University of Nevada, Reno

Identification of Circulating *Burkholderia pseudomallei* Antigens and Development of Chimeric Antibodies

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Cell and Molecular Biology

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May 2013

University of Nevada, Reno
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We recommend that the thesis
prepared under our supervision by

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entitled

Identification of Circulating Burkholderia pseudomallei Antigens and Development of Chimeric Antibodies

be accepted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

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May, 2013

Abstract

Burkholderia pseudomallei is the causative agent of melioidosis, an emerging tropical disease. Thousands contract this disease every year, mainly in Southeast Asia and northern Australia. Disease manifestations range from fatal acute septicemia to a chronic latent infection. Diagnosis is challenging because melioidosis shares symptoms commonly produced by other pathogens. The diagnostic standard remains culturing *B. pseudomallei* from patient samples. This can be challenging since the level of bacteria in patient samples is often very low. Our lab has previously used a novel technique termed In Vivo Microbial Antigen Discovery (InMAD) to identify *B. pseudomallei* diagnostic antigens secreted during a murine model of melioidosis. We have modified this technique by immunizing mice with filtered urine or sera from melioidosis patients. Mice generate an antibody response against the *B. pseudomallei* antigens present in the patient samples. The mouse serum is used to probe a *B. pseudomallei* lysate by 2D immunoblot; reactive proteins are identified by mass spectrometry. Proteins identified by mass spectrometry are then targeted by monoclonal antibodies for the rapid diagnosis of melioidosis.

Melioidosis is difficult to treat, due to its inherent resistance to common antibiotics. Therefore, novel methods of treatment are vital to increase patient survival from this disease. Immunotherapy is one option that could be used as an adjunct to antibiotics. Our laboratory and others have shown that monoclonal antibodies (mAb) specific to the *B. pseudomallei* capsular polysaccharide (CPS) can prevent disease in

mouse models of melioidosis. Our goal is to produce a chimeric mouse/human mAb specific to CPS that may be useful in treating human disease. The variable region of a capsule specific (mAb) was cloned and sequenced. This variable region was then cloned into a human IgG1 expression vector.

Acknowledgements

I would like to thank Dr. David AuCoin for the opportunity to work in his lab. The experience, the knowledge, and the guidance I have received along the way is invaluable along the path of my scientific career. I would also like to thank everyone in the Kozel Lab and AuCoin Lab, particularly Dr. Sindy Chaves, Breeana Hubbard, and Dr. Mark Hubbard for all of their help and guidance in unfamiliar techniques and concepts.

I would also like to thank Reva Crump, Dr Ruth Gault, and Dr.Kathy Schegg for all of their continued friendship and support.

Finally, I would like to thank my family members and friends for their encouragement and support.

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CHAPTER 1: BACKGROUND

Melioidosis is an emerging tropical disease, caused by the Gram-negative bacillus *Burkholderia pseudomallei*. Infection with *B. pseudomallei* is localized mainly in Thailand and northern Australia. Due to the increase in travel and improved diagnostics, however, a greater number of cases are being observed worldwide. *Burkholderia pseudomallei* can be aerosolized to cause a severe pulmonary infection. Its closest relative, *Burkholderia mallei*, has been used as a bioweapon during both World Wars (Lehavi, Aizenstien et al. 2002). This has placed *Burkholderia pseudomallei* the list of Tier 1 Select Agents of most concern to the United States government for weaponization.

Infection with *B. pseudomallei* occurs in a variety of ways. There are several routes of infection, including pulmonary, cutaneous, and rarely, ingestion. *B. pseudomallei* most commonly infects individuals in contact with soil and water in endemic areas (Wiersinga, Currie et al. 2012, Cheng, Currie et al. 2013). Pulmonary melioidosis occurs when the host inhales *B. pseudomallei,* cutaneous infection occurs through a penetrating injury or open wound, and gastrointestinal infection occurs through ingestion of contaminated soil or water. Most cases are thought to develop 1-21 days post-infection(Wiersinga, Currie et al. 2012). Patients present with coughing, malaise, fever, joint pain, abcesses, however, pneumonia is the most common and serious symptom of melioidosis (Cheng, Currie et al. 2013).

Two identified co-morbidities include diabetes (Hodgson, Govan et al. 2013), and alcoholism(Ashdown, Duffy et al. 1980). Even when a case of melioidosis is

diagnosed in time for treatment, 19-40% of patients with severe melioidosis die, depending on the treatment location (Limmathurotsakul and Peacock 2011). Less developed countries, like Thailand, generally lack personnel and training, so mortality rates are higher (~40%) (Limmathurotsakul, Wongratanacheewin et al. 2010). More developed countries, such as Australia, have a lower mortality (~19%) rate due to better equipment and trained personnel(Cheng, Hanna et al. 2003). Lack of immediate treatment increases the risk that the patient will develop sepsis and die.

The mechanism of pathogenesis of *B. pseudomallei* is not well understood, but several important factors have been elucidated. There are upwards of twenty virulence factors that *B. pseudomallei* employs upon infection of the host. One of the most important is the Type 3 Secretion System (T3SS). *B. pseudomallei* uses its T3SS to disrupt the vacuolar membrane it is contained in (Ulrich and DeShazer 2004). The late phagosome develops after the host cell's initial phagocytosis (Galyov, Brett et al. 2010). The bacterium largely relies on its BimA system to polymerize actin (Sitthidet, Korbsrisate et al. 2011). This way, it can evade host autophagy and avoid host killing. *B. pseudomallei* also encodes a Type VI secretion system (T6SS) that can promote bacterial replication, actin polymerization, and plasma membrane fusion. T6SS facilitates entry into adjacent cells, leading to the formation of multinucleated giant cells (MNGC) (Chen, Wong et al. 2011). It is also noted that *B. pseudomallei* polymerizes actin differently than any other bacterial pathogen (Breitbach, Rottner et al. 2003).

B. pseudomallei employs other techniques to avoid the host immune system. Capsular polysaccharide (CPS) (Masoud, Ho et al. 1997), expressed on the surface, allows *B. pseudomallei* to avoid immunodetection. CPS interferes with the complement activation cascade by reducing the amount of C3b complement that is deposited on its surface. *B. pseudomallei* also evades the immune system using biofilms. Biofilm production is essential to maintain antibiotic resistance for *B. pseudomallei*. In a study by Niumsup et al, the biofilm producing strain *B. pseudomallei* 377 showed a much slower or nonexistent diffusion of antibiotics ceftazidime (CAZ) and imipenem (IPM) though its biofilm than the biofilm deficient strain *B. pseudomallei* M6. Antibiotic resistance in *B. pseudomallei* is also assisted by the presence of many different drug efflux pump systems (Pibalpakdee, Wongratanacheewin et al. 2012). The BpeAB-OprB efflux pump in *B. pseudomallei* 1026b, for example, is used to mediate the efflux of aminoglycoside antibiotics (Mima and Schweizer 2010).

Another reason *B. pseudomallei* is a major concern is that there is no effective diagnostic method to diagnose this infection in a rapid manner. Currently, the gold standard of diagnosing melioidosis is a culture of a patient sample, which takes 3-7 days (Farkas-Himsley 1968). Patients who are non-bacterimic are generally left undiagnosed, and can develop a more severe infection (Limmathurotsakul and Peacock 2011). Tests in development include serodiagnostic tests, immunofluorescence, PCR and ELISA. The most common serodiagnostic test is indirect haemagglutination assay (IHA), which is hindered by seropositivity from

individuals who are native to endemic areas (Harris, 2011). Pongsunk et al created a sensitive direct agglutination (detects whole bacterial cells) in combination with a latex agglutination test (detects antigen), however, this also required the patient to be bacterimic (Pongsunk, Thirawattanasuk et al. 1999). Immunofluoresence has been used as a definitive test for melioidosis, however, this also requires a high amount of bacteria within patient samples(Wuthiekanun, Desakorn et al. 2005). It also requires expensive equipment that is lacking in resource poor laboratories. Many researchers have investigated PCR as a potential method of diagnostics (Koh, Tay et al. 2012, Hara, Chin et al. 2013). Koh et al reported that their multiplex PCR was cost effective and rapid, however, it depended upon an isolated, pure sample. Kaestli et al developed a TaqMan PCR for the detection of *B. pseudomallei* in clinical specimens. They identified seven open reading frames specific to *B. pseudomallei* for PCR detection directly from clinical samples. The study resulted in specificity near 100% and sensitivity averaging nearly 70% (Kaestli, Richardson et al. 2012). ELISAs are specific and sensitive (Cooper, Williams et al. 2013), but they require expensive equipment and training, both of which are generally lacking in endemic, resource poor areas. Recently, Nuti et al has investigated the possible use of an antigen capture ELISA for CPS (Nuti, Crump et al. 2011). This diagnostic has shown promise in detecting CPS directly from patient samples.

Melioidosis is difficult to treat, even if it is diagnosed early. The lack of a approved vaccine is a major obstacle to overcome. Vaccine research is ongoing, and a number of avenues are being pursued. The efficacy of vaccines using O-

polysaccharides(Burtnick, Heiss et al. 2012), as well as capsular polysaccharides(Burtnick, Heiss et al. 2012) are being investigated. Outer membrane vesicles have also been tested as a potential vaccine for melioidosis (Nieves, Asakrah et al. 2011). Currently, there are no vaccines that are able to induce sterilizing immunity (Sarkar-Tyson and Titball 2010).

