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Rapid identification and discrimination of bacterial strains by laser induced breakdown spectroscopy and neural networks

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ABSTRACT

Identification and discrimination of bacterial strains of same species exhibiting resistance to antibiotics using laser induced breakdown spectroscopy (LIBS) and neural networks (NN) algorithm is reported. The method has been applied to identify 40 bacterial strains causing hospital acquired infections (HAI), i.e. *Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium*, *Salmonella pullurum* and *Salmonella salamae*. The strains analyzed included both isolated from clinical samples and constructed in laboratory that differ in mutations as a result of their resistance to one or more antibiotics. Small changes in the atomic composition of the bacterial strains, as a result of their mutations and genetic variations, were detected by the LIBS–NN methodology and led to their identification and classification. This is of utmost importance because solely identification of bacterial species is not sufficient for disease diagnosis and identification of the actual strain is also required. The proposed method was successfully able to discriminate strains of the same bacterial species. The optimized NN models provided reliable bacterial strain identification with an index of spectral correlation higher than 95% for the samples analyzed, showing the potential and effectiveness of the method to address the safety and social-cost HAI-related issue.

1. Introduction

Hospital acquired infections (HAI) have been widely studied in the past 30 years and have been raised to top-priority issue due to the associated economic and social costs [1]. Therefore, many preventive campaigns as well as new protocols have been implemented [2,3]. On average 5–7% of hospitalized patients are affected by HAI, and 1% of such unwanted events result in the patient′s death [4]. Bacteria are responsible for 95% of HAI, *Escherichia coli* (18.2%), *Staphylococcus* (18.1%), *Pseudomonas* (6.0%), *Enterococcus* (15.4%), *Klebsiella* (3.7%), *Acinetobacter* (0.8%), and *Salmonella* (2.8%) being the most relevant ones.

An important issue highlighted in recent years has been the increasing emergence of bacteria that are resistant to many antimicrobial therapies, sometimes resulting in multidrug-resistant strains or "super bugs." One of the overriding reasons for this is the widespread indiscriminate use of antibiotics to treat infections [5]. This antibiotic resistance is evolved under the treatment regimens of single or multidrug combinations as a result of the mutations [6].

During the past decades several methods have been proposed to optimize the identification of bacterial strain, which are based on molecular techniques such as fluorescent probes [7], microarray assemblies [8,9] and polymerase chain reaction [10,11]. However, these methodologies present some difficulties and drawbacks such as use of consumables, primer, probes or fluorescently labeled RNA antibodies [12]. Moreover, sometimes the sequences in the database are not accurate or up-to-date and micro-heterogeneity is also found common in 16S rRNA gene sequence within a species [13,14]. The phenotypic similarities between the strains of the same bacterial species restrict their identification using routine diagnostic methods [15]. Although these methods provide a reliable and accurate bacterial identification, special sample treatment methods, the high costs and low speed to perform such analysis limit their use as rapid diagnostic methods in common laboratories in order to provide quick results which leads to an increase in the rates of infectious diseases in clinical settings. Further the direct handling of these potentially pathogenic bacterial samples poses health-associated security risks. At present, clinical safety procedures and cost-related considerations do not allow an easy routine analysis of highly dangerous pathogenic bacterial specimens causing hospital acquired infections. Nevertheless, bacterial identification within the first 24 h of infection allows the use of a more effective and less risky targeted-therapy decreasing unnecessary hospitalization days and costs.

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It is also necessary to point out the importance of sample preparation, which is an important step to achieve a significant result within a reasonable amount of time, thereby, avoiding or reducing the need for time-consuming culture enrichment steps. Thus, the increasing need for high speed and precision illustrates the importance of sophisticated methods for sampling and sample preparation within the overall process. The proper development and adaptation of sample preparation towards the detection method, is essential for exploiting the whole potential of the complete workflow of any diagnostic method.

Detection and identification of biological samples and, in particular bacteria, using laser induced breakdown spectroscopy (LIBS) analysis has been studied by several research groups [12,16–22]. The motivation of these studies was to evaluate the ability of LIBS to provide fast identification compared to traditional bioanalytical methods, benefitting from the possibility to combine it with chemometric methods in order to increase the performance of the technique. Morel et al. [18] investigated the detection of six bacteria by time resolved LIBS. They placed particular emphasis on *Bacillus globigii*, which acts as a non-pathogenic surrogate for *Bacillus anthracis* (anthrax), demonstrating the ability of LIBS to detect bacteria. Baudelet et al. [20] showed an unambiguous discrimination of different bacteria based on the concentration profile of trace elements. Recently, Rehse et al. [12,17] studied the effect of different experimental conditions (e.g. bacteria dilution and nutrient deprivation) on bacteria identification by discriminant functional analysis showing successful bacterial classification. Multari et al. [19] applied partial least squares regression analysis to differentiate *E. coli* from *Staphylococcus aureus* strains. Although these studies present good results, in some cases correct identification rate of bacterial strains or correlation falls below 85%. Thus, there is a clear need for more thorough and systematic studies that include new approaches making it possible to take this methodology in clinical setting for diagnosis of diseases and public health. Therefore, the motivation behind this study is to use a classification model for bacterial identification using artificial intelligence algorithms like neural networks (NN) to improve the accuracy and precision of the classification process. In a previous study by our group [23] artificial intelligence algorithms like neural networks (NN) have been used that have shown to be a promising chemometric methodology to classify and predict bacterial samples at genus level with a high degree of precision and accuracy. The full sets of variables (intensities at each wavelength) that constitute the sample spectrum are important in the process of comparison performed by the NN, which constitutes the basis of their ability to carry out discrimination. The NN is able to compute internal parameters (weights and bias) in the learning process for classifying a given set of input variables as belonging to particular sample with a high tolerance for noise and the presence of outliers [24].

