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TITLE

FTIR spectroscopy of chronic venous leg ulcer exudates: an approach to spectral healing marker identification.

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Keywords: Chronic venous leg ulcers, FTIR spectroscopy, Wound exudate.

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ABSTRACT

Chronic venous leg ulcer (CVLU) arises as a chronic venous insufficiency complication and is a major cause of morbidity throughout the world. Our hypothesis is that CVLU exudate composition is a biochemical representation of wound clinical state. Then, Fourier Transform Infrared (FTIR) spectroscopy could be a useful and less-invasive technique to study the clinical state of the ulcer. For this, the aim of this work was to perform a spectral characterization of exudate from CVLU using FTIR spectroscopy to identify potential healing markers.

45 exudate samples from CVLU, 95% of the strains isolated from CVLU in planktonic and biofilm phenotypes and other related biological samples as human plasma, serum, urine, blood cells, urea, creatinine, glucose and albumin were studied by FTIR spectroscopy. According to vibration frequency of biomolecules (lipids, protein, nucleic acids and carbohydrates) characteristic bonds at infrared region, different spectral windows were selected and spectral areas of each window were measured. Besides, Savitzky-Golay second derivative were obtained for all spectra and peaks from each standardized window were detected.

FTIR spectroscopy allowed to identify sample types (exudate, plasma, serum, urine) as each one present a unique relative composition and ratios ranges. Also, this technique could be useful to identify bacteria at phenotypic-ulcer state and allows to differentiate if bacteria are in biofilm or planktonic form which is unlikely by conventional methods.

In this work we found some spectral markers (areas, peaks) that allow to identify several parameters in exudate as: a) Total cellularity, b) Inflammatory cells load, c) Bacterial load, d) Fibrin amount, e) Inflammatory proteins. Because the measured areas or founded peaks are concentration-dependent this method could also serve to measure them.

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Therefore, FTIR spectroscopy could be useful to evaluate patient evolution as all these exudate parameters represent critical negative markers for wound healing.

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INTRODUCTION

Fourier Transform Infrared (FTIR) spectroscopy is being increasingly used in biomedical applications with high degrees of success[1-6]. Molecular bonds with an electric dipole moment that can change by atomic displacement owing to natural vibrations are IR active[7]. These vibrational modes are quantitatively measurable by IR spectroscopy, providing a unique, label-free tool for studying molecular composition and dynamics without perturbing the sample[7]. FT-IR spectroscopy is a non-destructive method for the analysis of cells, tissue and fluids[1-8]. However, there are not reported studies of wound fluid from Chronic Venous Leg Ulcers (CVLUs) by FT-IR spectroscopy.

CVLU arises as a chronic venous insufficiency complication and is a major cause of morbidity throughout the world[9-11], with an overall prevalence ranging up to 2% in the general population[13] and median ulcer durations that range from six - eight months to decades[13].

Several factors are involved on CVLU delayed healing process: venous insufficiency degree[14], infection[9,15], inflammatory molecules[9], etc. Due to all of these factors involved in the CVLUs development, the correct diagnostic, prognosis and its treatment are difficult, leading CVLUs to a long non-healing state[16]. Wound fluid or ulcer exudate may be used as a clinical state indicator, because its complex composition is reflex of the biochemical processes that occur on the wound bed and of its chronicity[17,18]. Exudate formation results from plasma ultrafiltrate as local inflammation consequence influenced by the wound healing process[19]. When the tissue is injured, inflammatory process begins along with the wound-healing process[20]. This promote the affluence of inflammatory cells, such as polymorphonuclears (PMN), lymphocytes, and macrophages that are key to the removal of contaminating microorganisms and infection[17,20]. Exudate has high viscosity and high protein

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amount (>30 g/L)[18] and several components from serum like glucose[18], urea[18,21], creatinine[18,21], lactate and salts[18,21]; tissue inflammatory molecules as cytokines, serine proteinase, cysteine proteinase, aspartic proteinase and matrix metalloproteinases (MMPs)[12,17,19]. Also, exudate contains bacteria and biofilm components as extracellular polysaccharide matrix (EPS)[22,23] and DNA[24]. Therefore, exudate might be considerate as a negative healing factor in chronic wounds because excessively proteolytic environment will continually degrade key growth promoting agents and thus will not allow normal wound healing to occur[17]. Chronic wound exudate has higher MMPs levels than acute exudate which produces tissue digestion[17]. There is a correlation between elevated levels of MMPs and delayed healing[12,25]. Also, MMP may cause inhibition of endothelial cell proliferation and angiogenesis[26]. Finally, exudate is a physical barrier for cell displacement in the re-epithelization process[26].

