

Award Number: W81XWH-10-2-0161

TITLE: Affinity Reagents for Multiplexed, Rapid Diagnosis of Bacterial Infections at the Point-Of-Care using Diagnostic Magnetic Resonance

PRINCIPAL INVESTIGATOR: Sarah Fortune, M.D.

CONTRACTING ORGANIZATION: Harvard College
Boston, MA 02115

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) October 2012		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 27 September 2011 - 26 September 2012	
4. TITLE AND SUBTITLE Affinity Reagents for Multiplexed, Rapid Diagnosis of Bacterial Infections at the Point-Of-Care using Diagnostic Magnetic Resonance				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-2-0161	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sarah Fortune Marta Fernandez-Suarez E-Mail: sfortune@hsph.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Harvard College Boston, MA 02115-6028				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Our long-term goal is to develop DMR (diagnostic magnetic resonance) into an automated, point-of-care diagnostic device of bacterial infections, which is portable and robust enough to be used in austere environments, and detects the presence of multiple pathogens with high sensitivity and within minutes. Our work focuses on developing DMR as a tool for the diagnosis of bacterial pneumonia. In DMR, pathogenic bacteria are labeled with magnetic nanoparticles that are conjugated to affinity reagents specific for each given organism, and detection sensitivity and specificity are mostly determined by the binding affinity and specificity of the selected affinity reagent. We have identified a suite of affinity reagents for a number of medically relevant pathogens. In addition, we propose that clinically useful diagnoses can be made by combining the specific identification of individual pathogens with the general diagnosis of classes of organisms that would provide an important guide to therapy. We continue to seek small molecule binders that are sufficiently sensitive or specific to serve as class specific binding agents. In addition, we are currently pursuing recombinant antibodies as potential class specific binding agents.					
15. SUBJECT TERMS Point-of-care diagnosis, bacterial infections, pneumonia, diagnostic magnetic resonance.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	8	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

We have developed a new diagnostic assay based on a miniaturized nuclear magnetic resonance system (DMR, for diagnostic magnetic resonance)^{1,2}, which can be used in settings with limited laboratory support. Our long-term goal is to use DMR to develop an automated, point-of-care diagnostic device of bacterial infections, which is portable and robust enough to be used in austere environments, and detects the presence of multiple pathogens with high sensitivity and within minutes.

By targeting pathogenic bacteria with magnetic nanoparticles conjugated to affinity reagents specific for given organisms, we had previously demonstrated that we can accurately identify bacteria, including *Staphylococcus aureus* and *Mycobacterium tuberculosis*, with startling speed and sensitivity. Here, we propose to expand this technology from a specialized detection system to a general microbiologic diagnostic platform, focusing first on developing a diagnostic for community acquired pneumonia. Specifically, we propose to develop a series of specific and general affinity ligands for the identification of individual organisms of medical importance and for broad classes of organisms with therapeutic implications.

BODY

The aims of this grant, first set forth in the approved proposal and then modified in the terms of the no-cost extension based on data generated are:

Aim 1. For the specific identification of medically important pathogens with unique treatment requirements, we will test the use of antibodies as DMR probes.

Aim 2. Develop DMR probes for the general detection of classes of bacteria, assessing both antibodies to common surface determinants and small molecules as probes.

Our milestones are:

1. Assemble panel of clinical isolates of *S. pneumoniae* and representative gram positive and gram negative pathogens.
2. Assemble panel of existing antibodies to *S. pneumoniae*. Develop antibody assays and conjugation chemistry to validate these antibodies in DMR detection.
3. Assemble and test panel of general antibodies to gram positive and negative organisms.
4. Assemble and test panel of existing small molecules with potential to bind to bacterial cell walls.
5. Assess technologies develop recombinant antibodies.
6. Assess the performance of the antibodies that we have identified in the context of the microfluidic specimen processing that we are simultaneously developing for DMR detection.

Below, we present the results for each of the proposed specific aims.

1. Assemble panel of clinical isolates of *S. pneumoniae* and representative gram positive and gram negative pathogens.

We first assembled a panel of laboratory reference strains of *S. pneumoniae* through collaboration with Professor Marc Lipsitch (Harvard School of Public Health). We also obtained from collaborators reference strains of a small number of bacteria to represent some of the most common causes of bacterial pneumonia. We chose *Streptococcus pneumoniae* and *Staphylococcus aureus* as the most representative gram positive bacteria, and *Haemophilus influenza* and *Pseudomonas aeruginosa* as common gram negative respiratory pathogens.

2. Assemble panel of existing antibodies to *S. pneumoniae* and other respiratory pathogens.

We first gathered a panel of four selected antibodies against the cell wall of *S. pneumoniae*. These antibodies were chosen based on their target specificity: they all bind to protein or saccharide targets that are present on the surface of all *S. pneumoniae* serotypes. This characteristic is essential to develop an antibody-based probe that is universal enough to enable diagnosis of pneumonia caused by any of the pathogenic strains. Selected antibodies targeted:

- (1) Pneumococcal C-polysaccharide (also called C-teichoic acid), a well-known immunologically active component of pneumococci.
- (2) A mixture of pneumococcal capsular components.
- (3) The pneumococcal surface adhesin A (PsaA) cell-wall protein.
- (4) A polyclonal antibody raised against a whole-cell blend of serotypes 3, 4, 6, 7, 9, 14, 18, 19, and 23.

