Applicability of Raman Spectroscopy for Characterization of Three Major Foodborne Pathogens

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Received Date: 05.09.2016 **Accepted Date: 16.10.2016**

Abstract: Due to its fastness, low-cost, and non-destructive features, the Raman spectroscopy has been found as a useful method to detect and identify microorganisms. In the present paper, the Raman spectral fingerprints of three major foodborne pathogens, including *Escherichia coli*, *Salmonella* typhimurium and *Staphylococcus aureus* were compared for the possible usefulness of this method for identification. Bacterial cultures grown into tryptic soy broth were inactivated with formaldehyde solution, and then freeze dried for analysis. A Renishaw Raman spectrometer system equipped with DDC camera and a Leica microscope was used in this study. Visual examination of the spectra showed significant differences. For example, intensity of bands around 856 and 877 cm^{-1} was much stronger in the spectra of *S. aureus* when compared to Gram negative organisms. In addition, the band at 917 cm^{-1} was only present in the spectra of *S. typhimurium*. It can be said that the Raman spectroscopy provides a powerful tool for the identification of foodborne pathogens at genus level, yet more research needs to be done to standardize the method for correct identification at species level.
Keywords: *E. coli, Foodborne pathogens, Raman spectroscopy, S. aureus, S. typhimurium*

Raman Spektroskopisinin Üç Önemli Gıda Kaynaklı Patojenin Karakterizasyonunda Uygulanabilirliği

Özet: Hızlı, düşük maliyetli ve örnek üzerinde tahribat yapmayan özelliklerinden dolayı Raman spektroskopisi mikroorganizmaların saptanmasında ve tanımlanmasında son zamanlarda yararlı bir metot olarak kullanılabileceği bildirilmiştir. Bu çalışmada, üç adet çok önemli gıda kaynaklı patojen olan *Escherichia coli*, *Salmonella* Typhimurium ve *Staphylococcus aureus*'un Raman spectral parmak izleri karşılaştırılarak bu metodun tanımlamada kullanılabilirliği araştırılmıştır. Tryptic soy broth içerisinde üretilen bakteri kültürleri formaldehit kullanılarak inaktive edildiler ve analize uygun hale getirmek için kurutuldular. DDC kamera ve Leica mikroskop cihazlarına sahip olan Renishaw marka Raman spektrometresi bu çalışmada kullanılmıştır. Elde edilen spektrumların görsel olarak yapılan incelemelerinde farklılıklar bulunmuştur. Örneğin, 856 ve 877 cm^{-1} bölgelerinde ortaya çıkan bantların yoğunluğu Gram negatif mikroorganizmalar ile kıyaslandığında *S. aureus*'dan elde edilen spektrumlarda daha fazla bulunmuştur. Buna ilaveten, 917 cm^{-1} bölgesinde elde edilen bant sadece *S.* Typhimurium'da bulunmuştur. Raman spektroskopisinin gıda kaynaklı patojenlerin genus seviyesinde tanımlanmasında güçlü bir araç olduğu söylenebilir, fakat metodun tür düzeyinde tanımlama yapabilmesi için standardizasyon çalışmalarına ihtiyaç duyulmaktadır.
Anahtar Kelimeler: *Gıda kaynaklı patojenler, Raman spektroskopisi, E. coli, S. aureus, S. typhimurium*

Introduction

Foodborne pathogens have been long recognized as one of the most severe public health problems worldwide. According to a previous report from Centers for Disease Control and Prevention (CDC), foodborne pathogens have been reported to be responsible for 48 million cases of human illnesses and about 5000 deaths in the United States annually (Scallan et al., 2011). It is crucial to assess the presence, types and abundance of microorganisms in foods because of not only threatening the health of the humans, but also play a role in the spoilage of foods (Adams and Moss, 2008). The sensitive, selective and rapid detection and identification of microorganisms in foods has therefore become an important but also challenging task for food microbiology laboratories (Velusamy et al., 2010). Up to now, there have been many methods utilized for this approach including but not limited to conventional culture, immunological and molecular genetic methods. Among these, standard culture following phenotypic characterization based methods, which can be carried out using either commercial kits or semi/fully automated systems, is the most commonly used reference method in most food microbiology laboratories (Castro-Escarpulli et al., 2015). Even though this method is easy to perform and highly sensitive, it is labor-intensive and usually requires long analysis time about 48 hours or sometimes several days for fastidious organisms or slow growing microorganisms like *Brucella* spp. (Al-Khaldi and Mossoba, 2004).

