DISSERTATION

INVESTIGATION INTO *MYCOBACTERIUM BOVIS* PHENOLIC GLYCOLIPID AS A POTENTIAL BIOMARKER OF BOVINE TUBERCULOSIS IN URINE

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ABSTRACT

INVESTIGATION INTO *M. BOVIS* PHENOLIC GLYCOLIPID AS A POTENTIAL URINARY BIOMARKER OF BOVINE TUBERCULOSIS

Mycobacterium bovis, the etiological agent of bovine tuberculosis (bTB) is reported to cause disease in man and animal alike on every continent aside from Antarctica. Although coordinated efforts have been made for over a century in the US to cease transmission of this pathogen, outbreaks still occur. It has been posited that the failure to eliminate transmission of this pathogen is partially due to the diagnostic in use, which lacks critical sensitivity and specificity. To address this gap, we investigated a potential new method of identifying infected animals that is through the detection of a pathogen-derived biomarker. *M. bovis* phenolic glycolipid (PGL) is a species-specific, highly abundant, and unique glycolipid that comprises up to 2.5% of the dry cell mass. Coupling an LC-ESI-TOF-MS, method with a solid phase extraction, we successfully detected PGL derived from the urine of naturally-infected cattle. With this knowledge, we aimed to generate a detector of PGL that could be applied in a rapid and field friendly diagnostic platform. Using phage display technology, we selected M13 bacteriophage capable of binding *M. bovis* PGL with specificity that differentiated between *M. bovis* PGL and *M. Canetti* PGL, as well as between total lipid fractions of various species of *Mycobacterium* and other lipids with similar biochemical properties to PGL. These *M. bovis* PGL specific phage were able to differentiate between unspiked cattle urine and urine spiked with PGL. Lastly, we assessed the relative stability of PGL, specifically contrasted to another highly abundant

mycobacterial glycolipid, phosphatidylinositol mannoside (PIM). We found that PGL was the more stable molecule when testing thermal and chemical stability, as well as when treated with protease K. However, when treating these glycolipids with whole cell lysates derived from fresh bovine organs, accurate detection on an LC-ESI-TOF-MS platform was lost. Further studies will be required to probe the stability of these molecules *in vivo*. Overall, this potential methodology to assess for infection status may be beneficial in improving the control of *M. bovis* if further developed, as this new approach has the potential to be more specific and sensitive than the currently used diagnostic.

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TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS iv
LIST OF TABLES ix
LIST OF FIGURES x
CHAPTER 1: LITERATURE REVIEW 1
1.1 Mycobacteria 1
1.2 Mycobacterial Diseases 2
1.3 Epidemiology of Bovine Tuberculosis 3
1.4 Pathology of *M. bovis* Infection 6
1.5 Diagnostics of bTB 7
1.6 Lipids as biomarkers of disease 11
1.7 Mycobacterial Lipids 14
1.8 Detection of lipids 18
1.8.1 Thin Layer Chromatography 18
1.8.2 Analytical instrumentation 19
1.8.3 Antibodies 20
1.9 Research rationale and summary of aims 21
REFERENCES 24
CHAPTER TWO: DETECTION OF PHENOLIC GLYCOLIPID BY MASS SPECTROMETRY PLATFORMS 36
2.1 Introduction 36
2.2 Materials and methods 37
Chemicals and reagents 37
PGL purification 37
Cattle urine samples 38
Saponification and lipid enrichment 38
Mass Spectrometry – LC-ESI-TOF-MS 38
Mass Spectrometry – MALDI-MS 39
Data Analysis 39
2.3 Results 40
ESI-TOF detection of PGL in comparison to MALDI-TOF detection 40
Enrichment for PGL from urine via a solid phase extraction and method detection limit 44
Saponification sample pretreatment improves detection of PGL by decreasing interfering signals 46
Detection of PGL in urine extracts of cattle naturally infected with *M. bovis* 49

2.4 Discussion 56
REFERENCES 60
CHAPTER THREE: DETECTION OF *M. BOVIS* PHENOLIC GLYCOLIPID WITH M13 BACTERIOPHAGE 62
3.1 Introduction 62
3.2 Materials and Methods 65
Chemicals 65
Phage display- 7mer and 12mer libraries and selection 65
Phage titering 66
Microtiter plate assay 67
DNA extraction and sequencing 68
Thin Layer Chromatography (TLC) 69
Statistical analyses 70
3.3 Results 70
Hydrophobic plate retains lipids through blocking and washing steps 70
Polyclonal microtiter plate assay shows enriched specificity to *M. bovis* PGL 71
Sequencing analysis reveals consensus in selected clones 73
Monoclonal phage microtiter plate assays show variable levels of PGL binding in contrast to background plastic binding 76
Monoclonal phage microtiter plate assays of specificity to *M. bovis* PGL 77
Binding kinetics of monoclonal phage 79
Limit of detection of five top performing clones 81
Phage Φ7.2 and Φ7.7 both show a statistically significant difference in signal between PGL-spiked and unspiked urine 81
3.4 Discussion 83
REFERENCES 86
CHAPTER FOUR: ASSESSMENT OF STABILITY OF BIOMARKER GLYCOLIPIDS 89
4.1 Introduction 89
4.2 Materials and Methods 90
Chemicals 90
PGL and PIM purification 90
Basic saponification and heating time points 91
Protease K treatment to assess lipid stability 91
Organ homogenate and supernatant assays of lipid stability 91
Detection of PGL and PIM by TLC 92
Detection of PGL and PIM by mass spectrometry 93
4.3 Results 93
Ac_2PIM_2 shows a greater sensitivity to thermal and chemical hydrolysis than PGL 93
Ac_2PIM_2 shows a greater sensitivity to protease K digestion than PGL 96
Neither lipid is sensitive to degradation by enzymes contained in organ homogenates or supernatants 99

Neither lipid is sensitive to degradation by kidney or liver homogenates, but Ac_2PIM_2 signal is ion suppressed when treated with homogenates and assessed by LC-ESI-TOF-MS detection 103
4.4 Discussion 106
REFERENCES 108
CHAPTER FIVE: FINAL DISCUSSION AND FUTURE DIRECTIONS 109
5.1 Final discussion 109
5.2 Future directions 113
REFERENCES 118
LIST OF ABBREVIATIONS 121

LIST OF TABLES

TABLE 2.1 SUMMATION OF CLINICAL SAMPLE RESULTS……..52

TABLE 3.1 LIMIT OF DETECTION OF PHAGE CLONES……81

LIST OF FIGURES

FIGURE 1.1 STRUCTURE OF Ac_2PIM_2……16

FIGURE 1.2 STRUCTURE OF PGL……17

FIGURE 1.3 *M. CANNETTI* PGL SUGAR MOIETY……18

FIGURE 2.1 STRUCTURE OF PGL……36

FIGURE 2.2 DETECTION OF PGL ON VARIOUS MS PLATFORMS……42

FIGURE 2.3 LIMITS OF DETECTION OF PGL ON VARIOUS MS PLATFORMS……43

FIGURE 2.4 SOLID PHASE EXTRACTION TLCS OF PGL-SPIKED URINE……45

FIGURE 2.5 METHOD DETECTION LIMIT OF PGL ON AN LC-ESI-TOF-MS PLATFORM……46

FIGURE 2.6 LC-MS OF POOLED, SPIKED CATTLE URINE……47

FIGURE 2.7 LC-MS OF SAPONIFIED AND UNTREATED PGL VERSUS TRIGLYCERIDE……48

FIGURE 2.8 LC-MS SPECTRA OF POSITIVE CLINICAL SAMPLE……50

FIGURE 2.9 LC-MS SPECTRA OF EQUIVOCAL CLINICAL SAMPLE……51

FIGURE 3.1 CARTOON DEPICTING MICROTITER PLATE ASSAY……64

FIGURE 3.2 TLC OF PLATE WASHES AND ELUTED PGL……71

FIGURE 3.3 POLYCLONAL ENRICHMENT FOR BINDERS OF PGL……72

FIGURE 3.4 CLUSTAL ALIGNMENT OF SEQUENCED PHAGE VARIABLE REGIONS……74

FIGURE 3.5 MONOCLONAL MICROTITER PLATE TESTING PHAGE CLONES AGAINST PGL……77

FIGURE 3.6 PHAGE CLONE SPECIFICITY ASSAY AGAINST INDIVIDUAL LIPIDS……78

FIGURE 3.7 PHAGE CLONE SPECIFICITY ASSAY AGAINST TOTAL LIPID EXTRACTS……79

FIGURE 3.8 TITRATION CURVES OF PHAGE CLONES……80

FIGURE 3.9 MICROTITER PLATE ASSAY OF SPIKED AND UNSPIKED URINE DETECTION BY PHAGE CLONES……82

FIGURE 4.1 TLC OF PIM SAPONIFICATION OVER TIMECOURSE……94

FIGURE 4.2 LC-MS OF SAPONIFIED AND UNTREATED PIM……..95

FIGURE 4.3 LC-MS OF SAPONIFIED AND UNTREATED PGL……..96

FIGURE 4.4 LC-MS AND TLC OF PIM TREATED WITH PROTEASE K AND UNTREATED PIM……98

FIGURE 4.5 LC-MS AND TLC OF PGL TREATED WITH PROTEASE K AND UNTREATED PGL……99

FIGURE 4.6 LC-MS OF PGL TREATED WITH LIVER AND KIDNEY SUPERNATANT……100

FIGURE 4.7 LC-MS OF PIM TREATED WITH LIVER AND KIDNEY SUPERNATANT……101

FIGURE 4.8 LC-MS OF ^{13}C TAG TREATED WITH LIVER AND KIDNEY SUPERNATANT……102

FIGURE 4.9 LC-MS OF PIM TREATED WITH LIVER AND KIDNEY LYSATE……..104

FIGURE 4.10 TLC OF PIM TREATED WITH WHOLE KIDNEY LYSATE OVER 12 HOURS……..105

FIGURE 4.11 TLC OF PIM TREATED WITH WHOLE LIVER LYSATE OVER 12 HOURS……..106

CHAPTER 1: LITERATURE REVIEW

1.1 Mycobacteria

The genus *Mycobacterium* contains over 170 species[1], including species that range from environmental saprophytes to species that cause significant disease in humans and animals. While only a fraction of mycobacterial species regularly cause infection and disease, the genus is well known for two historic human pathogens: *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium leprae*[2], the causative agents of tuberculosis (TB) and leprosy, respectively. With the exception of *M. leprae*, most human pathogens are contained within a subgroup of genetically closely related (>99%) mycobacteria called the *Mtb* complex[2]. Of particular interest, the *Mtb* complex contains the pathogenic species *Mtb*, *Mycobacterium bovis*, and *Mycobacterium canetti*[3].

Additionally, a group of opportunistic pathogens referred to as nontuberculous mycobacteria (NTM), cause disease most frequently in immunocompromised patients[4]. Some NTMs are environmental mycobacteria, such as *Mycobacterium chimaera*, which is found in soil, as well as contaminated healing and cooling units[5]. *Mycobacterium chelonae,* another NTM, has been isolated from tap water[6, 7] and also isolated from tattoo-associated dermal infections[8]. *Mycobacterium avium*, a pathogen known for causing pulmonary infection in patients with dysfunctional immunity or chronic lung disease, has been identified as causing infection in otherwise healthy individuals engaging in hot tub usage; these infections have been termed “hot tub lung”[9].

1.2 Mycobacterial Diseases

Mycobacterial diseases can have many different manifestations. *Mtb* and *M. bovis* infections lead to the formation of one or many granulomas. These granulomas are characterized by the presence of immune cells responding to the infection by effectively walling off the pathogen; however, this response may also result in inflammation and tissue damage[10, 11]. This can take place in one or more organ systems, with *M. bovis* more often disseminating throughout multiple organ systems. TB is largely a pulmonary disease, but pathology can also take place in extrapulmonary locations of the body, such as the vertebrae (Pott's disease) and in the joints (Poncet's disease)[12].

Other mycobacterial infections may not result in a well-defined granulomatous response, such as an *M. leprae* infection that can be either high bacterial burden (lepromatous and non-granulomatous) or low bacterial burden (tuberculoid with granulomas)[13, 14]. Likewise, *Mycobacterium ulcerans* causes Buruli ulcers, a predominantly dermatologic disease that results in skin necrosis due to the mycolactone toxin released by the bacteria; Buruli ulcers are associated with granulomas, particularly when expression of IFN-γ is high[15].

Specifically *M. bovis* infections have a propensity to develop "tuberculosis pearls," clusters of lesions that are the hallmark of miliary TB. Miliary TB is defined as disseminated infection wherein the bacteria proliferates and spreads throughout the body, infecting more than one organ system[16]. Miliary TB is known to occur as a result at least two conditions: immune depletion, such as in AIDS patients, or due to the virulence of the pathogen, such is the case

with *M. bovis* infection[17, 18]. The organ systems most commonly involved in miliary TB are the liver, lungs, bone marrow, kidneys, adrenal glands, and the spleen[19].

Treatments of mycobacterial diseases are dependent on the infecting *Mycobacterium* species and the inherent drug resistance of that pathogen. Treatments are usually cornerstoned by administration of antibiotics, with the potential for surgical intervention[20]. The four antibiotics isoniazid, ethambutol, rifampicin, and pyrazinamide are the first line drugs to treat drug-susceptible TB. These antibiotics target mycolic acid synthesis[21], cell wall biosynthesis[22], bacterial RNA polymerase[23], and membrane energetics[24, 25], respectively. The use of these antibiotics becomes limited when the bacterial strain or species is resistant to one or more of these drugs. *M. bovis* specifically is naturally resistant to pyrazinamide, as it does not produce pyrazinamidase, the enzyme responsible for activating the antibiotic[26]. Antibiotic intervention is also a lengthy process, as antibiotics are given for a minimum of 6 months, up to 2 years for persistent or drug-resistance cases[20]. Side effects from these therapeutic drugs can also be damaging and may include deafness, hepatitis, exanthema, severe nausea, and weight loss[27].

1.3 Epidemiology of Bovine Tuberculosis

The United States of America originally took steps to eradicate bovine tuberculosis (bTB) when the Bovine Tuberculosis Eradication Program was created in 1917[28]. Annually, 15 million dollars are utilized to combat the occurrence and transmission of bTB. The establishment of nation-wide pasteurization of dairy products during the tenure of the bTB Eradication Program decreased the incidence of bTB in humans in the United States[29]. Additionally, the bTB

Eradication Program implements stringent post-abattoir testing of carcasses to be consumed as meat. This also decreases the public health risk. Although this program has been successful in decreasing bTB prevalence from approximately 5% of US herds to <0.001% of US herds today[28], annual outbreaks still occur in cattle herds, wildlife, and humans[30-32].

A contributing factor for the sustained circulation of *M. bovis* is the current diagnostic test for bTB. The diagnosis of bTB is immune-based and inherently permissive to false positives and more importantly false negatives, which allow the pathogen to remain in the population[33]. Those at greatest risk for human infection with *M. bovis* are typically those in close contact with cattle herds (slaughterhouse employees, ranchers, large-animal veterinarians, etc.); however, cases also arise from consumption of "raw" (unpasteurized) dairy[34-37]. It also has been suggested that bTB is transmissible from person to person, similar to classical TB[38-40]. The actual prevalence of human TB due to *M. bovis* has yet to be truly identified and surveillance of *M. bovis* infection in humans and animals is lacking worldwide.

Statistics directly related to human infections with *M. bovis*, commonly referred to as "bovine tuberculosis" are underreported in many countries and can be more difficult to find than statistics related to prevalence of classical TB (infection caused by *Mtb*). In most cases, human infections are not identified by mycobacterial species and are automatically treated as *Mtb*. Incidence of *M. bovis* infection in cattle and man alike are higher in the developing world. The World Health Organization estimates that over 120,000 human cases of bTB occur worldwide annually, including over 10,000 human deaths due to bTB[41]. This is approximately 1% of the overall global TB burden reported by the US Centers for Disease Control (CDC) as 10.4 million new TB infections in 2016 and 1.7 million TB-related deaths[42]. The CDC also reports the

common statistic that an estimated one third of the world's population is infected with TB, largely latently however; and this population is also not differentiated between *Mtb* and *M. bovis* infected. The substantial amount of the population that is infected with mycobacteria underscores the highly adapted nature of the *Mtb* complex to human physiology and immunology.

In the United States, the burden of TB is smaller than in developing countries. In 2016, only 9,272 TB cases were reported in the US[43]. Of these cases, *M. bovis* is reported to be responsible for 1.4% of human TB cases. Yet, this is variable depending on the area surveyed[44]. A retrospective study based in San Diego, California, identified *M. bovis* as the cause of 45% of child TB cases and 6% of adult TB cases[45]. This study also compared mortality rates between *Mtb* TB human cases and *M. bovis* TB human cases, finding that TB caused by *M. bovis* was more likely to be fatal, a claim that is reasonable given what we know about the pathological and virulence differences between *Mtb* infection and *M. bovis* infection. Cases of *M. bovis* infection in cattle have been reported in every continent aside from Antarctica[46]. As such, this pathogen is relevant to international livestock industry, as well as a public health concern worldwide.

The host range of *M. bovis* is incredibly vast, with reported infections occurring in cattle and humans, as well as African lions[47, 48], white-tailed and red deer[49-56], and the European badger[57-60], in addition to many other mammalian species[52, 61-72]. A broad array of both domesticated and non-domesticated animals have been reported to be susceptible to *M. bovis* infection. The large host range of *M. bovis* creates an additional level of complexity in control of disease transmission, since this allows for generation of wildlife reservoirs[64, 73]. Although

cattle herds are regularly tested in the developed world for bTB, wildlife reservoirs largely go untested and continue to seed infection in domestic animal populations.

1.4 Pathology of *M. bovis* Infection

The pathology of an *M. bovis* infection is much akin to the pathology of *Mtb*-related disease, with some fundamental differences. Perhaps the most notable similarity is that both are granulomatous diseases. The granuloma is a dynamic structure composed primarily of macrophages and lymphocytes that wall off the site of infection from the rest of the body. T lymphocytes, B lymphocytes, dendritic cells, neutrophils, and fibroblasts assemble around the site of infection[74, 75]. These cells create a lymphocytic cuff, forming the wall of the granuloma[76]. This effectively walls off the infection from the rest of the body, albeit not killing the *Mycobacterium* spp., but containing the pathogen[77]. In terms of *Mtb* infection, this status would be classified as latent infection. On the contrary, *M. bovis* infection is not thought to be latent at any time, but rather slowly progressive. Granulomas in cattle are classified as stage I-IV, with stage I microscopic lesions being visible in 7-15 days post-experimental infection in cattle receiving 10^5 bacilli[78]. Stage I to stage IV granulomas differ in cellular composition, degrees of necrosis and caseation, and extent of fibrosis on the outer cuff[78]. By 60 days post-experimental infection, stage IV granulomas (mineralized and necrotic) are present. Stage I-III of granulomas may also be present in a tissue at this timepoint[79]. Granulomas of bTB also differ in cytokine expression, immune cell content, and iNOS levels, with stage IV granulomas showing a drop in levels of iNOS and CD8+ and γ/Δ T cells, which has been suggested as indicative of host failure to control the infection[78].

M. bovis is considered a more virulent pathogen than *Mtb* and has a greater proclivity to dissemination[80]. Dissemination occurs via two main pathways: haematogenous or lymphatic[81]. If the granuloma erodes into small blood vessels, bacilli may be released and spread through a haematogenous route[82]. If the granuloma erodes into a lymphatic vessels or if bacilli are spread through trafficking of phagocytic cells through the lymphatic system, lymphatic dissemination to other tissues occurs[83]. Bovine TB has also been called Pearl's disease because dissemination of the pathogen results in myriad small caseating granulomas, otherwise known as TB pearls[80]. In bTB, the primary lesion is often in the lungs, but haemotagenous spread leads to lesions in the liver, kidneys, meninges, serous cavities, and in the udders (when infection is in cattle). Because of this, *M. bovis* bacilli are shed through every orifice of the infected animal, in milk, urine, feces, and mucus[80, 84]. This allows for a broader selection of clinical samples for the development of new bTB diagnostics

1.5 Diagnostics of bTB

The current standard surveillance assay for bTB in cattle is analogous to a current diagnostic used for TB in humans in many developed countries, where TB vaccination with BCG does not occur. This diagnostic assay is an immune-based skin test, termed the Mantoux tuberculin skin test (TST), which relies on a predictable and robust immune response[85]. A positive diagnosis is based upon a strong antigen-specific delayed-type hyper sensitivity response.

In the case of bTB testing, the "antigen" is an extract of sterilized *M. bovis* Strain AN-5 filtrate containing a mixture of many proteins and denatured peptides[86, 87]. This mixture is called the purified protein derivative (PPD). Originally developed by Robert Koch in 1890,

tuberculin, the predecessor of PPD, was intended as a potential vaccine[88]. Tuberculin was not an effective vaccine and caused adverse inflammatory reactions in people exposed to *Mtb*. These adverse reactions were identified, however, to have diagnostic value. In the early 20th century Felix Mendel and Clemens von Pirquet worked independently to develop the first tuberculin-based skin test for diagnostic purposes. Inconsistency and batch variation led to mixed and sometimes false results[88].

In 1939, Henry Phipps Institute researcher Florence B. Seibert identified the major active compound in tuberculin as a protein and that the current preparation methods resulted in varied preparations with variable concentrations of the active proteins and contaminating polysaccharides and other macromolecules. Seibert refined the method of tuberculin production to improve consistency and purity and called it PPD[89]. Seibert's PPD showed improved consistency and standardization compared to classical tuberculin. In 1952, the World Health Organization accepted Seibert's standardized PPD as the international standard[90]. However, bovine tuberculin does is made by multiple manufacturers internationally and preparation is cruder than that of human tuberculin, varying between countries[91, 92].

If an individual has been exposed to mycobacteria, the subcutaneous injection of PPD should result in a type IV hypersensitivity response, also known as a delayed type hypersensitivity response. The reaction takes 48-72 hours to occur and is a cell-mediated response to the antigens in PPD. If the immune system has seen the antigens before and no immunocompromisation exists, CD4+ T_h1 helper T cells will recognize the antigen, as it presented complexed with MHC class II on the surface of antigen-presenting cells. CD4+ T cells will respond by secreting IL-3 and IFN-γ, which signals for more CD4+ T_h1 cells to be trafficked

to the region, further amplifying the immune signaling cascade[93]. This immune cascade results in erythema and dermal thickening around the site of injection, which is then measured and classified as positive if the swelling is beyond the size threshold (in mm) for a positive reading[85]. This threshold varies contingent upon the population being testing, i.e. presence of co-morbidities, immune status, age, etc.

Complications with this diagnostic exist. First, a robust and predictable immune response is necessary for this diagnostic to be efficacious. If an animal is immunocompromised, whether due to genetics or comorbidities, this robust immune response is dampened and a false negative results from this anergy. This is relevant to cattle in the United States, as the common liver fluke (*Fasciola hepatica*) is also endemic in the US. A liver fluke infection results in immune depletion that interferes with the mounting of a robust immune response to the PPD[94], thereby allowing for false negatives to stay in the population, perpetuating infection in the herd and potentially seeding infection in wildlife reservoirs.