We optimized the conjugation chemistry necessary for antibody detection, testing both biotin-streptavidin binding and click chemistry. We found that in general, biotin-streptavidin conjugation performs robustly. We also established a whole cell ELISA assay as well as the DMR assay to assess binding.

We found an antibody binder that enables identification of all tested serotypes of lab and clinical strains of *Streptococcus pneumoniae* with great sensitivity and specificity. Specifically, we identified an antibody against pneumococcal C-polysaccharide is indeed capable of binding all of the most common pneumococcal serotypes with adequate sensitivity and specificity.

In year two, we explored two antibody binders to another respiratory pathogen, *M. tuberculosis*, seeking antibodies that satisfy our specificity and sensitivity criteria, albeit with different trade-offs. Specifically, anti-PPD is a high affinity antibody that can discern between mycobacteria and other bacteria, but also binds non-tuberculosis mycobacteria to a great extent. On the contrary, anti-ESAT6 is highly specific to *M. tuberculosis*, but it binds MTB with less avidity than anti-PPD. We've tested binding affinity of these two antibodies against a panel of clinical strains of *M. tuberculosis*. Both antibody binders recognized all the clinical strains tested with similar avidity, suggesting that they are suitable as general diagnostic for tuberculosis.

3. Assemble a panel of general, class specific antibodies to gram positive and negative organisms.

Next, we obtained a number of antibodies that bind biomarkers, which are selectively present in either gram-positive or gram-negative bacteria.

- (1) Lipoteichoic acid (LTA): highly immunogenic membrane-anchored molecules in the cell envelope of gram positive bacteria.
- (2) Lipopolysaccharide (LPS): endotoxin found on the outer membrane of gram negative bacteria.
- (3) Lipid A: the lipid moiety of LPS.
- (4) Unknown epitope (gram negative bacteria): the antibody was raised against whole *E. coli* bacilli.

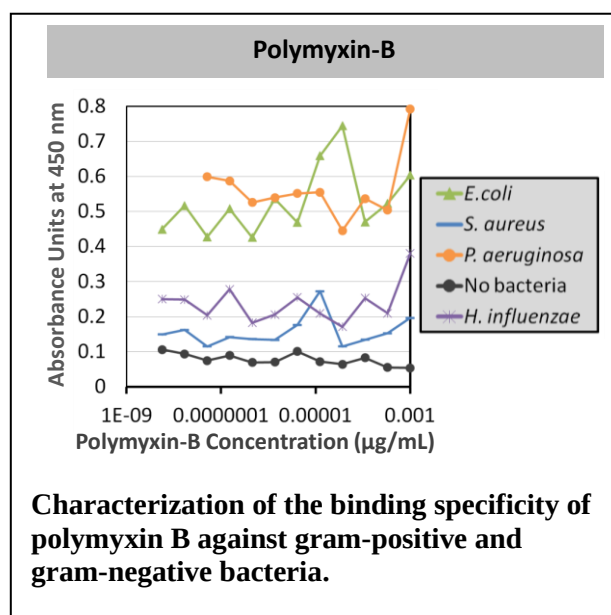
Tests with these antibodies demonstrated that antibody binders are, in general, not ideal for identification of broad classes of organisms, such as gram-positive versus gram-negative

4. Assemble panel of existing small molecules with potential to bind to bacterial cell walls.

We proposed that there are small molecules such as cell wall acting antibiotics, which commonly have more relaxed specificity than antibodies that could be useful to identify bacteria in groups with broad treatment requirements — such as gram-positive versus gram-negative bacteria.

We selected vancomycin and polymyxin B as our first antibiotics to be tested to bind gram-positive and gram-negative bacteria, respectively. We tested binding of the commercially available biotin-polymyxin B using a modified whole-cell ELISA. As shown, polymyxin B displayed greater affinity towards the gram-negatives *E. coli* and *P. aeruginosa*; with negligible binding to the gram-positive *S. aureus*. However, we failed to develop a universal conjugation strategy for vancomycin.

Recently, we investigated the antimicrobial agent Polydiallyl Dimethyl Ammonium Chloride (p-DADMAC) as affinity reagent (Figure 1). To facilitate specific targeting and concentration of bacteria, we are assessing a commercially available paramagnetic particles conjugated to p-DADMAC. As initial proof of concept, we examined binding affinity of these particles against Bacille Calmette Guerin (BCG) in presence of liquidified sputum. Preliminary qPCR results show that p-DADMAC is able to capture BCG post sputum processing. When compared to whole bacteria inoculum p-DADMAC beads retained only a portion of the spiked sample. In this initial assessment, the sample was not concentrated and may contribute to the reduced bacteria retention, suggesting the necessity of further optimization.