Antibiotic treatment for *B. pseudomallei* infection is an intensive process. The recommended treatment is 6 weeks of intravenous antibiotics, followed by 2 months of oral antibiotics (Estes, Dow et al. 2010), *B. pseudomallei* is inherently multi-drug resistant, complicating treatment options. It has a number of effective drug efflux pumps, so antibiotic development is limited. Recent studies have found potential antimicrobial peptides in natural toxins and venom, with promising results (Perumal Samy, Gopalakrishnakone et al. 2010). These tests, however, have yet to be confirmed in vivo.

Immunotherapy is a promising avenue for controlling infections of persistent, Gram-negative bacteria, including *B. pseudomallei*. Post-exposure CpG therapy may be an effective treatment for exposure to *B. pseudomallei* (Easton, Haque et al. 2011). A novel prophylaxis to prevent against highly virulent bacterial infections is the development of cationic liposome non-coding DNA complexes (CLDC) mixed with bacterial membrane protein fractions (Goodyear, Kellihan et al. 2009, Ireland, Olivares-Zavaleta et al. 2010). . Another avenue may be the use of chimeric or humanized antibodies as an adjunct to antibiotic therapy. Historically, humanized antibodies have helped treat many chronic diseases, such as rheumatoid

arthritis (Malviya, Salemi et al. 2013) and multiple sclerosis (Williams, Coles et al. 2013), as well as cancer (Miller, Foy et al. 2013). Multiple studies have been done on the effectiveness of antibodies as a therapeutic in treating melioidosis in an animal model (Zhang, Feng et al. 2011, AuCoin, Reed et al. 2012). AuCoin et al determined polysaccharide specific antibodies can protect mice against an intranasal challenge with *B. pseudomallei*. mAbs with specificity to CPS, in particular, demonstrated an 80% survival rate when used alone, and a 100% survival rate when combined with an mAb specific for *B. pseudomallei* lipopolysaccharide (LPS) (AuCoin, Reed et al. 2012). Jones et al have also described a study in which the combination of mAbs specific for polysaccharides protect mice against an intraperitoneal challenge with *B. pseudomallei* (Jones, Ellis et al. 2002), further supporting AuCoin et al data.

CHAPTER 2: TARGET DISCOVERY FOR THE IMMUNODIAGNOSIS OF MELIOIDOSIS

INTRODUCTION

Burkholderia pseudomallei is a Gram-negative saphrophytic bacterium endemic to southern Thailand and northern Australia. *Burkholderia pseudomallei* is the causative agent of melioidosis, an emerging tropical disease. Thousands contract this disease every year, mainly in Southeast Asia and northern Australia. Disease manifestations range from fatal acute septicemia to a chronic latent infection.

Diagnosis is challenging because melioidosis shares symptoms commonly produced by other pathogens. The diagnostic standard remains culturing *B. pseudomallei* from patient samples (Farkas-Himsley 1968). This can be challenging since the level of bacteria in patient samples is often very low(Wuthiekanun, Limmathurotsakul et al. 2007), and can take 3-7 days to confirm infection (Farkas-Himsley 1968). Despite advances in technology, infections of *Burkholderia pseudomallei* remain difficult to diagnose. Other methods are currently used to more rapidly diagnose infections; including molecular, serological and antigen capture techniques. Molecular techniques (PCR) have been limited by poor sensitivity, most likely stemming from low levels of *B. pseudomallei* in blood and the co-purification of PCR inhibitors with target DNA (Haase, Brennan et al. 1998). Serological tests can be performed rapidly, however a large percentage of healthy individuals in endemic areas are seropositive (Cheng and Currie 2005).

The focus of this research is to develop an assay for the detection of shed/secreted *B. pseudomallei* antigens in patient samples. Antigen detection has

been previously reported with some success. Our lab has previously used a novel technique termed In Vivo Microbial Antigen Discovery (InMAD) to identify *B. pseudomallei* diagnostic antigens secreted during a murine model of melioidosis (Nuti, Crump et al. 2011). We have modified this technique by immunizing mice with filtered urine or sera from melioidosis patients. Mice generate an antibody response against the *B. pseudomallei* antigens present in the patient samples. These antibodies are used to probe a *B. pseudomallei* lysate by 2D immunoblot; reactive proteins are identified by mass spectrometry. Proteins identified are then targeted by monoclonal antibodies for the rapid diagnosis of melioidosis.

MATERIALS AND METHODS

Bacterial Cultures - *B. pseudomallei* cells (strain 1026b) were grown overnight in BHI (Brain Heart Infusion broth) at 37°C with shaking (Bowen, Colorado State University). Cells were concentrated by centrifugation and resuspended in PBS. Cells were heat-killed at 80°C for 2.5 hours. To confirm the cells were heat killed, a sample was plated in high containment (BSL3) on BHI agar, incubated at 37°C overnight (Nuti, Crump et al. 2011).

Human Samples – Filtered urine samples from patients with culture-positive melioidosis or negative controls were obtained from sample archives (no identifiable private information supplied) at Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Samples were backplated upon arrival at UNR, and confirmed negative for presence of bacteria or fungi.

InMAD Procedure - Prior to immunization, a sample of sera was taken from each mouse to confirm the lack of antibodies reactive to *B. pseudomallei* lysate. Female BALB/c mice were immunized with an emulsion of human melioidosis patient urine (filtered) and Titermax adjuvant subcutaneously. 50 µl were brought to a final volume of 100µl with PBS, and finally mixed with 100µl of Titermax Gold Adjuvant (TiterMax US, Inc.) Mice were bled via the submandibular vein at 4, 6, and 8 weeks to assess reactivity to *B. pseudomallei* antigens. *B. pseudomallei* lysates were probed with serum collected from immunized mice by immunoblot. Final collection of sera was performed via cardiac puncture. Animal experiments were done in accordance

with the Guide for the Care and Use of Laboratory Animals, and the protocol used was approved by the IACUC Committee of the University of Nevada, Reno (Nuti, Crump et al. 2011).

1D Immunoblots- Heat-killed *B. pseudomallei* lysate was separated by SDS-PAGE on a 12% gel, and blotted onto PVDF membranes using standard Western blot procedure, using either a tank blot or a TurboBlot (Bio-Rad, Hercules CA). Membranes were blocked, then incubated with InMAd immune serum at a dilution of 1:30 using a blotter-gel. Detection of binding was done with goat anti-Mouse Ig (H+L) HRP (Southern Biotech) was used at a 1:10,000 dilution. Signal was visualized using a chemiluminescent substrate (Pierce).

Two Dimensional Immunoblot Analysis - Heat killed *B. pseudomallei* 1026b cells were pelleted and rehydrated with 440 ul of DeStreak Solution (GE). Cells were lysed in sonicating water bath for 20 minutes, then alternatively vortexed and stored on ice for an hour. Protein concentration was quantified using EZQ Quantitation Kit. IPG Ready-Strips, 11 cm 3-10NL from Bio-Rad were rehydrated with DeStreak Solution from GE and lysate mixture (200 µg), and 2% 3-10 Ampholytes (v/v)(Sigma, St. Louis MO).

Strips were focused using the IEF Cell from Bio-Rad for 20,000 Volt hours. The following protocol was used: 250 V, linear ramp for 20 minutes; 8000 V, linear ramp for 2 hours 30 minutes, and 8000 V final step. Strips were separated onto Bio-Rad Criterion Gels (8-16%), after being incubated for 10 minutes in 8M urea, 2% SDS, 0.05M Tris-HCL, pH 8.8, 20% glycerol, and 2% DTT for 10 minutes. This was

repeated for 10 minutes using 2.5% iodoacetimide (IAA) instead of DTT. Gels were blotted onto PVDF using Bio-Rad Turbo Transfer and stained to visualize total protein content. Blots were blocked overnight with TBST/milk, and probed with InMAD immune serum. Duplicate gels for protein extraction were incubated overnight in Sypro Gel stain, and imaged for total protein content the next day. Reactive protein spots were extracted from a duplicate gel using a spot cutting set by BioRad PDQuest version 8.0 software and BioRad ExQuest Spot Cutter.

Mass Spectroscopy and MALDI-TOF Analysis - Reactive spots from blots were transposed over images of total protein gel. Reactive spots were excised from gels and were analyzed by Orbitrap at the University of Nevada, Reno Proteomics Center. Sequence data was analyzed via Scaffold software, UniProt, Protean Lasergene and BLAST searches. Protein spots were analyzed as follows: spots were digested using the Investigator Proprep (Genomic Solutions, Ann Arbor MI) with several modifications. Protein spots were washed twice with 25mM ammonium bicarbonate (ABC) and 100% acetonitrile (CAN), reduced and alkylated using 10 mM dithiothreitol and 100 mM iodoacetamide, then incubated with 75 ng trypsin in 25 mM ABC for 6 hours at 37°C. Samples were eluted with 70% CAN, 0.2% formic acid and overlaid with 0.5 µl of 5 mg/ml MALDI matrix and 10 mM ammonium phosphate. All data was collected using an ABI 4700 MALDI TOF/TOF (Applied Biosystems, Foster City CA). Data was collected in reflector mode from a mass range of 700-4000 Daltons and an average of 1250 laser shots per mass spectrum. Each sample was internally calibrated on trypsin's autolysis peaks. Data was stored in an

Oracle database, and GPS Explorer Software (Applied Biosystems, Foster City CA) generated a peak list from the raw data. It was based on signal to noise filtering plus an exclusion list. The data were then searched by Mascot (Matrix Science, Boston MA), which used a tolerance of 20 ppm . Database search parameters included 1 missed cleavage, oxidation of methionines, and carbamidomethylation of cysteines.