Additionally, the PPD has shown to have substantial cross reactivity with multiple *Mycobacterium* species, including non-pathogenic environmental saprophytes. Nontuberculous mycobacteria contain macromolecules that are antigenically similar to those in *M. bovis* PPD. This can lead to false positives if an animal has been exposed to a nontuberculous *Mycobacterium* spp., such as *M. avium-intracellulare* complex, *M. haemophilum*, *M. scrophulaceum*, etc.[95]. Human or animal vaccination with the Bacillus Calmette-Guérin (BCG) vaccine also causes reactivity to the PPD test, invalidating its results[96]. There has been discussion and experimentation on vaccinating animals with BCG, both

domestic and wildlife, to assist bTB eradication efforts, but this leads to the same issue as human BCG vaccination: loss of the skin test diagnostic as a means to diagnose infection[97].

False positives can result in unnecessary financial loss to livestock producers, as these animals are culled. Additionally, a positive skin test is not indicative of active infection, rather it is indicative of exposure, and therefore follow-up testing is necessary where it is fiscally reasonable. Animals who are deemed positive for *M. bovis* are culled to protect the herd. Furthermore, false positives can be generated on skin tests by simple itching, via allergic reaction, host response variation (including age), and variation in the PPD preparation[98].

Other, more molecular, methods to diagnose bTB are available, though implemented less frequently. Bovigam (Europe), an interferon-gamma release assay, is an *in vitro* cellular diagnostic wherein blood is drawn and the immune cells in the serum are stimulated by mycobacterial antigens *in vitro* to assay for the release of IFN-y[99, 100]. A serological assay licensed in the US, the DPP VetTB Chembio assay, utilizes plasma, serum, or whole blood with recombinant mycobacterial antigens to assess for antibody presence[56]. Both of these assays negate the need for a secondary visit to assess for delayed-type hypersensitivity (DTH) response and are faster than the TST. They also negate the potential for sensitization to the assay itself and the possible negative consequences of repeat testing on the same animal. The results from these more molecular assays are more quantitative and clear cut than skin test results, and yet these assays still rely on robust and predictable immune response. Standardization of the antigenic preparations can still be variable and therefore problematic.

Other methods of diagnosis include post-mortem testing, bacterial culture, and other varieties of molecular testing. The post-mortem testing is facilitated by slaughterhouse employees or meat quality inspectors assessing the carcasses for signs of disease. Inspectors assess for presence of granulomas in the carcass, usually present in lymph nodes, lungs, spleen, liver, and the superficial linings of body cavities. This is relatively effective, although it poses a health risk to those inspecting carcasses and granulomas may be missed if they are too small to be seen with the naked eye.

Bacterial culture is considered the gold standard for diagnosing bTB[99]; however, isolating *M. bovis* from a clinical sample is a lengthy and difficult process, taking between several weeks to months to isolate, grow, and biochemically characterize the microbe[101]. Other competing microbes grow much faster than *M. bovis* and samples must be decontaminated from other bacteria before *M. bovis* can be isolated. The decontamination process can reduce the viability of the *M. bovis* in the specimen, further complicating this method of detecting bTB. Lastly, molecular testing has been used to identify *M. bovis* and more commonly *Mtb*[102-104]. Researchers have attempted to use nucleic acid amplification and PCR to identify *M. bovis* and some studies have demonstrated that PCR detection is more sensitive than bacterial culture[105, 106]; however this technique has yet to become commonplace and efficacy has largely been tested post-mortem[107].

1.6 Lipids as biomarkers of disease

A biological marker, i.e. a biomarker, is defined as a medical characteristic that can be measured accurately and reproducibly and utilized as a measure of normal biological processes,

as well as pathologies, including to diagnose and predict outcome of disease[108]. For the purpose of this publication, we will focus on diagnostic biomarkers, or biomarkers that distinguish a diseased from non-diseased status. An example of this is the current method of screening for type II diabetes, wherein plasma glucose and glycated hemoglobin levels are assessed after a period of fasting[109]. Diagnostic biomarkers have also been investigated for utilization in assessment of cancers and genetic disorders, such as cystic fibrosis[110, 111]. A biomarker example relevant to infectious disease would be the use of galactomannan detection in serum to diagnose invasive infection of *Aspergillus*[112, 113]. Tests that function by detection of a genomic mutation or genomic material from an infectious organism are another example of biomarker-based assays.

As mycobacteria are lipid-rich organisms and possess some unique lipids, the potential for the use of a mycobacterial lipid as a biomarker was present. The concept of lipids as biomarkers is not novel. High density lipoprotein (HDL) -cholesterol concentrations have been correlated to cancer risk in women[114]. Similarly, HDL cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol, and triglyceride content serve as biomarkers of cardiovascular health[115]. Omega 3- and omega-6 polyunsaturated fatty acids have been investigated as a biomarker of major depressive disorder[116]. In the same vein, phospholipids, fatty acids, oxysterols, and cholesterols have been investigated as biomarkers for Alzheimer's disease, likely stemming from the pathological dysregulation of lipid metabolism[117].

Lipid signatures have also been established to track cyanobacteria that form environmentally harmful blooms in the Baltic Sea. To trace the spatiotemporal distribution of *Aphanizomenon* and *Dolichospermum*, the composition of fatty acid methyl esters, normal and

mid-chain branched alkanes, and bacteriohopanepolyols is determined using a Bligh Dyer lipid extraction technique prior to mass spectrometry analysis[118]. Relevant to tuberculosis diagnostics are the studies focused on assessing a mycobacterial lipoglycan, lipoarabinomannan (LAM) as a potential diagnostic biomarker. Studies have failed to reach a consensus on the efficacy of LAM as a biomarker; and studies suggest the usefulness of LAM as a diagnostic biomarker is limited to certain populations[119].

Children, who are unable to produce sputum for a sputum smear test, were investigated as a population that may benefit from a LAM-detection diagnostic. In a study of 61 children who were suspected to be TB positive, LAM detection in urine with a cutoff of 0.98 mg/L was shown to have 83% sensitivity and 85% specificity[120]. Higher cutoff values resulted in a decrease in sensitivity and specificity[121]. LAM has shown the greatest efficacy as a diagnostic biomarker in the context of patients co-infected with *M. tuberculosis* and HIV, and in patients with disseminated tuberculosis[122-124]. Recent publications have shown improvements to this assay. When LAM detection was assessed via a capture ELISA, in conjunction with detection of mycobacterial cell wall markers on gas chromatography mass spectrometry, namely α-D-arabinofuranose and tuberculostearic acid, sensitivity and specificity was improved. Sensitivity of the GC/MS assay was >99% and specificity = 84%. The capture ELISA had a sensitivity of 98% and a specificity of 92%[125]. LAM has also been investigated as a serum biomarker of bTB, showing similar efficacy detecting *M. bovis* infection as to what has been reported with *Mtb*[126, 127].

The clear advantage that a pathogen-derived biomarker diagnostic would have over the currently utilized immune-response based diagnostics is avoiding the complication of an

inadequate or overly robust immune response. This would decrease the number of false positives due to anergy, as the diagnostic would not hinge on immune response. Likewise, if the biomarker is species-specific, concerns pertaining to cross-reactivity with other mycobacterial species no longer apply. A successful example of this is the use of PGL as a biomarker for *M. leprae* infection. *M. leprae* PGL has been identified in the sera and urine of leprosy patients[128-130]. Antibodies to the *M. leprae* PGL have been assessed in saliva as a biomarker of transmissibility of *M. leprae*[131]. Similarly, *M. avium* infection status has been probed in terms of presence of antibodies to the species-specific glycopeptidolipid (GPL) core[132]. Antibodies to the GPL core have been found in the sera of patients with *M. avium* infections[133].

Lastly, mycolic acids have been used in conjunction with detection of mycobacterial DNA for purposes of TB in human remains[134]. As stable biomarkers that can survive thousands of years intact, mycolic acids have been established as powerful diagnostic biomarkers for paleoepidemiologic purposes. Mass spectrometric detection of mycolic acids has also been investigated in patient sputum and lung tissue of infected mice[135]. In these studies, mycolic acids have shown some efficacy as biomarkers of infection.

1.7 Mycobacterial Lipids

Approximately 40% of mycobacterial dry cell weight is comprised of cell wall lipids[136]. As such, mycobacteria are very lipid-rich bacteria. These lipids have been studied extensively. In addition to the mycobacterial lipids mentioned above (LAM, GPL, and mycolic acids), two lipids will be introduced here, as they are the focus of this work. First, the mycobacterial glycolipids

phosphatidylinositol mannosides (PIMs) have some structural similarity to the phosphatidylinositol found in eukaryotic cell walls, in that they contain a domain with a diacylglycerol linked via a phosphate to inositol; however, the mycobacterial PIM structure is also capped with 1 to 6 mannose residues. Up to two additional long chain fatty acids can be esterified to either the inositol residue or a mannose of PIM. As such, PIMs can be characterized by their number of fatty acid chains and the number of mannoses on the molecule, ranging from PIM_1 to diacyl (Ac_2) PIM_6[137]. The most abundant PIM in the mycobacterial cell wall is Ac_2PIM_2, which contains two acyl chains (one on the inositol sugar and one on a mannose), in addition to the two fatty acids esterified onto the glycerol backbone[138]. Ac_2PIM_2 is shown in Figure 1.1. Thus far, all *Mycobacterium* spp evaluated produce PIMs; thus PIM species are not specific to any single *Mycobacterium* species[136].

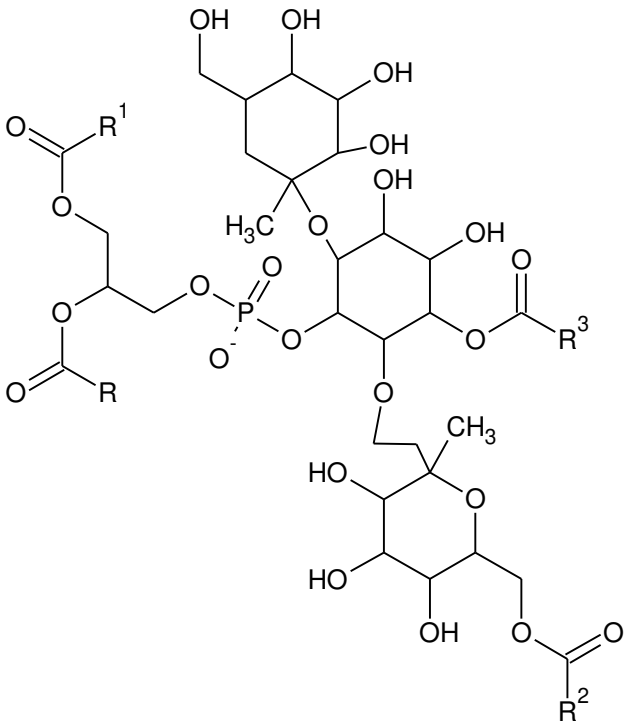

Figure 1.1: Diacyl-phosphatidylinositol dimannoside (Ac_2PIM_2) shown here, containing two acyl chains (R^2 and R^3) ester bound to a mannose and the inositol, respectively. Also two fatty acids (R and R^1) are ester bound to the glycerol backbone.

Another mycobacterial glycolipid, phenolic glycolipid (PGL) has species-specificity in some *Mycobacterium*. PGLs are produced by a handful of *Mycobacterium* spp including, but not limited to, *M. leprae, M. bovis,* and *M. canetti*[139]. The PGL produced by *M. bovis* (Figure 1.2) is functionally species-specific, and was originally the product termed "mycoside B." *M. canetti* has been reported to produce the same PGL species as *M. bovis* in very minute concentrations. The *M. bovis* PGL found in *M. canetti* is thought to be a biosynthetic intermediate or a product of breakdown of the more abundant triglycosyl PGL in *M. canetti*, the sugar region of which is shown in figure 1.3 for comparison to *M. bovis* PGL[140, 141]. All PGL species are based on a backbone of phenol-phthiocerol, upon which two mycocerosic acids are ester bound. Mycocerosic acids are methyl branched fatty acids. The species-specificity of

PGLs is attributed to the carbohydrate structures, glycosidically bound to the phenol. The saccharide moiety of *M. bovis* PGL is a 2'-O methyl rhamnose[142]. Because this is the region with inherent specificity, the glycosyl motif was targeted for specific detector development, in the research described in chapter three of this dissertation. The mass of PGL measures between 2-2.5% of the dry cell weight of *M. bovis* BCG cells[143]. With so much of the cell being lipids, this lends to the idea of finding a relatively specific and abundant lipid to use as a biomarker.

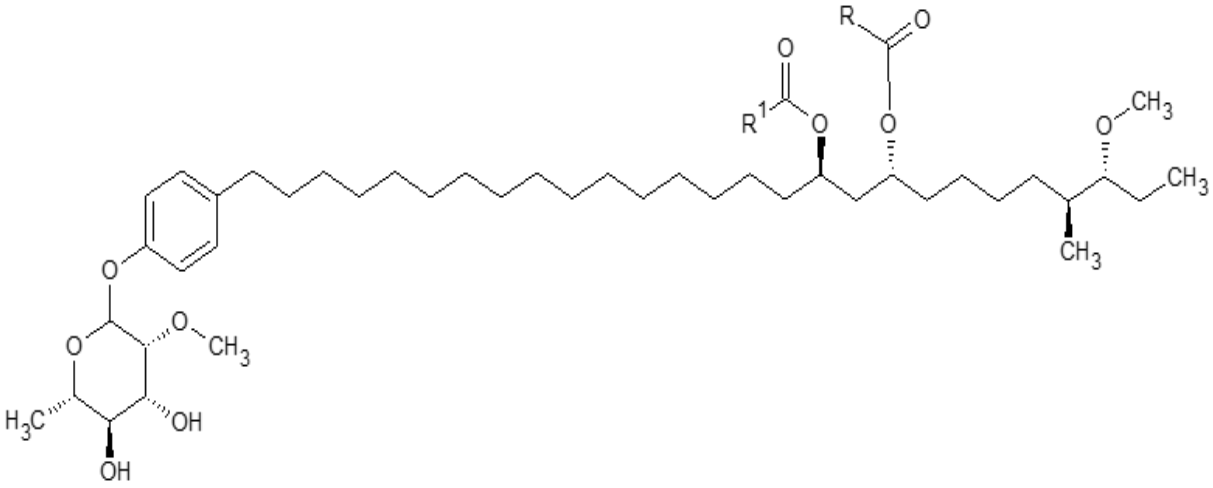

Figure 1.2: *Mycobacterium bovis* phenolic glycolipid. This structure is composed of a C27 phthiocerol backbone, two variable length mycocerosic acids, and a 2-O-methyl rhamnose glycosidically linked to the phenol.

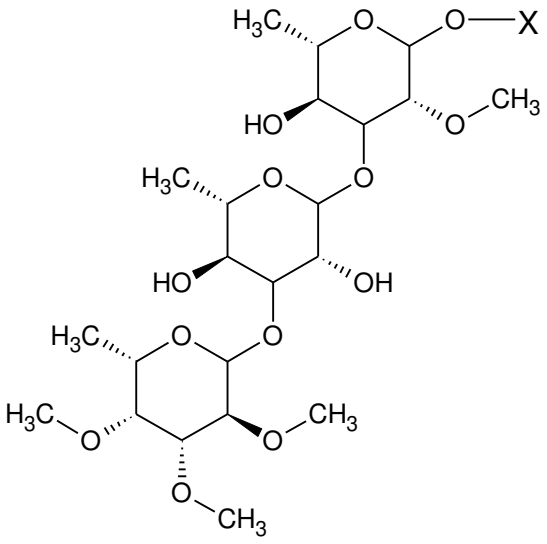

Figure 1.3: *Mycobacterium Canetti* phenolic glycolipid sugar moiety. X depicts where the triglycosyl moiety is glycosidically bound to the phenol-pthiocerol backbone. The sugar region of the *M. Canetti* PGL is 2,3,4-tri-O-methyl fucose-rhamnose-2-O-methyl rhamnose.

1.8 Detection of lipids

1.8.1 Thin Layer Chromatography

There are a host of methods for detecting and quantifying lipid species. Prior to more advanced instrumentation, lipids were historically detected on thin layer chromatography. Thin layer chromatography (TLC) methodology was developed in the first decade of the 20th century by Italian scientist Mikhail Tswett, who published a set of papers on the method and its usefulness in separating photosynthetic pigments in plants[144-146]. By adsorbing a sample onto a plate coated in silica, then applying a mobile phase with different polarity to the system, it was discovered that molecules were separated by relative polarity using TLC. Lipids have been analyzed this way and many different stains have been developed to visualize molecules with different biochemical functional groups[147, 148]. TLC is one way to assess for the presence of phenolic glycolipid and this method has been utilized for this purpose for many decades[142, 143,

[149-154]. TLC has even been explored as a means to identify *M. bovis* by its lipid profile[155]. TLC is used in this publication as an alternative to mass spectrometry for the detection of PGL, utilizing an α-naphthol stain, specific for carbohydrate-containing molecules[156].

1.8.2 Analytical instrumentation

In addition to thin layer chromatography, the literature is replete with lipid detection methods on mass spectrometry (MS) instrumentation. Two labs have independently created mycobacterial lipidomic databases via MS detection[157, 158]. Phenolic glycolipid, in particular, has previously been detected on several MS platforms, some of which are now obsolete. Gas chromatography mass spectrometry (GC-MS) was used in the 1990s to detect and analyze *tert*-butyldimethylsilyl derivatives of the aglycone region of PGL; this aided in analysis of synthetic intermediates of PGL in *M. microti*[159]. GC- electron impact MS and gas-liquid chromatography mass spectrometry were used to identify the mycocerosic acid substituents of PGL that are esterified to the pthiocerol backbone of the glycolipid[160, 161]. Matrix-assisted laser desorption mass spectrometry (MALDI-MS) has been used to measure synthesis of the disaccharide moiety of *M. leprae* PGL[162]. MALDI-MS has also been used to monitor synthesis of PGL-tb to determine which mycobacterial proteins were responsible for the *O*-methylation that takes place on the fucosyl residue[163] and was also used to generate a lipid biosignature of intact bacilli from the *Mtb* complex[164]. Proton nuclear magnetic resonance (NMR) methods have also been used to elucidate the structures of different PGL species[140, 159, 165-167]. Infrared spectroscopy has also been used for structural elucidation of mycobacterial lipids[168].

1.8.3 Antibodies

There is evidence that some lipids may be detectable with antibodies. Antibodies to lipopolysaccharide (LPS), an endotoxin present in the cell wall of gram-negative bacteria, have been identified. Specifically, antibodies to the lipid region (Lipid A) of LPS have been found and characterized[169]. Interestingly, in a study by Kuhn et al., murine monoclonal antibodies to Lipid A were characterized by the portion of Lipid A recognized, all antibodies mapped to the hydrophilic sugar backbone of Lipid A and none of them mapped to the acyl chains of the macromolecule[169]. Acylation of Lipid A did impact the physical spatial availability of the epitopes that interacted with the antibodies[169]. A human monoclonal antibody has also been developed against LPS endotoxin and furthermore investigated as a treatment for gram-negative bacterial sepsis. This human antibody also maps to the Lipid A region of LPS and was shown to increase survival rate of laboratory animals experiencing bacteremia[170].

As noted in section 1.6.1, numerous mycobacterial lipids are known antigens that induce a strong antibody response. Antibodies to a variety of mycobacterial lipids have been identified, including: the core of *M. avium* GPL[132, 133], *M. leprae* PGL[131, 150], *Mtb* trehalose-6, 6'-dimycolate[171, 172], and *Mtb* LAM[173]. Antibodies to *M. leprae*'s PGL-1 have been quantitated to assess for exposure to the pathogen. Anti-PGL-1 IgM and IgA antibodies were strongly indicative of transmission and have been suggested as having potential diagnostic value for leprosy[131]. Furthermore, when rabbit antiserum to *Mtb* phenolic glycolipid (PGL-Tb1) was assessed for specificity against four other purified mycobacterial PGLs, including *M. bovis* PGL, the antiserum did not react to other PGL species[150]. This antigenic nature indicates that the

production of monoclonal antibodies to these structures, as well as the relative specificity, could potentially be exploited.

One means to accomplish direct selection of a lipid binder is phage display, a method that utilizes recombinant antibody fragments displayed on the protein coat of a bacteriophage. In 1985, George Smith published his seminal paper on phage display, detailing the utilization of a large library of phage expressing variable peptides on the virion surface to pan for binders to a specific target[174]. Phage display has since been used for a variety of purposes including, but not limited to: to create binders to cell surface receptors that recapitulate the activity of the *in vivo* receptor ligands[175], to generate specific colorimetric biosensors[176], to select for phage that are biomimetics of endogenous compounds[177], to identify phage particles with therapeutic potential, particularly receptor agonists and antagonists[178-182], and to find phage that bind biomarker molecules[183-186], including lipid biomarkers[183]. Phage-display derived antibodies give scientists an additional means to specifically detect a lipid[187]. As phage display does not require animal immunization, animal culling, or myeloma hybridization, it was an attractive candidate method for selecting binders of *M. bovis* PGL.

1.9 Research rationale and summary of aims

The rationale behind this project was that the currently used diagnostic for bTB in the United States of America and other countries is not suitable to cease transmission of the pathogen, *M. bovis*. The tuberculin skin test (TST) is permissive to false negatives and false positives, which result in perpetuation of the transmission cycle via evasion of culling and unnecessary fiscal loss, respectively. It has been posited that improved diagnostics and

development of efficacious vaccines would improve control of the spread and perpetuation of *M. bovis*[188-190]. If a diagnostic were developed that negated necessity of a robust and predictable immune response, and rather relied upon detection of a highly abundant pathogen-derived biomarker, this novel diagnostic would be inherently less permissive to false negatives and false positives as it would eliminate a major source of variation: diversity in immune response between individuals and all the factors that can affect immune responses. Additionally, if the selected biomarker was species-specific, the updated diagnostic would be more specific than the TST, which is plagued by complications stemming from cross reactivity with saprophytic mycobacterial species.

PGL was previously investigated as a biomarker for infection with *M. leprae*[128, 129, 191]. Stemming from original work by Brennan and associates[128, 130, 149, 192], as well as the disseminated nature of *M. bovis* infections, we hypothesized that PGL produced by *M. bovis* could be a possible diagnostic biomarker of bTB and detectable in urine of naturally-infected cattle. We selected urine based on the facile and non-invasive nature of its acquisition. This dissertation delves into the feasibility of this glycolipid as a diagnostic biomarker by assessing the potential for detection, developing a specific detector of PGL, and contrasting the relative stability of this molecule in the host and through the kinetics of clearance. The specific aims devised to assess these questions and evaluate our hypotheses are as follows:

Specific Aim I: Assess the detectability of *M. bovis* PGL recovered from infected cattle urine on multiple mass spectrometry platforms (Chapter 2)

Specific Aim II: Select for a phage that is a specific binder to *M. bovis* PGL via M13 phage display (Chapter 3)

Specific Aim III: Assess the stability of *M. bovis* PGL within the host via cell free and *in vitro* studies (Chapter 4)

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CHAPTER TWO: DETECTION OF PHENOLIC GLYCOLIPID BY MASS SPECTROMETRY PLATFORMS

2.1 Introduction

Mycoside B, otherwise known as the phenolic glycolipid (PGL) of *M. bovis*, is a candidate biomarker of *M. bovis* infection (Figure 2.1). The *M. bovis* PGL is a highly abundant lipid, measuring between 2% and 2.5% of the cell dry weight[1, 2]. Many mycobacterial species produce phenolic glycolipids[2-5]; however the 2' O-methyl rhamnose carbohydrate moiety of mycoside B provides specificity for *M. bovis*. Due to the species-specificity of this glycolipid and its highly abundant nature, we selected PGL to investigate the feasibility of this lipid as a biomarker of bTB.