In summary, exudate from a chronic wound contains plasma components, inflammatory cells, proteins from the inflammatory response, bacteria and components from the bacterial biofilm matrix (**Figure 1**). Our hypothesis is that exudate composition is a biochemical representation of the clinical state of a chronic wound. Therefore, FTIR spectroscopy associated to other clinical parameters could be a useful technique that provides a less-invasive and simply way to represent the clinical state of the ulcer and that allows the identification of prognosis/diagnostic markers. For this, the aim of this work was to perform a spectral characterization of exudate from CVLU using FTIR spectroscopy to identify potential healing markers.

EXPERIMENTAL.

1. Ethics.

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This research protocol was approved and monitored by Independent Medic Ethic Committee from Argentinian Norwest (CIEM-NOA). Patients under observation signed an informed consent before being included to this protocol.

2. Patients

For the mentioned protocol, 45 patients from Dermatology Service of Nicolas Avellaneda Hospital (San Miguel de Tucuman, Tucuman – Argentina) were selected. Patients with CVLU were diagnosed by venous doppler and clinical criteria.

Inclusion criteria: a) Ulcer location: Lower-third of lower limbs. b) Both Sexes. c) Age: between 40 – 80 years. d) ulcer size: 20 to 150 cm^2. e) Ulcer evolution time: 1 to 3 years

Exclusion criteria: Patients with background of 1) systemic infection, 2) cancer and/or under chemotherapy treatment, 3) autoimmune disease, 4) drugs abuse, were excluded from this study.

As relevant clinical information, patient's clinical association pathologies and ulcer evolution time were analyzed from its clinical records.

3. Exudate samples

Exudate was obtained by gentle aspiration with a syringe (without needle and avoiding to produce pain and bleeding) from 6 different points of the CVLU and stored at -20ºC until processing.

4. Spectral contribution controls

In order to evaluate spectral contributions on exudate that come from plasma ultrafiltrate, the following lyophilized controls were used (Figure 1):

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Serum (n = 8): Obtained by whole blood extraction from random patients. Sera were left to clot for 15 minutes and then centrifuged for 10 min at 3000 rpm.

Plasma (n = 8): Obtained by whole blood extraction from random patients on sodium citrate 1.2 % w/v (ratio:9/1) and centrifuged 10 min at 3000 rpm.

Free-protein serum (n = 8): This control is useful to find if there are protein contributions on exudate spectra that not come from plasma, analyzing specifically at protein spectral regions. Polson *et al*. protocol was followed to obtain this free-protein serum[27]. A serum aliquot was separated and then treated with absolute ethanol 99.5% v/v (Cicarelli) (1/0.5), incubated at -20ºC during 12 h and then centrifuge at 8000 rpm for 20 minutes. This process was repeated twice to ensure serum deproteinization and corroborated with UV spectroscopy (200 – 400 nm)[28] and Bradford method[29].

Urine (n = 8): Urine is a plasma ultrafiltrate and for this reason could be a useful control for exudate study as a free-protein control (**Figure 1**). Urine samples were taken after day-first urine from 8 volunteer human subjects in order to reduce the amount of filtered proteins to the maximum. Urine samples come from 8 different human volunteers between 30 – 40 years old with no kidney disease history, hepatic disease or use of chronic medication to ensure the correct glomerular function. Free-cells urines were obtained by centrifugation at 3000 rpm for 10 min.

Blood cells control (n = 8): was used to analyze cellular contribution from inflammatory response (white blood cells - WBC) and bleeding (red blood cells - RBC) on exudate (**Figure 1**). To obtain this control, a whole blood anticoagulated (citrate 1.2% w/v) aliquot was separated and centrifuged at 3000 rpm for 10 min, then plasma was separated and remaining cells (WBC + RBC) were washed with saline three times. Finally, cells were re-suspended in saline and stored at 4 ºC until its processing.

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Other serum controls: Different controls were carried out to analyze individual contributions of most important seric molecules (**Figure 1**). Glucose (Cicarelli-Argentina), Urea (Cicarelli-Argentina), Creatinine (Anedra-Argentina) and inorganic $H_2PO_4^{1-}$/HPO_4^{2-} mix (Cicarelli-Argentina), Human Albumin (Sigma-Aldrich-USA) were used as individual drug controls.