RESULTS

Previously, the AuCoin lab has developed a technique termed InMAD (Figure 1A) in order to identify circulating microbial antigens shed/secreted during *B. pseudomallei* infection (Nuti, Crump et al. 2011). Originally, BALB/c mice were infected with *B. pseudomallei*. Sera was harvested, and filtered, and used to immunize a set of BALB/c mice. Six weeks later, InMAD immune sera was harvested from the mice, and target antigens were identified by 2D immunoblot.

The current study used a modified InMAD approach(Figure 1B) by immunizing a set of BALB/c mice with filtered human melioidosis patient urine. The urine samples were confirmed culture positive for *B. pseudomallei* (Table1). Prior to immunization, urine was filtered to remove any live *B. pseudomallei* cells. Culture positive urine was used in this study, in hopes that there were more secreted antigens present. Five mice were immunized per each patient sample.

Western blots of heat-killed whole cell lysates were probed with the pre-immune sera and the InMAD sera in order to identify secreted *B. pseudomallei* antigens. For most mice, the pre-immune sera was unreactive with the *B. pseudomallei* lysate. Pre-immune sera from mice that was reactive with the lysate was excluded from further analysis. Representative InMAD Immune Serum binding to *B. pseudomallei* lysate is shown in Figure 2. The InMAD immune sera from Mice 1-5 were reactive to the *B. pseudomallei* lysate (Figure 2). Mice 1 and 2 were immunized with an emulsion of UID3 and Titermax, whereas mice 3-5 were immunized with an emulsion of UID7 and Titermax.

Antigen Detection by 2D Immunoblot. A 2D membrane of *B. pseudomallei* lysate was probed with reactive pooled InMAD immune sera from mice (Figure 3). The pre-immune sera were unreactive to *B. pseudomallei* proteins (Figure 3). The InMAD immune sera contained antibodies reactive with multiple *B. pseudomallei* proteins (Figure 3). Each of these is a candidate diagnostic protein.

Candidate Diagnostic Antigens Identified by InMAD. Proteins reactive by 2D immunoblot were excised from a duplicate gel and sequence with mass spectrometry (Orbitrap). Protein sequences were analyzed using Scaffold software and BLAST searches. Fourteen diagnostic antigens were identified (Table 2). Two spots excised from the 2D gel contained additional proteins (Table 2). Among them was co-chaperonin GroES, which is known to associate with chaperone GroEL as a heat shock and stress protein. GroEL has been implicated in multiple other studies as a potential diagnostic and therapeutic target, because of its high expression levels in vitro (Woo, Leung et al. 2001, Felgner, Kayala et al. 2009). Hypothetical protein is a functionally unknown protein that is anticipated to be located within the bacterial cell(cello.org). 50s ribosomal subunit L7/L12 is an abundant, immunogenic protein that has also been implicated in other studies as a protein shed during *B. pseudomallei* infection (Suwannasaen, Mahawantung et al. 2011). Phasin and transcription elongation factor NusA are proteins that have been identified as shed during infection in the previous murine InMAD study (Nuti, Crump et al. 2011). Although NusA itself was not present in the list of reactive proteins from the murine InMAD study, there were several other elongation factors present.

Polyclonal Antibodies (pAb) Raised to Protein Targets. Target protein sequences were analyzed by BLAST to find peptide sequences that were not homologous to other, clinically relevant pathogens. GroES and Hypothetical protein were chosen as our highest priority targets for polyclonal antibody production. GroES is known to associate with GroEL, a common, antigenic protein that has been previously explored as a target for diagnostics (Woo, Leung et al. 2001, Felgner, Kayala et al. 2009). Hypothetical protein was chosen because of its unique characteristic of being unique to *B. pseudomallei*. Another criteria for choosing these proteins was based on the nature of *B. pseudomallei* pathogenesis. Many melioidosis patients have kidney failure, and accordingly, kidney problems and diabetes are an indication of susceptibility to disease. Healthy kidneys will filter any proteins that are above 30kDa. Antigens that are below 30 kDa, therefore, will theoretically be present in the urine of all melioidosis patients. In this study, several protein targets are found around 10 kDa, and seem to be highly reactive and immunogenic (Figure 3). In the interest of finding a diagnostic target that would diagnose melioidosis in otherwise healthy patients, lower kDa targets were pursued.

Additionally, target protein sequences were analyzed by Protean Lasergene software to identify immunogenic sequences. Immunogenic sequences were then analyzed by BLAST to identify *B. pseudomallei* specific sequences. The pAbs to these peptide sequences were raised in rabbits and purified. The pAbs were confirmed to bind to *B. pseudomallei* targets via 2D immunoblot (Figure 4, GroES shown as an example). Reactive proteins were excised from a duplicate gel and sequenced using

mass spectrometry (Orbitrap). Of particular interest is Spot #4 (Figure 4), which is the same spot that InMAD sera identified as a candidate (Spot#4, Figure 3).

Confirmation of Target Proteins Presence in Human Patient Urine Samples. Due to the lack of available human patient urine, the presence of GroES and Hypothetical protein were unable to be confirmed in the human samples that the mice were initially immunized with. Other proteins located in this InMAD study, however, have been confirmed to be in human patient samples, such as GroEL (Data not shown).

DISCUSSION:

Previously, the AuCoin lab has utilized a platform called InMAD in mice in order to discover circulating antigen secreted by *B. pseudomallei* (Nuti, Crump et al. 2011). The results of Nuti et al (Nuti, Crump et al. 2011) concluded that InMAD was a feasible approach. This study determined that directly immunizing mice with filtered human urine, and possibly sera, is a valid approach to identify diagnostic targets. The results suggest that there are a number of possible protein targets for the development of antigen capture immunoassays for the diagnosis of melioidosis, in addition to the previously discovered polysaccharide targets described in Nuti et al. Additional research, including experiments on melioidosis positive non-bacteremic patient urine, need to be done to confirm the presence of these proteins in human urine. Some of the proteins mentioned in this study overlap with the previous study from a murine InMAD study by Nuti et al. GroEl, phasin, as well as transcription factors were identified in the murine InMAD study. This suggests that studies may overlap, and that for human samples that are difficult to attain, an animal model may be an appropriate model.

Previously, collaborators at University of Arizona had indentified twelve *B. pseudomallei* proteins present in the pooled human urine via Multi-Dimensional Protein Identification Technology (MuDPIT). Three were identified with a high degree of confidence: GroEL, BipC, and FliC (Bush et al, unpublished data). BipC is a protein involved in the T6SS, described previously, FliC is a protein that is used in the flagella, and has been noted as an immunogenic virulence factor during *B.*

pseudomallei infection (Chua, Chan et al. 2003). GroEL was the only *B. pseudomallei* protein discovered in this study, possibly due to increased expression of the protein. GroEL has been identified in numerous studies on *B. pseudomallei* and the proteins that are identified during infection. A study by Lertmemongkolchai et al. (Suwannasaen, Mahawantung et al. 2011), as well as Felgner et al. both of these previous studies have identified *B. pseudomallei* antigens by proteome microarrays. Several proteins that were not found in MudPIT or in the previous InMad study appeared as reactive proteins in the microarray, including 50s ribosomal subunit L7/L12. For future experiments, 50s ribosomal subunit would be an excellent target to pursue, because of its abundance in bacterial lysates, immunogenicity, and its size. 50s ribosomal subunit is under 30 kDa, which would also make it a reasonable candidate to pursue for the diagnosis of melioidosis patients who do not have compromised kidneys or diabetes.

Unique polyclonal antibodies specific to antigenic peptides predicted to be present on the surface of GroES and Hypothetical Protein bound to their intended targets. The 2D blots indicate that the pAbs bound to other proteins as well as their intended proteins. These spots were not excised and analyzed. It is not clear whether the other proteins are alternatively spliced or modified forms of the intended targets. Specificity to protein targets could be improved by creating monoclonal antibodies to these targets. Additionally, monoclonal antibodies will need to be produced for use on an immunodiagnostic assay.

Future directions include testing a larger panel of human melioidosis patient urine samples to confirm the presence of these identified protein candidates, as well as the presence of GroES and Hypothetical Protein. Testing will include 1D Western blots, 2D blots, or ELISAs to confirm the presence of the target protein. Creation of new pAbs to 50s ribosomal subunit L7/L12, as well as phasin and GroEL could also be used to target proteins present in human urine.