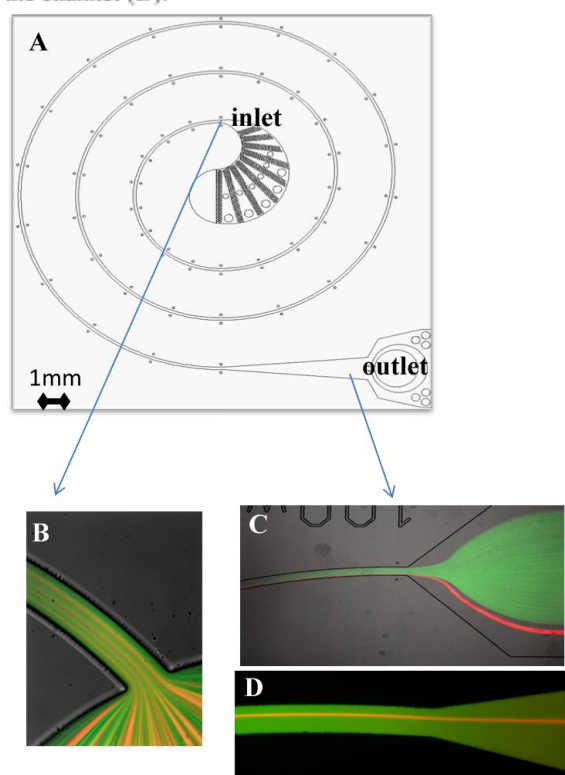


5. Assess technologies develop recombinant antibodies. Our data to date suggest that we need to develop scalable class and pathogen specific antibodies. Therefore, in our NCE, we are assessing cutting edge yeast display technology to develop recombinant antibodies. We will assess the quality of these antibodies in with a single proof of concept pathogen for which we have polyclonal antibodies that provide a point of comparison. If satisfactory, we will develop a

class specific affinity reagent for gram-negative organisms using lipopolysaccharide as an affinity target to fulfill one of the major goals in Aim 2.

6. Assess the performance of the antibodies that we have identified in the context of the microfluidic specimen processing that we are simultaneously developing for DMR detection. Successful detection of whole bacteria using the DMR requires efficient isolation and concentration of bacteria from patient sample. In our NCE, we further developed a microfluidic platform for automated isolation of whole bacteria. Specifically, we have explored the performance of different microfluidic based isolation and concentration strategies to isolate respiratory pathogens. We have had success with inertial focusing to isolate bacteria from larger cells (Figure 2) and are now optimizing concentration parameters.

Figure2. First stage of bacteria concentration microfluidic device. Inertial focusing separates large eukaryotic cells from bacteria. When a mixed population containing E.coli (green) and larger white blood cells (WBC, red or orange) is flowed through a spiral device, forces within the channel forces cells to align to distinct region. Within aqueous buffer, WBCs align to the inner wall of the device (C). However, in more viscous matrix (liquidified sputum), this cell population align towards the center of the channel (D).



KEY RESEARCH ACCOMPLISHMENTS

- We have identified a robust antibody that recognizes all tested serotypes of lab and clinical strains of *Streptococcus pneumoniae* with great sensitivity and specificity.
- We have identified antibody binders that recognize a second respiratory pathogen, *Mycobacterium tuberculosis*, with moderate sensitivity and specificity.
- Existing antibodies perform poorly for identification of broad classes of organisms, such as gram-positive versus gram-negative bacteria.
- We have demonstrated small molecule binding for class detection (polymyxin and potentially PDADMAC). However, find that broad use of small molecules is limited by the ability to modify the small molecule with a chemical handle for conjugation.
- We have optimized inertial focusing of respiratory pathogens in sputum as a first step of a sample processing pipeline.

REPORTABLE OUTCOMES (PUBLICATIONS)^{3,4}

- Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. Aldridge BB, Fernandez-Suarez M, Heller D, Ambravaneswaran V, Irimia D, Toner M, Fortune SM. *Science*. 2012 Jan 6; 335(6064):100-4. Epub 2011 Dec 15.
- Specific pathogen detection using bioorthogonal chemistry and diagnostic magnetic resonance. Liong M, Fernandez-Suarez M, Issadore D, Min C, Tassa C, Reiner T, Fortune SM, Toner M, Lee H, Weissleder R. *Bioconjug Chem*. 2011 Dec 21; 22(12):2390-4. Epub 2011 Nov 7.

CONCLUSION

In sum, during this award period, we have demonstrated that individual and general affinity ligands can be found with the ability to identify specific bacterial species and broad classes of bacteria, respectively. This work brings us one step closer to turning DMR into a general diagnostic tool in microbiological analysis. To continue working towards this goal, we seek to develop scalable solutions for antibody generation. We also seek to develop better class specific antibodies. Finally, we seek to develop an optimized sample processing pipeline for sputum in order to test binding agents and DMR detection in clinical material.

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