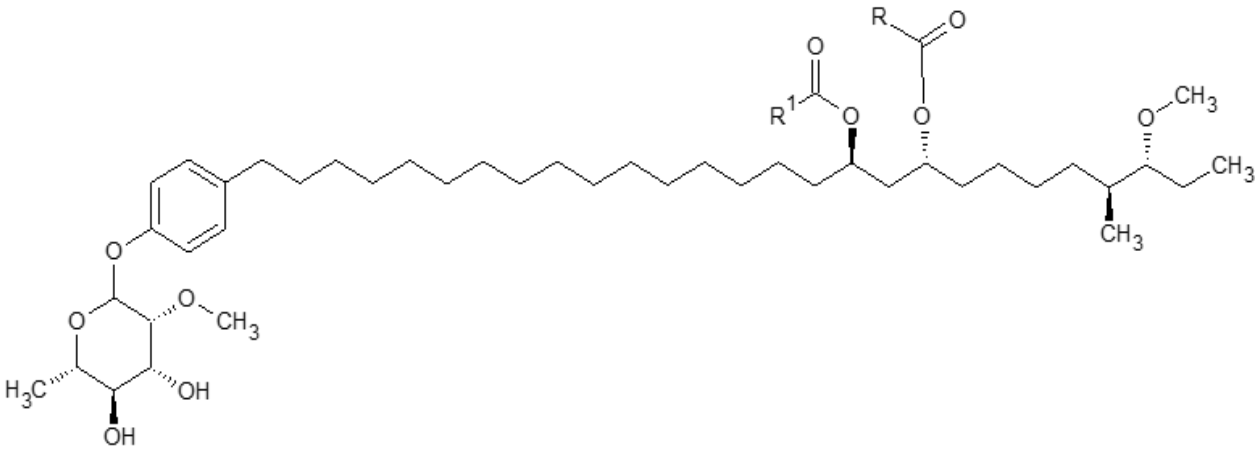


Figure 2.1: Phenolic glycolipid of *M. bovis.* Esterified to a phthiocerol-phenol backbone are two mycocerosic acids, which may vary in chain length, denotes here as R and R^1. A single 2'O-methyl rhamnose is glycosidically linked to the phenol, providing species-specificity for this glycolipid.

Analytical detection of PGL has been predominantly by thin layer chromatography[1-4, 6-12], but matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) has also been applied to detect and assess purified PGL [8, 13, 14]. To ascertain whether PGL is a potential biomarker and detectable in the urine of *M. bovis* infected cattle, we applied liquid chromatograph electrospray ionization mass spectrometry (LC-ESI-MS). In doing this, we also devised a method to enrich for PGL in cattle urine and created methodologies that overcame limitations of accurately detecting urine derived PGL by MS. As a proof-of-concept, we demonstrated that PGL is detectable in urine from infected cattle, and therefore holds promise as a biomarker for bovine tuberculosis.

2.2 Materials and methods

Chemicals and reagents

Methanol, acetonitrile, hexane, and MS-grade water were obtained from Honeywell Burdick & Jackson (Muskegon, MI, USA). Isopropanol, sodium hydroxide, and formic acid were obtained from Fischer Scientific (Pittsburgh, PA, USA). Chloroform was obtained from JT Baker (Center Valley, PA, USA). Glycerol trioleate was obtained from Sigma-Aldrich (St. Louis, MO, USA).

PGL purification

PGL was purified from lyophilized *M. bovis* BCG cells as previously described[15] with the following modifications. Lipids were overnight extracted from lyophilized *M. bovis* BCG cells kindly provided by Karen Dobos (CSU) using 10:10:3 chloroform:methanol:water on a stir plate at a concentration of 30 ml solvent per gram of cells. Cells were filtered with Whatman paper

twice. The organic filtrate was transferred to a round bottom flask and dried to 10 mL volume on a rotary evaporator. Concentrated lipid extract was combined with 90 mL cold acetone, and allowed to precipitate overnight at -20^{o}C. The precipitate was collected by centrifugation at 20,000 g for 30 minutes at 4^{o}C, dried under nitrogen gas, and suspended in 2:1 chloroform:methanol. The final lipid preparation was applied to 2 mm silica gel 60 preparative TLCs (Millipore Sigma; Darmstadt, Germany) with a mobile phase of 95:5 chloroform:methanol. PGL was extracted from the preparative TLC[16, 17].

Cattle urine samples

Filter-sterilized cattle urine collected post-mortem by catheterization from an infected cattle herd in Michigan was kindly provided by Jordi Torrelles of the Ohio State University. Confirmation of *M. bovis* infection was performed by histopathology, as well as culture and gross pathology.

Saponification and lipid enrichment

Two mL of cattle urine was treated with NaOH (final concentration of 200 mM) at 80^{o}C for 1 h. The solution was neutralized to pH 7 with acetic acid. The saponified urine was loaded onto a 6 cc (500 mg) Waters C8 Sep-Pak Vac © cartridge. The lipids were eluted sequentially with acetonitrile (2 mL), 6:4 acetonitrile:isopropanol (2 mL), 8:2 isopropanol:hexane (8 mL), and hexane (2 mL), under pressure. The final two fractions were combined, dried under nitrogen, and suspended in isopropanol (30 µL).

Mass Spectrometry – LC-ESI-TOF-MS

Chromatography was based on a method previously described[18] using a Poroshell 120 reverse-phase C8 2.7 µm column (2.1 mm x 100 mm; Agilent Technologies; Palo Alto, CA) and an Agilent 1200 series high pressure liquid chromatography (HPLC) system (Agilent Technologies; Palo Alto, CA). Sample injection volumes of 5 µL were used for limit of detection (LOD) and method detection limit (MDL) analyses, and sample injection volumes of 10 µL were used for clinical samples. PGL was eluted with a linear gradient of 100% acetonitrile, 0.1% formic acid to 100% 8:2 isopropanol:hexane, 0.1% formic acid (also called solvent B). The eluent was introduced directly into an Agilent 6220 Accurate-Mass TOF mass spectrometer equipped with an electrospray ionization source. The parameters for the mass spectrometer were as follows: positive mode ionization; fragmentor voltage, 250V; capillary voltage, 5.5 kV; column compartment temperature, 30^{o}C; gas temperature, 250^{o}C; gas flow rate, 6.5 L/min. Data were collected in both centroid and profile modes in 4-GHz high-resolution mode. MS data were processed in Agilent MassHunter Qualitative Analysis software to assess for PGL presence and relative abundance.

Mass Spectrometry – MALDI-MS

Urine lipid extracts were assessed for the presence of PGL by MALDI-MS mass spectrometer using a Bruker Microflex LRF as previously described[8]. Lipid extracted from urine (0.5 µL) was mixed 1:1 with 10 mg/mL DHB matrix in 1:1 chloroform:methanol, and 0.5 µL was crystallized on a MALDI plate at room temperature. Spectra were acquired in positive reflectron mode, with a total of 1500 shots. Laser power was set to 75%.

Data Analysis

Urine samples were defined as PGL positive if the intensity and/or peak area of the most predominant species of PGL were 3x greater than the limit of the blank, i.e. background signal observed at the same retention time and mass as PGL in a negative control. Isotopic peak patterns were assessed to distinguish between contaminating triglycerides and true PGL signal. Samples were denoted as equivocal if PGL peak intensity/peak area was less than 3 times the limit of the blank. Samples were negative if the intensity/peak area of PGL peak(s) were at or below the limit of the blank.

2.3 Results

ESI-TOF detection of PGL in comparison to MALDI-TOF detection

MS detection of the mycobacterial PGL have previously been demonstrated with a MALDI-time of flight (TOF) platform[8, 13, 14]. To assess whether detection by MS could be improved using a different ionization method, we compared the detection of purified PGL on an ESI-TOF mass spectrometer with the MALDI-TOF platform. As shown in Figure 2.1a and 2.1b, both platforms detected multiple species of PGL based on variations in the alkyl chain length. However, ESI-TOF MS provided greater mass resolution and accuracy than the MALDI-TOF MS, as is inherent in the parameters of these instruments. The sodium adducts of PGL predominated the MALDI-TOF spectrum with the most abundant species being observed at an *m/z* of 1530.309; this ion had a mass error of 34.97 PPM from the calculated sodium adduct of this PGL species (*m/z* 1530.363). In contrast, an ammonium adduct of the same PGL species (*m/z* 1526.407) was observed as the most dominant product by ESI-TOF MS 1526.407. This ion had a mass error of 5.15 PPM. The increased mass resolution provide by the ESI-TOF platform

allowed for the identification of multiple adducts for a single PGL species as well as the mono-isotopic and the ^{13}C isotopic masses (Fig. 2.2c)

To further assess these two MS platform for detection of PGL, the LOD was determined based on the signal generated for the most abundant PGL species. For this evaluation the ESI-TOF mass spectrometer was interfaced with an HPLC unit and the PGL resolved by reversed phase (RP) chromatography. LOD was defined by the Wisconsin Department of Natural Resources Laboratory Certification Program as the signal to noise ratio is greater than 5, i.e. the LOD is the concentration that generates a signal 5x greater than the limit of the blank[19] (n=3). The LOD for ESI-TOF was based on the *m/z* 1526.407 ammonium adduct species of PGL and was determined to be 1.95 ng (1.28 pmol; figure 2.3a). The *m/z* 1530.309 sodium adduct ion was used to establish the LOD for the MALDI-TOF platform as 10.0 ng (6.56 pmol; figure 2.3b). The limit of the blank, i.e. the noise generated by an injection of solvent only at the relevant retention time and *m/z*, on the ESI-TOF is shown for reference in figure 2.3c.

Because of the increased mass accuracy and resolution provide by ESI-TOF, as well as the lower LOD, this platform was selected for further evaluation of PGL as a potential biomarker that can be detected in cattle urine extracts. Additionally, high pressure liquid chromatography (HPLC) can be directly interfaced with the ESI-TOF platform. This would allow for an additional measure (retention time, RT) to increase the accuracy of PGL detection, as well as means to enrich for PGL in a complex biological sample.

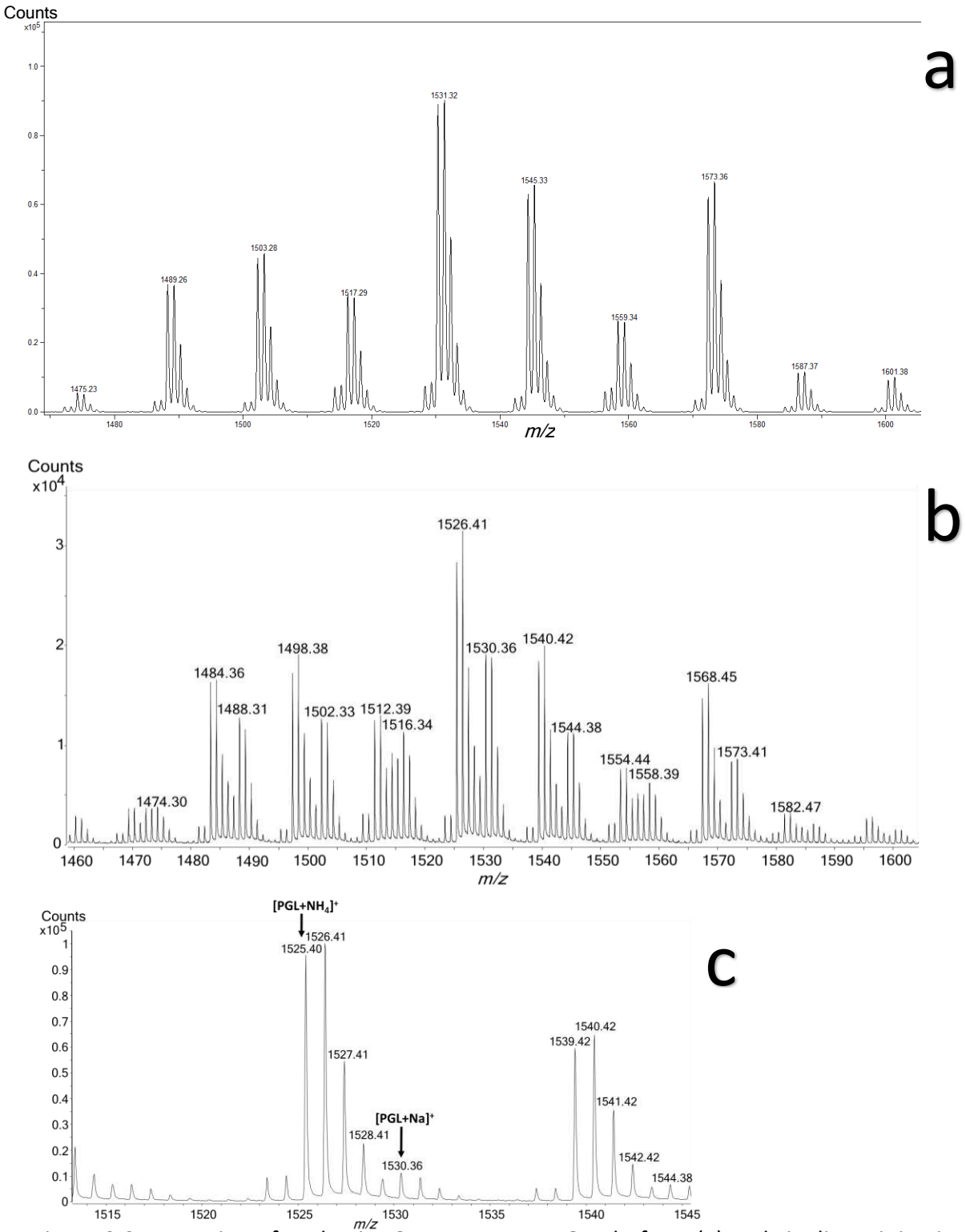
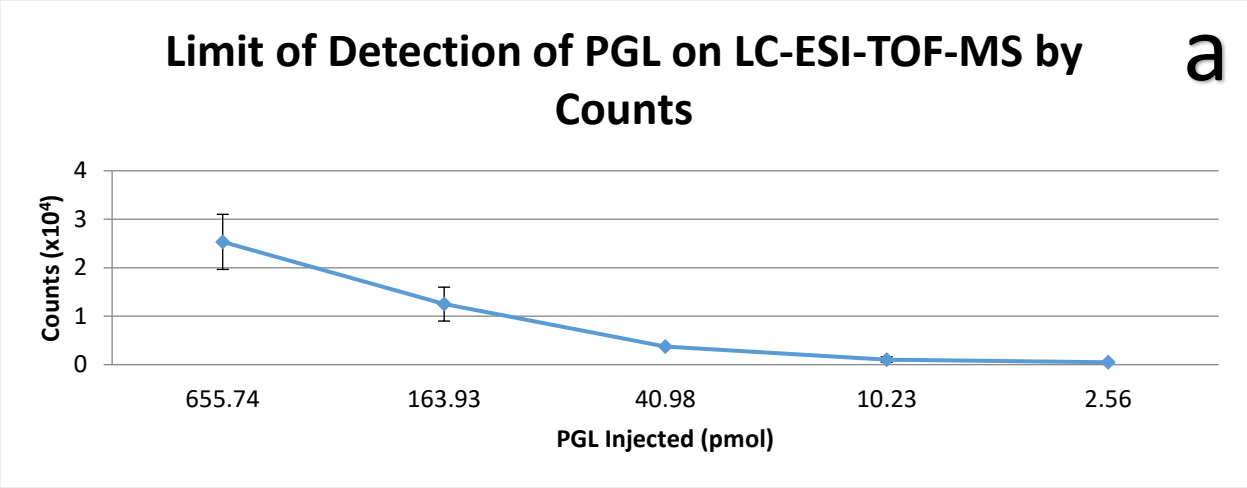
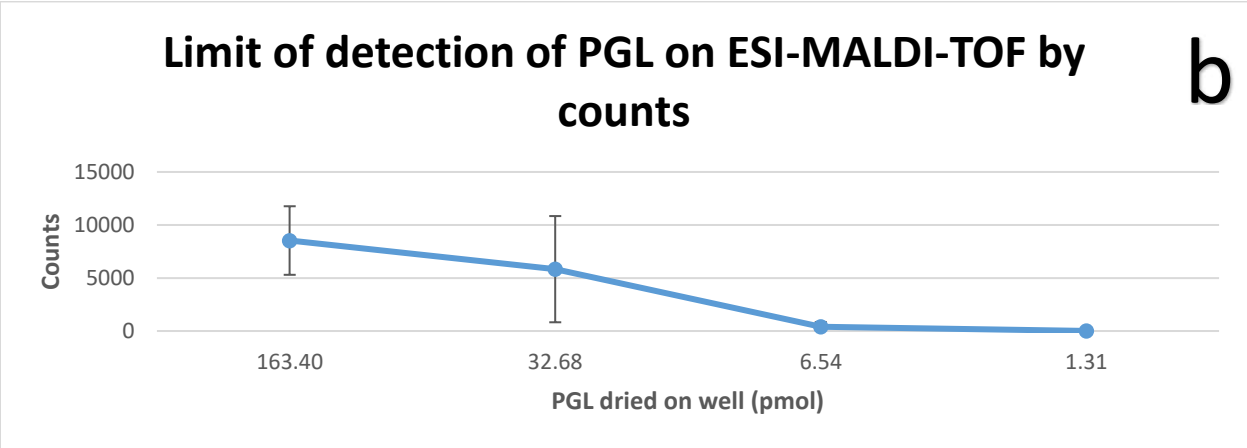
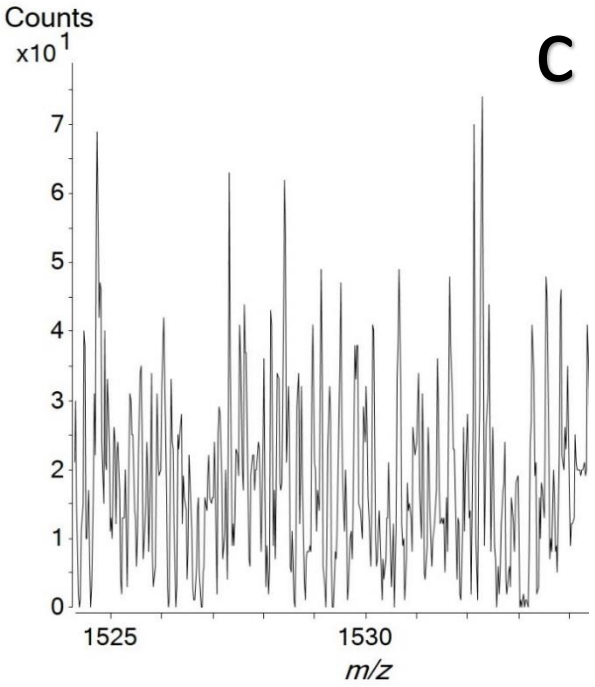


Figure 2.2: Detection of *M. bovis* PGL on a MALDI-TOF platform (a) and via direct-injection on an LC-ESI-TOF-MS platform (b). The most abundant ion on both platforms' spectra is the PGL species containing mycocerosic acids of C26 and C29 chain lengths. The length of the phthiocerol backbone in both these ions is C27. Two adducts of a single PGL species were revealed in the LC-ESI-TOF-MS spectra (c).





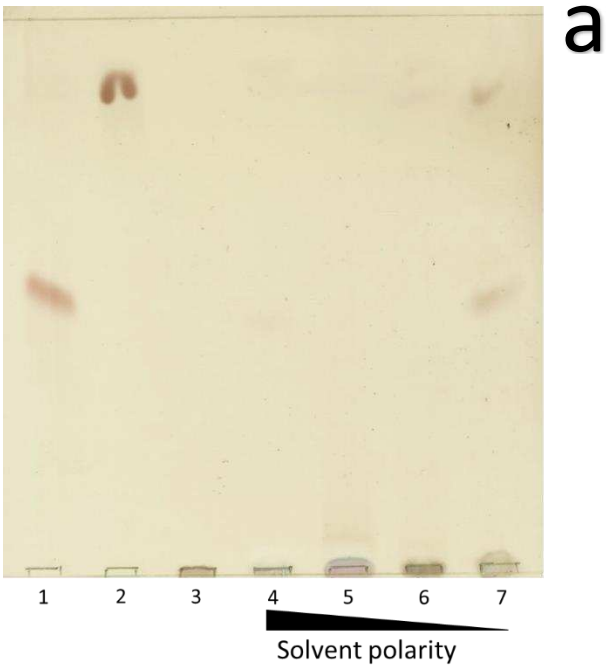


c

Figure 2.3: Limits of detection of PGL on two MS platforms. The LOD of PGL on LC-ESI-TOF-MS (a) and on MALDI-TOF (b). The noise generated by the injection of solvent only on an LC-ESI-TOF-MS platform, i.e. limit of the blank (c), was used to calculate LOD.

Enrichment for PGL from urine via a solid phase extraction and method detection limit

To reduce the complexity of the urine, as well as concentrate a biological sample to a volume compatible with LC-MS, solid phase extraction of PGL with a C8 solid phase extraction cartridge was optimized. By applying series of increasingly apolar solvents with various volumes, it was possible to reduce sample complexity of the sample and concentrate urine 100 fold. Specifically, PGL (50 μg) spiked into 2 ml of urine from healthy cattle was applied to a C8 solid phase extraction cartridge and recovered in two hydrophobic fractions, isopropanol:hexane (8:2) and hexane (Fig. 2.4). These fractions were combined, dried, and suspended in 30 μl of isopropanol. A large percentage (97%) of the PGL yielded off the cartridge was recovered in the combined isopropanol:hexane (8:2) and hexane eluants, as measured by LC-MS based on the extracted ion chromatogram for the peak area of the *m/z* 1530.36 ion (data not shown).