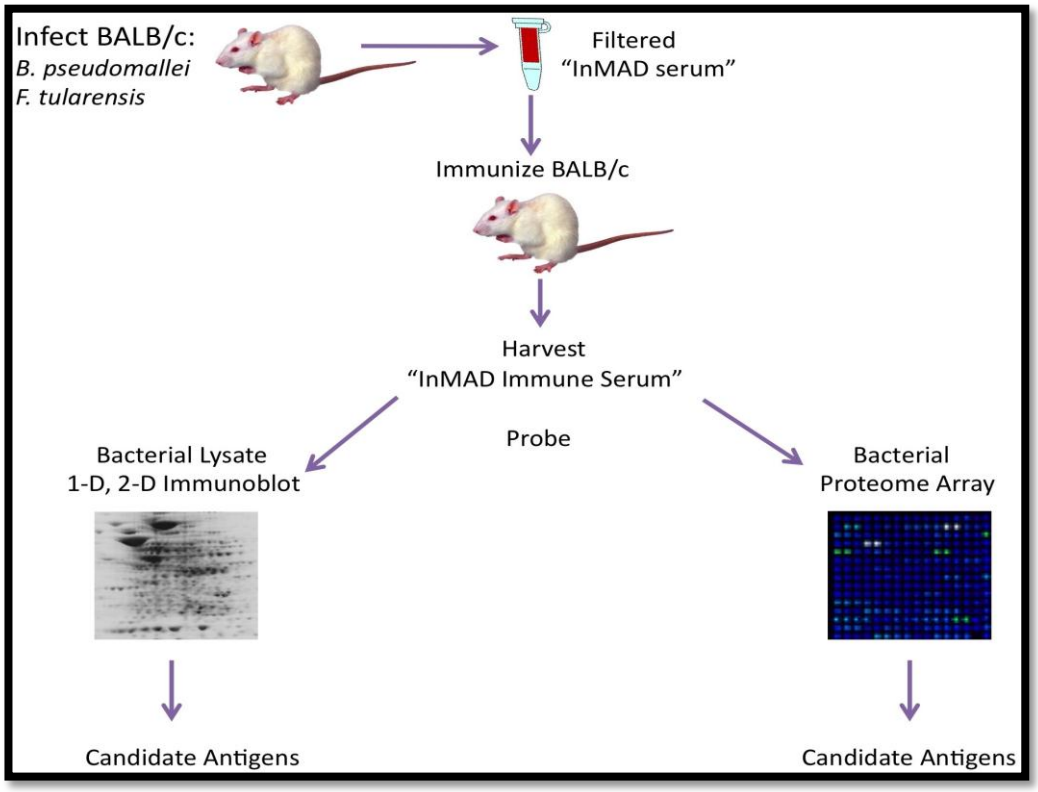


Figure 1A. **Original InMAD Schematic.** Balb/c mice were infected with heat-killed B. pseudomallei, and InMAD sera was harvested after 6-8 weeks. The InMAD sera was used to immunize Balb/c mice. After 6-8 weeks, InMAD Immune Serum was collected. The InMAD Immune Serum was used to probe a 2-D Immunoblot for reactive antigens.

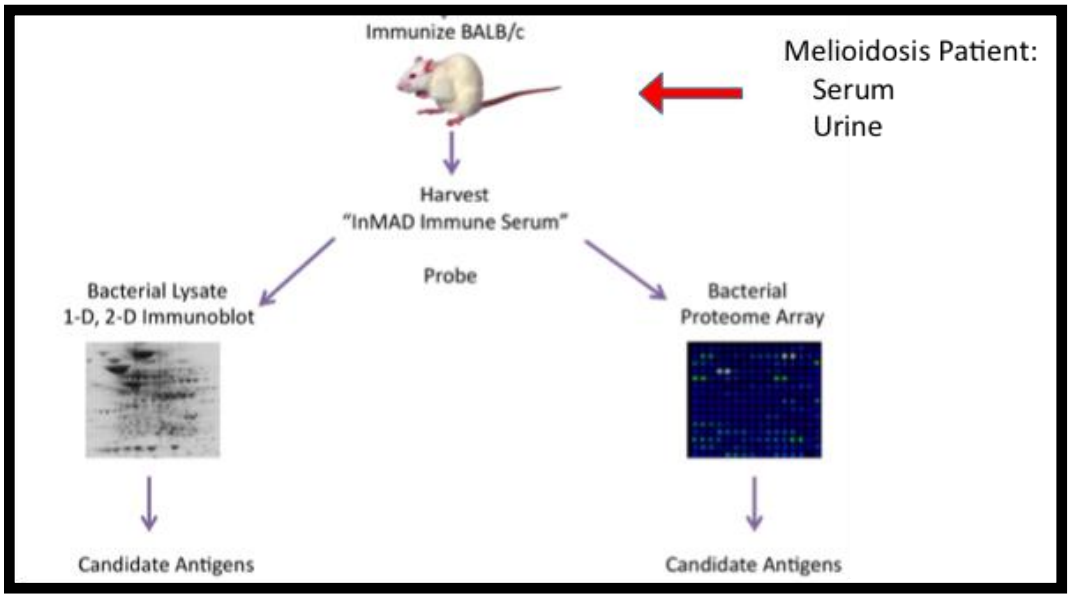


Figure 1B. **Modified InMAD Schematic**. Mice were immunized with filtered human melioidosis patient urine. At 6 weeks, InMAD immune serum was harvested from the mice. The InMAD immune serum was used to probe a 2-D immunoblot of *B. pseudomallei* lysate for reactive antigens.

	CFU/ml	Patient Status
UID1	22,500	Survived
UID2	>100,000	Survived
UID3	75,000	Died
UID5	>100,000	Died
UID7	>100,000	Died

Table 1. **Melioidosis Patient Samples Used in Study**. CFU/ml of *B. pseudomallei* present in patient urine prior to filtration was determined by quantitative culture, Patient Status (Survived or Died).

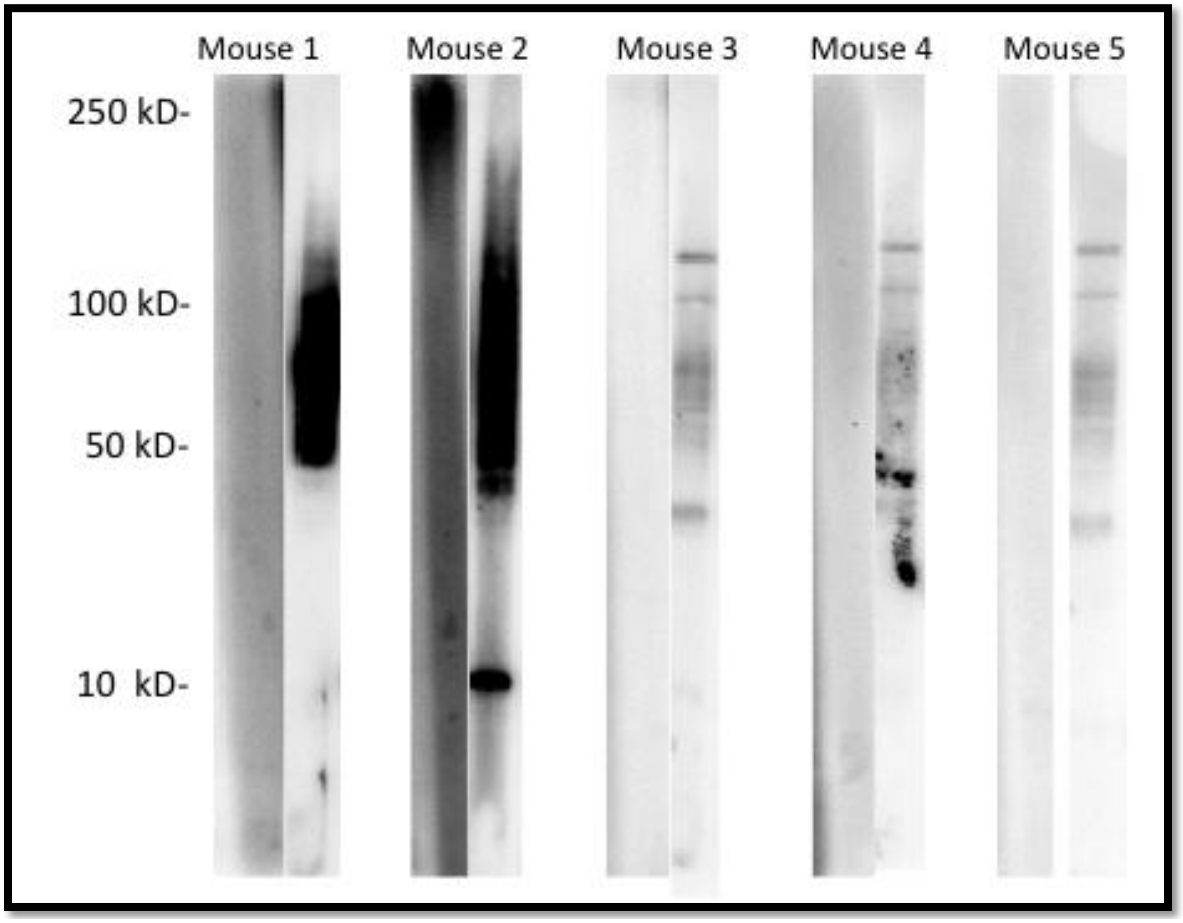


Figure 2. **B. pseudomallei proteins are reactive with InMAD immune serum.** Pre-immune serum, pictured at the left per each mouse, does not react with *B. pseudomallei* proteins in 1D Western blots. At 6 weeks post-immunization, InMAD immune serum is reactive with *B. pseudomallei* proteins.

