resistant, when those antibiotics are ineffective, based on the gold standard. The ensemble analysis was carried out based on identifying an effective antibiotic, when N antibiotics exist.

RESULTS AND DISCUSSION

In this study, the potential of FT-IR microscopy combined with multivariate analysis was evaluated for determination of the susceptibility of *E. coli* samples to antibiotics, in the time span of minutes. There are no significant safety hazards or risks associated with this research.

Many studies have reported that mutation of the bacterial genome can result in resistivity of the bacteria.[82,83] These biochemical changes are small; thus, we need a highly sensitive method to monitor these changes. FT-IR spectroscopy is well-known for its high sensitivity for the biochemical changes.[84,85]

The protocol of this study was designed as illustrated in Figure 1, under the approval of the Institutional Review Helsinki Board at SUMC, in Beer Sheva, Israel. "Ground truth" determination of the susceptibility of *E. coli* isolates from patient samples was determined by the disk diffusion method for a number of the commonly used antibiotics.

Average spectra of all *E. coli* isolates, sensitive and resistant to *Ceftazidime* as determined by classical methods, are shown in Figure 2a in the regions 900−1795 cm^{-1} and 2800−3000 cm^{-1}. The ranges 1795−2800 cm^{-1} and 3000−4000 cm^{-1} are essentially devoid of spectral features and were excluded as a part of the manipulations procedure. Lipids are the main contributors of the infrared absorptions bands centered at 2859 and 2926 cm^{-1} due to their CH_2 and CH_3 vibrations.[86] Proteins are the main contributors to the amide I (C═O stretching) and amide II (N−H bending and C−N stretching) absorption bands centered at 1654 and 1543 cm^{-1}, respectively.[87] Proteins absorb with weaker bands at 1452 and 1379 cm^{-1}, which are due to their antisymmetric and symmetric CH_3 bending vibrations. The bands centered at 1238 and 1080 cm^{-1} are contributed mainly by the amide III mode and glycogen, respectively.[86,88]

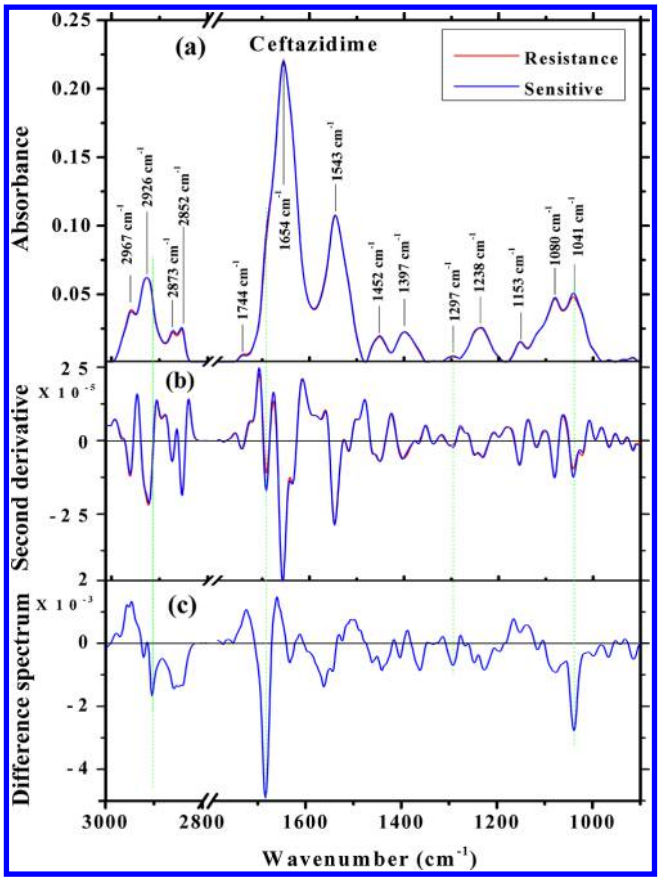

Figure 2. Average infrared absorption spectra of all sensitive and resistant *E. coli* isolates determined as sensitive and resistant to ceftazidime by classical methods, respectively (a), second derivative spectra (b), and difference spectrum (c). These spectra are displayed in the region 2799−3000 and 900−1795 cm^{-1} after manipulation. The main features and their assignments are labeled in the figure. A few vertical green lines were added to show correlation between the minima in the difference spectrum and the absorption bands in the absorption spectra in part a.