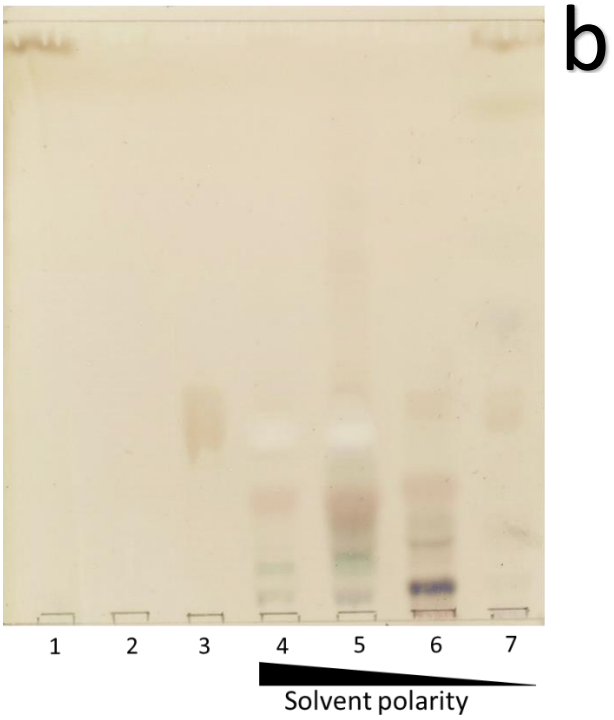


Figure 2.4: TLC of standard lipids and solid phase extraction (SPE) of PGL-spiked bovine urine using mobile phases of 95:5 chloroform:methanol (a) and 60:30:6 chloroform:methanol:water (b). (1) PGL; (2) triglyceride; (3) phosphatidylcholine; (4) SPE flow through; (5) SPE fraction 1: acetonitrile; (6) SPE fraction 2: 6:4 acetonitrile:isopropanol; (7) SPE fractions 3 and 4 combined: 8:2 isopropanol:hexane, and 100% hexane.

With the establishment of a PGL enrichment protocol, a method detection limit (MDL) was determined for PGL in urine based on LC-MS detection. The MDL was defined by the US Environmental Protection Agency as a minimum concentration at which an analyte can be detected with 99% confidence that the analyte is present in the sample[20]. MDL was calculated by contrasting the average peak area of the most abundant PGL species spiked samples versus method blanks, i.e. unspiked, pooled negative urine (n=7). As with determination of the LOD, the ion corresponding to the most abundant PGL species (*m/z* 1530. 363 sodiated adduct) was used to establish an MDL of 15.63 ng, (10.21 pmol) (Fig 2.5).

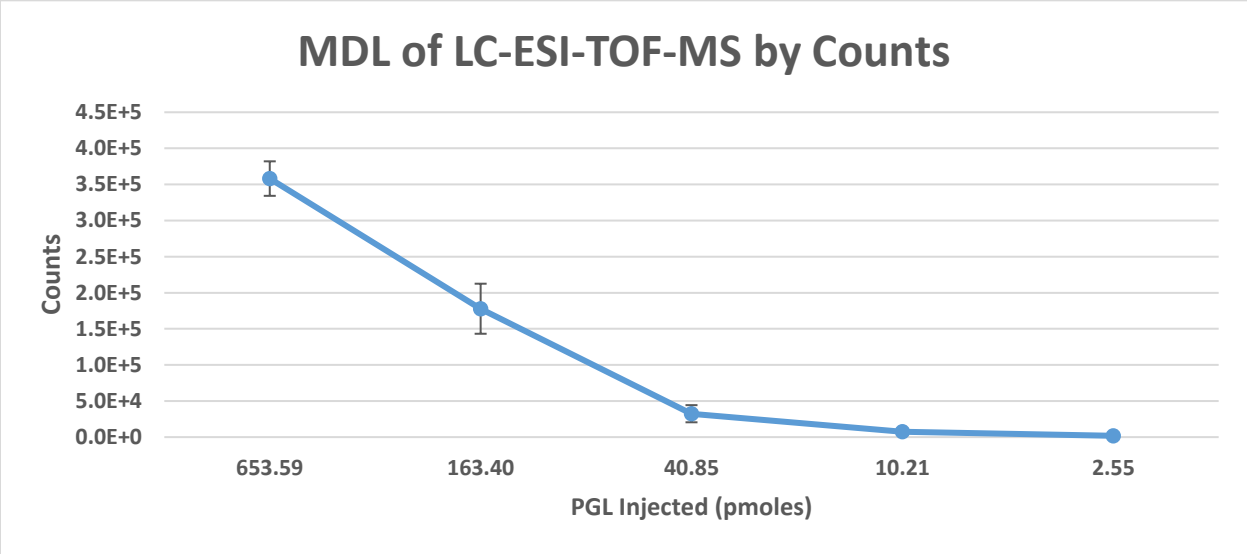


Figure 2.5: Method detection limit of *M. bovis* PGL on LC-ESI-TOF-MS is established at 10.20 pmols, or 15.63 ng within a 5 µL injection volume.

Saponification sample pretreatment improves detection of PGL by decreasing interfering signals

During the analysis of urine extracts by LC-MS, it was observed that host endogenous triglyceride species resulted in ion species with *m/z* values similar to those for the PGL species. Additionally, these triglycerides had a RT similar to PGL, further confounding data analysis.

These interfering triglycerides could be identified by the different isotopic peak they produced in comparison to PGL (Fig. 2.6). To address this potential limitation to accurate PGL detection in urine, saponification was evaluated as a pretreatment to reduce the ion signal from triglycerides.

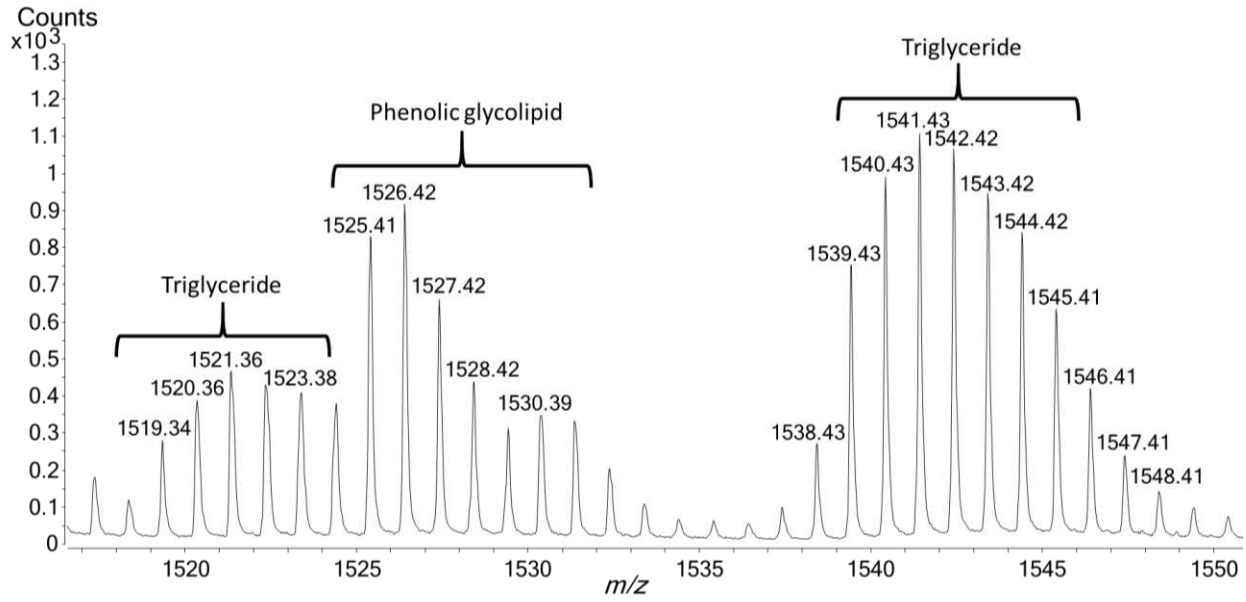


Figure 2.6: LC-MS of pooled, spiked cattle urine. Interfering signal from host triglycerides complicates detection of PGL in a spiked positive control sample of pooled urine. The signal generated by triglycerides occurs at the same retention time and similar *m/z* values. Triglyceride signal can be distinguished from PGL signal based on isotopic peak ratios, wherein PGL species show the largest peak with a single ^{13}C, *m/z* 1526.42 in spectra above.

A range of saponification conditions were evaluated and the minimal condition required to hydrolyze the triglycerides was determined to be treatment for 1 h with 200 mM sodium hydroxide at 80^{o}C. This protocol reduced the triacylglycerol ion signal by 99.6% as calculated by the ion peak area (Fig 2.7a and b). In contrast, PGL subjected to the same treatment did not result in a significant loss in detection by LC-MS (Fig 2.7c and d).

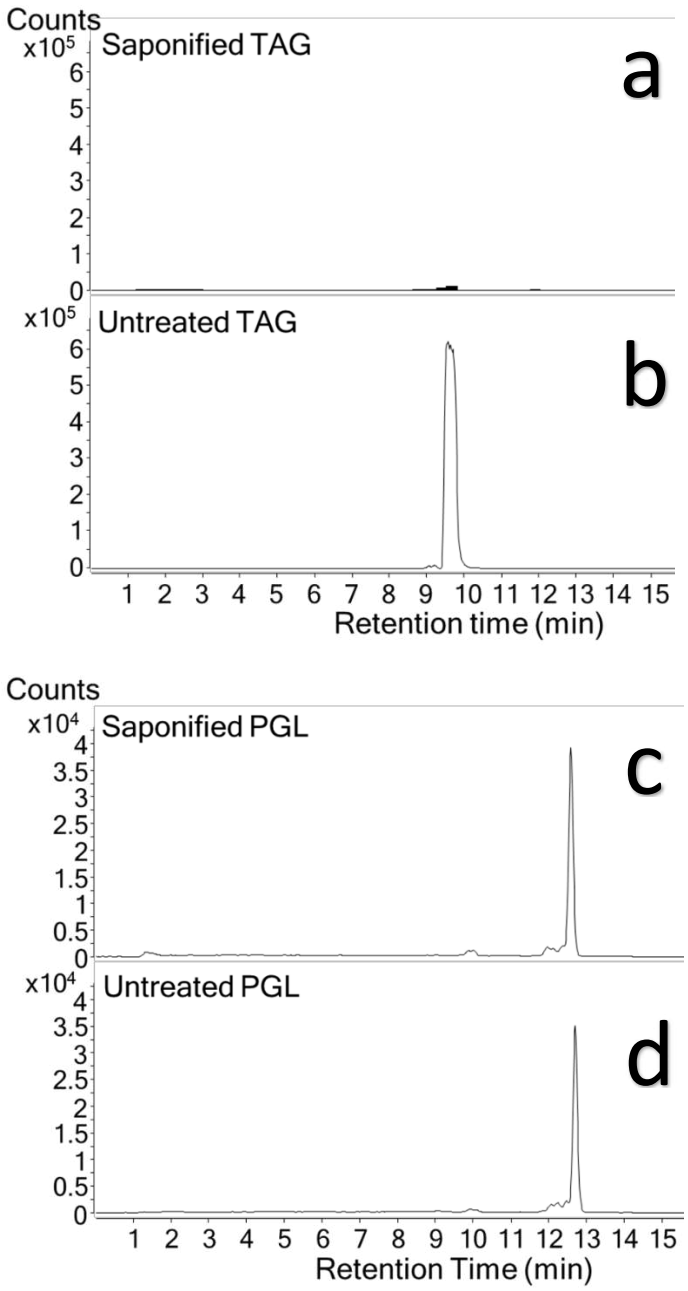


Figure 2.7: LC-MS of treated (a) and untreated (b) triacylglyceride standard, and treated (c) and untreated (d) PGL. With saponifying pretreatment, the amount of triglyceride, as measured by peak area of the extracted ion chromatogram (*m/z* 905.82), is reduced by 99.6%. Extracted ion chromatograms of PGL are for *m/z* 1526.40.

Detection of PGL in urine extracts of cattle naturally infected with *M. bovis*

Urine was obtained from 40 head of cattle that were suspected of bovine tuberculosis and confirmed to be infected with *M. bovis* upon slaughter. Additionally, urine collected from ten head of uninfected cattle were evaluated for comparison and as a negative control. Infected urine was filter sterilized prior to receipt by our lab for the analysis for the presence of PGL. Aliquots of urine (2 ml) were subjected to saponification and SPE, and the resulting fractions predicted to possess PGL were analyzed by LC-ESI-MS. For an animal to be considered positive for PGL presence, the detected product ion was required to have a RT between 12 and 13 min, a PPM error of < 10, and a minimum counts of 5x the noise at RT = 12-13 min and *m/z* of the negative control. The minimum counts was based on the results from the MDL. Based on these criteria PGL was positively detected in 5 of the 40 urine samples from infected animals, and in none of the urine from the 10 uninfected animals.

Figure 2.8 provides the LC-MS the spectrum of an animal determined to be PGL positive. Specifically, the sodium adducts of the C29/C26 and C29/C27 PGL species were detected above the MDL, and equivocal signals (below the MDL cutoff) were observed for the C30/C27 and C27/C27 PGL species. When all the animals' spectra were manually interrogated, six additional samples from the infected cohort were classified as equivocal (i.e. an ion series corresponding to a PGL species was detected at the correct retention time; however the signal was below the MDL). Figure 2.9 is an example of the LC-MS spectrum from an animal designated as equivocal for PGL presence. None of the uninfected animal were found to be equivocal for the presence of PGL. Results of each clinical sample are summated by PGL species in Table 2.1.

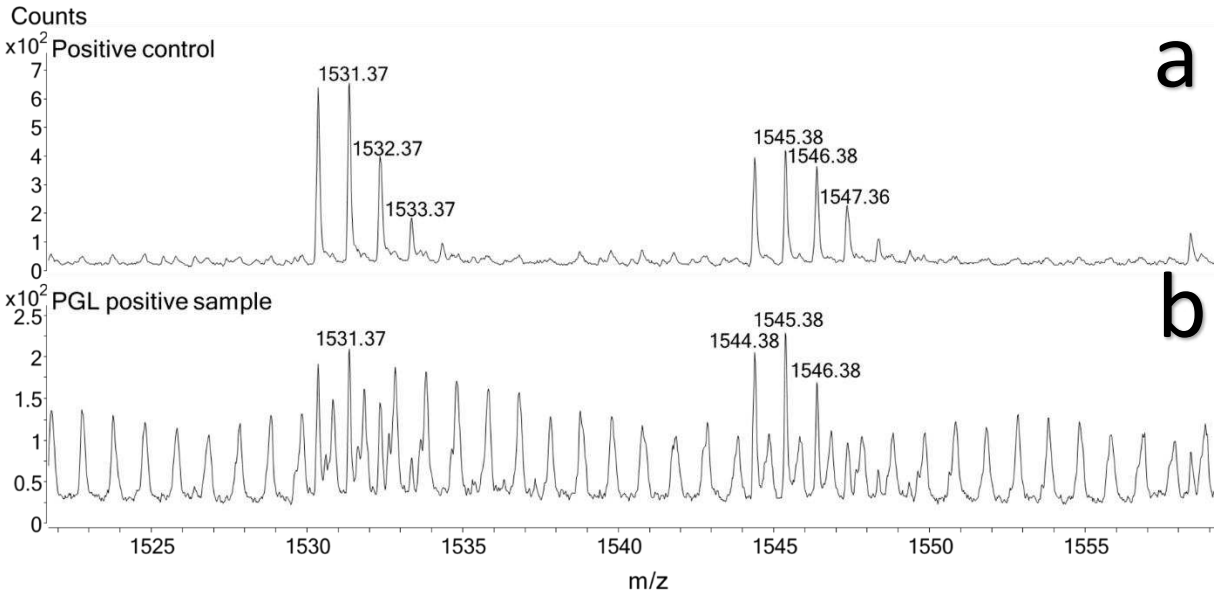


Figure 2.8: LC-MS spectra of a positive clinical sample at a retention time of 12-13 minutes (panel a) compared with a spiked positive control at the same retention time (panel b). Two PGL species are reliably identified in this sample, PGL C29/C26 and C29/C27. Triglyceride signal was reduced by saponification; however, TAG signal is still present at the retention time where PGL was found and PGL species C27/C27 and C30/27 were deemed equivocal due to this interference.

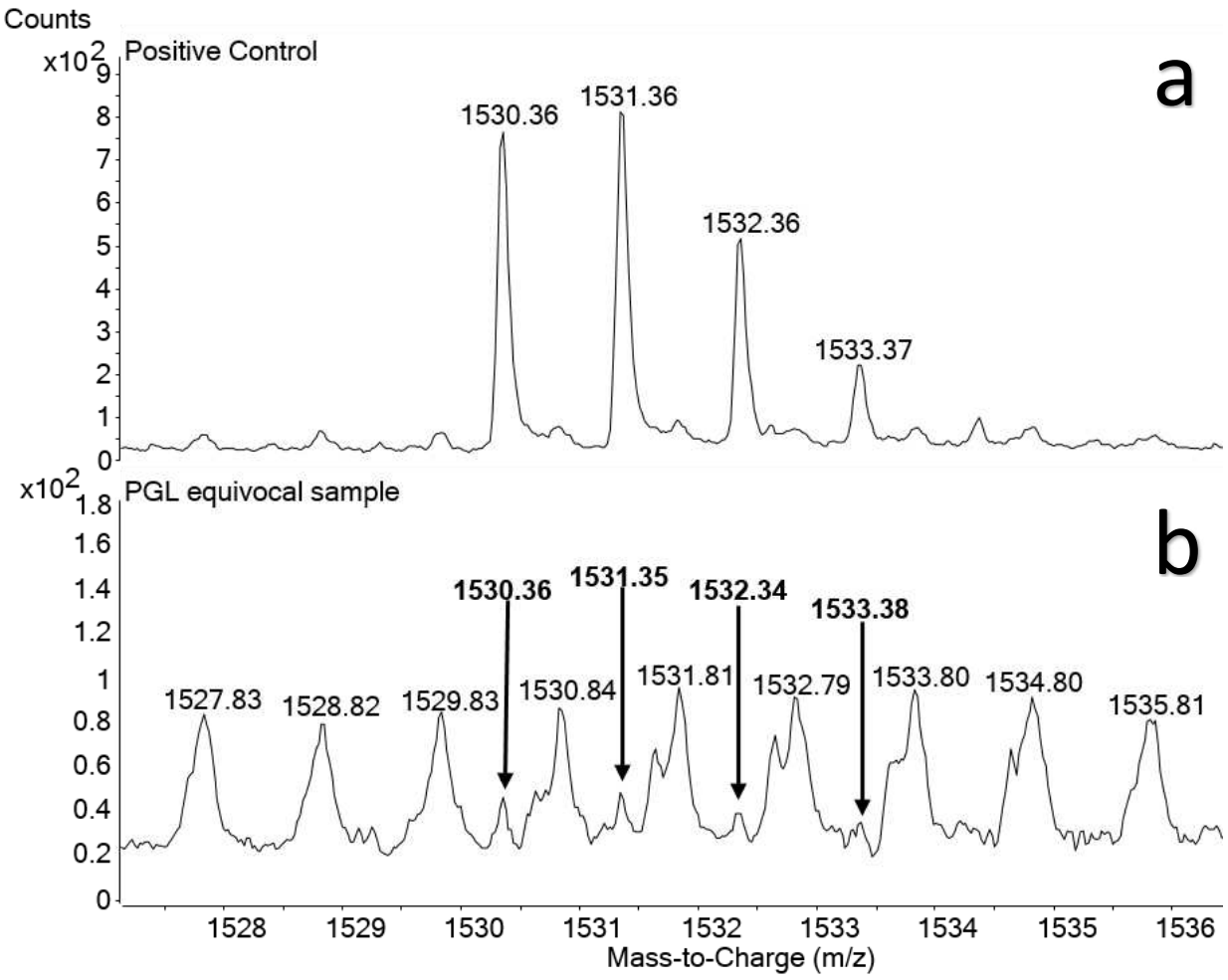


Figure 2.9: LC-MS spectra of an equivocal clinical sample at a retention time of 12-13 minutes (panel b) compared with a spiked positive control (panel a). Panel b is a representative example of the animals that had no conclusive positive signals for any PGL species, but did generate equivocal signals for one or more PGL species. The bolded arrows and *m/z* values denote PGL signals.

Table 2.1: Summation of clinical sample results

Cow #	PGL species (mono mass)	Monoisotopic (PPM and counts)	$^{13}C_1$ (PPM and counts)	$^{13}C_2$ (PPM and counts)
1	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
2	1530.363	PPM = 11.9; counts = 32 EQUIVOCAL	PPM = 10.0; counts = 30 EQUIVOCAL	PPM = 12.9; counts = 28 EQUIVOCAL
	1544.378	Negative	Negative	Negative
5	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
7	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
8	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
10	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
11	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
12	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
13	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
15	1530.363	PPM = 13.3; counts = 24 EQUIVOCAL	PPM = 10.1; counts = 28 EQUIVOCAL	PPM = 6.7; counts = 22 EQUIVOCAL
	1544.378	PPM = 10.2; counts = 19 EQUIVOCAL	PPM = 8.1; counts = 18 EQUIVOCAL	PPM = 29.3; counts = 16 EQUIVOCAL
16	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
17	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
18	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
19	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
20	1530.363	Negative	Negative	Negative

	1544.378	Negative	Negative	Negative
21	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
22	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
23	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
24	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
25	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
26	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
27	1530.363	PPM = 8.8; counts = 40 EQUIVOCAL	PPM = 25.7; counts = 43 EQUIVOCAL	PPM = 11.0; counts = 27 EQUIVOCAL
	1544.378	PPM = 5.7; counts = 35 EQUIVOCAL	PPM = 31.5; counts = 40 EQUIVOCAL	PPM = 39.4; counts = 32 EQUIVOCAL
28	1516.346	PPM = 0.3; counts = 80 POSITIVE	PPM = 1.1; counts = 80 POSITIVE	PPM = 10.6; counts = 62 POSITIVE
	1530.363	PPM = 2.2; counts = 107 POSITIVE	PPM = 0.5; counts = 105 POSITIVE	PPM = 0.4; counts = 80 POSITIVE
	1544.378	PPM = 1.5; counts = 111 POSITIVE	PPM = 1.0; counts = 128 POSITIVE	PPM = 5.8; counts = 96 POSITIVE
	1572.409	PPM = 6.9; counts = 60 POSITIVE	PPM = 8.6; counts = 62 POSITIVE	PPM = 10.9; counts = 58 POSITIVE
29	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
30	1516.346	PPM = 1.8; counts = 90 POSITIVE	PPM = 3.3; counts = 85 POSITIVE	PPM = 0.9; counts = 60 POSITIVE

	1530.363	PPM = 1.3; counts = 200 POSITIVE	PPM = 1.1; counts = 220 POSITIVE	PPM = 1.4; counts = 150 POSITIVE
	1544.378	PPM = 3.0; counts = 215 POSITIVE	PPM = 1.0; counts = 235 POSITIVE	PPM = 3.2; counts = 176 POSITIVE
	1558.394	PPM = 3.9; counts = 85 POSITIVE	PPM = 4.8; counts = 82 POSITIVE	PPM = 13.5; counts = 90 POSITIVE
	1572.409	PPM = 2.1; counts = 122 POSITIVE	PPM = 2.9; counts = 165 POSITIVE	PPM = 4.2; counts = 115 POSITIVE
31	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
32	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
33	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
34	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
35	1516.346	PPM = 6.1; counts = 46 POSITIVE	PPM = 1.2; counts = 59 POSITIVE	PPM = 11.1; counts = 39 POSITIVE
	1530.363	PPM = 3.7; counts = 105 POSITIVE	PPM = 3.8; counts = 115 POSITIVE	PPM = 5.7; counts = 70 POSITIVE
	1544.378	PPM = 0.1; counts = 118 POSITIVE	PPM = 1.7; counts = 142 POSITIVE	PPM = 8.9; counts = 95 POSITIVE
	1558.394	PPM = 3.4; counts = 52 POSITIVE	PPM = 4.4; counts = 58 POSITIVE	PPM = 12.8; counts = 56 POSITIVE
	1572.409	PPM = 1.8; counts = 70 POSITIVE	PPM = 2.8; counts = 75 POSITIVE	PPM = 1.3; counts = 56 POSITIVE
36	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative