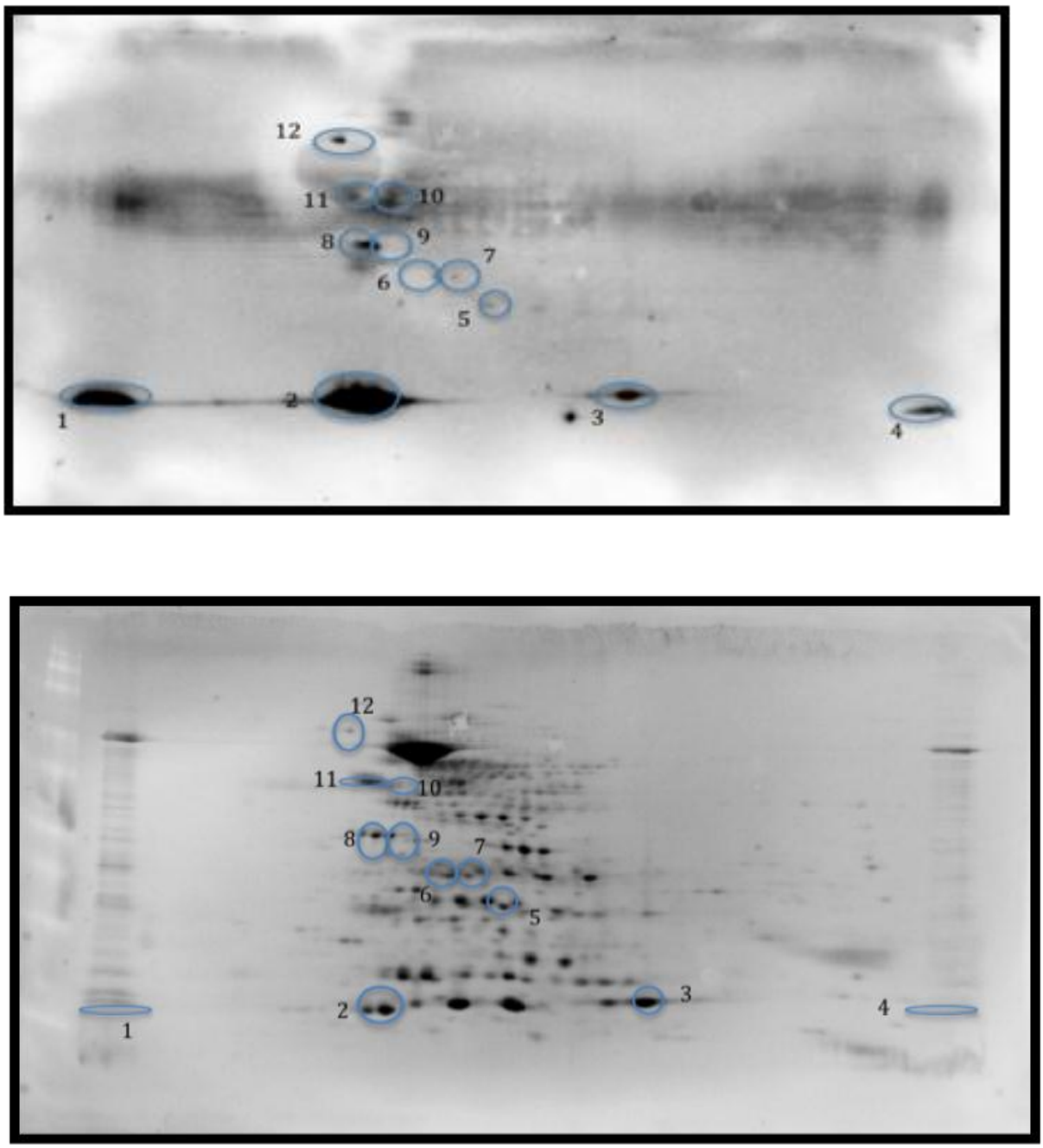
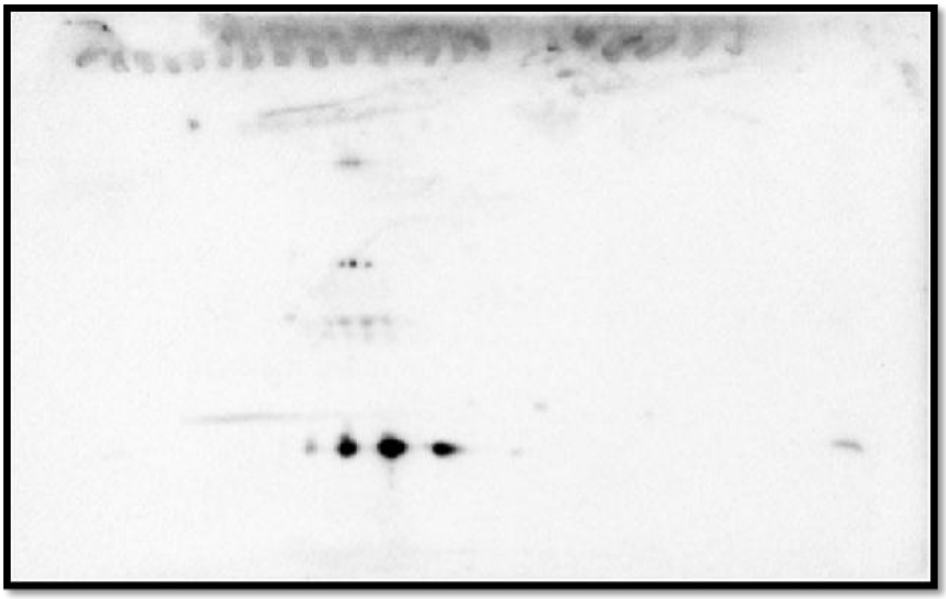


Figure 3. **Target antigen detection by 2D immunoblot**. (A) Total protein gel of a *B. pseudomallei* lysate. (B) Immunoblot of total protein probed with InMAD immune serum produced from mice immunized with patient urine. The circled spots are the proteins found to be reactive with InMAD immune serum, which are potential secreted diagnostic targets. Reactive proteins were excised from a duplicate protein gel and identified by mass spectrometry.

	Protein ID	Size (kDa)
1	Co-chaperonin GroES- gi 107021935	11
2	50s Ribosomal Subunit- gi 53723863	13
3	Hypothetical Protein- gi 217419383	14
4	Co chaperonin GroES- gi 107021935	11
5	Phasin- gi 167581317	20
6	Hypothetical Protein/OsmY- gi 53718900/ 76819679	20/22
7	Glutathione S-transferase domain containing protein- gi 76808775	23
8/9	Electron transfer flavoprotein subunit alpha- gi 53720108	32
10	FtsA/FtsZ- gi 53720630/ 53720631	43/42
11	GroEL/Phosphopyruvate hydratase- gi 167912219/ 53719880	55/45
12	Transcription Elongation Factor NusA- gi 53719533	55

Table 2. Candidate diagnostic proteins identified via 2D gel electrophoresis

A.



B.

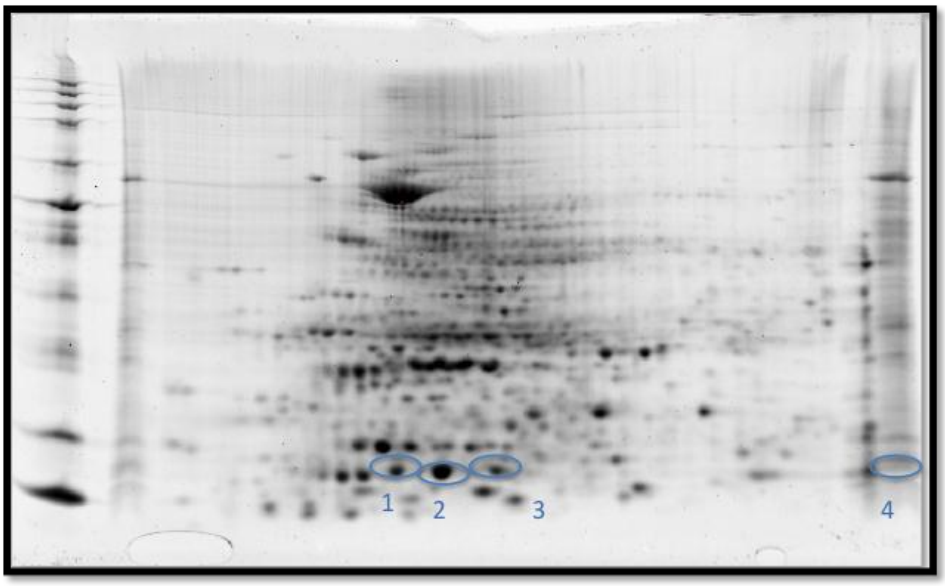


Figure 4. **Binding of GroES specific pAbs**. *B. pseudomallei* lysate was probed with pAb raised in rabbits against GroES immunogenic peptide. (A) Immunoblot of B.pseudomallei lysate probed with GroES (B) Duplicate total B. pseudomallei protein gel reactive antigens shown in (A) were excised and confirmed to be GroES via Orbitrap.