Table 1. Bacterial Susceptibility of Bacterial Samples Included in This Study Regarding Five Antibiotics[a]

isolate numbers as they appear in the medical files	antibiotics				
	gentamicin	ceftazidime	nitrofurantoin	nalidixic acid	ofloxacin
311218	S	S		S	S
311224	R	S		S	S
311124	R	R	S	R	R
311157	S	S		S	S
310927	S	S			
310973	S	S			S
310982	S	S	R	S	S
310881	R	S		S	R
310974	R	S	R	R	S
311031	R	S		S	S

[a]S = sensitive; R = resistant.

The centroids of the absorption bands were derived using second-derivative spectra, as shown in Figure 2b. The difference

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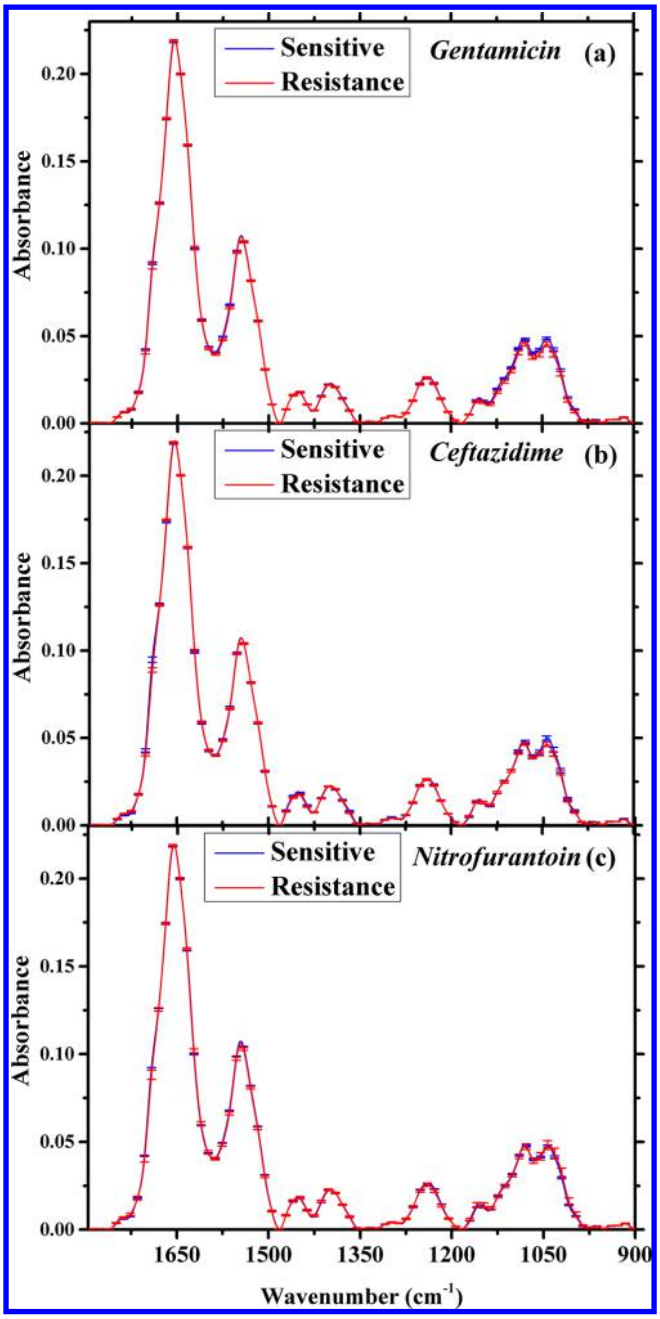

