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● *Original Contribution*

ULTRASOUND IMAGING AND CHARACTERIZATION OF BIOFILMS BASED ON WAVELET DE-NOISED RADIOFREQUENCY DATA

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Abstract—The ability to non-invasively image and characterize bacterial biofilms in children during nasopharyngeal colonization with potential otopathogens and during acute otitis media would represent a significant advance. We sought to determine if quantitative high-frequency ultrasound techniques could be used to achieve that goal. Systematic time studies of bacterial biofilm formation were performed on three preparations of an isolated *Haemophilus influenzae* (NTHi) strain, a *Streptococcus pneumoniae* (Sp) strain and a combination of *H. influenzae* and *S. pneumoniae* (NTHi + Sp) in an *in vitro* environment. The process of characterization included conditioning of the acquired radiofrequency data obtained with a 15-MHz focused, piston transducer by using a seven-level wavelet decomposition scheme to de-noise the individual A-lines acquired. All subsequent spectral parameter estimations were done on the wavelet de-noised radiofrequency data. Various spectral parameters—peak frequency shift, bandwidth reduction and integrated backscatter coefficient—were recorded. These parameters were successfully used to map the progression of the biofilms in time and to differentiate between single- and multiple-species biofilms. Results were compared with those for confocal microscopy and theoretical evaluation of form factor. We conclude that high-frequency ultrasound may prove a useful modality to detect and characterize bacterial biofilms in humans as they form on tissues and plastic materials. (E-mail: helguera@cis.rit.edu) © **2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Acute otitis media, Biofilms, Characterization, High-frequency ultrasound, *Haemophilus influenzae*, *Streptococcus pneumoniae*, Wavelet de-noising.**

INTRODUCTION

Bacteria are microscopic organisms that reproduce by binary fission. At the average 37°C human body temperature, an entire population of bacteria can double in only 9.8 min (Eagon 1962). Many of these rapidly growing populations of bacteria are also capable of growing in an exopolymer-encased biofilm, a type of aggregative growth attached to a surface. The exopolymer is an organism-produced substance that may be composed of a variety of components that encase the bacteria to function as a shield, against which antibiotics are ineffective in curing infection (Ehrlich et al. 2002). Antibiotics can exterminate planktonic organisms, but cannot penetrate biofilms.

Biofilm-related infections are a major health problem. Biofilms often form on the surface of catheters inserted into ill persons to monitor blood pressure and urine output and on catheters used to infuse medicines into cancer patients (Bookwalter et al. 2008; Kujundzic et al. 2007). Biofilms also form on the surface of tissues. Bacteria embedded in biofilms cause infections observed in persons with cystic fibrosis. Previous research found that biofilms also form in the middle ear space during chronic and recurrent ear infections, thereby making the infections recalcitrant to antibiotic treatment (Bakaletz 2012; Ehrlich et al. 2002; Post 2001).

Two bacteria species account for 90% of all ear, sinus and lung infections in children and adults: *Haemophilus influenzae* and *Streptococcus pneumoniae* (Casey et al. 2010). These same bacteria start the disease process by occupying the nose and throat (Bookwalter 2008; Jurcisek et al. 2007; Muñoz-Elias et al. 2008) before ascending to the middle ear (Hong et al. 2007; Jurcisek 2005; Weimer et al. 2010), sinuses or lungs to cause infections, so their detection as biofilms in the nasopharynx would provide valuable medical

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information to the clinician. Currently, there are no available technologies to detect biofilms *in vivo* or, specifically, to detect the maturity or bacterial composition of a biofilm. There is an urgent medical need for such technology.

To correctly discern a bacterial biofilm from the various structures within the body, the acoustic properties of biofilms, in response to ultrasound, would need to be known. Once known, these characteristics could then be used to identify a biofilm while it was still *in vivo*, either on a plastic catheter or attached to the epithelial cells lining the nose, throat, middle ear, sinuses or lungs. Shemesh et al. (2007) have proven that 50-MHz ultrasound can be used to image *Streptococcus mutans* biofilms on tooth enamel. They used the internal software of a Vevo 770 transducer (VisualSonics, Toronto, ON, Canada) to create 2-D and 3-D structural images of the biofilm and verified that from the second to seventh day of growth, the thickness of the biofilm increases (Reid et al. 2009).