Nucleic acid based methods such as PCR amplification, DNA hybridization for bacterial detection and identification on the other hand attracted increasing attentions and are being supplanted by rapid, highly sensitive and specific

molecular genetic methods for the last decade (Gasanov et al., 2005; Sanchez, 2006). However, despite the some advantages in particular fast turnaround time, molecular analysis require tedious sample pretreatments, expensive equipment and reagents, and as well as rich technical expertise (Carbonnella et al., 2011). In addition to these shortcomings, the results might be hindered by the interference such as PCR inhibitors which can lead to false negative results (Lee and Fairchild, 2006). Moreover, matrix-assisted laser desorption time-of-flight/ionization mass spectrometry (MALDI-TOF/MS) is an emerging tool that has already found utility for the analysis of a variety of biological samples including detection and identification of bacteria (Guo et al., 2014; Wieser et al., 2012). Despite the high sensitivity and speed of analysis, several requirements of MALTI-TOF/MS such as expensive cost obviously hamper its application in routine analysis (Wieser et al., 2012).

As current detection methods do not fully address the challenges for bacterial analysis, it is therefore highly desirable to develop methods with some advantages as low cost, high sensitivity and less analysis procedure. The use of vibrational spectroscopy methods e.g., Raman and infrared spectroscopy (IR) have become one of the most widely applied techniques in order to provide intrinsic information regarding the molecular structure of the cells (Li et al., 2012). With the rapid developments and advancements and as well as some obvious advantages like simple sample handling, being non-destructive and obtaining results in seconds, the Raman spectroscopy is now widely used tools in numerous applications in biological purposes including identification of unknown molecular structures and tissue imaging, surface analysis at *in vitro* and *in vivo* level (Baena and Lendl, 2004). The potential usefulness of Raman spectroscopy for identification of bacteria yeast and fungi have been clearly demonstrated (Çulha et al., 2010; Meisel et al., 2012). Although the investigation of bacterial cells by using Raman spectroscopy has been carried on for almost two decades by some researchers, there is a need to collect more spectral data regarding the major foodborne pathogens before the establishment of this method as a routine technique for microbiological diagnosis. This study was aimed to build on the existing knowledge by analyzing and comparing the Raman spectral fingerprints of important foodborne pathogens, namely *Escherichia coli*, *Salmonella* typhimurium and *Staphylococcus aureus*.

Material and Methods

Bacterial species and preparation: The bacterial species selected for this experiment are *E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028 and *S. aureus* ATCC 25923. All bacterial species were cultured on tryptic soy agar at 37 °C for 24 h. Overnight culture of all bacterial species were then used to inoculate 100 ml of tryptic soy broth and incubated at 37 °C with shaking at 150 rpm for 24 hours. Independently prepared sets (n=3) of each bacterial isolates were used as a replicate through this experiment. The bacterial cultures were then inactivated as described previously (Meisel et al., 2012) with some modifications recommended by Liu et al., (2009). Briefly, formaldehyde solution (37%) was added into bacterial cultures to obtain a final concentration of 10% formaldehyde. The mixture was mixed continuously and spun down at 5000 x *g* for 10 min and then supernatant was discarded. The resulting pellet was washed with ultrapure water three times. In order to dehydrate bacterial cells, ethanol solutions at different concentrations (10%, 30%, 50%, 70%, 90% and 100%) were applied and cells were harvested by centrifugation (5000 x *g* for 10 min). After these treatments, the bacterial pellets were dried by using freeze dryer (Telstar, LyoQuest -85).