Figure 3. Average infrared absorption spectra of all sensitive and resistant *E. coli* isolates determined, by classical methods, as sensitive and resistant to (a) gentamicin, (b) ceftazidime, and (c) nitrofurantoin, in the region 900−1795 cm^{-1} after manipulation. The determinations of sensitive and resistant *E. coli* isolates to each antibiotic were performed independently. The error bars were calculated as standard errors of the measurements.

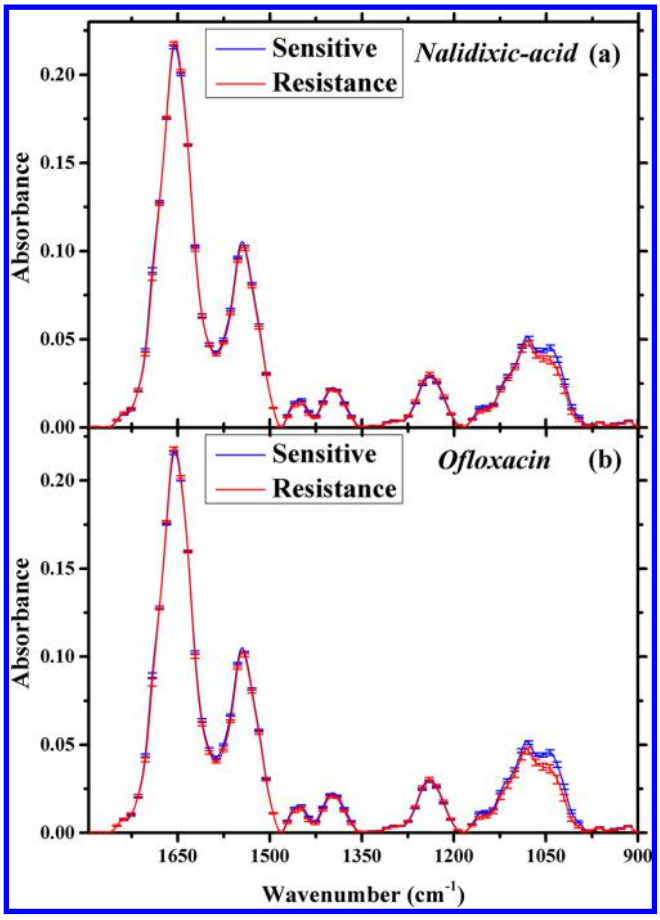

Figure 4. Same as Figure 3 for the (a) nalidixix acid and (b) ofloxacin antibiotics.

spectrum of resistant minus sensitive is plotted in Figure 2c. As can be seen from the figure, the spectral differences do exist, although they are subtle. Repeated measurements in numerous patient samples indicate that these subtle differences are reproducible.

In this study, we included 494 different *E. coli* as sensitive or resistant to different antibiotics. Table 1 lists a few bacterial samples, and their susceptibility results for specific antibiotics as designated by the microbacteriology lab.

The average spectra of the bacterial isolates, sensitive and resistant to the antibiotics included in this study are shown in Figure 3a−c, (gentamicin, ceftazidime, and nitrofurantoin) and Figure 4a,b (nalidixic acid and ofloxacin). The error bars were calculated as the standard error of the spectra. Second-derivative spectra (data not shown) were generated for the sensitive and resistant *E. coli* isolates to each antibiotic separately, and no band shifts were observed. The spectral differences are subtle changes in relative intensities and shapes of spectral features, which we hypothesize to be indicative of changes in molecular secondary and tertiary structure. The spectral differences between the resistant and sensitive spectra for gentamicin, ceftazidime, nitrofurantoin, and ofloxacin are mainly in the carbohydrate 990−1170 cm^{-1} region; and for nalidixic acid and ofloxacin the changes are in the carbohydrate and protein bands at 1452 cm^{-1}.

