Ultra Wide Band Based Quantitative and Qualitative Method for Bacterial Endotoxin Detection

Muhammad Elsayeh, Ahmed H. Kandil
Systems and Biomedical Engineering Department, Faculty of Engineering, Cairo University, Cairo University Road, Giza, Egypt

Received: December 11, 2014
Accepted: February 16, 2015

ABSTRACT
The existence of quick and accurate identification and detection methods for bacteria and bacterial endotoxin plays an important role in delivering high quality biomedical products. Healthcare institutions (Medical and Pharmaceutical) realized the importance of a quality control system to ensure the absence of pathogens and pyrogens in the medical products and equipment. The quality control systems used to identify and detect the bacteria and bacterial endotoxins lack speed or accuracy. This work presents a method that uses electromagnetic waves in the Ultra Wide Band (UWB) region of the microwave spectrum to detect and identify bacteria and bacterial endotoxins. The developed method is based on the properties of interaction between organic materials and electromagnetic waves. The interaction is measured quantitatively and qualitatively. The scattered parameters of sample networks are measured and cepstrum coefficients are estimated for the analysis of the scattered parameters signals' energies. Experimental results proved effective identification and detection of bacterial endotoxin even with concentrations as low as 0.0003 EU/ml; the developed method can be extended to detect and identify the presence of different bacteria.

KEYWORDS: Rapid Microbial detection; rapid pyrogen detection; microwave spectroscopy; dielectric spectroscopy; Ultra Wide Band; Cepstrum Analysis.

INTRODUCTION
Endotoxaemia results from releasing large amounts of endotoxins into the blood stream. It may cause a septic shock and circulatory failure. Endotoxaemia and sepsis are the leading cause of death in surgeries [Eisele et al., 1998; Esteban et al., 2013]. It may be an indicator for a poor quality system in pharmaceutical industry of intravenous injection product [FDA, 2012; Daneshian et al., 2006]. Endotoxins are Lipopolysaccharide (LPS) pyrogens produced by negative gram bacteria outer cell walls [Rietschel et al., 1994]. According to quality control guidelines and patient safety require, endotoxins detection as an essential test for intravenous instruments and /or product’s pyrogen free verifications [FDA, 2012].

There are several endotoxins’ assays such as: Thiobarbituric acid -assay, Rabbit Pyrogen Test(RPT), Human blood test, Endotoxin Activity Assay and the well-known (gel clotting or photometric) Limulus Amebocyte Lysate (LAL) assay [Eisele et al., 1998; Bui et al., 2011]. Different methods and instrumentation, used in detection of endotoxin, already exist such as Capillary electrophoresis, Laser Induced Fluorescence (CE-LIF), Gas chromatography -Mass
Spectroscopy (GC-MS), Matrix - Assisted Laser Desorption Ionization - Time of Flight Mass spectroscopy (MALDI-TOF-MS), Ion trap mass spectroscopy and Fourier Transform Ion Cyclotron Resonance Mass Spectroscopy [Bui et al., 2008; Bui et al., 2011; Dörnyei et al., 2011]. However, since using the mentioned instrumental methods are very artful and complicated methods, as they need samples’ preparations and derivatization as preparatory steps for the instrument to detect the LPS. As a result, these methods are time consuming. Moreover, they need complex procedures to ensure accurate measurements and have expensive implementations. The main objective of this research is to establish an alternative, accurate method for rapid endotoxin detection and quantification with low running cost.

Since the materials containing charged particles produce secondary fields when they come in contact with electric or magnetic fields. They result in conduction, polarization, or magnetization of the particles in that material. Polarization of the particles forces the material to act as a dielectric [Rao, 1987]. The permittivity is determined by the dielectric material’s ability to polarize its particles under the influence of an electric field [Mijovic and Fitz, 1998].

The polarization effect or dielectric mechanism is related to the material properties and the interaction of the electromagnetic waves, especially electric field components of the wave.