37	1516.346	PPM = 4.0; counts = 300 POSITIVE	PPM = 3.0; counts = 285 POSITIVE	PPM = 2.8; counts = 180 POSITIVE
	1530.363	PPM = 4.2; counts = 800 POSITIVE	PPM = 3.4; counts = 880 POSITIVE	PPM = 5.9; counts = 505 POSITIVE
	1544.378	PPM = 3.9; counts = 790 POSITIVE	PPM = 3.7; counts = 880 POSITIVE	PPM = 4.0; counts = 505 POSITIVE
	1558.394	PPM = 5.6; counts = 195 POSITIVE	PPM = 7.6; counts = 235 POSITIVE	PPM = 12.5; counts = 150 POSITIVE
	1572.409	PPM = 7.0; counts = 275 POSITIVE	PPM = 5.4; counts = 305 POSITIVE	PPM = 4.9; counts = 180 POSITIVE
38	1530.363	PPM = 5.6; counts = 98 POSITIVE	PPM = 3.0; counts = 103 POSITIVE	PPM = 7.0; counts = 72 POSITIVE
	1544.378	PPM = 0.3; counts = 107 POSITIVE	PPM = 0; counts = 108 POSITIVE	PPM = 4.9; counts = 80 POSITIVE
	1572.409	PPM = 11.3; counts = 74 POSITIVE	PPM = 9.5; counts = 83 POSITIVE	PPM = 4.0; counts = 80 POSITIVE
39	1530.363	PPM = 5.2; counts = 30 EQUIVOCAL	PPM = 15.7; counts = 36 EQUIVOCAL	PPM = 26.4; counts = 30 EQUIVOCAL
	1544.378	PPM = 35.8; counts = 30 EQUIVOCAL	PPM = 16.7; counts = 28 EQUIVOCAL	PPM = 56.7; counts = 30 EQUIVOCAL
40	1530.363	PPM = 1.1; counts = 41 EQUIVOCAL	PPM = 1.9; counts = 40 EQUIVOCAL	PPM = 2.7; counts = 41 EQUIVOCAL
	1544.378	Negative	Negative	Negative
41	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative

42	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
43	1530.363	PPM = 1.9; counts = 44 EQUIVOCAL	PPM = 5.6; counts = 50 EQUIVOCAL	PPM = 1.0; counts = 40 EQUIVOCAL
	1544.378	PPM = 16.6; counts = 43 EQUIVOCAL	PPM = 18.0; counts = 40 EQUIVOCAL	PPM = 1.2; counts = 34 EQUIVOCAL
44	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative
45	1530.363	Negative	Negative	Negative
	1544.378	Negative	Negative	Negative

2.4 Discussion

In this chapter, we have established that the phenolic glycolipid of *Mycobacterium bovis* is detectable in naturally-infected cattle urine; and therefore PGL may be a potential biomarker of bovine tuberculosis. Via detection on an LC-ESI-TOF-MS platform, the method detection limit is 13 ng, which translates to approximately 5.13×10^{12} molecules of PGL. Each *M. bovis* cell is approximately 2% PGL (dry mass). This means that 1.89×10^{6} cells would provide a sufficient amount of PGL to detect on an LC-ESI-TOF-MS. In terms of urine volume, assuming a comparable amount of bacilli in cattle urine as to what has been reported in badger urine[21, 22], this would equate to 6.28 mL of urine. In this work, we used 2 mL aliquots of urine. It is likely that with 6.28 mL of urine, additional samples would have been positive.

A fundamental hindrance to using mass spectrometry to detect PGL however, is the interference from signal generated by host-derived triacylglycerides. Using a base saponification to minimize this background, in conjunction with a solid-phase extraction lipophilic enrichment, we were able to detect phenolic glycolipid from naturally-infected cattle

urine samples on an LC-ESI-TOF-MS platform. When we contrasted our methods for detecting PGL on an LC-ESI-TOF-MS platform with a MALDI-TOF-MS platform, we found that MALDI-TOF-MS was not feasible for detection of PGL derived from urine of naturally infected cattle, as the limited volume of sample one can load on a MALDI chip was confirmed to be too small to detect PGL concentrated from cattle urine at anticipated physiological concentrations. Detection of infected urine-derived PGL on a MALDI-TOF platform may be possible if a larger volume of urine were concentrated and applied to the MALDI chip. As the LOD of the sodiated adduct of PGL on the MALDI-TOF was established at 10 ng per well, i.e. 40 ng per µl (as the sample is diluted 1:2 in MALDI crystallization matrix DHB and only ½ µL is dried per well), this equates to 600 ng per mL of urine. With only 2 mL of urine, even if the urine extract was concentrated down to 1 µL, rather 30 µL, this would still be 20 ng/mL urine, a concentration higher than the expected physiological concentrations of PGL excreted by sub-clinical cattle with low bacterial titers.

As such, of the methods assessed, an LC-ESI-TOF-MS platform was the most efficacious method for detecting urine-derived PGL on a mass spectrometer, particularly when small volumes of urine are utilized. When this detection method is used in succession with the solid phase enrichment method, detection of PGL is possible. Furthermore, when sample pretreatment is used to hydrolyze endogenous triacylglycerides, detection is improved through the associated reduction in background. This study has supported the notion that PGL can potentially serve as a diagnostic biomarker for bovine tuberculosis, providing a new possible target for accurate diagnosis of this zoonotic disease. This is a substantial step towards

validating a new diagnostic target, as urine-derived PGL from naturally infected cattle has not been detected previously.

Nonetheless, there are substantial limitations from using any of these mass spectrometry platforms in the scope of a diagnostic. Mass spectrometry is not feasible as a field diagnostic, which would be most advantageous for national surveillance and minimizing transmission of *M. bovis*. Additionally, the volume of urine used in this study is much smaller than urine volumes used in previous studies detecting mycobacterial PGLs. Yet the smaller volume is more relevant to a field diagnostic and allows for easier sample pretreatment than a larger volume. Perhaps one of the most problematic hindrances in this study was the sterilization method used for the infected urine. The urine samples used here were all filter-sterilized, which is not ideal due to the potential for loss of material on the filter. Gamma irradiation would have been preferable for the purposes of detecting PGL; however these samples were filter sterilized prior to shipment to our lab for analysis. We were limited in the amount of samples, depth of clinical information about the infected cattle, and the sterilization and packaging of the urine; nonetheless, the proof-of-concept detailed here that naturally-infected cattle urine contains PGL is substantial.

Concerning continuing this project in the direction of development of a field diagnostic, a different means of detection must be applied. Potential detection mechanisms include the use of phage detector or the more common use of antibodies to detect an analyte, in this case PGL. Both phage and antibodies provide a means of specific and sensitive detection. Likewise, both of these detectors could be practically applied to a field diagnostic and can reach binding affinities in the nanomolar range. Phage, however, are more favorable because they are easier

to produce, requiring only *E. coli* culture to amplify the viral particles, rather than immunization of an animal[23-27]. Phage are also generally considered to be more stable than monoclonal antibodies[28] and can be genetically manipulated to fuse a reporter protein onto the phage, such as a GFP or luciferase[29-31]. It is possible that a lower limit of detection may be achievable with phage or antibodies than with mass spectrometry, as difficult ionization is not required for detection with phage or antibodies and resolution of PGL from contaminants is easier achieved with a more specific detector like phage or antibodies.

In summation, this study reveals that PGL of *M. bovis* may be a potential target for a pathogen-derived biomarker-based diagnostic. In terms of detection on MS platforms, the use of an LC-ESI-TOF-MS platform is more efficacious than MALDI-TOF for detecting PGL from small amounts of naturally-infected cattle urine. Using a SPE method in conjunction with a saponifying pretreatment, detection of PGL extracted from naturally-infected cattle urine is possible. PGL has thus far shown to be stable enough to survive host clearance intact, and unique enough to potentially develop a species-specific diagnostic through its detection.

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CHAPTER THREE: DETECTION OF *M. BOVIS* PHENOLIC GLYCOLIPID WITH M13 BACTERIOPHAGE

3.1 Introduction

With the knowledge that phenolic glycolipid (PGL) specific to *Mycobacterium bovis* is detectable in the urine of infected animals, the following series of experiments were designed to develop and test a biological detector that is both sensitive and specific to *M. bovis* PGL. The long term goal is the development of a diagnostic or test that could be applied as a point-of-care diagnostic. Thus, we sought to explore biological detectors that could be applied to a glycolipid structure.

Phage display, originally described in 1985 by George Smith, utilizes the basic principles of molecular interactions to probe for specific binders of a target molecule[1]. Phage display is based upon the fusion of small variable peptides to an envelope protein at one pole of the virion. Built into the design of this process is the fact that the phenotype and genotype are inherently coupled within the phage[2, 3]. When undergone with the proper selection conditions, counter selection, and controls, phage display has been an efficacious method for selecting a binder specific to a target with binding kinetics similar to or better than conventional hybridoma monoclonal antibodies[2, 4, 5]. One such successful instance of phage display that is currently a commercially available product for inflammation-related diseases on the international medical market is Humera®, also known as adalimumab[6-9]. However, phage display developed diagnostics remain absent in the diagnostic market.

The phage libraries used in this study are based on M13, one with a variable region of seven amino acids spliced into the pIII protein (the minor coat protein), and the second with a variable region of twelve amino acids spliced into the pIII protein. The pIII protein is present in five copies on one pole of the M13 phage and allows for binding of the phage to the host cell F pilus, mediating entry of the viral particle into the bacterial cell. The genetic engineering of these variable regions does not impact pIII function, as the variant peptides are located at the N terminus of the pIII protein, next to a short linker region, before the full, functional pIII sequence. Both the 7mer and 12mer libraries are provided at a diversity level of 10^9 different phage particles, only genetically deviating from each other in the variable regions described.

Key to this method is the ability to immobilize the target molecule. Once the target is sufficiently immobilized on a matrix, a large library of phage containing a variable region displayed on the virion surface can be washed over the target with increasingly stringent wash buffers to select for a tight binder of the desired target. Similar to hybridoma-based antibody development, phage display has traditionally been used to select for binders to hydrophilic, proteinaceous compounds. Therefore, critical adjustments were made to the standard protocol to allow for binding of the hydrophobic PGL to a 96 well titer plate, washing of the phage library over the target without elution of the target molecule, and elution of the bound phage from the target adhered to the titer plate without also eluting the target. Critically, the 96 well microtiter plate must have a hydrophobic surface capable of tightly binding the target lipid. Additionally, caution was implemented when detergents were used, as a concentration of detergent above a desirable threshold would dislodge the target lipid from the plate. Furthermore, although PGL may bind to the surface of hydrophilic cell culture type plates via its

glycosyl moiety, we speculated that it would be more advantageous to allow the acyl chains of the PGL molecule to be immobilized, as this presumably leaves the more biologically unique phenol 2'-OMe rhamnose region of the molecule to be available for interaction with the phage. Phage binding was measured via a microtiter plate based assay, wherein phage were recognized via an α-gpVIII antibody. A secondary antibody coupled to an HRP domain was utilized for quantitation. The final assay scheme is depicted by the cartoon in figure 3.1.

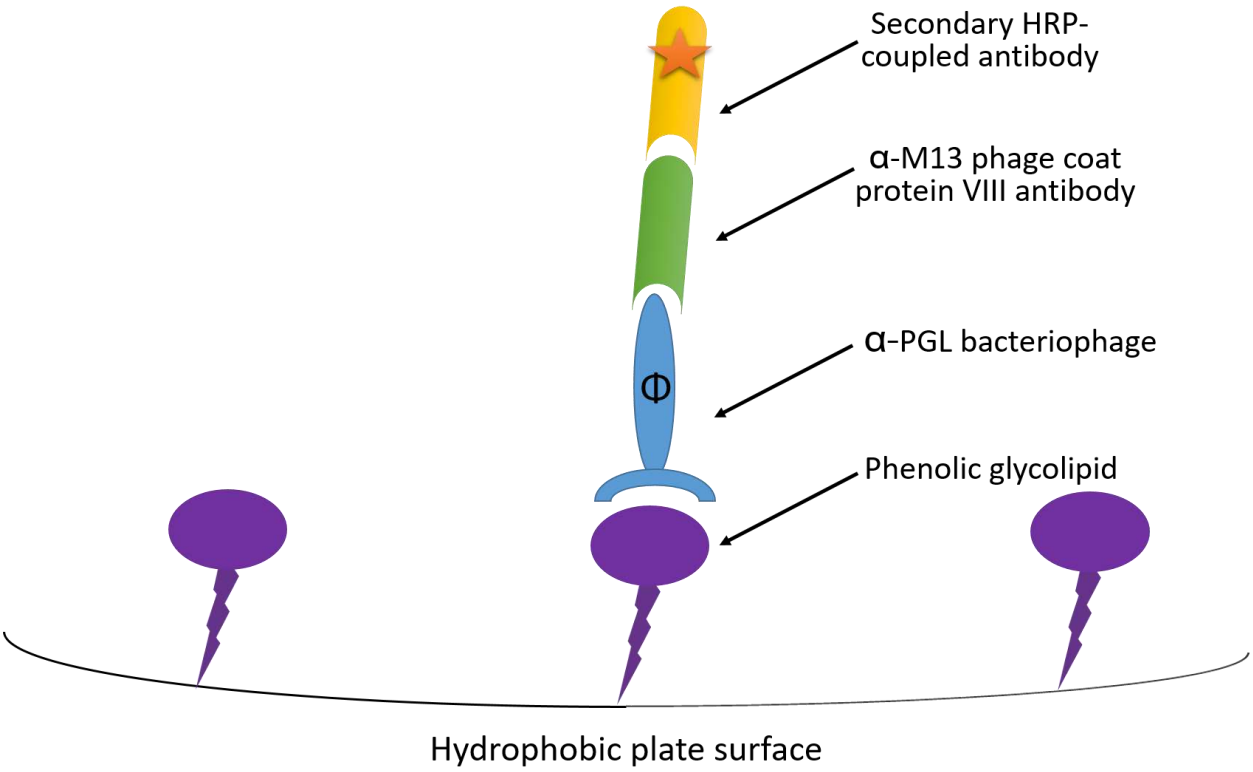

Figure 3.1: Cartoon depicting microtiter plate assay for quantitation of phage binding. Phenolic glycolipid (purple) is dried overnight onto a hydrophobic plate surface. After blocking the plate, phage (blue) are incubated on the plate, followed by an antibody against the M13 gpVIII coat protein (green), and a secondary antibody that is tagged with horseradish peroxidase (yellow).

Using a modified method based off the standard phage display method provided by New England Biolabs, we hypothesized that we could select for a phage that binds to PGL. If

such a binder is identified, it may further be implemented into a urine-based point of care diagnostic for bovine tuberculosis, as well as serve as a research tool in the lab for detection of *M. bovis* phenolic glycolipid or potentially distinguishing *M. bovis* culture from other mycobacterial species.

3.2 Materials and Methods

Materials and methods are provided by the manufacturer of the phage display kit (New England Biolabs) with some modifications described below.

Chemicals

Methanol, acetonitrile, hexane, and MS-grade water were obtained from Honeywell Burdick & Jackson (Muskegon, MI, USA). Isopropanol, sodium hydroxide, formic acid, Tween-80, hydrochloric acid, luria broth and luria broth agar, sodium citrate, 30% hydrogen peroxide, and EDTA were obtained from Fisher Scientific (Pittsburgh, PA, USA). Chloroform, Tris-base, and sodium chloride were obtained from JT Baker (Center Valley, PA, USA). Glycerol trioleate, polyethylene glycol-8000, ABTS salt, sodium iodide, and ethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Phage display- 7mer and 12mer libraries and selection

Both the 7mer and 12mer phage library kits were purchased from New England BIolabs Inc. (Ph.D. -7 and -12, NEB #E8100S and NEB #E8110S, respectively).

M. bovis phenolic glycolipid was solubilized in isopropanol and 5 µg of PGL per well was dried on the 96 well plate (Thermo Polysorp) by evaporation overnight in a fumehood. In lipid-negative wells, an equal loading volume of isopropanol was dried. The positive control for phage selection used 225 µg of streptavidin bound to a Greiner One Bio 96 well tissue culture

plate. Three rounds of panning to select for PGL specific phage were performed on PGL-coated plates with increasing concentrations of detergent at each consecutive round of panning (TBS-0.1% Tween-80, TBS-0.3% Tween-80, and 0.5% Tween-80) with an input of 1×10^{11} phage molecules per round of panning. Phage were incubated on plates for 1 h at room temperature, rocking. Streptavidin-binding phage were eluted with 0.1 mM biotin in TBS for at least 30 min. PGL-binding phage were eluted with a general elution buffer (0.2M Glycine-HCl (pH 2.2) and 1 mg/mL BSA) for no more than 10 min before neutralizing with 150 µL of 1m Tris-HCl, pH 9.1. Phage eluted from the target molecule were amplified in 20 mL ER2738 *E. coli* ($O.D._{600} \approx 0.05$) for 5 h at 37^{O}C, shaking, before culture was twice clarified via centrifugation at 12,000 g and 4^{O}C for 10 minutes. The upper 80% of the final supernatant was precipitated overnight in 0.17 vol of 20% PEG-8000/2.5 M NaCl at 4^{O}C. Phage were pelleted by centrifugation at 12,000 g at 4^{O}C for 15 minutes. The pellet (containing amplified phage) was then washed and suspended in TBS. An equal volume of sterile glycerol was added to the stock for long-term storage at -20^{O}C.

Phage titering

An aliquot of phage (1 µL) was serially diluted in LB broth. An aliquot (10 µL) of each dilution was used to infect 200 µL aliquots of ER2738 *E. coli* culture ($OD_{600} = 0.5$) for 5 min at room temperature. The inrfected cultures were applied to 3.5 mL top agar and spread on $IPTG^{+}$/$Xgal^{+}$/LB agar plates and incubated at 37^{O}C overnight. Blue pseudoplaques were counted to quantify the phage concentration.

Microtiter plate assay

Microtiter plate assays were performed in Thermos Polysorp© plates (colorimetric microtiter plate assay) or Costar white polystyrene plates (chemiluminescent microtiter plate assay, product number #3912). Lipids suspended in isopropanol were dried in plate wells overnight in a volume of 50 µL. Negative or "empty" wells contained only dried isopropanol. Plates were blocked with 200 µL PBS-0.1% Tween-80 for 30 min at room temperature, rocking. Blocking agent was removed and 1 x 10^{12} phage (100 µL) were added to wells in TBS and incubated for 1h at room temperature, rocking. Unbound phage were removed and the plates were washed six times with 200 µL TBS-0.5% Tween-80. Primary antibody (mouse anti-M13 Φ gpVIII; #ab9225 Abcam) was diluted 1:3,000 in blocking buffer and 200 µL was added to each well. The plates were incubated for 1 h at room temperature, rocking, and washed six times with TBS-T. Secondary antibody (anti-mouse IgG HRP antibody; #ab6728 Abcam) was diluted 1:500,000 in blocking buffer and 200 µL was added to each well. The plates were incubated for 1 h at room temperature, rocking, and washed six times with TBS-T. For colorimetric microtiter plate assays, ABTS substrate was prepared by dissolving 22 mg of azino-bis(3-ethylbenzothiazole sulfonic acid) diammonium salt (ABTS; Sigma Aldrich catalog # 10102946001) in 100 mL of 50 mM sodium citrate buffer (pH 4.0), and 21 mL of this ABTS stock was mixed with 36 µL of 30% hydrogen peroxide immediately prior to use. An aliquot of ABTS stock (200 µl) was added to each well for development and timepoint readings were taken on a microplate reader set to 405 nm. For chemiluminescent microtiter plate assays, Thermo SuperSignal® ELISA Femto Maximum Sensitivity Substrate was used (#37074). An equal volume of luminol/enhancer solution was combined with the stable peroxide solution and 100 µL of

this substrate was used per well. Plate was incubated for 1 min and immediately read on a plate reader set to 425 nm for relative luminescence.

Monoclonal microtiter plate assays for specificity were performed with a chemiluminescent substrate. Assays were performed in triplicate and values were averaged and graphed. The lipids tested include: *M. bovis* PGL, *M. canetti* PGL, *P. aeruginosa* rhamnolipid, linoleic acid, and total lipid extracts from *M. bovis, M. tuberculosis* H37Ra, and *M. canetti*. Individual lipids tested were assessed for signal generated against 5 µg of lipid. Total lipid extracts contain 50 µg of their respective extract.

Microtiter plate assays of urine extracts included pooled, spiked urine aliquots (2 mL urine spiked with 5 µg PGL), which were subjected to saponification pre-treatment (100mM NaOH at 80^{O}C for 1 h) prior to neutralization with approximately 3 drops of acetic acid. Samples were then washed and the organic layer was removed into a new tube. This was then dried under N_2 gas and suspended into a volume of 100 µl of isopropanol. Lipid-containing isopropanol was then dried in wells of a 96 well white CoStar plate, alongside un-spiked controls pretreated in parallel.

A sample size of n=3 applied to all microtiter plate analyses.

DNA extraction and sequencing

An aliquot (500 µL) of amplified phage were precipitated with 200 µL of 20% PEG-800 in 2.5 M NaCl for 10-20 min at room temperature, and centrifuged at 14,000 rpm, 4^{O}C for 10 min. The supernatant was discarded, and the phage pellet was suspended in 100 µL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4M NaI. DNA was precipitated with 250 µL of ethanol for 20 min at

room temperature. Tubes were centrifuged at 14,000 rpm, 4^{o}C for 10 min and the supernatant discarded. Ice cold 70% ethanol (500 µL) was used to wash the pellet. Tubes were re-centrifuged and the supernatant discarded. DNA pellets were briefly dried under vacuum. Pellets were resuspended in 30 µl of nuclease-free water, of which 5 µL were run on an agarose gel to check DNA quality and quantity alongside a purified single-stranded M13mp18 DNA standard (NEB #N4040) and the other 25 µL were sent to Quintara Bio for standard Sanger sequencing with the -96 gIII primer provided in the New England Biolabs Ph.D. kits. Data were analyzed on Chromas software. Sequence consensus was assessed by cluster analysis via Clustal Omega (European Bioinformatics Institute).

Thin Layer Chromatography (TLC)

TLC was performed to assess for lipid retention on the 96 well titer plates. Lipids solubilized in isopropanol were dried on the plate overnight. Wells were washed six times with 200 µL TBS-0.5% Tween-80, and each wash was collected and dried on a savant. Lipids were eluted with 200 µL isopropanol, transferred to a test tube, and dried under nitrogen gas. All samples were suspended in 30 µL of 2:1 chloroform:methanol, sonicated, and subjected to TLC, alongside standards. To assess for PGL retention (of any mycobacterial species), the TLC was performed on an aluminum-backed silica gel 60 plate with a mobile phase of 95:5 chloroform:methanol. To assess for total lipid retention, the TLC mobile phase was 60:30:6 chloroform:methanol:water. All TLCs were stained with 1% α-Napthol and 5% sulfuric acid in absolute ethanol (Sigma Aldrich). A heating gun was used to char the TLC plates.