CHAPTER 3: PRODUCTION AND CHARACTERIZATION OF A CHIMERIC MONOCLONAL ANTIBODY SPECIFIC FOR THE CAPSULAR POLYSACCHARIDE OF BURKHOLDERIA PSEUDOMALLEI

INTRODUCTION

Melioidosis is difficult to treat, due to its inherent resistance to common antibiotics. In endemic areas mortality rates remain high, even when proper antibiotics are administered. Therefore, novel methods of treatment are vital to reduce mortality rates from this disease. The use of immunotherapy, in addition to antibiotics, could be potentially useful in treating melioidosis. Immunotherapy is a broad field, and has been used for decades in many other areas of research, such as in the development of therapeutics for cancer and genetic disorders(Malviya, Salemi et al. 2013, Miller, Foy et al. 2013). Our laboratory and others are investigating the passive transfer of antibodies specific to *B. pseudomallei* antigens in treating infection.

One of the antigens we are targeting is the manno-heptose capsular polysaccharide (CPS) of *B. pseudomallei* and creates a large immune response in melioidosis survivors (Reckseidler-Zenteno, DeVinney et al. 2005, Reckseidler-Zenteno, Moore et al. 2009, Heiss, Burtnick et al. 2012). mAb 3C5 has also been shown to be protective in challenge studies in a murine model of pulmonary melioidosis (AuCoin, Reed et al. 2012). In the study done by AuCoin et al., mice were passively immunized with low doses of mAb 3C5 and mAb 4C7, specific for the

lipopolysaccharide of *B. pseudomallei*. Mice were then challenged with a lethal dose of *B. pseudomallei*. Mice that had been immunized with mAb 3C5 or mAb 4C7 had a much higher survival rate than the control group, 80% and and 55%, respectively. Mice that were immunized with a combination of 3C5 and 4C7 had a one hundred percent survival rate.

The goal of the project is to develop a chimeric mouse/human antibody, utilizing the antibody variable region of MAb 3C5, in order to produce a potential therapeutic MAb for the treatment of melioidosis. Therefore, the variable region of both heavy and light chains from MAb 3C5 have been sequenced and cloned into a human IgG1 expression vector for expression in CHO cells.

MATERIALS AND METHODS:

Hybridoma Production: Spleens from mice immunized with heat killed *B. pseudomallei* were harvested and fused with murine myeloma cells to create a hybridoma cell line as previously described(Nuti, Crump et al. 2011). Clones were selected by ELISA and Western blot for reactivity with the CPS.

RNA Purification: 3C5 hybridoma cells were harvested (1 x 10^6) by centrifugation. They were lysed, and the total RNA was collected (Qiagen RNA preparation kit). RNA was aliquoted and Mouse Ig Primers were added according to protocol (Mouse Ig Primer Kit, Novagen)

cDNA Synthesis: cDNA was synthesized with a collection of Mouse Ig Primers from Novagen. cDNA was PCR amplified with the primers specific for Murine IgG variable regions (Novagen), both Kappa and Lambda chains. PCR products were analyzed by gel electrophoresis, and were purified using a gel extraction kit (Qiagen).

TA Cloning: PCR products were then cloned into the pGEM T-Easy vector (Promega). Clones were sequenced and variable regions were analyzed through the IMGT database (http://www.imgt.org/) for productive heavy and light chains. The variable heavy chain region was amplified from T-Easy vectors using 5'-ACATACTCTAGAGCCACCATGGACTTCGGGTTGATCTGGGTTTTCCTTGTC-3' and 5'-GATGGGCCCTTGGTGCTAGCTGAGGAGACGGTGAC-3' primers. The variable light chain region was amplified from T-Easy vectors using 5'-CATACCTCTAGAGCCACCATGGAAAAGACAGCTATCGCGATTGCAGTGGCACTG-3' and 5'- AGATGGTGCGGCCGCAGTCCGAGCCCGTTTCAGCT-3' primers.

OptiVec/pcDNA Cloning: Variable regions were cloned into pcDNA (light chain) using restriction enzymes XbaI and NotI, and OptiVec vector (heavy chain), using restriction enzymes XbaI and NheI. Plasmids were then transformed into DH5α competent *E. coli* cells, and screened for positive clones. Positive clones were sequenced, and plasmids that produced the variable region were purified using a MaxiPrep kit (Qiagen).

CHO S Cell Line Transfection- 1x 10^6 CHO S cells were transfected according to the Freestyle (Invitrogen) protocol. One ml aliquots were taken daily until Day 7. Supernatant was harvested by centrifugation. Supernatant was tested using a direct ELISA with purified CPS, 10 mg/ml, in the solid phase.

RESULTS:

Previously, the AuCoin lab had produced a monoclonal antibody, mAb 3C5, specific to the capsular polysaccharide of *B. pseduomallei*. Heat-killed *B. pseudomallei* was used to immunize BALB/c mice, and several hybridoma lines were generated. One of the clones, termed 3C5, gave a strong signal by Western blot and ELISA when purified capsule was used in the solid phase. MAb 3C5 was also shown to be protective in a passive immunization study in BALB/c mice that were challenged intranasally with *B. pseudomallei*. Thus, mAb 3C5 was chosen for chimerization in the hopes that the chimeric antibody would also be protective in a murine challenge study, in hopes for eventually producing a therapeutic.

Subcloning and Sequencing 3C5. RNA was purified from 3C5 hybridoma cells. cDNA was created using reverse primers (Table 2). cDNA was used in a PCR reaction with degenerative Murine Ig Primers. PCR products were visualized via agarose gel (data not shown). PCR products were purified and clones into T-Easy Vector systems. PCR products were sequenced at UNR Genomics, and data was analyzed via IMGT databases for productive heavy and light chains. PCR products that contained a productive light and heavy chain, respectively, were subcloned using primers from IDT (primer sequences listed in Results Section). PCR products for the heavy chain were digested with XbaI and NheI, and PCR products from the light chain were digested with XbaI and NotI. Heavy and lights chains were then cloned into the OptiVec and pcDNA vector systems. Heavy and light chains were transfected into CHO S cells according to the Freestyle protocol. CHO S cells were

incubated for 7 days. Aliquots of supernatant were taken each day. An ELISA was done on the supernatant against purified CPS in the solid phase (Figure 5). Due to the low quantity of antibody present in the supernatant, the heavy and light chains were optimized to improve production.

Cloning into OptiVec and pcDNA. Kozak sequences and leader sequences were introduced into the vector to enhance secretion from DG44 CHO cells. Currently, antibody expression from DG44 cells is still in progress.

DISCUSSION:

Previously, AuCoin laboratory produced a mAb specific for the CPS of *B. pseudomallei*. In passive immunization studies, the mAb 3C5 protected mice from pulmonary melioidosis (AuCoin, Reed et al. 2012). Previous studies have shown that humanized monoclonal antibodies are effective against highly virulent bacteria with capsules (Chen, Schneerson et al. 2011). Notably, this study reported the protective effect of administering humanized monoclonal antibody 20h challenge with *Bacillus anthracis*. To date, challenge studies with chimeric or humanized antibodies against *B. pseudomallei* have not been performed, and the difficulty of treating melioidosis infections demonstrates a need for effective immunotherapeutics with the ability to protect when administered after infection.

In this study, the variable regions of the heavy and light chain of mAb 3C5 were sequenced and analyzed with the IMGT database, showing the similarity to a human germline antibody sequence. A small amount of chimeric mAb was produced and expressed. Expression and purification is currently being optimized.

Future directions include producing the full-length chimeric antibody from DG44 CHO cell line. Following production, antibody would be purified and the stable affinity will be characterized using Surface Plasmon Resonance (SPR). It will be interesting to determine if the affinity of the chimeric mAb is similar to the mouse mAb 3C5. The chimeric mAb will be evaluated for protection in a murine model of pulmonary melioidosis and compared to mAb 3C5.

mAb 3C5	Heavy Chain		Light Chain	
	Forward	Reverse	Forward	Reverse
Name	MuIgVh5'-F	MuIgGVh3'-2	MuIgkVl5'-D	MuIgkVl3'-1
Bases	35	35	42	30
Sequence	ACTAGTCG ACATGAAC TTYGGGYT SAGMTTGR TTT	CCCAAGCT TCCAGGGR CCARKGGA TARACIGRT GG	ACTAGTCG ACATGAGG RCCCCTGC TCAGWTTY TTGGIWTCT T	CCCAAGCT TACTGGAT GGTGGGAA GATGGA

Table 2. Mouse Ig-Primer Set. The primers used to generate in frame, productive light and heavy chains from the cDNA of 3C5 hybridoma cells.