Instrumental analysis: The Raman spectra were recorded on bacterial samples using Renishaw in Via mapping microRaman spectrometer outfitted with a Leica microscope and equipped with a CCD detector. A 50x objective was used to focus on the sample. For excitation of samples, 1-s integration time was used at laser lines of 785 nm edge (50% laser power). For this study, a mobile diffraction grating of 1200 line/mm was used and the measured Raman spectral range was between 101 and 4001 wavenumbers/cm^{-1} with the central wavenumber at 1111 cm^{-1}. Spectra were processed using Wire 4.1 software of Renishaw. The spectral areas of each sample were normalized and baseline correction was applied to all spectrums.

Results and Discussion

Accurate and rapid detection and identification of microorganisms is fundamental role of the clinical and food microbiology laboratories. However, the available methods for this purpose are either time consuming, costly or laborious. The Raman vibrational spectroscopy has had extensive

use in biological research in many different fields, which provided an excellent means for fingerprint of the chemical composition of a variety of biological samples at molecular level (Baena and Lendl, 2004; Li et al., 2012). In the current study, the obtained spectrums from the Raman spectroscopy were compared for the characterization of three foodborne pathogenic microorganisms.

The RAMAN spectrum in the range of 500-3100 cm for bacterial species is shown in the Figure 1 and 2. Figure 1 provides a direct comparison of the spectra from three replicate cultures of the same *E. coli* strain. As seen in Figure 1, Raman peaks of *E. coli* obtained from three replicate cultures are highly consistent. In addition, the spectra from replicate samples of *S. aureus* and *S.* typhimurium also showed identical spectrum (data not given). These findings are in good agreement with previous reports, in which strong correlation for vibrational spectra of *E. coli* was found between different studies (Meisel et al., 2014; Chan et al., 2007; Liu et al., 2009; Schuster et al., 2000). In the current study, the main bands at 723, 781, 827, 1004, 1033, 1092, 1126, 1330, 1452, 1582, 1666, 2888 and 2934 cm^{-1}, that were obtained from the all bacterial species tested, can be ascribed to adenine, cytosine or uracil, tyrosine, phenylalanine, carbohydrates (C-C deformation), DNA ($O\text{-}P\text{-}O^-$) stretching, C-N stretching, adenine or guanine, lipids ($C\text{-}H_2$ deformation), adenine or guanine, amide I, lipids and fatty acids, and protein and lipids, respectively (Liu et al., 2009; Meisel et al., 2014). However, it was noted that measured spectral fingerprints from the same bacterial species can be altered by a variety of factors such as growth culture conditions and incubation time etc (Chan et al., 2007; Maquelin et al., 2002). Despite the fact that vibrational variations can occur from the same bacterial sample, Maquelin et al., (2000) shown that single bacterial cell has been used in construction of spectra of Raman spectroscopy indicating that this method can provide an excellent means for bacterial detection. Moreover, Raman spectroscopy has also been reported to provide unique insights into physiological status of bacterial culture in the measurement of chemical composition changes in response to environmental influences (Chan et al., 2007; Schuster et al., 2000). Liu et al., (2009) has also employed the Raman spectroscopy to analyze antimicrobial mechanisms of essential oils towards *E. coli cells.*

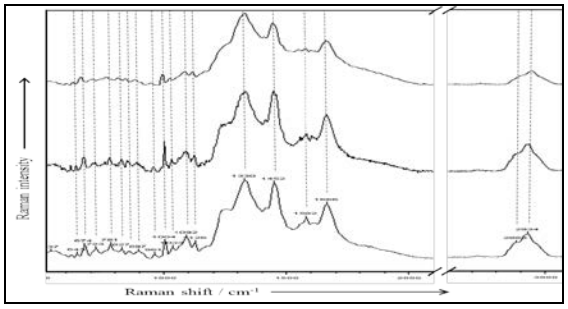

Figure 1. Raman spectral graphs of *E. coli* (3 replicates) analyzed on different days. Main signals are positioned (Raman shift /cm^{-1}) and matched for each graphs with dashed vertical lines.