The first step toward detecting biofilms *in vivo* is the study of their properties in an *in vitro* environment based on quantitative parameters. Useful information about the unique properties of materials that interact with ultrasound waves can be found in the spectral content of the received echo radiofrequency (RF) signals. Processing the spectral content of these RF data can illuminate unique properties that are otherwise indiscernible. Knowledge of these properties may help lead to the detection of the presence of a monospecies biofilm, differentiate between monospecies biofilms formed by different strains of those bacteria or even detect multispecies biofilms.

Although many research groups have investigated the capability of ultrasound imaging of biofilms, the acoustic properties are still relatively unknown. This study comprises a systematic time evaluation of isolated LB86-028 non-typeable *H. influenzae* biofilms, *S. pneumoniae* 02-001-V3 biofilms and biofilms produced by the combination of *S. pneumoniae* and *H. influenzae*.

The overarching goal was to develop a high-frequency, pulse-echo ultrasound system to non-invasively image and characterize biofilms in children during nasopharyngeal colonization by potential otopathogens and during acute ear infections (acute otitis media).

Spectral parameters such as peak frequency, bandwidth and integrated backscatter coefficient were investigated as a function of time. These metrics helped quantify gradual changes in the development of the biofilm and could be used to highlight unique signatures of the species-specific biofilm being investigated. In our preliminary work, we also investigated changes in biofilm thickness as a function of maturity (Vaidya et al. 2011).

Confocal microscopy of three independent repeats of longitudinal studies for each case under investigation was used to confirm the ultrasound findings.

METHODS

Ultrasound biofilm preparation

Growth of 24-h-old LB86-028 non-typeable H. influenzae *biofilms on agar.* An inoculum of a reference strain of non-typeable *H. influenzae* (NTHi), LB 86-028, was aseptically quadrant-streaked for colony isolation onto the surface of a chocolate agar plate and incubated for 24 h at 37°C in 5% CO_2 and 10% oxygen in a Heracell 150 i dual gas incubator (ThermoFisher Scientific, Pittsburgh, PA, USA) for 24 h. Afterward, a single isolated colony was inoculated into brain-heart infusion broth supplemented with 20 μg/mL final concentrations of hemin and β-nicotinamide adenine (sBHI, Sigma, St. Louis, MO, USA) and incubated again for 24 h under identical conditions. One hundred milliliters of 1% sBHI agar was prepared, and 1 mL was used to overlay the well bottom of a six-well sterile tissue culture plate (Corning, Corning, NY, USA) and allowed to solidify. Subsequently, a 1:200 dilution of the overnight culture was made into fresh sBHI broth, and 8 mL of this diluted culture was pipetted into the agar-overlaid wells of the tissue culture plate. This culture plate was then placed into the incubator and allowed to grow for 24 h under the same micro-aerophilic conditions mentioned above.

Growth of 24-h-old S. pneumoniae *02-001-V3 biofilms on agar.* A 5% sheep's blood agar plate was streaked to obtain isolated colonies of a clinical isolate of *S. pneumoniae* 02-001-V3, (Sp), obtained from the −80°C repository stocks of the Rochester General Research Institute. The plate was incubated in a Heracell 150 i dual gas incubator at 37°C in 5% CO_2 and 10% oxygen for 24 h. A single isolated colony was then inoculated into sBHI and incubated for 24 h under identical conditions. One hundred milliliters of 1% sBHI agar was prepared, and 1 mL was used to overlay the well bottom of a six-well sterile tissue culture plate and allowed to solidify. Subsequently, a 1:100 dilution of the overnight culture was made into fresh sBHI broth, and 8 mL of this diluted culture was pipetted into the agar-overlaid wells of the tissue culture plate. This culture plate was then placed into the incubator and allowed to grow for 24 h under the same micro-aerophilic conditions mentioned above.

Growth of 24-h old-co-cultured LB86-028 NTHi and S. pneumoniae *02-001-V3 biofilms on agar.* Simultaneous growth of LB86-028 NTHi and *S. pneumoniae* 02-001-V3, (NTHi + Sp) biofilm was accomplished by mixing equal volumes of respectively 1:200 and 1:100 dilutions