A

Fab	HCDR3	LCDR3
3C5	T R G G S A M D Y	G Q S Y S Y P L T

B

	Heavy Chain				Light Chain		
mAb	V-gene	Identity (%)	J-gene	D-gene	V-gene	Identity (%)	J-gene
3C5	IGHV3-15*08	78.91	IGHJ6*01	IGHD3-10*01	IGKV4-1*01	72.76	IGKJ4*02

Table 3. Analysis of mAb 3C5.(A) Alignment of the amino acid sequence of the heavy chain CDR3 and the Light Chain CDR3 of mAb 3C5. (B) Assignment of 3C5 mAb variable regions to its closest human germ line counterpart, based on nucleotide sequence homology, identified by search of the IMGT database (http://www.imgt.org/)

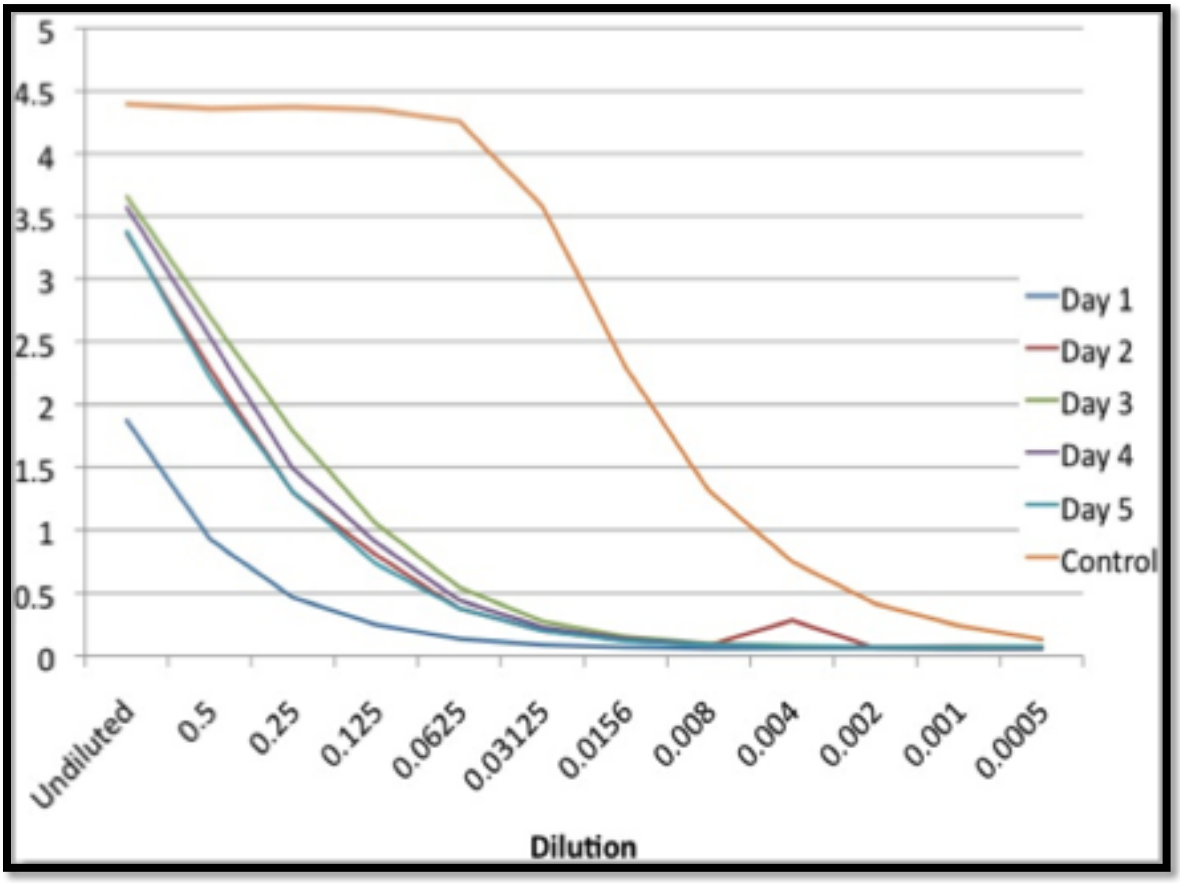


Figure 5. ELISA with supernatant from transfected CHO S cell line. CHO S cells were transfected with the chimeric heavy chain plasmid and the chimeric light chain plasmid. One ml aliquots of supernatant were collected per day until day 7, when CHO S supernatant was harvested via centrifugation. Supernatant was tested against purified CPS in the solid phase.

REFERENCES

Ashdown, L. R., V. A. Duffy and R. A. Douglas (1980). "Meliodosis." Med J Aust **1**(7): 314-316.

AuCoin, D. P., D. E. Reed, N. L. Marlenee, R. A. Bowen, P. Thorkildson, B. M. Judy, A. G. Torres and T. R. Kozel (2012). Polysaccharide specific monoclonal antibodies provide passive protection against intranasal challenge with Burkholderia pseudomallei. PLoS One. United States. **7:** e35386.

Breitbach, K., K. Rottner, S. Klocke, M. Rohde, A. Jenzora, J. Wehland and I. Steinmetz (2003). Actin-based motility of Burkholderia pseudomallei involves the Arp 2/3 complex, but not N-WASP and Ena/VASP proteins. Cell Microbiol. England. **5:** 385-393.

Burtnick, M. N., C. Heiss, R. A. Roberts, H. P. Schweizer, P. Azadi and P. J. Brett (2012). "Development of capsular polysaccharide-based glycoconjugates for immunization against melioidosis and glanders." Front Cell Infect Microbiol **2**: 108.

Burtnick, M. N., C. Heiss, A. M. Schuler, P. Azadi and P. J. Brett (2012). "Development of novel O-polysaccharide based glycoconjugates for immunization against glanders." Front Cell Infect Microbiol **2**: 148.

Chen, Y., J. Wong, G. W. Sun, Y. Liu, G. Y. G. Tan and Y. H. Gan (2011). "Regulation of Type VI Secretion System during Burkholderia pseudomallei Infection ▽ ." Infect Immun **79**(8): 3064-3073.

Chen, Z., R. Schneerson, J. Lovchik, C. R. Lyons, H. Zhao, Z. Dai, J. Kubler-Kielb, S. H. Leppla and R. H. Purcell (2011). Pre- and postexposure protection against virulent anthrax infection in mice by humanized monoclonal antibodies to Bacillus anthracis capsule. Proc Natl Acad Sci U S A. United States. **108:** 739-744.

Cheng, A. C. and B. J. Currie (2005). "Melioidosis: Epidemiology, Pathophysiology, and Management." Clin Microbiol Rev **18**(2): 383-416.

Cheng, A. C., B. J. Currie, D. A. Dance, S. G. Funnell, D. Limmathurotsakul, A. J. Simpson and S. J. Peacock (2013). Clinical definitions of melioidosis. Am J Trop Med Hyg. United States. **88:** 411-413.

Cheng, A. C., J. N. Hanna, R. Norton, S. L. Hills, J. Davis, V. L. Krause, G. Dowse, T. J. Inglis and B. J. Currie (2003). "Melioidosis in northern Australia, 2001-02." Commun Dis Intell Q Rep **27**(2): 272-277.

Chua, K. L., Y. Y. Chan and Y. H. Gan (2003). "Flagella are virulence determinants of Burkholderia pseudomallei." Infect Immun **71**(4): 1622-1629.

Cooper, A., N. L. Williams, J. L. Morris, R. E. Norton, N. Ketheesan and P. M. Schaeffer (2013). "ELISA and immuno-polymerase chain reaction assays for the sensitive detection of melioidosis." Diagn Microbiol Infect Dis **75**(2): 135-138.

Easton, A., A. Haque, K. Chu, N. Patel, R. A. Lukaszewski, A. M. Krieg, R. W. Titball and G. J. Bancroft (2011). Combining vaccination and postexposure CpG therapy provides optimal protection against lethal sepsis in a biodefense model of human melioidosis. J Infect Dis. United States. **204:** 636-644.

Estes, D. M., S. W. Dow, H. P. Schweizer and A. G. Torres (2010). "Present and future therapeutic strategies for melioidosis and glanders." Expert Rev Anti Infect Ther **8**(3): 325-338.

Farkas-Himsley, H. (1968). "Selection and rapid identification of Pseudomonas pseudomallei from other gram-negative bacteria." Am J Clin Pathol **49**(6): 850-856.

Felgner, P. L., M. A. Kayala, A. Vigil, C. Burk, R. Nakajima-Sasaki, J. Pablo, D. M. Molina, S. Hirst, J. S. Chew, D. Wang, G. Tan, M. Duffield, R. Yang, J. Neel, N. Chantratita, G. Bancroft, G. Lertmemongkolchai, D. H. Davies, P. Baldi, S. Peacock and R. W. Titball

(2009). A Burkholderia pseudomallei protein microarray reveals serodiagnostic and cross-reactive antigens. Proc Natl Acad Sci U S A. United States. **106:** 13499-13504.

Galyov, E. E., P. J. Brett and D. DeShazer (2010). "Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis." Annu Rev Microbiol **64**: 495-517.

Goodyear, A., L. Kellihan, H. Bielefeldt-Ohmann, R. Troyer, K. Propst and S. Dow (2009). Protection from pneumonic infection with burkholderia species by inhalational immunotherapy. Infect Immun. United States. **77:** 1579-1588.