To corroborate protein amount and confirm the origin of the protein contributions on spectra, two assays for protein determination were performed over albumin, urea, creatinine, urine, free-protein serum and serum controls: UV spectra obtained at 200 – 400 nm[26] and Bradford method[27].

5. Bacteria

To find bacterial contributions on exudate FTIR spectra (**Figure 1**), strains isolated from CVLU were studied in its planktonic and biofilm forms. Selected strains were isolated from CVLU exudate samples by conventional methods and represent 95% of the aerobic isolations[16,31,32]. Isolated Gram-positive bacteria were: *Staphylococcus aureus,* Meticilin Resistance *S. aureus* (MRSA)*, S. haemolyticus,* Coagulase Negative Staphylococcus (CNS), *Beta-hemolytic Streptococcus* and *Enterococcus faecalis.* Isolated Gram-negative bacteria were: *Pseudomonas aeruginosa, Pseudomonas sp, Escherichia coli, Serratia marscecens, Proteus mirabilis, Enterobacter aerogenes, Enterobacter sp, Klebsiella pneumoniae*, *Burkholderia cepacia, Providencia sp* and *Citrobacter sp.* All bacteria were stored at -20ºC in BHI media + glycerol (30%).

Planktonic form: Each strain was activated at room temperature for 30 min, cultured in BHI broth and then incubated for 6 h at 37ºC. Cultures were centrifuged at 8000 rpm for 10 min and planktonic cells pellets were washed 3 times (saline) to remove culture medium. Planktonic pellet was lyophilized before its spectroscopic study.

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Biofilm formation: Each strain was activated at room temperature for 30 min, cultured in BHI broth or BHI broth (plus 5 % v/v) human serum for nutritionally highly demanding bacteria) (1/10 v/v) and then incubated 37ºC until biofilm formation (12 to 24 h depending on the strain). In some cases, bacteria were stressed to allow biofilm formation (nutritional stress, UV radiation, thermic stress). Cultures were centrifuged at 3000 rpm for 10 min (to obtain mainly a biofilm pellet). Biofilm pellets were washed 3 times (saline) to remove culture medium and planktonic bacteria. Biofilm pellet was lyophilized before its spectroscopic study.

6. FTIR Spectroscopy

To collect FTIR spectra, a Perkin-Elmer GX 1 spectrophotometer was used. Exudate samples and controls were processed as liquid samples, 5 µl of exudate sample and controls were dried by N_2 flow and vacuum over AgCl circular optical windows. Planktonic and biofilm bacteria were processed for duplicate as solid samples on KBr pills of spectroscopic grade (1:20). Spectra were collected with 64 scans and a 4cm^{-1} of resolution in the range of mid-infrared 4000 cm^{-1} – 400 cm^{-1}. For spectral pre-processing: smoothing, baseline correction and normalization with Amide I band were used. Pre-processing is useful to compensate differences in sample quantity or a different optical pathlength[7,32].

6.1 Infrared regions

According to vibration frequency of main biomolecule bonds[2,7,33,34], different spectral windows were selected according to main bonds characteristic of each biomolecule: W1: CH_3 and $>CH_2$ of Lipids (2800–3000 cm^{-1}); W2: Ester bonds (1770–1720 cm^{-1}); W3: Amide I (1700–1600 cm^{-1}); W4: Amide II (1600–1480 cm^{-1}); W5: Phosphates of nucleic acids (1270–1190 cm^{-1}); W6: Carbohydrate bonds of polysaccharides (1190–900 cm^{-1}). Mixed region (1480–1280 cm^{-1}), unspecific region (4000–3000 cm^{-1}) and fingerprint region (900–

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400 cm^{-1}) were not taken into account for this study **(Figure 2)**. **Table 1** shows the proposed assignments for different biomolecules studied.

6.2 Spectral Area.

Spectral area was measured by OMNIC 8.0 software at all standardized biomolecules windows of each normalized spectrum. Total spectral area corresponds to the sum of the individual area values. From these measurements, different ratios values were calculated and percentages of biomolecules showed on spectra were estimated.