In this paper we have intended to extend the previous study to investigate the application of LIBS–NN to discriminate different antibiotic resistant strains of same bacterial species and address its use as a rapid potential diagnostic methodology. The aim is to determine if genetic variations between bacterial strains of the same bacterial species, even when there is a difference in only one gene, generate sufficient or significant changes in their atomic composition which can be detected by LIBS–NN method in order to achieve their discrimination and identification.

2. Material and methods

2.1. LIBS set-up

The LIBS technique and the methodology used in the present work together with the most significant experimental conditions have been previously described [23]. Briefly, LIBS measurements were obtained using a *Q*-switched Nd:YAG laser (Quantel, Brio model) operating at 1064 nm, with a pulse duration of 4 ns full width at half maximum (FWHM), 4 mm beam diameter and 0.6 mrad divergence. Samples were placed over an X–Y–Z manual micro-metric positionator with a 0.5 μm stage of travel at every coordinate to ensure that each laser pulse impinged on a fresh sample. The laser beam was focused onto the sample surface with a 100 mm focal-distance lens, producing a spot of 100 μm in diameter. The laser fluence was fixed to 20 J/cm^2 and the repetition rate was 1 Hz. Emission from the plasma was collected with a 4 mm aperture, and 7 mm focus fused silica collimator placed at 3 cm from the sample, and then focused into an optical fiber (1000 μm core diameter, 0.22 numerical aperture), coupled to a spectrometer. The spectrometer system was a user-configured miniature single-fiber system EPP2000, StellarNet (Tampa, FL, U.S.A.) with a CCD detector. A grating of 300 l/mm was selected; a spectral resolution of 0.5 nm was achieved with a 7 μm entrance slit. The wavelength range used was from 200 to 1000 nm. Therefore, 2048 data points were recorded for each sample. The detector integration time was set to 1 ms. In order to prevent the detection of bremsstrahlung, the detector was triggered with a 5 μs delay time between the laser pulse and the acquired plasma radiation using a digital delay generator (Stanford model DG535). The spectrometer was computer-controlled using an interface developed with Matlab, which allowed for data processing and real-time analysis.

2.2. Bacterial samples

A total of 40 strains of different bacterial species i.e. *E. coli* (Ec) [25], *Pseudomonas aeruginosa* (Pa) [26], *Klebsiella pneumoniae* (Kp) [27], *Salmonella typhimurium* (St) [28], *Salmonella pullorum* (Sp) [28] and *Salmonella salamae* (Ss) [28] were included in the study. Kp, Ec and Pa strains showed multidrug antibiotic resistance and multiple genes mutations (Table 1), whereas St, Sp and Ss were resistant to kanamycin and differed in only one gene (Table 2). Two bacterial strains of the *Salmonella* species (strains 2 and 3 of Table 2) were constructed by inactivation by directed mutagenesis of specific gene in the wild type background (strains 1 of Table 2). All the bacterial strains were cultivated in LB agar (Difco Microbiology, Lawrence, KS, U.S.A) at 37 °C for 12 h in three Petri dishes (8.9 cm in diameter).

Sample ID has been represented in XYZ format, where X is the genus, Y the bacterial species and Z the type of strain. Thus, for example, Kp1 refers to the first strain of *K. pneumoniae* (K21P).

2.3. LIBS measurements and spectral libraries

Bacterial samples were measured directly in the Petri dish at room experimental conditions. The water content of the bacterial samples was reduced by flowing air before LIBS spectra acquisition. For each bacterial strain four replicate Petri dishes were measured. Eighty single-laser-shot spectra from one Petri dish and twenty single-laser-shot spectra each from the remaining three Petri dishes were acquired. The acquisition time of these 140 spectra was approximately 2 min. Because the emission intensity signal may vary with laser pulse, spectra were normalized by the most intense emission line, i.e. Na(I), to avoid instrumental variations (Fig. 1). The 80 spectra from the first Petri dish were used to train the NN model for each bacterial strain (spectral training library), whereas the 20 spectra from the remaining three Petri dishes each were used to test the model (spectral test library). Although the matrix dataset was considerably large, the computation time for training each NN model was below 10 s.