The vibrational mode of the molecule depends on the frequency of the applied field. The vibrational motion of the molecules induce a displacement in molecule equilibrium position, called electric displacement and can be expressed by Equation (1)

\[ D(\omega) = \varepsilon_0 E(\omega) + P \]  

(1)

Where, \( \omega \) is the angular frequency \( 2\pi f \), \( E(\omega) \) is the electric field of the microwave relative to oscillation frequency, \( P \) is the Polarization density corresponding to dipole moment density is expressed by Equation (2)

\[ P = \alpha E \]  

(2)

So the displacement occurring is related to the applied electric filed which induced by the dipole moment, where \( \alpha \) is the molecular polarizability which is a material property that depend on the material structure and bond nature. The polarizability has a direct link with

<table>
<thead>
<tr>
<th>Technology / Method</th>
<th>Special Sample preparation needed</th>
<th>Estimated Process time</th>
<th>Accuracy</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAL assay (Gel clot)</td>
<td>Yes (Vortex, serial dilution, incubation... etc.)</td>
<td>3 hrs</td>
<td>&lt; 0.01 Eu/ml</td>
<td>False positive and false negative results due to LAL reagent interaction with other compounds i.e. (β↑D-glucans) from fungus or cellulose derivatives</td>
</tr>
<tr>
<td>LAL assay kinetic chromogenic</td>
<td>Yes (Vortex, incubation, etc.)</td>
<td>1-5 hrs</td>
<td>&lt;0.005 Eu/ml</td>
<td>False positive due to LAL reagent interaction with other compounds i.e. (β↑D-glucans) from fungus or cellulose derivatives</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Yes Sample derivatization</td>
<td>18-20 hrs</td>
<td>10^{-16}-10^{-17} mol</td>
<td>Time consuming and very expensive</td>
</tr>
<tr>
<td>CE-LIF</td>
<td>Yes Sample derivatization</td>
<td>1-5 hrs</td>
<td>10^{-15}-10^{-17} mol</td>
<td>Time consuming and very expensive</td>
</tr>
<tr>
<td>MALDI-TOF-MS</td>
<td></td>
<td>4-8 hrs</td>
<td>10^{-16}-10^{-17} mol</td>
<td>Time consuming and very expensive</td>
</tr>
</tbody>
</table>
material dielectric properties as expressed in Equations (3) and (4)

\[
\alpha = \chi_e \varepsilon_0
\]  

(3)

Where \( \chi_e \) is the electric susceptibility of the material and can be expressed as Equation (4)

\[
\chi_e = \varepsilon^* (\omega) - 1
\]  

(4)

Where, \( \varepsilon^* (\omega) \) is the complex permittivity relative to each frequency variation.

According to Equations (3) and (4), \( \alpha \) can be expressed as follow in Equation (5)

\[
\alpha = (\varepsilon^* (\omega)^{-1}) \varepsilon_0
\]  

(5)

By substituting in Equation (2) using Equation (5), we can express the dipole moment in terms of complex permittivity and electric field relative to frequency variation in Equation (6)

\[
P(\omega) = (\varepsilon^* (\omega)^{-1}) \varepsilon_0 E(\omega)
\]  

(6)

Dielectric spectroscopy depends on the dielectric mechanism which can divided into relaxation and resonance processes [Afsar et al., 1986; Baker-Jarvis et al., 1993]. Dielectric spectroscopy depends on the measurements of Scattering parameters (S-Parameters) using Vector Network Analyzer (VNA), this is followed by calculating the relative permittivity for each frequency [Afsar et al., 1986]. Moreover, The impedance is determined after converting S-parameters into Impedance parameters (Z-Parameters) [Eldosoky and Moustafa, 2011]. According to the Nicolson-Ross-Weir (NRW) mathematical model which is the most common model used to perform the calculation of complex permittivity and permeability of the material using S-parameter data [Nicolson and Ross, 1970], complex permittivity can be calculated using NRW model as in Equation (7).

\[
\varepsilon^*(\omega) = \sqrt{\frac{\frac{c}{\omega d} \ln(\frac{1}{1 + f_L})^2}{\frac{1 + f_L}{1 - f_L}}}
\]  

(7)

Where \( \Gamma \) is the reflection coefficient which is calculated by Equation (8) and Equation (9)

\[
\Gamma = K \pm \sqrt{K^2 - 1}
\]  