Haase, A., M. Brennan, S. Barrett, Y. Wood, S. Huffam, D. O'Brien and B. Currie (1998). "Evaluation of PCR for diagnosis of melioidosis." J Clin Microbiol **36**(4): 1039-1041.

Hara, Y., C. Y. Chin, R. Mohamed, S. D. Puthucheary and S. Nathan (2013). Multiple-antigen ELISA for melioidosis - a novel approach to the improved serodiagnosis of melioidosis. BMC Infect Dis. **13:** 165.

Heiss, C., M. N. Burtnick, Z. Wang, P. Azadi and P. J. Brett (2012). "Structural analysis of capsular polysaccharides expressed by Burkholderia mallei." Carbohydr Res **349**: 90-94.

Hodgson, K. A., B. L. Govan, A. K. Walduck, N. Ketheesan and J. L. Morris (2013). Impaired early cytokine responses at the site of infection in a murine model of type 2 diabetes and melioidosis comorbidity. Infect Immun. United States. **81:** 470-477.

Ireland, R., N. Olivares-Zavaleta, J. M. Warawa, F. C. Gherardini, C. Jarrett, B. J. Hinnebusch, J. T. Belisle, J. Fairman and C. M. Bosio (2010). "Effective, broad spectrum control of virulent bacterial infections using cationic DNA liposome complexes combined with bacterial antigens." PLoS Pathog **6**(5): e1000921.

Jones, S. M., J. F. Ellis, P. Russell, K. F. Griffin and P. C. Oyston (2002). "Passive protection against Burkholderia pseudomallei infection in mice by monoclonal antibodies against capsular polysaccharide, lipopolysaccharide or proteins." J Med Microbiol **51**(12): 1055-1062.

Kaestli, M., L. J. Richardson, R. E. Colman, A. Tuanyok, E. P. Price, J. R. Bowers, M. Mayo, E. Kelley, M. L. Seymour, D. S. Sarovich, T. Pearson, D. M. Engelthaler, D. M. Wagner, P. S. Keim, J. M. Schupp and B. J. Currie (2012). "Comparison of TaqMan PCR Assays for Detection of the Melioidosis Agent Burkholderia pseudomallei in Clinical Specimens." J Clin Microbiol **50**(6): 2059-2062.

Koh, S. F., S. T. Tay, R. Sermswan, S. Wongratanacheewin, K. H. Chua and S. D. Puthucheary (2012). Development of a multiplex PCR assay for rapid identification

of Burkholderia pseudomallei, Burkholderia thailandensis, Burkholderia mallei and Burkholderia cepacia complex. J Microbiol Methods. Netherlands, 2012 Elsevier B.V. **90:** 305-308.

Lehavi, O., O. Aizenstien, L. H. Katz and A. Hourvitz (2002). "[Glanders--a potential disease for biological warfare in humans and animals]." Harefuah **141 Spec No**: 88-91, 119.

Limmathurotsakul, D. and S. J. Peacock (2011). Melioidosis: a clinical overview. Br Med Bull. England. **99:** 125-139.

Limmathurotsakul, D., S. Wongratanacheewin, N. Teerawattanasook, G. Wongsuvan, S. Chaisuksant, P. Chetchotisakd, W. Chaowagul, N. P. Day and S. J. Peacock (2010). Increasing incidence of human melioidosis in Northeast Thailand. Am J Trop Med Hyg. United States. **82:** 1113-1117.

Malviya, G., S. Salemi, B. Lagana, A. P. Diamanti, R. D'Amelio and A. Signore (2013). "Biological Therapies for Rheumatoid Arthritis: Progress to Date." BioDrugs.

Masoud, H., M. Ho, T. Schollaardt and M. B. Perry (1997). "Characterization of the capsular polysaccharide of Burkholderia (Pseudomonas) pseudomallei 304b." J Bacteriol **179**(18): 5663-5669.

Miller, M. J., K. C. Foy and P. T. Kaumaya (2013). "Cancer immunotherapy: present status, future perspective, and a new paradigm of peptide immunotherapeutics." Discov Med **15**(82): 166-176.

Mima, T. and H. P. Schweizer (2010). The BpeAB-OprB efflux pump of Burkholderia pseudomallei 1026b does not play a role in quorum sensing, virulence factor production, or extrusion of aminoglycosides but is a broad-spectrum drug efflux system. Antimicrob Agents Chemother. United States. **54:** 3113-3120.

Nieves, W., S. Asakrah, O. Qazi, K. A. Brown, J. Kurtz, D. P. Aucoin, J. B. McLachlan, C. J. Roy and L. A. Morici (2011). A naturally derived outer-membrane vesicle vaccine protects against lethal pulmonary Burkholderia pseudomallei infection. Vaccine. Netherlands, 2011 Elsevier Ltd. **29:** 8381-8389.

Nuti, D. E., R. B. Crump, F. Dwi Handayani, N. Chantratita, S. J. Peacock, R. Bowen, P. L. Felgner, D. H. Davies, T. Wu, C. R. Lyons, P. J. Brett, M. N. Burtnick, T. R. Kozel and D. P. AuCoin (2011). "Identification of circulating bacterial antigens by in vivo microbial antigen discovery." MBio **2**(4).

Perumal Samy, R., P. Gopalakrishnakone, H. Bow, P. N. Puspharaj and V. T. Chow (2010). Identification and characterization of a phospholipase A2 from the venom of

the Saw-scaled viper: Novel bactericidal and membrane damaging activities. Biochimie. France, 2010 Elsevier Masson SAS. **92:** 1854-1866.

Pibalpakdee, P., S. Wongratanacheewin, S. Taweechaisupapong and P. R. Niumsup (2012). Diffusion and activity of antibiotics against Burkholderia pseudomallei biofilms. Int J Antimicrob Agents. Netherlands, A 2012 Elsevier B.V. and the International Society of Chemotherapy. **39:** 356-359.

Pongsunk, S., N. Thirawattanasuk, N. Piyasangthong and P. Ekpo (1999). "Rapid Identification of Burkholderia pseudomallei in Blood Cultures by a Monoclonal Antibody Assay." J Clin Microbiol **37**(11): 3662-3667.

Reckseidler-Zenteno, S. L., R. DeVinney and D. E. Woods (2005). The capsular polysaccharide of Burkholderia pseudomallei contributes to survival in serum by reducing complement factor C3b deposition. Infect Immun. United States. **73:** 1106-1115.

Reckseidler-Zenteno, S. L., R. Moore and D. E. Woods (2009). "Genetics and function of the capsules of Burkholderia pseudomallei and their potential as therapeutic targets." Mini Rev Med Chem **9**(2): 265-271.

Sarkar-Tyson, M. and R. W. Titball (2010). Progress toward development of vaccines against melioidosis: A review. Clin Ther. United States, 2010 Excerpta Medica Inc. **32:** 1437-1445.

Sitthidet, C., S. Korbsrisate, A. N. Layton, T. R. Field, M. P. Stevens and J. M. Stevens (2011). "Identification of Motifs of Burkholderia pseudomallei BimA Required for Intracellular Motility, Actin Binding, and Actin Polymerization▽ ." J Bacteriol **193**(8): 1901-1910.

Suwannasaen, D., J. Mahawantung, W. Chaowagul, D. Limmathurotsakul, P. L. Felgner, H. Davies, G. J. Bancroft, R. W. Titball and G. Lertmemongkolchai (2011). Human immune responses to Burkholderia pseudomallei characterized by protein microarray analysis. J Infect Dis. United States. **203:** 1002-1011.

Ulrich, R. L. and D. DeShazer (2004). "Type III Secretion: a Virulence Factor Delivery System Essential for the Pathogenicity of Burkholderia mallei." Infect Immun **72**(2): 1150-1154.

Wiersinga, W. J., B. J. Currie and S. J. Peacock (2012). "Melioidosis." N Engl J Med **367**(11): 1035-1044.

Williams, T., A. Coles and L. Azzopardi (2013). "The Outlook for Alemtuzumab in Multiple Sclerosis." BioDrugs.

Woo, P. C., P. K. Leung, S. S. Wong, P. L. Ho and K. Y. Yuen (2001). "groEL encodes a highly antigenic protein in Burkholderia pseudomallei." Clin Diagn Lab Immunol **8**(4): 832-836.

Wuthiekanun, V., V. Desakorn, G. Wongsuvan, P. Amornchai, A. C. Cheng, B. Maharjan, D. Limmathurotsakul, W. Chierakul, N. J. White, N. P. J. Day and S. J. Peacock (2005). "Rapid Immunofluorescence Microscopy for Diagnosis of Melioidosis." Clin Diagn Lab Immunol **12**(4): 555-556.

Wuthiekanun, V., D. Limmathurotsakul, G. Wongsuvan, W. Chierakul, N. Teerawattanasook, P. Teparrukkul, N. P. Day and S. J. Peacock (2007). Quantitation of B. Pseudomallei in clinical samples. Am J Trop Med Hyg. United States. **77:** 812-813.

Zhang, S., S. H. Feng, B. Li, H. Y. Kim, J. Rodriguez, S. Tsai and S. C. Lo (2011). In Vitro and In Vivo studies of monoclonal antibodies with prominent bactericidal activity against Burkholderia pseudomallei and Burkholderia mallei. Clin Vaccine Immunol. United States. **18:** 825-834.