Each sample was represented by an average spectrum of at least 16 spectra, measured from different sites of the same sample culture. The SNR of each spectrum was higher than 100. The reproducibility of spectra was evaluated by determining the dispersion of the spectra around the average spectrum. As can be seen from the Figure S-1, the spectra are almost overlaid, illustrating the good reproducibility of our measurements.

To monitor the spectral changes among the different isolates associated with susceptibility of the bacterial isolate, we require a high signal-to-noise ratio and reproducible spectra. Thus, the preparation of the samples is critical; the concentration of the bacterial cells should be determined carefully to produce

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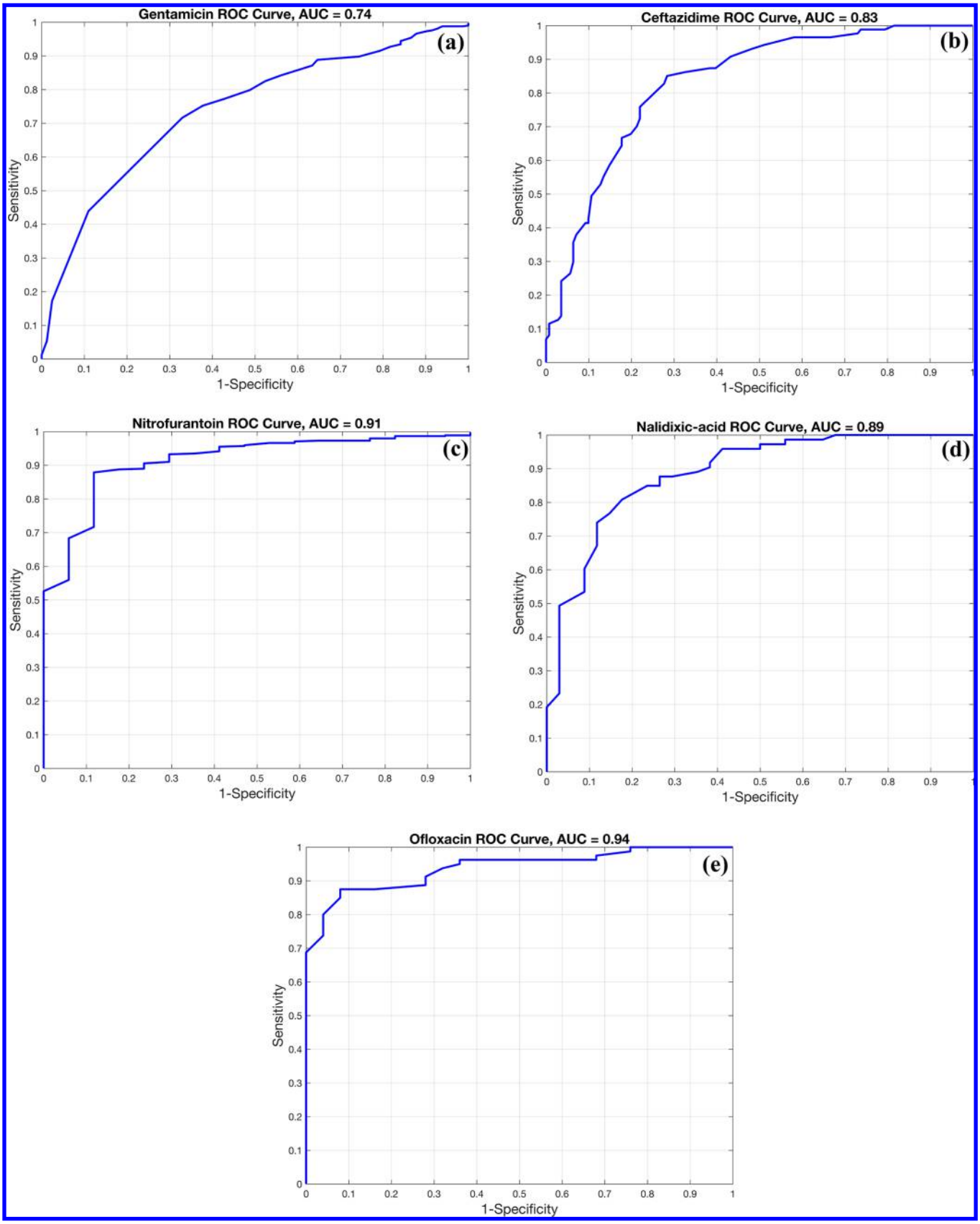