(8)

\[
K = \frac{S_{12}^2(\omega)+S_{11}(\omega)+S_{12}(\omega)}{2S_{11}(\omega)}
\]  

(9)

\( T \) is the transmission coefficient which is expressed in Equation (10)

\[
T = \frac{S_{11}(\omega)+S_{12}(\omega)-\Gamma}{1-S_{11}(\omega)+S_{12}(\omega)\Gamma}
\]  

(10)

We can conclude that the dipole polarization is highly dependent on the dielectric properties of the material, and the Complex permittivity changes according to the applied microwave’s frequencies[Bottcher et al., 1970]. Hence, the scattering parameters values change according to material interaction with the microwave.

MATERIALS AND METHODS

In this research, we used Ultra wide Band (UWB) frequencies with sweeping step of 10 MHz in the microwave spectrum to cover the \( \beta \) and \( \gamma \)-dispersion frequencies [Schwan and Takashima, 1993; Pethig, 1979]. The developed method depends on measuring the energy loss due to the presence of endotoxin inside the samples’ medium. This loss is defined as the endotoxin’s signature.

Experiment Setup

As shown in Figure 1a the experiment setup, Consists of a cuboidal housing, two UWB microstrip patch antennas and sample tube. The Antennas are connected to Rohde & Schwarz VNA (model: R&SZVA110) via two Sub Miniature version A (SMA) connectors for two port network analysis. The antennas fabrication are based on the design configuration introduced by [Zasowski et al., 2006] as shown in Figure 1b. The antennas have a dimension of (2.2 x 2.0 cm²), each antenna consists of a rectangular
patch with two steps, a single patch window slot of dimension \((1.5 \times 0.05 \text{ cm}^2)\) and a partial ground plane of dimension \((4.0 \times 0.9 \text{ cm}^2)\) and 50 \(\Omega\) feeding microstrip line. The antenna is fabricated on FR4 substrate with thickness of 1.6 mm and dimension of \((4.0 \times 4.0 \text{ cm}^2)\).

**Samples preparations and measurments**

The reference sample was prepared from LAL Reagent water which is endotoxin free distilled water. Serial dilutions of different concentrations were prepared by a control spike which is a lipopolysaccharide from *E. coli* 055:B5 as certified by Charles River Endosafe®. The samples are divided into standard samples and testing samples as shown in Table II. For The bacteria culturing and preparation, we used Nutrient broth and agar slants to prepare lyophilized cultures of *(Kocuria rhizophila (Micrococcus luteus) ATCC 9341, Pseudomonas aeruginosa ATCC 9027, Staphylococcus aureus ATCC 6538P, Salmonella typhimurium ATCC 14028 and Staphylococcus haemolyticus ATCC 29970D-5.* The S-Parameters \(S_{11}\) and \(S_{12}\) are collected for each sample. The parameters of interest, such as \(S_{11}\), indicates the energy loss from scattering and reflection (return loss). \(S_{12}\) indicates (Transmission loss). The antennas are placed in a symmetrical pattern relative to each other. The antennas position are placed to operate in the radiative Fresnel near field region. The signals are measured in dB and represents Power Spectrum of the network across different frequencies from 2 GHz to 10 GHz. The power spectrum can be expressed as in Equations (11) and (12)

\[
S_{11} = -20 \log_{10} \frac{V_{\text{Reflection}}}{V_{\text{Incident}}} \tag{11}
\]

\[
S_{12} = -20 \log_{10} \frac{V_{\text{Transmission}}}{V_{\text{Incident}}} \tag{12}
\]

Where \(V_{\text{Reflection}}\) is the measured reflection voltage, \(V_{\text{Transmission}}\) is the measured transmission voltage and \(V_{\text{Incident}}\) is the initial incident voltage.