6.3 Second derivative.

Second derivative Savitzky-Golay (SG) (9 points and order 3) were obtained by OMNIC 8.0 software for all spectra. Each standardized biomolecule window was analyzed to find peaks in its corresponding region. As selection criteria 100% of sensitivity and threshold of 0.010 were used for peaks selection (software parameters). Before second derivative obtaining, the original absorbance spectra were previously converted to transmittance units in order to match the second derivative peaks with the original spectrum bands (software requirement for peak selection).

7. Statistics

Statistical significance was evaluated using the Mann-Whitney-Wilcoxon U test for non-parametrical variables. Data analysis was performed with GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA).

RESULTS AND DISCUSSION.

1. Exudate, plasma, serum, free-protein serum and urine spectral areas

In this study, relative composition of each biological polymer in exudates and controls (serum, plasma, free-protein serum, urine, blood cells and other related controls) (**Figure 3 a-**

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3 **l**) were studied by measurement of spectral areas and ratios between them. Also It was found that each analyzed sample by FTIR (exudates and controls) show a unique relative composition and ratios. This could be used to identify sample types (exudates, plasmas, serum, urine) **(Figure 4)**. **Table 2** shows relative composition of each exudate.

1.1 CH_3 / CH_2 window (lipids)

These areas are mainly determined by C-H asymmetric and symmetric stretching of >CH_2 and CH_3 groups, that are typically present on fatty acids and lipids in biological samples. **(Table 1)**. There are no significant differences between spectral areas of exudate and plasma, serum, free-protein serum and cells which indicates a similar lipid concentration **(**Figure 4**)**. In these samples, fatty acids and lipids are possibly represented by membrane phospholipids from prokaryotic and eukaryotic cells and/or from VLDL, LDL, HDL and chylomicrons[35]. In exudates, we expect contributions mainly from cell membrane lipids, which was demonstrated by the great similarity of areas between exudates and cells **(**Figure 4**)**. Therefore, exudate cellularity could be estimated from this window area. Logically, urines do not have absorbance in this region, not only because are free-cell urines but also because are free from lipoprotein.

Oppositely, albumin have C-H asymmetric and symmetric stretching of >CH_2 and CH_3 from its hydrocarbon chain.

1.2 Ester bonds window (lipids)

This area is mainly determined by >C=O stretch of ester bonds in fatty acids **(**Table 1**)**. Exudates and free-protein serum were the only samples that showed this spectral band **(**Figure 4**)**. In the case of exudates, the presence of this band (and the associated peaks in second derivative) could represent membrane phospholipids concentration in the sample. Moreover, free- protein serum controls exhibit absorbance, which may be due to ester bond formation by the alcohol used during deproteinization method.

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1.3 Amide I and amide II windows (Proteins)

Amide I areas are mainly determined by >C=O stretching of peptidic bond of proteins and peptides, followed by C-N swinging and other vibrations of secondary protein structure components. Amide II areas are mainly determined by vibrations of NH bend, C-N stretch, CO bend and N-C stretch **(**Table 1**)**.

The protein absence of free-protein controls, like free-protein serum and urine, was demonstrated by UV spectra **(Figure 5)** and Bradford method (data not shown). These samples show lower amide I areas respect exudate, serum and plasma ($\rho<0.001$). However, despite being free-protein samples, still having absorbance in amide I window **(**Figure 4**)**. This might be caused by the contribution of C=O stretch and N-H stretch and deformation of urea[36], strong C=O stretch, C–C–N bending of creatinine bonds[37] as urea and creatinine spectra also showed **(Figure 3h and 3j)**.

It was previously demonstrated that wound fluid protein concentration (measured by biochemical methods) is lower than serum protein concentration[18,21]. However, there were no significant differences between amide I areas of exudate, serum and plasma **(**Figure 4**).** As all these samples have similar concentrations of urea and creatinine[18,21], this would indicate also a similar protein concentration which is not correct. This may be due to in amide I region contribution of the inflammatory, bacterial and serum proteins all together on exudates were detected. This compensates the difference detected by biochemical methods that only measure inflammatory and seric proteins[18,21].

Amide II areas represent the real protein content of the samples, because free-protein serum and urine show null or minimum absorbance and serum and plasma were significantly higher than exudate ($\rho<0.001$) as expected **(**Figure 4**)**.

1.4 Phosphates windows (nucleic acids)

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In this window, areas are mainly determined by $>PO_2^-$ stretch absorbance present in RNA and DNA (Table 1). However, in this region also may be present vibrations of NH bend, C-C stretch, C-N stretch and CO bend of the called Amide III[2].