Figure 5. Performance of the developed SVM classifier in terms of ROC for the classification of the *E. coli* bacterial samples into sensitive and resistant using FT-IR spectra for (a) gentamicin, (b) ceftazidime, (c) nitrofurantoin, (d) nalidixix acid, and (e) ofloxacin.

optimum thickness on the ZnSe slides, yielding a strong IR signal without saturating the IR detector.

The resistant and sensitive spectra of all *E. coli* isolates to certain antibiotics, as validated by the gold-standard method,

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Table 2. Classification Performance of Machine Learning Classifier of the Bacterial Samples According to Their Susceptibilities

antibiotic	sensitive	resistant	sensitive	(%) SE	SP	PPV	NPV	AUC
gentamicin	412	82	0.83	0.72	0.67	0.92	0.32	0.74
ceftazidime	87	141	0.38	0.79	0.75	0.66	0.85	0.83
nitrofurantoin	445	17	0.96	0.88	0.88	0.99	0.22	0.91
nalidixic acid	73	34	0.68	0.81	0.82	0.91	0.67	0.89
ofloxacin	80	25	0.79	0.87	0.92	0.97	0.70	0.94

Table 3. Classification Results of the Ensemble Analysis

	SP	SE (at least 1)	SE (1/1)	SE (1/2)	SE (2/2)
ensemble (accuracy)	0.53	0.91	0.89	0.90	0.61
weighted ensemble (accuracy)	0.53	0.91	0.89	0.90	0.60
ensemble (PPV)	0.68	0.86	0.82	0.86	0.40
weighted ensemble (PPV)	0.68	0.86	0.85	0.86	0.41

[a]Ensemble (accuracy) = optimized for accuracy; weighted ensemble (accuracy) = optimized for accuracy weighted by the fraction of sensitive samples for a given antibiotic (a priori probability); ensemble (PPV) = optimized for PPV; weighted ensemble (PPV) = optimized for PPV weighted by the fraction of sensitive samples for a given antibiotic (a priori probability).

are similar, with much overlap but exhibit subtle spectral differences in shape and intensity (Figure S-2). Given this circumstance, we chose a statistical classification approach (rather than model-based), and we employ multivariate pattern recognition and machine learning methods to differentiate among the sensitive and resistant bacterial samples.

The best performance of our classifier was obtained when analyzing the low-wavenumber region, 900−1795 cm^{-1}. Figure 5 shows the receiver operating characteristic (ROC) curve for different antibiotics (a) ceftazidime, (b) gentamicin, and (c) nalidix acid. The area under the curve (AUC) of the ROC curve determines the accuracy of the classification for distinguishing the sample as sensitive or resistant to a specific antibiotic. An AUC of 1.0 means a perfect test (100% correct classifications), whereas an AUC of 0.5 would represent random chance classification, as in flipping a coin.

Clinically, the important issue is to identify at least one antibiotic among the available options, to which there is high confidence that the bacteria will respond. Using the data and the developed classifier for each antibiotic, a multidimensional class boundary was determined. The performance of the classifier within these boundaries for the complete data set was used to obtain the classification results summarized in Table 2. The confidence level of the classification of a bacterial sample using the classifier depends on its distance from the boundary; thus, the confidence may be higher or lower for different classifiers (antibiotics).