Statistical analyses

Paired, two-tailed student's t-tests were used to generate p values via the Microsoft Excel spreadsheet formula function (=T.TEST). Critical α was pre-set at 0.05.

3.3 Results

Hydrophobic plate retains lipids through blocking and washing steps

Before beginning the phage display panning process, it was essential to select a microtiter plate for panning that sufficiently bound PGL to its surface and furthermore retained PGL through the washing steps. To assess for PGL retention, PGL (20 μg; 13.11 nmol) suspended in isopropanol was dried overnight on Thermo Polysorp, Grenier One Bio, and Thermo Nunc Immuno 96 well plates. The plates were washed as depicted in the phage panning protocol and the lipid extracted with 200 μL isopropanol. TLC analyses of the washes and isopropanol elution demonstrated that the Thermo Polysorp retained PGL on the surface through iterative washes (Figure 3.2) Plates with non-hydrophobic surfaces did not bind and retain PGL as well (data not shown). Specifically the PGL was fully or partially eluted with the wash buffer on less hydrophobic plate surfaces.

Figure 3.2: Assessment of PGL retention on Thermo Polysorp microtiter plate through blocking and washing steps. (1) PGL standard; (2) blocking buffer; (3) wash 1 (x6 with TBS-0.1% tween-80); (4) wash 2 (x6 with TBS-0.3% tween-80); (5) wash 3 (x6 with TBS-0.5% tween-80); (6) isopropanol extract.

Polyclonal microtiter plate assay shows enriched specificity to *M. bovis* PGL

The phage pool obtained after three rounds of biopanning was assessed for enrichment of phage that bound to PGL. We assessed round three pooled phage preps for signal generated by binding to surface-immobilized PGL derived from *M. bovis* total lipid extract, contrasted to non-specific signal generated by the polyclonal pool of phage against *M. canetti* total lipid,

which is negative for presence of the PGL species phage were selected against. Two quantities of phage were assessed: 1 x 10^{12} phage virions or 1 x 10^{11} phage virions. In each case, we observed that the phage tested against a total lipid fraction containing *M. bovis* PGL generated a statistically significant higher signal than *M. canetti* total lipid (Figure 3.3). With 1 x 10^{11} phage, signal against *M. bovis* total lipid (reported as OD_{405}) was 0.991 and 0.179 for *M. canetti* total lipid ($p=2.61 \times 10^{-3}$). Similarly, with 1 x 10^{12} phage virions per well, the OD_{405} for *M. bovis* total lipid was 2.683, contrasted to an OD_{405} of 0.327 for *M. canetti* total lipid ($p=4.96 \times 10^{-4}$). Through this analysis, we were able to confirm that through our iterative panning process, we were able to enrich the phage pools for binders of *M. bovis* PGL.

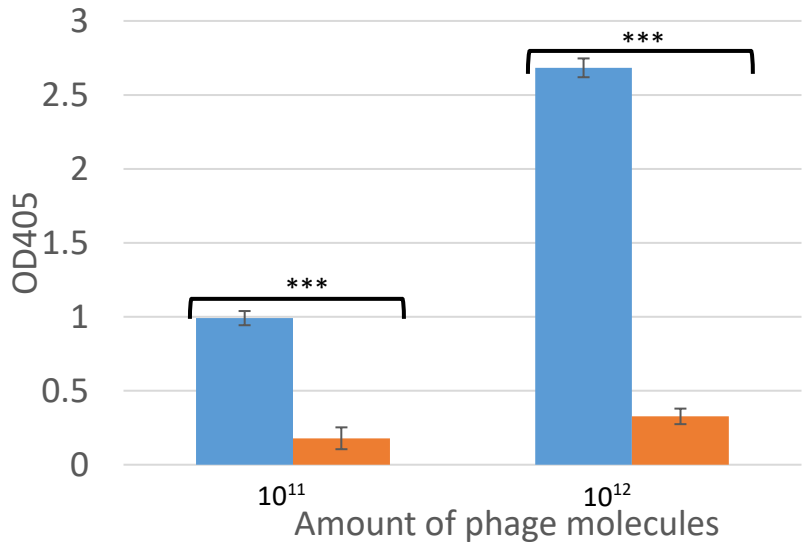

Figure 3.3: Polyclonal microtiter plate assay with a colorimetric substrate to assess for PGL-selected phage signal against two different species' total lipid extracts as measured by OD405. Blue bars represent signal against *M. bovis* total lipid and orange bars represent signal against *M. canetti* total lipid. In the case of 10^{11} and 10^{12} virions per well, *M. bovis* total lipid containing *M. bovis* PGL produces a higher signal, demonstrating enrichment of phage pools for binders to *M. bovis* PGL. *** = P<0.001

Sequencing analysis reveals consensus in selected clones

To assess whether our biopanning process resulted in phage clones with distinct consensus sequences, twenty pseudoplaques per phage pool were selected and sequenced. Seventeen unique sequences (13 from the 7mer library and 4 form the 12mer library) were identified. A clustal analysis performed with Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) revealed a consensus alignment between these 17 phage clones selected against PGL (Figure 3.4).

Phage ID																							
7.5	-	-	-	-	-	-	-	-	M	P	R	L	P	P	A	-	-	-	-	-	-	-	-
7.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	D	A	R	Y	K	S
7.14	-	-	-	-	-	-	-	-	Y	R	A	I	P	S	P	-	-	-	-	-	-	-	-
12.3	-	-	-	-	-	-	-	-	-	-	A	H	N	H	T	P	I	K	Q	K	Y	L	-
7.7	-	-	-	-	-	-	-	-	-	-	H	H	K	H	M	A	K	-	-	-	-	-	-
7.2	-	-	-	-	-	-	-	-	-	F	H	H	K	H	K	Q	-	-	-	-	-	-	-
12.2	-	-	-	-	-	-	-	-	V	P	R	H	S	H	P	L	I	N	M	R	-	-	-
7.3	-	-	-	-	-	-	-	-	V	P	R	H	S	H	P	-	-	-	-	-	-	-	-
12.12	M	K	A	H	H	S	Q	L	Y	P	R	H	-	-	-	-	-	-	-	-	-	-	-
7.6	M	K	A	H	H	S	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7.R1.3	-	-	-	-	-	H	V	S	H	V	R	H	-	-	-	-	-	-	-	-	-	-	-
7.R1.1	-	-	-	A	S	S	H	I	H	H	-	-	-	-	-	-	-	-	-	-	-	-	-
7.10	-	-	-	-	-	A	G	I	H	S	R	V	-	-	-	-	-	-	-	-	-	-	-
12.15	-	-	-	-	H	G	A	G	H	H	R	L	H	Q	I	V	-	-	-	-	-	-	-
7.1	-	-	-	-	-	-	-	-	H	H	R	T	N	Q	D	-	-	-	-	-	-	-	-
7.4	-	-	-	-	-	-	-	H	H	L	R	L	N	T	-	-	-	-	-	-	-	-	-
7.11	-	-	-	-	-	-	-	H	H	L	R	Q	N	H	-	-	-	-	-	-	-	-	-

Figure 3.4: Clustal analysis of PGL-selected unique phage variable regions sequenced out of round three pooled phage. Phage clone IDs are listed to the left of the sequence.

Two of the 7mer amino acid sequences (Φ_7) were found within the 12mer amino acid sequences (Φ_{12}). These are VPRHSHP (Φ7.3) and MKAHHSQ (Φ7.6). Additionally, an

enrichment for histidine residues is seen, including the following motifs: HH, HXHH, HHXH, and HSH. This aligns with the current knowledge on amino acids binding motifs for carbohydrates[10-12]. Previous studies on lectins, carbohydrate binding proteins, reveal that histidine residues are the functional portion of the active site and modification of the imidazole ring of the histidine oblates function of the lectin.

Sequencing of the positive control pool of phage (selected again streptavidin as suggested by the manufacturer) revealed the HPQ consensus expected (NEB Ph.D. Instruction Manual). Sequencing of the negative control pool of phage was also performed, and when aligned with the PGL-binding phage sequences, minimal consensus was found (data not shown). Any sequences that were found in both the negative pool and the PGL-selected pool were excluded from future analysis, unless to serve as a control. The phage excluded from future analysis at this stage were: Φ7.3 and Φ7.9. Φ12.2 was also excluded, as a sequence having high homology to a phage found in the negative pool. Two of three sequences that do not follow the general pattern of H enrichment of the PGL-selected pool were identified in the negative pool. This is indicative of some unrelated plastic binding phage in the polyclonal pool. However, most clones sequenced in the negative pool and in the PGL pool are different, indicative that the PGL binder pool is mostly different than the negative selected pool, further indicative that these two pools of phage are binding different binders (i.e. plastic binders versus PGL binders).

Monoclonal phage microtiter plate assays show variable levels of PGL binding in contrast to background plastic binding

To assess for non-specific plastic binding and establish levels of background binding, a microtiter plate assay was performed for each phage clone for signal to PGL (3.28 nmol) compared to that of a control, a well with dried isopropanol. This revealed varying levels of background between the PGL-binding phage clones tested. In Figure 3.5, signal generated in wells with PGL are shown in contrast to background signal. A streptavidin binding phage functioning as a negative control (denoted by "- control") was also tested and had the lowest levels of signal to noise, as anticipated. Phage producing low overall signal were eliminated and the PGL selected phage with the highest signal to noise ratios (Φ7.1, 7.2, 7.5, 7.7, and 12.3) proceeded into specificity testing.

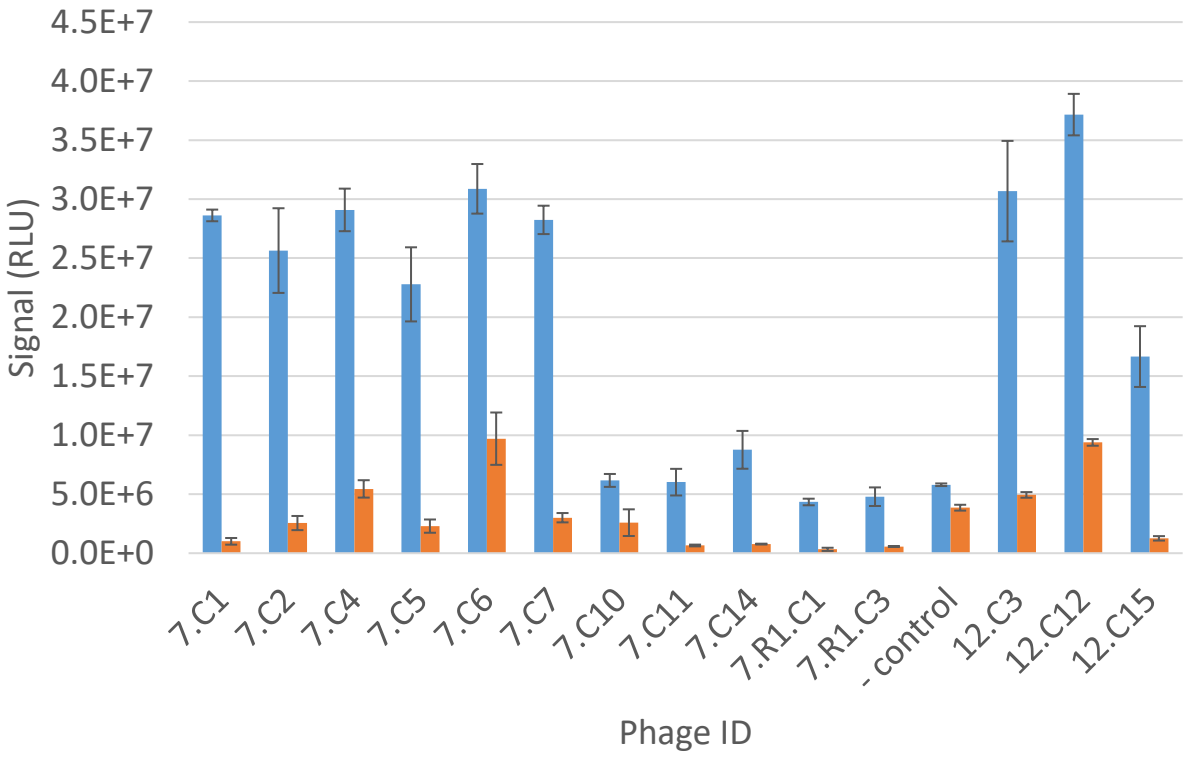

Figure 3.5: Phage generated-signal to PGL versus background signal in empty, isopropanol-coated well, as measured by a chemiluminescent substrate. Signal generated by phage in wells containing 5 µg (3.28 nmol) of PGL are shown in blue. Signal generated by phage in empty wells are shown in orange. Individual phage clones show different signal to noise ratios.

Monoclonal phage microtiter plate assays of specificity to *M. bovis* PGL

The specificities of the monoclonal phage to single lipids and total lipid extracts from various mycobacterial species were determined. We tested our phage clones against four individual lipids: the PGL of *M. bovis*, the PGL of *M. canetti*, rhamnolipid of *Pseudomonas aeruginosa,* and a fatty acid (linoleic acid). Rhamnolipid served to test nonspecific binding to rhamnose-containing amphipathic lipids. Linoleic acid served to assess for non-specific binders of hydrophobic molecules. Additionally, because a saponification step was included in the sample preparation method, which generates free fatty acids as a result of triglyceride hydrolysis, it was important to ascertain whether phage would generate a signal to fatty acids.

It should also be noted that retention of lipids on the plate through washing was assessed via TLC and it was confirmed that the lipids tested sufficiently bound the plate surface (data not shown). As shown in Figure 3.6, clones 7.1, 7.2, 7.5, 7.7, and 12.3 showed marked specificity for *M. bovis* PGL. The streptavidin-selected clone was included as an additional control in this assay.

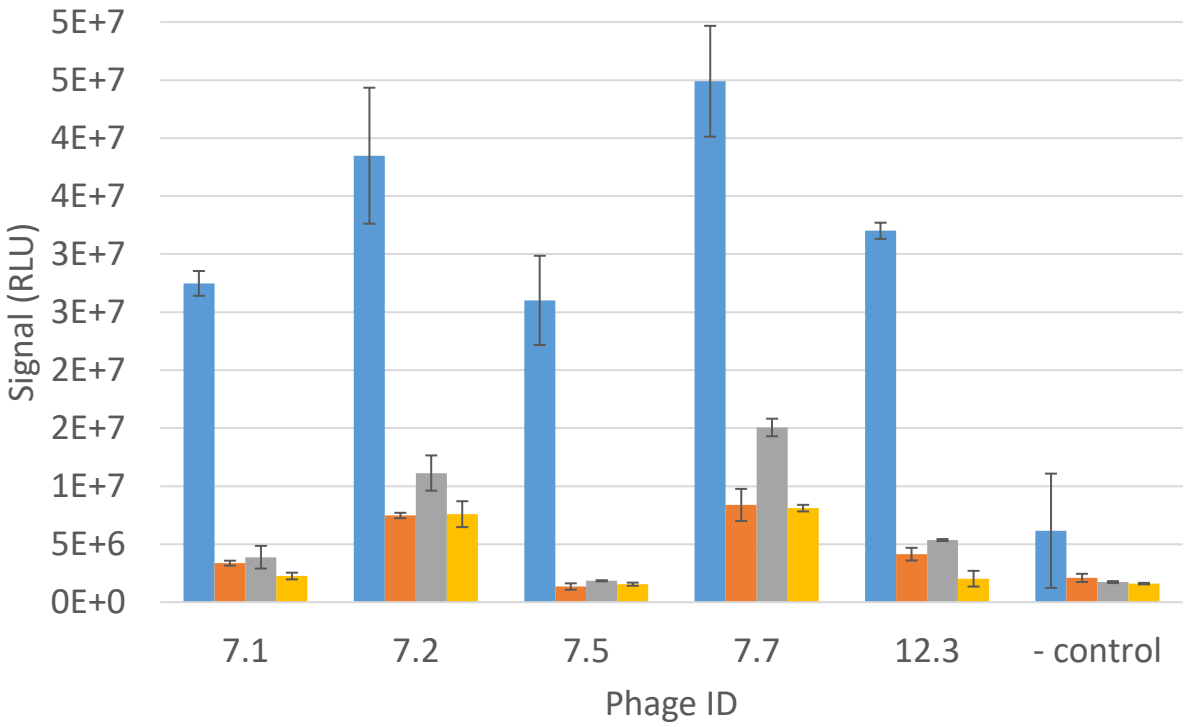

Figure 3.6: Specificity of phage clones as assessed by signal generated against four lipids. Chemiluminescent signal generated by phage clones to various lipids (5 µg) including: *M. bovis* PGL (blue), *M. canetti* PGL (orange), *P. aeruginosa* rhamnolipid (grey), and linoleic acid (yellow).

Phage clones were also tested against total lipid extracts from *M. bovis*, *M. canetti*, and *M. tuberculosis* H37Ra. These data are shown in Figure 3.7. The phage clones generated the highest signal to the lipid extracts of *M. bovis*, while the signals to *M. canetti* and *M. tuberculosis* H37Ra total lipid were 5.5x (Φ12.2 *M. bovis* vs. *Mtb*) to 155.8x (Φ7.2 *M. bovis* vs.

M. canetti) times lower than those for *M. bovis* total lipid. The clones selected to be tested for binding kinetics based upon their overall signal to *M. bovis* PGL and their relative specificity were Φ12.3, 7.1, 7.2, 7.5, and 7.7.

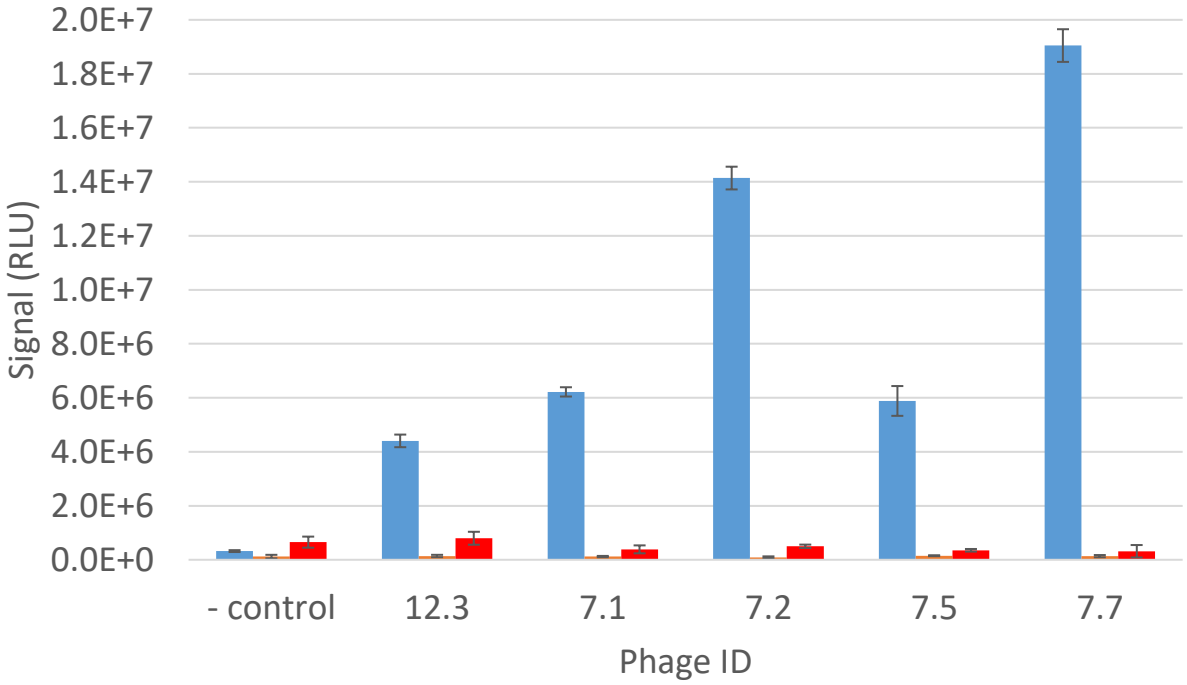

Figure 3.7: Specificity assay with top performing phage clones tested against total lipid extracts of three mycobacterial species. Chemiluminescent signal generated by phage clones to various total lipid extracts (50 µg) from different mycobacterial species including: *M. bovis* (blue), *M. canetti* (orange), and *M. tuberculosis* H37Ra (red) was measured.

Binding kinetics of monoclonal phage

To assess binding kinetics of the monoclonal phage, a titration of six phage clones (Φ12.3, 7.1, 7.2, 7.5, and 7.7) was performed and the signal to noise ratios for PGL binding were determined. The overall trend was the same for each clone assessed. A decrease in number of phage virions was associated with a decrease in the signal to noise ratio. This trend is illustrated in Figure 3.8, which shows the signal of each clone to PGL (shown in blue) versus an

empty well (shown in orange). The relative signal to noise ratio of all clones tested decreased as the concentration of phage decreased, with a concentration of 10^{12} phage particles producing the largest signal to noise ratio.

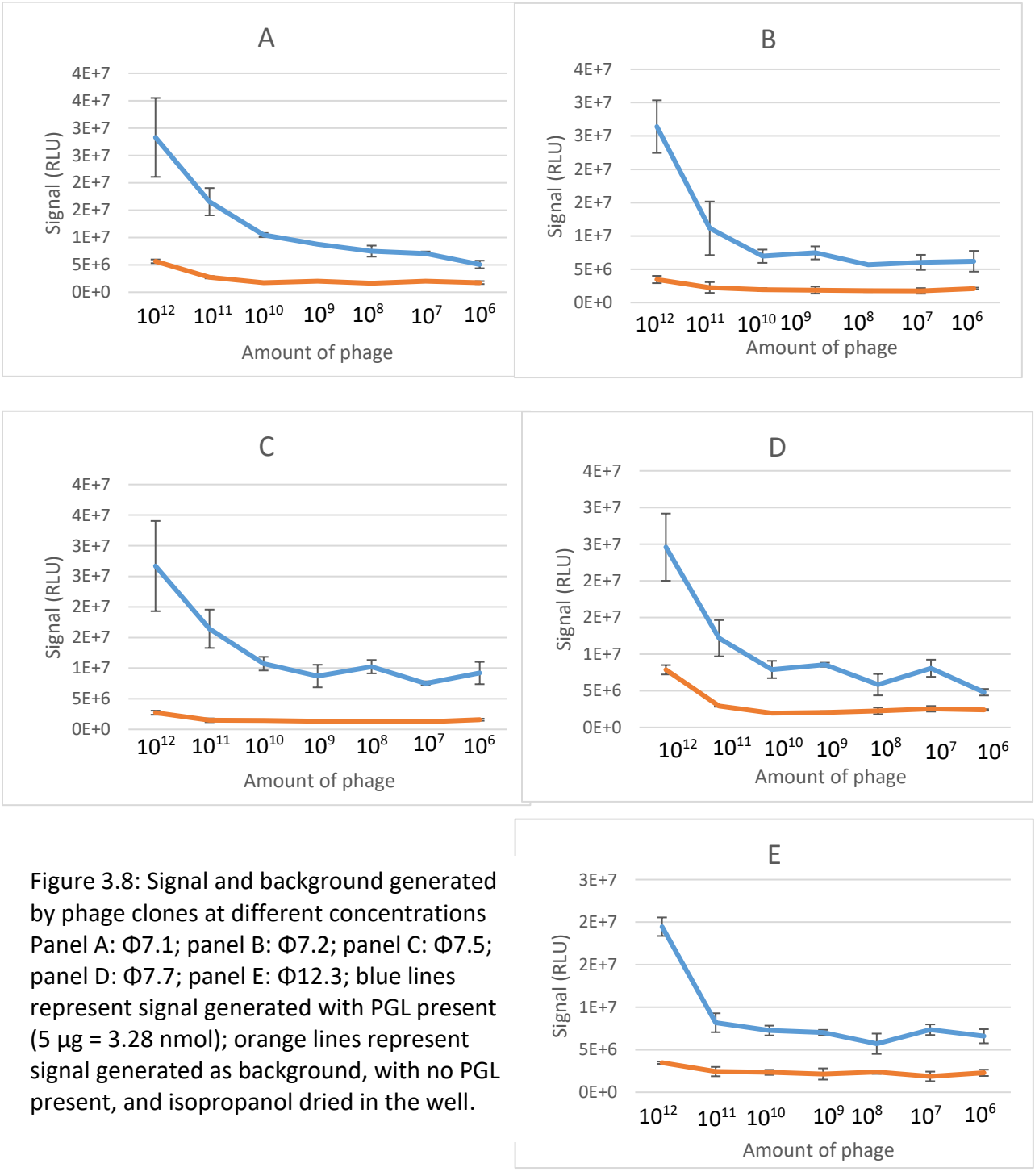

Figure 3.8: Signal and background generated by phage clones at different concentrations Panel A: Φ7.1; panel B: Φ7.2; panel C: Φ7.5; panel D: Φ7.7; panel E: Φ12.3; blue lines represent signal generated with PGL present (5 µg = 3.28 nmol); orange lines represent signal generated as background, with no PGL present, and isopropanol dried in the well.