Urine and free-protein serum show null absorbance in this region, which is logic as both samples are DNA/RNA/protein free (Figure 4 – Phosphate bonds). In contrast, areas observed in serum and plasma could be caused by proteins while the observed areas in cells and exudates could be as a result of to the sum of nucleic acids and proteins. Taking into account that: 1) exudate protein content is lower than serum protein content (Figure 4 – Amide II)[18,21]; 2) there is no significant differences between phosphate bonds as there are in serum and exudate, and 3) phosphate areas in cells are significantly lower than phosphate areas in exudates ($\rho<0.001$); we could assume that the difference between phosphate areas from exudates and cells are mainly determined by nucleic acids indirectly related to exudate cellularity (Figure 4 – Phosphate bonds).

1.5 Carbohydrate bonds (Polysaccharides)

In this window, areas are mainly determined by C-O, C-C stretch, C-O-H, C-O-C deformation of carbohydrates or C-OH stretch of serine, threonine, tyrosine in proteins (Table 1).

On serum and plasma, areas values probably came from vibrational modes of glycoproteins, glucose and other sugars. Cells presented absorbance because of membrane glycoproteins presence. Albumin has absorbance in this window because C-OH stretch and vibrational modes of serine, threonine and tyrosine[2]. On exudates, areas values probably came from vibrational modes of cellular glycoproteins, glucose, seric glycoproteins, lipopolysaccharides from planktonic bacteria and exopolysaccharides from bacterial biofilm matrix. There is an important variability in polysaccharides concentration among all samples which is demonstrated by the elevated standard deviation (SD) in polysaccharides area from exudate

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samples (Figure 4 – Carbohydrate bonds). Taking into account that all controls present low SD we could assume that the elevated SD in exudates may be due to different biofilm matrix exopolysaccharides contribution from infecting bacteria[23,33]. Because of this polysaccharides areas in exudates could indirectly represent its biofilm load.

Free-protein serum carbohydrates areas was significantly higher than exudates areas ($\rho<0.001$). This may be due to spectral contributions of remnant alcohol (C-OH) from deproteinization method **(Figure 3l and 4)**.

2. Spectral areas of bacteria

We analyzed 95% of the aerobic clinical isolates[16] from CVLU exudates. Each strain showed a unique relative composition for planktonic and biofilm phenotypes **(Table 3)**. This could be useful to identify bacteria at phenotypic-ulcer state. Also allow to differentiate if bacteria are in biofilm or planktonic phenotype, which is unlikely by conventional methods. Besides, as mentioned above, a broad variability among polysaccharides areas for all bacteria spectra were observed. This could be as result of the different exopolysaccharides composition in each case.

3. Peaks

A deep study of the peaks founded in 2nd derivative spectra from exudate samples, controls and bacteria in both phenotypes was performed. Here we only show the typical sample peaks that could have clinical significance.

In the CH_3/CH_2 region (W1) a characteristic peak at ~2933 cm^{-1} was founded on exudate spectra (100%) and on blood cells spectra (100%). This peak could represent the presence of cellular membrane phospholipids from inflammatory cells since this peak is absent in bacteria and controls.

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In the ester bonds region (W2) a peak between 1716-1713cm^{-1} was found in exudates (75%) and bacteria both biofilm (100%) and planktonic (83%) phenotypes. Since this peak is absent in blood cells spectra and other controls, it could represent membrane Phospolipids from bacterial cells.

In the amide I region (W3), 100% of plasma and exudate samples showed a peak at 1690 cm^{-1}. Furthermore, 100% of serum samples present a peak at 1695 cm^{-1} with lower absorbance. This displacement and lower absorbance could be owed to fibrinogen that is the only proteic difference between plasma and serum **(Figure 6)**. Therefore, this peak could be useful to measure fibrin amounts in exudates.

In all bacteria spectra (planktonic and biofilm), peaks between 1633-1629 cm^{-1} and 1623-1616 cm^{-1} were the ones with higher absorbance than others amide I peaks **(Figure 6)**. Besides, a specific peak between 1682 and 1680 cm^{-1} was founded only in exudate samples (100%). Because this peak is absent in plasma, serum and bacteria, it would represent tissue pro-inflammatory proteins. Among them we can find proteases as serine proteinase, cysteine proteinase, aspartic proteinase and matrix metalloproteinases (MMPs)[12,17,19]. If it is demonstrated that this peak belongs to exudate proteases, it would be extremely useful for the ulcers prognosis, as there is a correlation between elevated levels of proteases and delayed healing[12,25,26].