As mentioned above, a new type of ensemble analysis was performed, aimed at providing doctors with recommendations for antibiotics that are likely to work with a high confidence level. For example, when one antibiotic is included in the ensemble then the ensemble sensitivity Sen(1/1) equals the top-ranked antibiotic sensitivity as appears in Table 2. When the two top-ranked sensitive antibiotics (Table 2) are included in the ensemble, then Sen (1/2) is calculated by identifying *one* of the two antibiotics as effective when one of them is effective based on the gold standard, while Sen (2/2) is calculated by identifying both antibiotics as effective when both are effective based on the gold standard. Table 3 summarizes the results of ensemble classification performance optimized for different classification measures.

As can be seen from Table 3, the agreement rate for the case of the highest statistical sensitivity to be 90% and 94% for one of the top two choices of antibiotics.

Although the potential of infrared spectroscopy to determine *E. coli* bacteria susceptibility to cephalothin was successfully evaluated by Lechowics et al., in this study, the number of isolates was much larger (494 isolates), and the susceptibility was determined to five different antibiotics. Ours and Lechowics et al. studies both emphasize the great potential of infrared spectroscopy in the field of bacterial susceptibility assessment.

Recently, the method of MS selected reaction monitoring (SRM) was reported to be used for the identification of bacterial susceptibility of *S. aureus* to antibiotic in 60−80 min, to determine the susceptibility directly from extremely infected patients' samples, without culturing.[73] In principle, in these extreme cases, infrared microscopy can also be used to determine the susceptibility of the bacteria without culturing. This is true, since the MS-SRM method requires at least 10^7 − 10^8 CFU/mL[89] bacterial colony forming units, while the FT-IR microscopy method requires only 3×10^5 CFU/mL for good measurements.

In the current study, the infrared microscopy method was used to identify the susceptibility of *E. coli* to antibiotics from patients' urine. Bacterial concentrations over 10^4 CFU/mL[90] of urine are considered as clinical and require treatment. The high concentration of bacteria needed to perform an SRM measurement inhibits its use for direct examination of patient samples with low titers of pathogenic bacteria.

MS is based on the ribosomal protein spectra; thus, resistant mutants that do not differ sufficiently in their ribosomal protein sequences from their original sensitive isolate will not be determined correctly[91] by MS. On the other hand, the FT-IR spectroscopic technique is independent of the protein mass difference, and the expansion of the database to include these new mutation spectra will assist in management of this problem. Furthermore, infrared microscopy has additional advantages over MS-SRM: it is simpler, less expensive, and can be used for all types of bacteria.

While using MS for the identification of bacteria at the species level has become a routine test, it is a long way from being applied for detection of antibiotic resistance of bacteria. The issue of bacterial resistance is both important and urgent; thus, more studies are required to establish the potential of infrared spectroscopy for bacterial susceptibility identification.

CONCLUSIONS

Infrared spectroscopy, combined with advanced multivariate analysis, offers the potential to provide a sensitive and reliable method for rapid determination of the susceptibility of *E. coli* samples to antibiotics based on the fingerprint region 900−

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1795 cm^{-1}. Our estimate of the overall time to determine the susceptibility of the examined bacterial isolate, since receiving the bacterial colonies from the bacteriological laboratory, is about 25 min. We intend to continue our research with enlarged the databases and to expand the study to include different types of bacteria, leading to a method that offers the potential for clinical implementation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01025.

Several infrared absorption spectra acquired from different sites of the sample to represent the high reproducibility of the spectra and some of the sensitive and resistant *E. coli* isolates regarding ceftazidime antibiotic as an example (PDF)

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Notes

The authors declare no competing financial interest.

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