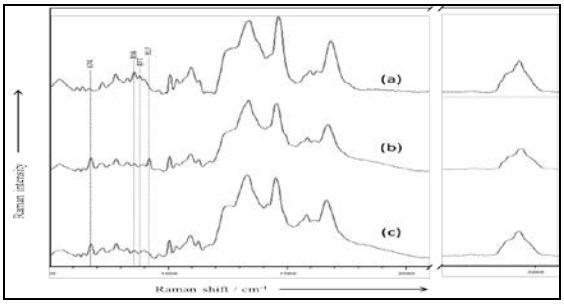

Figure 2. Comparison of Raman spectra obtained from three bacterial species (a; *S. aureus*, b; *S. Typhimurium* and c; *E. coli*).

Based on the results obtained in the current study, *E. coli* share many Raman peaks in common with other investigated species. In Figure 2 it can be seen that *S. aureus* however possesses an enhanced band at 856 cm^{-1} assigned to tyrosine previously (Liu et al., 2009) when compared to spectrum of these obtained in Gram negative organisms. This was also observed by Maquelin et al., (2000), who however reported that *Staphylococcus* strains could be separated by predominantly strong band at 780 cm^{-1}, which did not appear in the current measurement. Interestingly, one peak at 877 cm^{-1} assigned to carbohydrates (He et al., 2011) was clearly appeared in the spectrum of *S. aureus* showing a clear separation from Gram negative organisms, as it was not present in the spectrum of *E. coli* and *S.* typhimurium. In addition, the intensity of one peak at 674 cm^{-1} corresponding to C-S stretching (Maquelin et al., 2002) was higher in *E. coli* and *S.* typhimurium when compared to *S. aureus*. Moreover, *S.* typhimurium spectra were clearly differentiated with the band at 917 cm^{-1}, which was not observed in either *E. coli* or *S. aureus* spectrum. In view of these results, Raman spectroscopy was found to be of value to in discrimination of foodborne pathogens that was reported by several investigators for bacteria, fungi and yeast (Çulha et al., 2010; Meisel et al., 2012). For instance, in a recent study of 20 strains of bacteria from five species including *Brucella* spp.,

the Raman spectroscopy was shown to produce results with overall 92.9% accuracy in short time to discriminate strains at species level (Meisel et al., 2012). Authors also verified the ability of RAMAN for direct detection of *Brucella* species from milk samples indicating a great potential of this method for practical application in real food model (Meisel et al., 2012). Similar findings were also reported from the studies of Çulha et al., (2010), who have applied the surface enhanced Raman scattering (SERS) method to differentiate a number of bacterial species including *E. coli*, *Shigella sonnei*, *Erwinia amylovora* and *Proteus vulgaris.* The authors reported that silver colloidal nanoparticles increased the vibrational intensities enabling to detect bacteria of interest at the single cell level (Çulha et al., 2010). In addition to bacterial cells, the SERS method has also been shown to be effective in identifying a number of yeasts such as *Hyphopichia burtonii*, *Candida parapsilosis* and *Filobasidiella neoformans* (Çulha et al., 2010). However, one of the major problems in using Raman spectroscopy is to obtain highly homogenous bands from different species of bacteria (Maquelin et al., 2002; Meisel et al., 2012).

In conclusion, the results of the current study demonstrated that there are some obvious differences between the spectra of foodborne pathogens tested, which might provide a novel approach in order to detect and identify major foodborne pathogens as well as clinically relevance bacterial species. On the other hand, it is well-known fact that bacteria evolves over time in terms of their genotypic and phenotypic characteristics, which definitely complicates their discrimination by Raman spectroscopy. The current study was carried out under controlled conditions such as adjusted timing and temperature and using a standard growth medium as well and different spectral fingerprints form even the same bacterial species can be obtained if the bacteria of interest is grown under different conditions or isolated from the real food matrix. Hence, much work needs to be done in order to address the some major drawbacks such as low discriminatory power at species level and a standard sample preparation for routine analysis.

Acknowledgement

I am thankful to Dr. Ersen Göktürk (Mustafa Kemal University) for his technical help to operate Raman spectroscopy and also would like to thank Dr. Muharrem Akcan (Dumlupınar University) for his critical comments to improve the manuscript.

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