Limit of detection of five top performing clones

Limit of detection for the top five performing phage was determined by serially diluting PGL and assessing for signal generated by 10^{12} phage particles in PGL-coated wells, contrasted to isopropanol-coated wells. High background in an isopropanol-coated wells introduced a bias in the assessment of a LOD. True LOD is defined as 5x the limit of the blank, i.e. five times the signal generated by phage in a well containing only dried isopropanol. With this definition of LOD, the LOD is greater than 3.28 nmol for all phage tested, as shown in table 3.1.

Table 3.1: The limit of detection was greater than 3.28 nmoles for each phage tested.

Phage ID	LOD (nmol)
7.1	>3.28
7.2	>3.28
7.5	>3.28
7.7	>3.28
12.3	>3.28

Phage Φ7.2 and Φ7.7 both show a statistically significant difference in signal between PGL-spiked and unspiked urine

Although the limit of detection was not as low as desired, we proceeded to test the two consistently top performing phage clones (Φ7.2 and Φ7.7) with spiked and unspiked pooled urine to evaluate whether the phage clones would produce statistically significant different signals for positive and negative pooled urine. The phage binding was assessed in triplicate with 2 mL of PGL-spiked saponified preps of urine that were subjected to a post-saponification

extraction. Using a two-tailed student's t-test, Φ7.2 was found to have significantly higher signal in wells containing PGL-spiked urine extract (p=0.0196). Phage Φ7.7 was also found to have significantly higher signal in wells containing PGL-spiked urine extract (p=0.000565). We were able to conclude that our phage were capable of detecting PGL spiked in urine with statistical significance (α = 0.05), as shown in Figure 3.9.

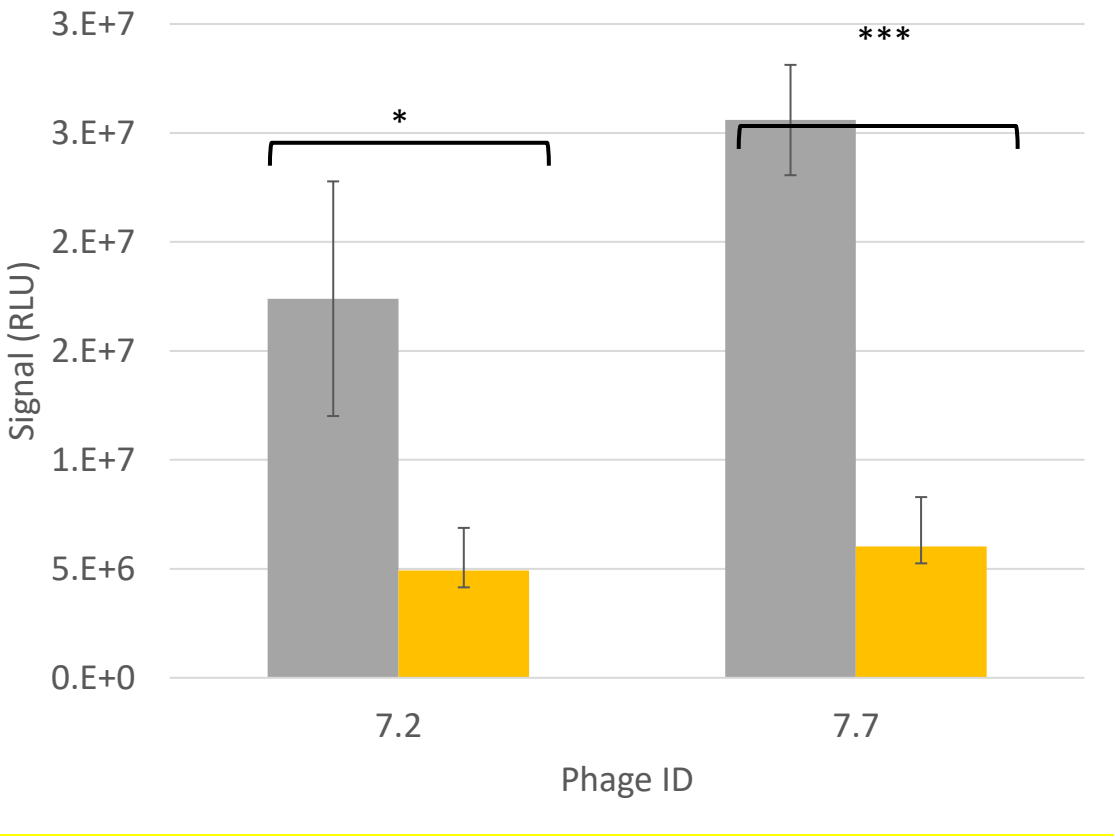

Figure 3.9: Assessing phage-generated signal in negative pooled, and positive spiked pooled urine extracts. Chemiluminescent signal generated by phage clones detect PGL spike (3.28 nmol) in pooled cattle urine. Signal generated against negative urine extract is shown in yellow; signal generated against spiked urine extract is shown in gray. * = P< 0.05; *** = P<0.001

3.4 Discussion

There is a need for an improved diagnostic for bTB that is more sensitive and specific, with less reliance on a predictable and robust immune response[13-28]. To address this need, we attempted to develop a biological detector of our putative biomarker of infection with *M. bovis*, PGL, an abundant and unique cell wall glycolipid synthesized by the pathogen. The approach we utilized was phage display, wherein a 10^{12} library of M13 phage displaying variable regions on their virion coat is panned over immobilized PGL iteratively, with increasing stringency in wash buffers. Using this method, we were able to select M13 phage that bind specifically to *M. bovis* PGL. Between these phage clones, we were able to identify consensus sequences that align with findings from previous studies on the active site of carbohydrate-binding lectins, namely that histidine residues are critical for carbohydrate binding[10-12, 29].

Phage Φ7.2 and Φ7.7 were consistent top performers in our assays and both of these clones possess the motif HHKH within their variable displayed region, indicative that this motif is important for carbohydrate biding and the recognition of PGL. When specificity was assessed using both individual lipids and total lipid extract from various mycobacterial species, we found that the selected phage did have specificity for PGL and *M. bovis* total lipid extract. Although our limit of detection is higher than what would be desirable for diagnostic purposes, when we tested our top two performing phage against pooled urine extract spiked with PGL, statistically significant difference were seen between the signal generated by Φ7.2 or Φ7.7 against urine extract containing PGL versus negative urine extract ($p=0.0196$ and $p= 0.0006$, respectively).

To further improve upon feasibility of using a phage clone for detection of PGL, a few possibilities exist that would have resulted in the poor limit of detection observed. Using a

greater amount of phage virions per well may improve signal for low amounts of PGL, as these phage do not appear to be tight binders of PGL. Additionally, the consensus sequence HHKH may be used to generate a nanobody antibody, which may improve binding to PGL[30]. Furthermore, the platform and treatment method used in this study, microtiter plate assay on a 96 well plate and saponification/biphasic extraction, may not be ideal. Further investigation is needed to confirm which sample pretreatment method leaves most PGL intact and recovers the greatest amount of PGL from the urine. It is possible that C8 magnetic beads may be useful for extraction of PGL from aqueous urine and reducing the complexity of the clinical sample.

Limitations of this study include the use of a different brand of plate for counter-selection of the phage. During selection, we used Thermo Polysorp clear plates; however during the analysis of phage clones, we shifted to a chemiluminescent reporting system and thus switched to Costar white plates. This likely led to some additional background as these plates were not selected against, leaving room for non-specific plastic binding. Similarly, the solid phase extraction and saponification method used in chapter 2 for reducing triglyceride content and recovering PGL from urine may have introduced artifacts into the system, artificially increasing the signal of the saponified samples. This has been reported in the literature and is likely a result of the saponifying base catalyzing chemilumescence of luminol via deprotonation of hydrogen peroxide[31].

Additionally, previous studies have stated that there are unknown compounds in urine that may inhibit horseradish peroxidase (HRP)[32]. Thus, in future experiments it would be important to assess alkaline phosphatase, rather HRP, conjugated to the secondary antibody. Furthermore, the phage clones identified as binders of PGL in this study must be tested with

naturally-infected cattle urine. However, in summation, this study leaves hope for using phage as detectors of pathogen-derived biomarkers, as well as expansion upon the original methods developed by Smith to be applicable to an amphipathic glycolipid as a target, immobilized in a hydrophobic surface 96 well plate.

Furthermore, a physical principle known as the Marangoni effect was likely a limitation of our methodology. The Marangoni effect, also known as "the coffee ring phenomenon," is the physical principle wherein differential rates of evaporation across a liquid surface lead to uneven distribution of the localization of solutes deposited on a surface as the solvent evaporates[33, 34]. As the edge of a droplet evaporates, liquid from the center of the droplet is pulled outwards, carrying dissolved particles with it. It is likely that this uneven deposition of solutes through the process of isopropanol evaporation impacted our results by driving the molecules of PGL to the periphery of the well via the aforementioned capillary action. The literature describes means of overcoming this phenomenon that include the addition of surfactants such as sodium dodecyl sulfate[35] or a surfactant-like polymer such as polyethylene glycol[36], increasing the speed of droplet drying such that dry speed is greater than solute movement in the evaporating solvent[37], or via the application of electrowetting to counteract internal molecular flux during drying[38]. Controlling the rate of droplet evaporation via temperature regulation would likely be the easiest means of controlling PGL distribution and overcoming the Marangoni effect. Additionally, future studies may also consider the potential effects that isopropanol may have on the biochemistry of the plate surface. Although the plate manufacturer states that isopropanol is compatible with the plastic of the plate, exactly how and if the chemistry of the surface is affected by exposure to isopropanol is unknown.

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CHAPTER FOUR: ASSESSMENT OF STABILITY OF BIOMARKER GLYCOLIPIDS

4.1 Introduction

Several challenges confound finding suitable biomarkers. First, biomarkers must possess structural and biochemical uniqueness to distinguish disease from non-disease with high sensitivity and specificity. Secondly, pathogen-derived biomarkers must withstand degradation or modification by host and pathogen enzymes to remain detectable in clinical samples. Alternatively, breakdown or modified products must retain structural uniqueness and can be produced in a consistent manner. Lastly, a pathogen generated biomarker must be produced sufficient concentrations to be detected amongst the host-produced compounds that are naturally more abundant. It is with these aspects in mind that we contrasted two abundant mycobacterial glycolipids (phenolic glycolipid and phosphatidylinositol mannoside, PGL and PIMs respectively) for their stability in a surrogate host environment. Because PIMs have not been detected in urine or sera, unlike PGL[1-3], we hypothesized that PGL would be a more suitable biomarker than PIM in that PGL would show greater stability to enzymatic breakdown. As a representative PIM for comparative assessment, we chose the highly abundant species, Ac_2PIM_2[4-6]. We addressed this hypothesis on multiple levels including: sensitivity to chemical saponification and thermal degradation, sensitivity to protease k digestion, and sensitivity to breakdown when exposed to fresh cattle organ homogenates of bovine kidney and liver. Through these varied assays, we were able to contrast the stability of two abundant mycobacterial lipids in evaluation of the potential as biomarkers of infection.

4.2 Materials and Methods

Chemicals

Methanol, acetonitrile, hexane, and mass spectrometry grade water were obtained from Honeywell Burdick & Jackson (Muskegon, MI, USA). Isopropanol, sodium hydroxide, and formic acid were obtained from Fischer Scientific (Pittsburgh, PA, USA). Chloroform was obtained from JT Baker (Center Valley, PA, USA). Glycerol trioleate was obtained from Sigma-Aldrich (St. Louis, MO, USA). [^{13}C] Glycerol triolein was obtained from Cambridge Isotope Laboratories Inc. (Tewksburg, MA, USA).

PGL and PIM purification

PGL and PIMs were purified from lyophilized *M. bovis* BCG cells as previously described[7]. Lipids from lyophilized *M. bovis* BCG cells (provided by Karen Dobos, CSU) were extracted overnight in 10:10:3 chloroform:methanol:water at a ratio of 30 ml solvent per gram of cells. The lipid extract was filtered twice with grade 42 Whatman paper. The filtrate was dried to 10 mL under nitrogen gas and combined with 90 mL cold acetone, and allowed to precipitate overnight at -20^{o}C. The acetone precipitation was centrifugated at 20,000 g for 30 min at 4^{o}C, and the supernatant decanted. The pellet, containing PIM and PGL, was dried under nitrogen gas and weighed prior to suspension in 2:1 chloroform:methanol. The PGL was purified by preparative TLC with a mobile phase of 95:5 chloroform:methanol and extracted from the preparative TLC plate, as previously described[8]. PIMs were purified by application to a chromatography column (Chemglass 1000mL) and elution with stepwise gradient of 10% methanol in chloroform to 100% methanol. Each step represented a 10% increase in the gradient and 800 mL of solvent were applied at each step. The 70:30 chloroform:methanol

fraction was applied to a 2 mm Silica gel 60 preparative TLC (Millipore Sigma; Darmstadt, Germany) and Ac_2PIM_2 was extracted.

Basic saponification and heating time points

PGL and Ac_2PIM_2 (50 µg in 25 µL isopropanol) were spiked into 500 µl of pooled urine and saponified with either 100 mM or 10 mM NaOH in methanol for various timepoints, ranging from 10 min to overnight at temperatures ranging from 22^{O}C to 80^{O}C. Saponifications were neutralized with approximately two drops of acetic acid. Lipids were retrieved via a biphasic separation after addition of 2 mL 2:1 chloroform:methanol. In a new tube, the organic fraction was dried under nitrogen gas and suspended in 50 µL of 2:1 chloroform:methanol. An aliquot (40 µL) of the lipid was evaluated by TLC and 10 µL was dried and suspended in 70µL of solvent B (per MS methods) for analysis by an LC-ESI-TOF-MS, as described in chapter two.

Protease K treatment to assess lipid stability

PGL and Ac_2PIM_2 (50 µg in 25 µL isopropanol) were spiked into 500 µl of pooled clarified urine. Protease K (50 µg) was added to each sample and incubated at 50^{O}C for 4 h. Reactions were terminated by the addition of an equal volume of protease k inhibitor cocktail (Roche cOmplete inhibitor tablets, #11697498001). After 1 h incubation at room temperature, samples were washed with chloroform:methanol (2:1). The organic layer was removed, dried under nitrogen gas, and suspended in 50 µL chloroform:methanol (2:1). An aliquot (40 µL) was evaluated by TLC, and 10 µL was dried and suspended in 70 µL isopropanol for analysis by LC-ESI-TOF-MS, as described in chapter two.

Organ homogenate and supernatant assays of lipid stability

Bovine liver and kidney tissue were collected from freshly slaughtered cattle by Dr. Paul Morley (CSU) and immediately placed on ice. Organs were aliquoted at 4^{O}C into 1 g tissue portions and stored at -80^{O}C. Organ aliquots were thawed on ice for 1 to 2 h and minced into 5 mm by 5 mm slices. Homogenate medium (HM) (PBS, 10 mM HEPES, pH 7.4) or HM with 1 mM EDTA (9 ml) was added to 1 g of minced tissue and the tissue was homogenized. The homogenate was directly used to assess lipid stability or was centrifuged at 13,000 g for 10 min. The supernatant was transferred to a new tube.

PGL, [^{13}C] glycerol trioleate, and Ac_2PIM_2 (2 µg) solubilized in 5 µL isopropanol were spiked into 500 µL organ homogenates or supernatants, in triplicate. Samples were incubated at 38.5^{O}C for 60 min. The reactions were stopped with a biphasic wash. The organic layer was retrieved, dried under nitrogen gas, and suspended in 70 µL of solvent B (referring to solvent B of LC system, per the MS method) and run on LC-ESI-TOF-MS, as described in chapter two.

Note homogenates and supernatants prepared in HM + EDTA were supplemented with additional enzymatic inhibitors: 0.08 µg/mL Ebelactone A (esterase inhibitor; Cayman #19163) and 180 µL of 25x protease k inhibitor cocktail in diH_2O (Roche cOmplete inhibitor tablets; #04693116001) prior to treatment of lipids. Likewise, homogenates and supernatants prepared in HM + EDTA were treated at 80^{O}C for 20 min to enhance inactivation of enzymes.

Detection of PGL and PIM by TLC

PIMs was evaluated by TLC using aluminum-backed silica gel 60 plates (Millipore Sigma; Darmstadt, Germany) with a mobile phase of 60:30:6 chloroform:methanol:water. PGL was evaluated by TLC on aluminum-backed silica gel 60 plates (Millipore Sigma; Darmstadt,

Germany) with a mobile phase of 95:5 chloroform:methanol. The lipids were visualized by spraying the TLC plates with 1% α-napthol + 5% sulfuric acid in absolute ethanol and charring.

Detection of PGL and PIM by mass spectrometry

Mass spectrometry for PIM analysis was performed with an XBridge™ BEH C18, 2.1 x 100 mm, 2.5 micron Waters Acquity UPLC chromatography column (Waters; Milford, MA) and an Agilent 1200 series HPLC system mass spectrometer (Agilent Techonologies; Palo Alto, CA). Sample injections of 10 µL were analyzed in negative ion mode. Ac_2PIM_2 was eluted with a linear gradient of 50% 5:2:3 water:methanol:isopropanol + 5 mM ammonium acetate (solvent A) to 70% 9:1 isopropanol:methanol + 5mM ammonium acetate + 0.1% acetic acid (solvent B) over 15 min, to 100% B over the final 5 min with a flow rate of 0.25 mL/min.

For PGL and the [^{13}C] glycerol trioleate standard, samples were assessed for lipid presence in a positive ion mode LC-ESI-TOF-MS Agilent mass spectrometer as described in chapter two.

4.3 Results

Ac_2PIM_2 shows a greater sensitivity to thermal and chemical hydrolysis than PGL

To assess the relative stability of Ac_2PIM_2 and PGL, varying degrees of base saponification were applied, a chemical condition that should hydrolyze the esterified fatty acids of both structures. PIMs showed a greater proclivity to degradation as compared to PGL. The base hydrolysis of Ac_2PIM_2 began within 5 minutes at room temperature with a low molarity base (10 mM NaOH; Figure 4.1). Products of hydrolysis were evident at 5 minutes into treatment, and by 12 hours PIM was completely degraded (Figure 4.1). The data obtained by

TLC was corroborated by evaluation of saponification products by mass spectrometry. As shown in Figure 4.2, no distinguishable signal for Ac_2PIM_2 remained after 12 hours of hydrolysis.

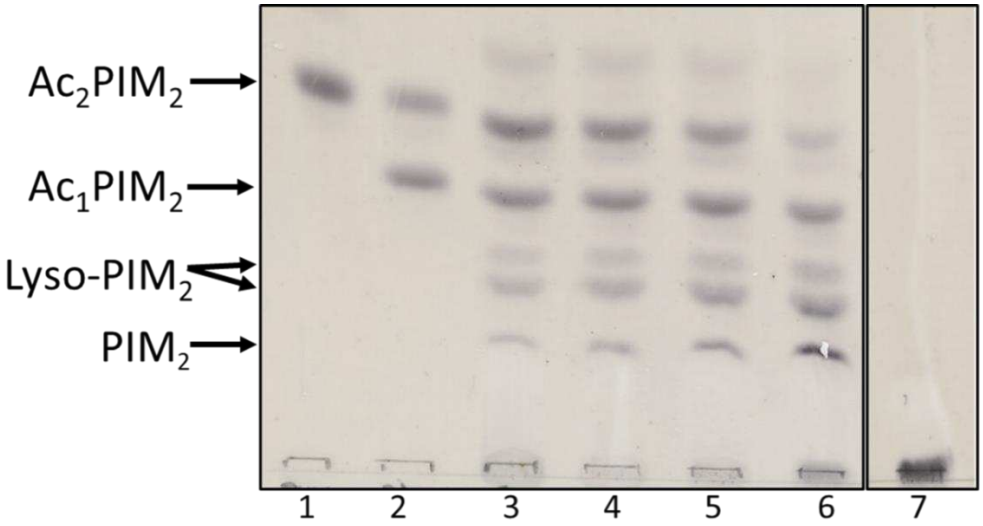


Figure 4.1: TLC tracking saponification of Ac_2PIM_2 over time. Hydrolysis products observed are Ac_1PIM_2, lyso-PIMs, and unacylated PIM_2. (1) Ac_2PIM_2 standard; (2) Ac_1PIM_2 and Ac_2PIM_2 standard; (3) 5 min incubation; (4) 10 min incubation; (5) 15 min incubation; (6) 30 min incubation; (7) 12 h incubation.