**Detection Algorithm**

The algorithm in detection and quantification depends on the Cepstrum Analysis for both \(S_{11}\) and \(S_{12}\) signals. Cepstrum Analysis Algorithm is a well-known algorithm in speech recognition applications and analysis of radar signal returns applications [Noll, 1967]. The idea of this algorithm is

<table>
<thead>
<tr>
<th>Standard samples</th>
<th>Concentration EU/ml</th>
<th>Testing Samples</th>
<th>Concentration EU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0003</td>
<td>1</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.03</td>
<td>2</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.125</td>
<td>3</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>6</td>
<td>10000</td>
</tr>
<tr>
<td>7</td>
<td>100000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
to find the signature pattern of endotoxin existence in a medium. This pattern is such a mean of energy variation for each frequency. By converting the captured power spectrum of $S_{11}$ and $S_{12}$ using the inverse Fourier transform of the power spectrum as in Equation (13), we obtain the signal Cepstrum form, so we can define the Cepstrum as the inverse Fourier transform of the log-magnitude Fourier Spectrum [Proakis and Manolakis, 2007]

$$C_{S_{11},S_{12}} (Q) = F^{-1} \{S_{11} (f), S_{12} (f)\}$$  \hspace{1cm} (13)

Where $F^{-1}$ is the inverse of fast fourier transform, $f$ is the frequency and $Q$ is the quefrecy which is independent variable measures of time of the cepstral graph.

After applying time gating liftering (filter in frequency domain) the signal energy for each band lifter is calculate by as Equation (14).

$$E_{[t]} = \frac{bw}{S} \sum_{j=1}^{S} |C_{S_{11},S_{12}} (Q_j)|^2$$ \hspace{1cm} (14)

Where $bw$ is the number of the bands in the quefrecy range and can be calculated by equation

$$bw \sim \frac{\text{total no.of samples}}{S}$$ \hspace{1cm} (15)

$S$ is the window width which can be calculated as follows

$$S = \frac{\text{time gate}}{\text{initial sampling time}}$$ \hspace{1cm} (16)

Where initial sampling time $0.1 \mu$ sec (10 MHz) and the time gate is 1 nsec (1 Ghz).

$w(n)$ is a rectangle windowed lifter with window width of $0.0125 \mu$ sec (80 MHz) and can be expressed by Equation (17)

$$w(n) = \begin{cases} 1 & n \leq 8 \\ 0 & n > 8 \end{cases}$$ \hspace{1cm} (17)

The Total Signal Energy Expansion (TSEE) can be calculated as follow

$$TSEE = \frac{\sum_{k=1}^{bw} |E_k[C_{S_{11}} (Q)] + E_k[C_{S_{12}} (Q)]|^2}{\sum_{k=1}^{bw} |E_k[C_{REF_{S_{11}} (Q)}] + E_k[C_{REF_{S_{12}} (Q)}]|^2}$$

The calculated TSEE is Normalized across the reference sample signal energy expansion. The Concentration prediction model will use the calculated TSEE in recognizing the unknown test samples. The model is polynomial model of sixth degree or knows; its coefficients are solved using the curve fitting for the standard sample TSEE values. The predicated output will be compared with actual concentration of the test samples and the error will be calculated to ensure model accuracy.

Results and Discussion

Figures 2 and 3 show the power spectrum of $S_{11}$, $S_{12}$ signal for the lowest (0.0003 EU/ml) and highest endotoxin concentration against reference pyrogen free distilled water.

Figure 2. $S_{11}$ power spectrum for lowest and highest endotoxin concentration against reference pyrogen free distilled water.

Figure 3. $S_{12}$ power spectrum for lowest and highest endotoxin concentration against reference pyrogen free distilled water.
concentration (100,000 EU/ml) against the reference sample. Figures 4 and 5 are the cepstrum transform of $S_{11}$, $S_{12}$ signal for endotoxins concentrations and reference sample. The lowest quefrequencies represent the highest frequencies and vice versa. The peaks occur in the cepstrum because the harmonics in the spectrum are periodic, and the period corresponds to certain signature. As shown in the figures, the difference between each signal for each frequency indicates the endotoxins concentrations

![Cepstrum Transform of S11](image)

**Figure 4.** Cepstrum transformation of $S_{11}$ for lowest and highest endotoxin concentration against reference pyrogen free distilled water.

![Cepstrum Transform of S12](image)

**Figure 5.** Cepstrum transformation of $S_{12}$ for lowest and highest endotoxin concentration against reference pyrogen free distilled water.