In the amide II region (W4) a peak at 1497 cm^{-1} was found in 100% of exudate and serum controls although we couldn't find a possible assignment for it.

In the phosphate bonds region (W5), a specific peak between 1262 and 1260 cm^{-1} only in 100% of exudates (with an important absorbance) was found. Hence, it could be another representative peak for proteases as was previously assigned to Amide III vibrations (Table 1). Planktonic bacteria present a peak between 1244 and 1242 cm^{-1} and eukaryotic cells present a peak between 1236 and 1234 cm^{-1}. These peaks represent a DNA A-form marker

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3 for antisymmetric PO_2^- stretch[37]. Therefore, these peaks could represent
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5 prokaryotic/eukaryotic load in the sample as both are present in exudates.
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7 In the carbohydrates bonds region (W6) an extraordinary variability of peaks was founded.
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9 There are only a few peaks that were sample-characteristic as 1171-1174 cm^{-1} for exudate
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11 (100%) and 1097-1093 cm^{-1} for biofilm and planktonic bacteria (100%). The rest of the
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13 founded peaks might represent the variability produced by biofilm matrix exopolysaccharides
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15 and glycoproteins in exudates and glycoproteins in plasma and serum.
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18 **CONCLUSION**
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20 FTIR spectroscopy allows to identify sample types (exudates, plasmas, serum, urine,
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22 planktonic bacteria, biofilm bacteria) as each one present a unique relative composition and
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24 ratios ranges. Also, this technique could be useful to identify bacteria at phenotypic-ulcer
25
26 state and allows to differentiate if bacteria are in biofilm or planktonic form which is unlikely
27
28 to do so by conventional methods.
29
30
31 Because the measured areas or the located peaks are concentration-dependent, this method
32
33 could serve to study several parameters in exudate as:
34
35 1) Exudate cellularity
36
37 a) Total cellularity could be estimated from the CH_3 / CH_2 window area.
38
39 b) Inflammatory cells load could be estimated from ester bonds window area or by measuring
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41 2933 cm^{-1} and/or 1236-1234 cm^{-1} peak areas from 2nd derivative (SG).
42
43 c) Bacterial load could be estimated by measuring 1716-1713 cm^{-1} and/or 1244 - 1242 cm^{-1}
44
45 peak areas from 2nd derivative (SG).
46
47 2) Exudate total protein content
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49
50 a) In complex human fluid samples like exudates, urine, serum or plasma is advisable to use
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52 amide II areas to estimate total protein content.
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d) Fibrin amount could be estimated by measuring 1690 cm^{-1} peak area from 2nd derivative (SG).

b) Inflammatory proteins could be estimated by measuring 1682-1680 cm^{-1} and/or 1262-1260 cm^{-1} peak areas from 2nd derivative (SG).

3) Exudate biofilm load could be indirectly estimated by measuring carbohydrate bonds area.

All of these exudate parameters could be useful to evaluate patient evolution as cells and proteins from inflammatory response, fibrin and planktonic or biofilm bacterial load represent critical negative markers for wound healing. Hence, FTIR spectroscopy could be a useful technique that provides a less-invasive and simple way to represent the clinical state of the wound.

In the future, the use of other spectral contribution controls could allow the identification of more specific markers in exudate. For example, hemoglobin as bleeding marker, purified specific phospholipids from eukaryotic membranes as inflammatory cellularity marker, lipopolysaccharides and peptideglycan as bacterial cellularity marker, matrix metalloproteinases (MMP-2, MMP-8, MMP-9) as protease activity markers and different exopolysaccharides from bacterial biofilm matrix (i.e. alginate) as specific biofilm infection marker.