Effect can be determined. The endotoxin material shows specific response in a certain frequency bands (4 - 5 GHz) of $S_{11}$. The signal's wave form and amplitude changes according to the endotoxin concentrations as shown in Figure 7. Figure 6 shows the estimated TSEE against the endotoxins concentrations. In Table III describe the prediction model output against the actual test samples concentration. This error could be minimized if the standard curves
cover more concentrations, which make it a calibration curve dependent method.

Figure 8 shows the difference between two negative gram bacteria (Pseudomonas aeruginosa and salmonella typhimurium) in distilled water with an endotoxin concentration < 0.25 EU/ml the difference between two positive gram (staphylococcus haemolyticus and micrococcus luteus) bacteria also studied to show the ability of the technique in detection and differentiation of bacteria as shown in Figure 9.

This method can be used in detecting other particles and materials. An experiment is conducted to differentiate between Staphylococcus aerus bacterial exotoxin exopolysaccharide (EPS) and E.Coli bacterial endotoxin (LPS) which has the relative similarity in chemical and molecular structure [Abraham et al., 2009]. Figure 12 shows the relative similarity between the highest and lowest concentration of LPS $S_{11}$ and Staphylococcus aerus EPS $S_{11}$ in which the signals have a slight difference in wave form and amplitude, hence this trail proof that the method can detect and differentiate endotoxin and exotoxin and showed how sensitive is this method. This method proves and shows the commonalities between EPS and LPS. In addition this experiment shows

<table>
<thead>
<tr>
<th>S/N</th>
<th>Test sample Concentrations in EU/ml</th>
<th>Predicted concentration in EU/ml</th>
<th>Absolute Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.003</td>
<td>0.0057</td>
<td>0.0027</td>
</tr>
<tr>
<td>2</td>
<td>0.06</td>
<td>0.0806</td>
<td>0.0206</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>0.0359</td>
<td>0.2141</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>8.3968</td>
<td>5.8968</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>192.2764</td>
<td>92.2764</td>
</tr>
<tr>
<td>6</td>
<td>10000</td>
<td>9471.2</td>
<td>528.7720</td>
</tr>
</tbody>
</table>

Table III. Perdiction output against test sample actual concentration
Figure 11. $S_{11}$ signal for gamma radiated Poly ethylene glycol powder with gram negative Pseudomonas aeruginosa.

Figure 12. A- $S_{11}$ power spectrum Lowest endotoxin conc. and Staphylococcus aeurus EPS against Reference (Pyrogen free Distilled water) B- $S_{11}$ power spectrum highest endotoxin conc. and Staphylococcus aeurus EPS against Reference (Pyrogen free Distilled water).

Figure 13. Calculated Signal Energy of $S_{11}$ Cepstrum for different endotoxin concentration at time gate = 0.0125 $\mu$sec and rectangle window width of one sample (0.1 $\mu$sec).
the feasibility of using this technique to differentiate between positive and negative gram Bacteria as shown in Figures 8 and 9.

However, $S_{11}$ shows a certain endotoxin response in frequency domain also can be shown using the computed signal energy of the cepstrum transformed signal using different values of time gate lifting and rectangle windowed filter as shown in Figures 13 and 14.

Figure 15 shows a certain response for distilled water natural occurrence endotoxin (NOE) with concentration of (less than 0.25 EU/ml). The NOE is mixed with different bacteria species to see if there is any masking effect for endotoxin due to the presence of the mixture.

CONCLUSION

A novel technique for bacterial endotoxin detection and quantification is developed. It shows good sensitivity in LPS detection; however, its quantification accuracy is dependent on developing calibration procedure to provide more accurate measurements. It is a low cost, simple and rapid method introduced in microbial and pyrogen detection applications. This method will be expanded to differentiate, identify and classify bacteria species in our future works.

REFERENCES


components by APTS”. Journal of Biochemical and Biophysical Methods, 70 (6), pp. 1313-1316.


Eldosoky Mohamed A. A., Moustafa and Haytham M. (2011) “Detection of the blood leukemia by using the ultra wide band pulses”. General assembly and scientific symposium, XXX URSI.


Muhammad Elsayeh and Ahmed H. Kandil,  Ultra Wide Band Based Quantitative and Qualitative Method for...