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TABLES

Window	Denomination	Wavenumber (cm^{-1})	Proposed vibrational mode	Proposed primary source
W1	CH_3; CH_2	3000 - 2800	C-H asymmetric and symmetric stretch of $>CH_2$ and CH_3 present on fatty acids and lipids	Lipids, membrane phospholipids.
W2	Ester bonds	1770 - 1720	>C=O stretch of ester bonds in fatty acids	Lipids, membrane phospholipids.
W3	Amide I	1715 - 1600	>C=O stretch, C-N stretch, CCN deformation in peptide bonds	Proteins and peptides
W4	Amide II	1600 - 1480	NH bend, C-N stretch, CO bend, N-C stretch	Proteins and peptides
W5	Phosphate bonds	1270 - 1200	$>PO_2^-$ stretch in RNA/DNA or NH bend, C-C stretch, C-N stretch, CO bend (Amide III)	Nucleic acids and proteins.
W6	Carbohydrates bonds	1190 - 900	C-O, C-C stretch, C-O-H, C-O-C deformation of carbohydrates or $>PO_2^-$ sym. stretch of phosphodiester group in nucleic acids	Carbohydrates, polysaccharides and nucleic acids

Table 1. Proposed vibrational modes at different frequencies to define work regions (windows) in this study[1,2,3,5,7,32,33]. The primary source proposed are biomolecules which present high particular bonds amount that present absorbance at each window. That allow

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define each region as a windows where a particular biomolecule concentration could be measured.

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Exudate	W1	W2	W3	W4	W5	W6
001	11.21	0.58	22.18	12.19	2.29	14.81
002	13.50	0.00	23.56	11.67	3.37	13.63
003	11.70	0.00	27.12	10.00	1.50	16.27
004	12.97	0.00	32.90	15.00	3.31	18.16
005	19.66	1.75	28.02	16.17	2.90	17.82
006	16.12	0.83	29.76	15.24	3.71	24.66
007	20.07	1.76	34.26	14.82	3.95	28.06
008	18.60	1.53	34.36	14.15	4.13	29.37
009	9.59	0.00	36.95	17.42	1.85	5.68
010	8.61	0.00	35.14	15.83	1.54	4.50
011	14.88	0.23	36.02	17.70	2.61	16.84
012	32.68	4.81	36.60	17.43	4.37	25.49
013	16.99	0.00	37.22	19.65	2.39	13.34
014	11.78	0.02	38.12	16.93	3.31	22.36
015	19.69	0.64	28.68	14.63	2.78	17.02
016	26.54	3.50	33.72	18.72	2.76	12.71
017	10.37	0.00	49.91	22.36	1.83	4.88
018	7.77	0.04	29.77	16.01	1.61	9.07
019	19.84	0.15	32.69	15.91	5.90	42.69
020	14.25	0.00	33.23	17.01	3.61	22.10
021	17.36	0.36	36.55	19.44	2.99	21.10
022	17.92	0.16	39.36	18.69	3.92	20.08
023	12.84	0.01	36.39	17.13	2.87	16.66
024	12.01	0.37	26.85	11.67	0.95	14.60
025	7.97	0.12	33.30	16.95	1.59	8.09
026	15.97	0.15	38.86	20.65	2.96	21.05
027	10.74	0.00	35.82	16.84	2.28	26.92
028	9.75	0.05	35.51	16.51	1.35	17.98
029	18.99	0.55	37.06	15.54	5.11	44.04
030	12.19	0.00	39.63	18.19	1.86	10.47
031	10.21	0.00	39.11	17.10	1.22	9.64
032	8.41	0.00	40.35	18.98	1.96	38.19
033	20.10	0.00	34.04	11.59	2.42	49.04
034	9.26	0.00	27.40	13.17	0.97	10.98
035	10.89	0.05	35.85	17.67	2.85	15.27
036	6.67	0.00	35.11	17.32	1.32	6.38
037	8.60	0.09	35.45	13.34	2.09	15.94
038	10.57	0.05	32.84	12.97	1.17	24.31
039	10.14	0.00	35.38	17.76	2.21	27.06
040	7.64	0.00	38.39	21.18	1.75	5.09
041	9.45	0.02	42.22	20.88	2.91	13.17
042	17.02	0.33	36.76	17.06	3.78	29.22
043	9.44	0.09	36.24	16.67	1.62	22.28
044	26.60	2.41	26.04	7.17	1.97	42.22
045	14.62	0.82	38.72	18.44	4.77	35.42
Mean	14.05	0.48	34.52	16.26	2.63	20.10
SD	5.64	0.98	5.13	3.05	1.15	11.06
MIN	6.67	0.00	22.18	7.17	0.95	4.50
MAX	32.68	4.81	49.91	22.36	5.90	49.04

Table 2. FTIR spectral characterization of exudates. The observed values correspond to relative spectral areas in each window and therefore represent exudate biopolymers relative composition.

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	Bacteria	W1	W3	W4	W5	W6	2nd derivative peaks
Planktonic phenotype	*Staphylococcus haemolyticcus*	10.22	65.80	1.35	17.12	43.73	**1750, 1095**
	Staphylococcus aureus	22.30	42.22	6.77	21.52	97.23	**1259, 1239, 1090**
	MRSA	17.11	55.06	9.02	11.66	54.63	**968**
	Enterococcus faecalis	12.76	48.41	14.02	18,00	40.53	**1719, 1614, 1546, 1074**
	Enterococcus faecalis	25.52	56.20	7.09	11.27	65.84	**1719, 1090**
	Beta-hemolytic Streptococcus	7.06	45.64	10.08	3.70	42.39	**1695, 1545, 1230, 1078, 1015, 970**
	Proteus mirabilis	13.69	47.82	12.77	8.09	30.82	**1060**
	Proteus mirabilis	12.97	49.84	16.28	6.56	30.02	-
	Enterobacter sp.	12.45	58.33	16.7	8.34	31.88	-
	Enterobacter aerogenes	16.43	56.51	10.65	8.60	56.45	-
	Pseudomona sp.	14.93	53.93	13.28	9.77	53.02	**1731, 1227, 1177, 1127**
	Pseudomona sp.	12.82	50.22	10.18	4.70	37.13	**1731, 1227, 1097**
	Pseudomona. aeruginosa	25,50	50,20	3,00	3,90	44,70	**1665**
	Providencia sp.	8.91	45.65	9.96	4.68	20.31	-
	Citrobacter sp.	13.75	49.88	13.85	5.63	36.25	**1716**
	Klebsiella pneumoniae	13.45	45.30	12.02	5.53	40.96	**1641**
	Klebsiella pneumoniae	16.74	53.97	8.92	6.90	59.65	**1641**
	Serratia marcescens	17.29	55.37	15.08	8.90	36.78	**2874**
	Escherichia coli	9.79	48.46	15.66	4.89	23.08	**1236, 1120**
	Escherichia coli	14.98	33.81	14.99	5.45	36.82	**1236, 1120**
	Burkholderia cepacia	15.26	56.20	18.16	7.97	29.43	-
Biofilm phenotype	*Staphylococcus haemolyticcus*	12.95	56.15	17.60	8.44	33.67	-
	Staphylococcus aureus	15.09	56.79	15.04	9.36	47.67	**2744, 984**
	Beta-hemolytic Streptococcus	14.05	61.75	13.69	7.44	52.79	**969**
	Beta-hemolytic Streptococcus	11.12	34.2	13.25	6.57	45.63	**969**
	Enterococcus faecalis	13.08	45.26	12.02	7.10	41.70	**1634, 1212**
	Enterococcus faecalis	20.31	49.39	5.45	10.92	95.41	**1212**
	MRSA	13.78	59.48	12.17	8.01	50.41	-
	MR-CNS	9.90	34.04	11.36	5.63	44.37	**1075**
	CNS	10.04	61.39	8.58	11.83	41.58	**971**
	Proteus mirabilis	13.08	58.88	15.17	8.00	30.20	**1511, 1637, 1619, 1238, 1089**
	Proteus mirabilis	13.07	47.27	14.23	4.415	27.34	**1511, 1637, 1619, 1238, 922**
	Enterobacter sp	14.89	46.87	16.23	8.23	24.29	-
	Enterobacter aerogenes	16.47	48.02	13.50	11.14	77.33	-
	Pseudomona aeruginosa	17,00	43,80	11,30	6,90	27,20	**1085**
	Pseudomona sp.	14.33	45.83	16.60	5.74	28.29	**1223**
	Pseudomona sp.	16.55	55.84	17.32	7.77	34.7	-
	Citrobacter sp.	10.24	47.99	10.77	5.20	26.76	-
	Klebsiella penumoniae	15.48	47.55	12.19	8.39	49.68	**2873, 1163, 1104, 1068, 990**
	Klebsiella penumoniae	16.97	49.61	11.50	7.70	57.43	**2873**
	Escherichia coli	11.55	50.5	16.40	6.54	30.43	-
	Burkholderia cepacia	12.53	47.76	15.5	5.39	25.25	-

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Table 3. FTIR spectral characterization of bacteria. The observed values correspond to relative spectral areas in each window and therefore represent bacterial biopolymers relative composition in planktonic and biofilm phenotype. Also, the table shows second derivative peaks that were present only in such strains.

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