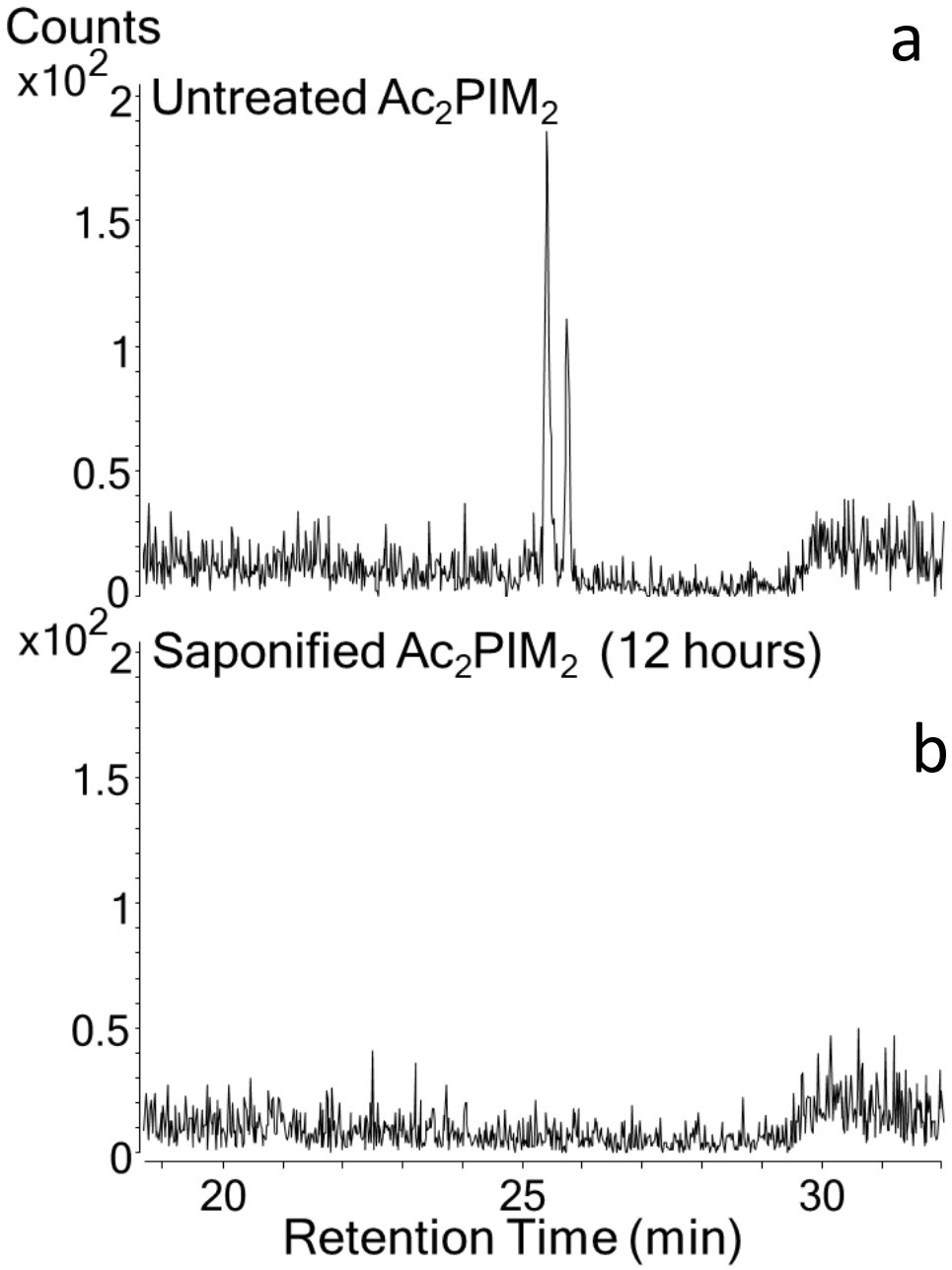


Figure 4.2: LC-MS of untreated Ac_2PIM_2 (a) and Ac_2PIM_2 subjected to saponification with 10 mM NaOH at 20^{o}C for 12h (b). The extracted ion chromatograms shown are the combined signal of the most abundant Ac_2PIM_2 ions (*m/z* 1652.13, 1680.19, and 1694.19). Signal for these species is lost by 12h.

In contrast, saponification of PGL failed to result in the hydrolysis of this lipid, even with 100 mM NaOH at 80^{o}C for 30 minutes. As shown in Figure 4.3, despite treatment, no reduction

in PGL abundance was observed, indicative of PGL's stability to thermal and chemical degradation. This robustness was in stark contrast to the lability of PIM in weak basic conditions at room temperature.

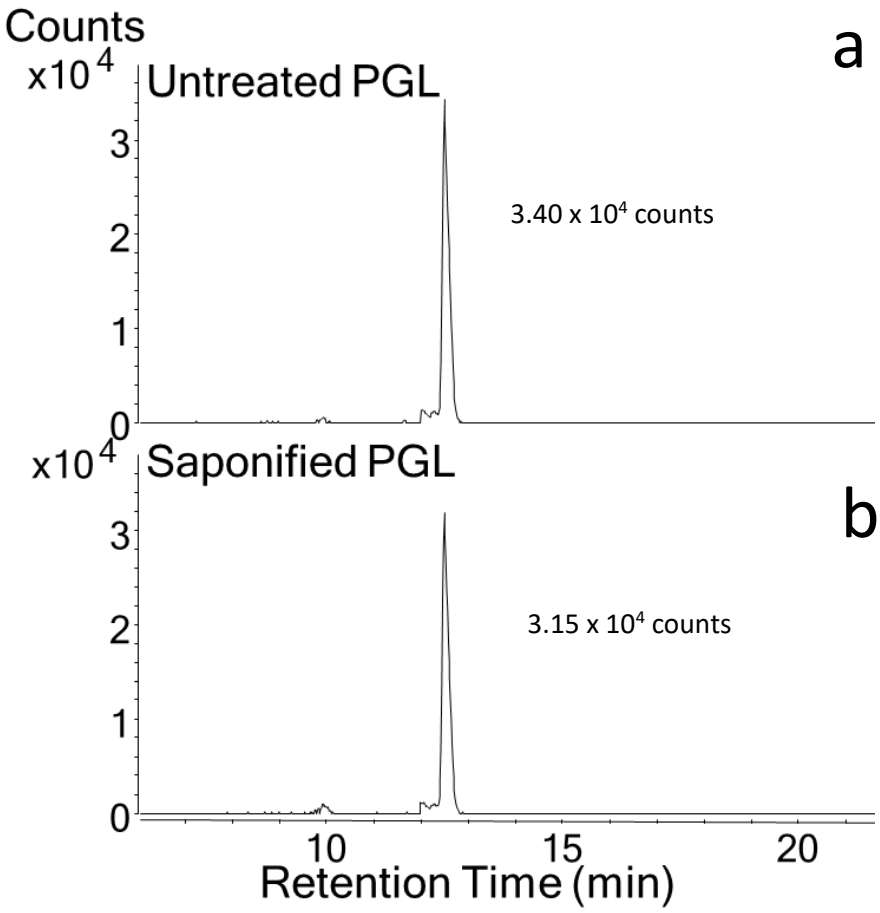


Figure 4.3: LC-MS of untreated PGL (a) and PGL subjected to saponification with 100 mM NaOH at 80^{O}C for 30 min (b). The extracted ion chromatograms shown is that of the most abundant PGL ion (*m/z* 1526.40).

Ac_2PIM_2 shows a greater sensitivity to protease K digestion than PGL

To assess relative sensitivity to degradation by enzymatic treatment, protease K was used to treat both Ac_2PIM_2 and PGL. It is noted that non-specific proteases also possess

esterase activity[9]. Similar to saponification, Ac_2PIM_2 showed a greater vulnerability to degradation by protease K than PGL. Complete degradation of Ac_2PIM_2 was observed by LC-ESI-TOF-MS (Figure 4.4). A TLC of untreated and treated Ac_2PIM_2 corroborated the MS data (Figure 4.4C).

In contrast, treatment of PGL with protease K under the same conditions did not alter the intensity of PGL on the EIC showing PGL treated with protease K (Figure 4.5B) in comparison with the untreated control (Figure 4.5A). A TLC analysis confirmed these results (Figure 4.5C).

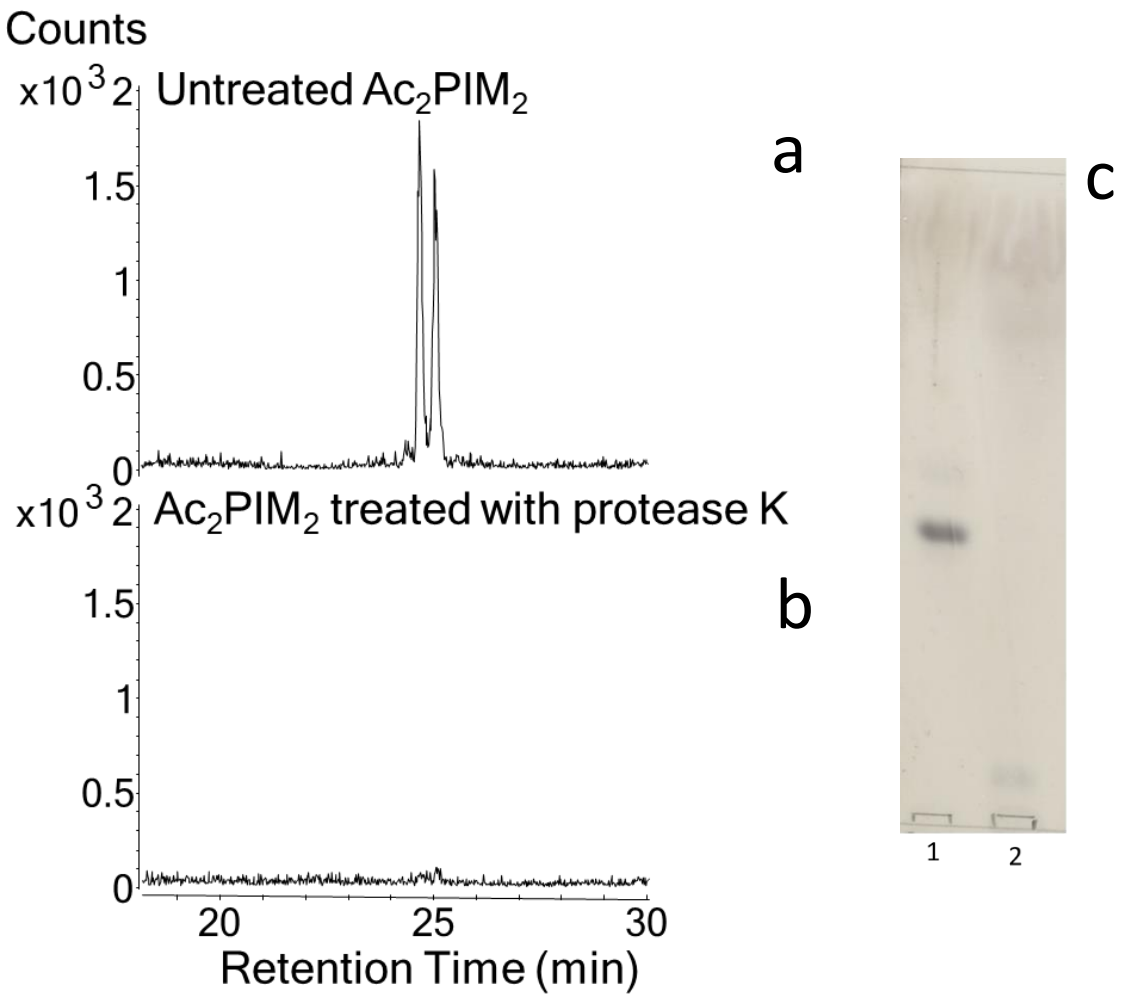


Figure 4.4: Ac_2PIM_2 digestion by protease K via LC-ESI-TOF-MS and TLC. Extracted ion chromatograms of the LC-MS data for Ac_2PIM_2 untreated (a) and treated (b) with protease K. The extracted ion chromatograms were generated using the *m/z* values of 1652.13, 1680.19, and 1694.19. The results yielded by LC-MS were further corroborated by the TLC analysis (c), Lane 1, Ac_2PIM_2 standard; Lane 2, Ac_2PIM_2 + protease K.

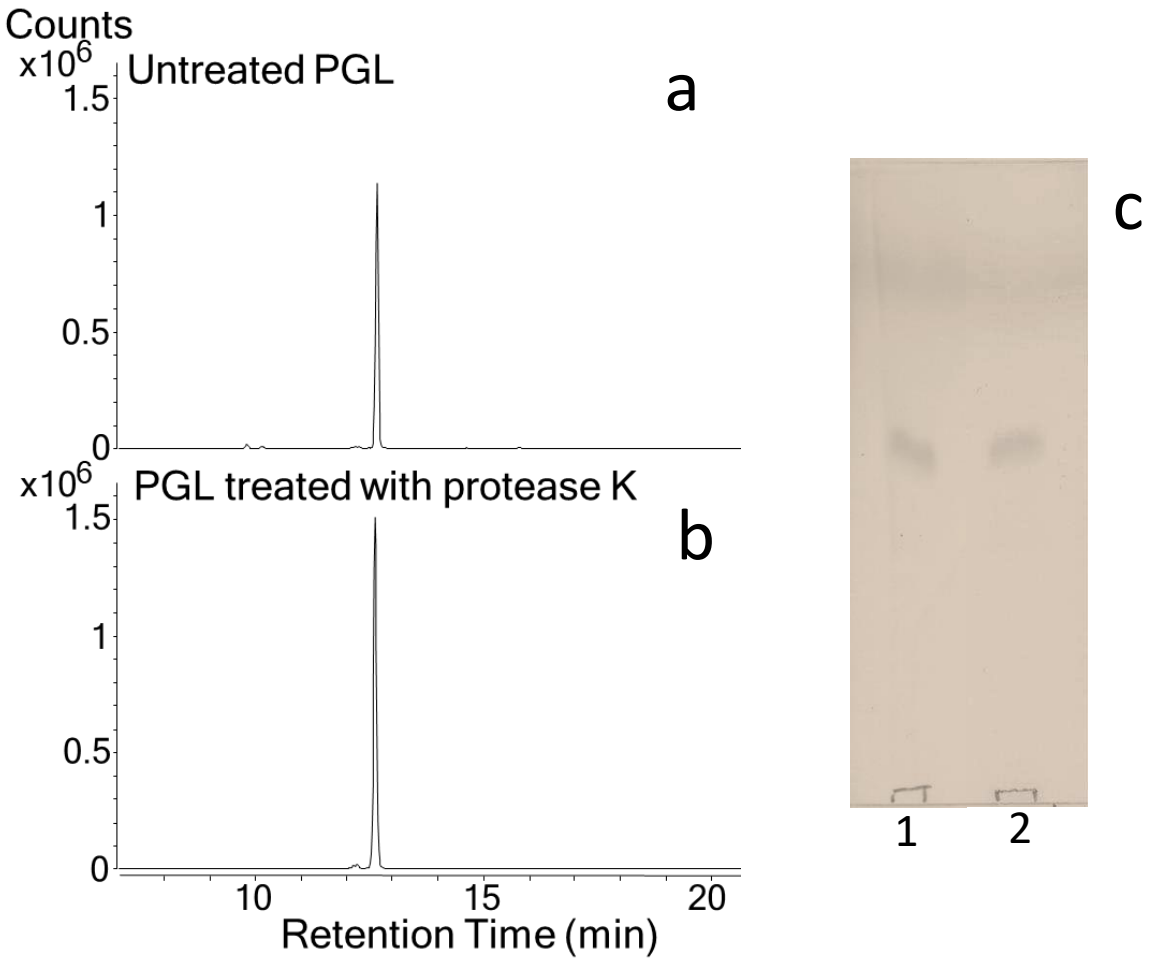


Figure 4.5: PGL digestion by protease K via LC-ESI-TOF-MS and TLC. Extracted ion chromatgrams of the LC-MS data for PGL untreated (a) and treated (b) with protease K. The extracted ion chromatograms were generated using the *m/z* value of 1526.40. These LC-MS results were corroborated by the TLC shown (c), Lane 1, PGL standard; Lane 2, PGL + protease K.

Neither lipid is sensitive to degradation by enzymes contained in organ homogenates or supernatants

When Ac_2PIM_2 and PGL were treated with the supernatants of bovine liver and kidney homogenates, neither lipid was degraded (Figures 4.6 and 4.7). To ensure the supernatants possessed lipolytic enzymes, isotopically labelled triacylglycerol (TAG) was used as a substrate. As demonstrated in Figure 4.8, treatment with both bovine liver and kidney supernatants

resulted in the degradation of the TAG standard. Specifically, treatment with liver supernatant reduced the TAG signal by 87.3% and the kidney supernatant led to a 47.1% signal reduction.

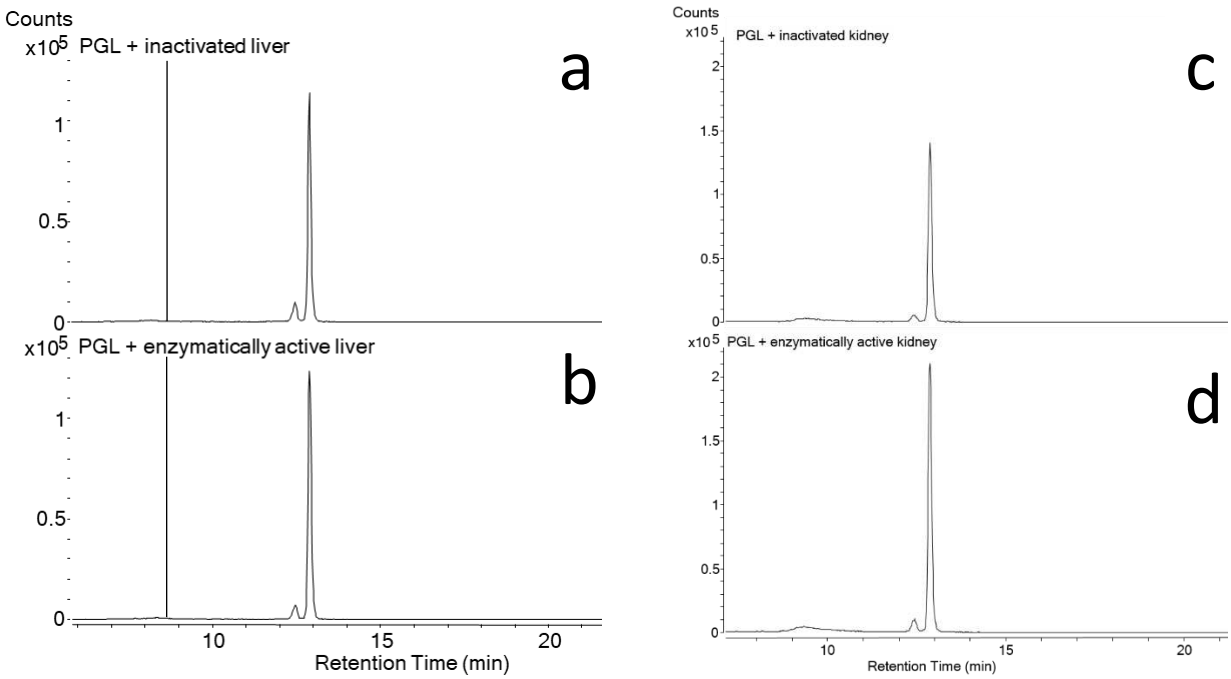


Figure 4.6: LC-MS of PGL treated with inactivated bovine liver homogenate supernatant (a), active liver homogenate supernatant (b), inactivated bovine kidney homogenate supernatant (c), and active kidney homogenate supernatant (d). The extracted ion chromatograms shown are that of the most abundant PGL species' ion, both the ammoniated and sodiated adducts (*m/z* 1526.40 and 1530.40, respectively).

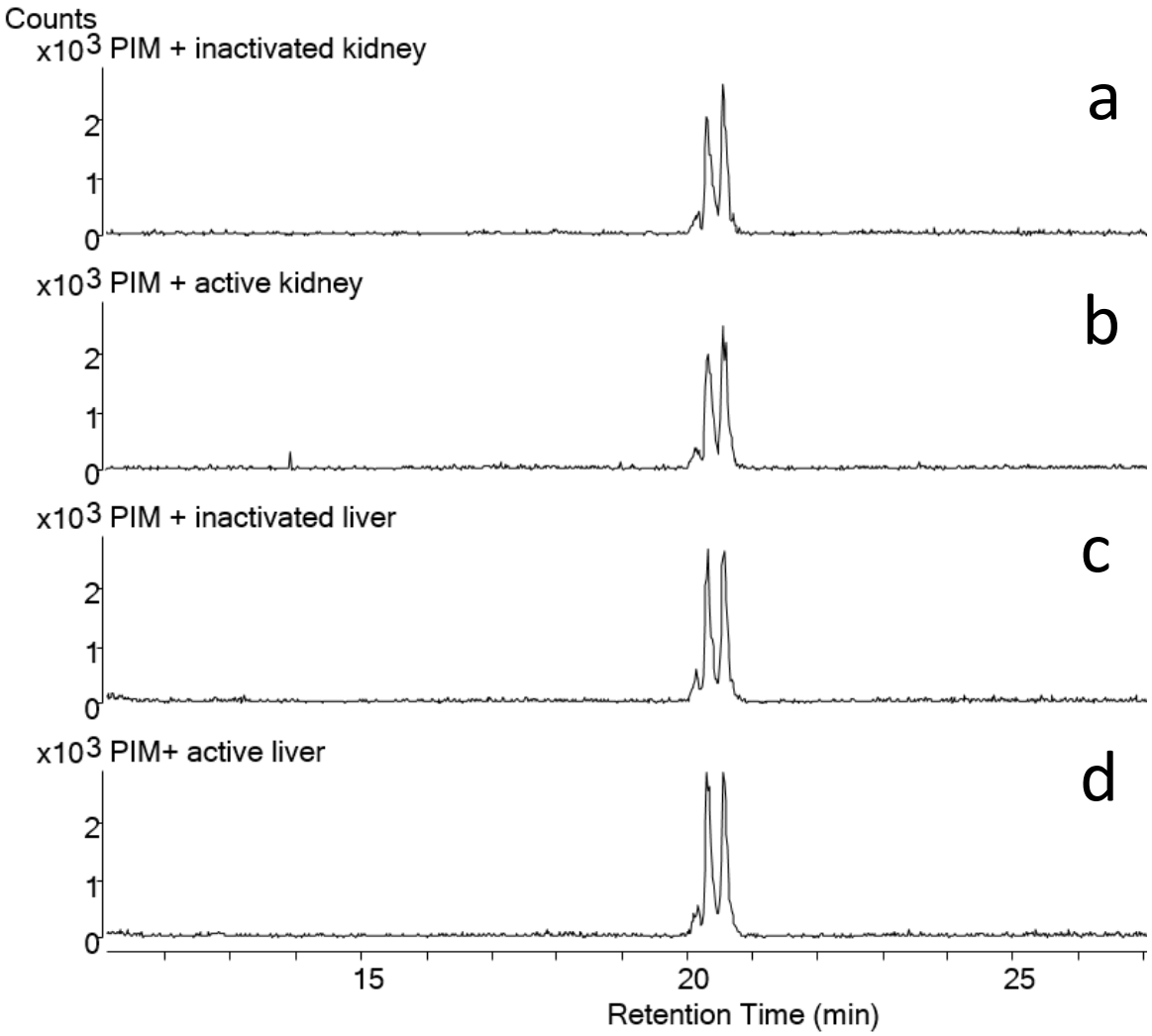


Figure 4.7: LC-MS of Ac_2PIM_2 treated with inactivated bovine kidney homogenate supernatant (a), active kidney homogenate supernatant (b), inactivated bovine liver homogenate supernatant (c), and active liver homogenate supernatant (d). The extracted ion chromatograms shown are that of the most abundant Ac_2PIM_2 species' ions (*m/z* 1652.13, 1680.15, and 1695.18 respectively

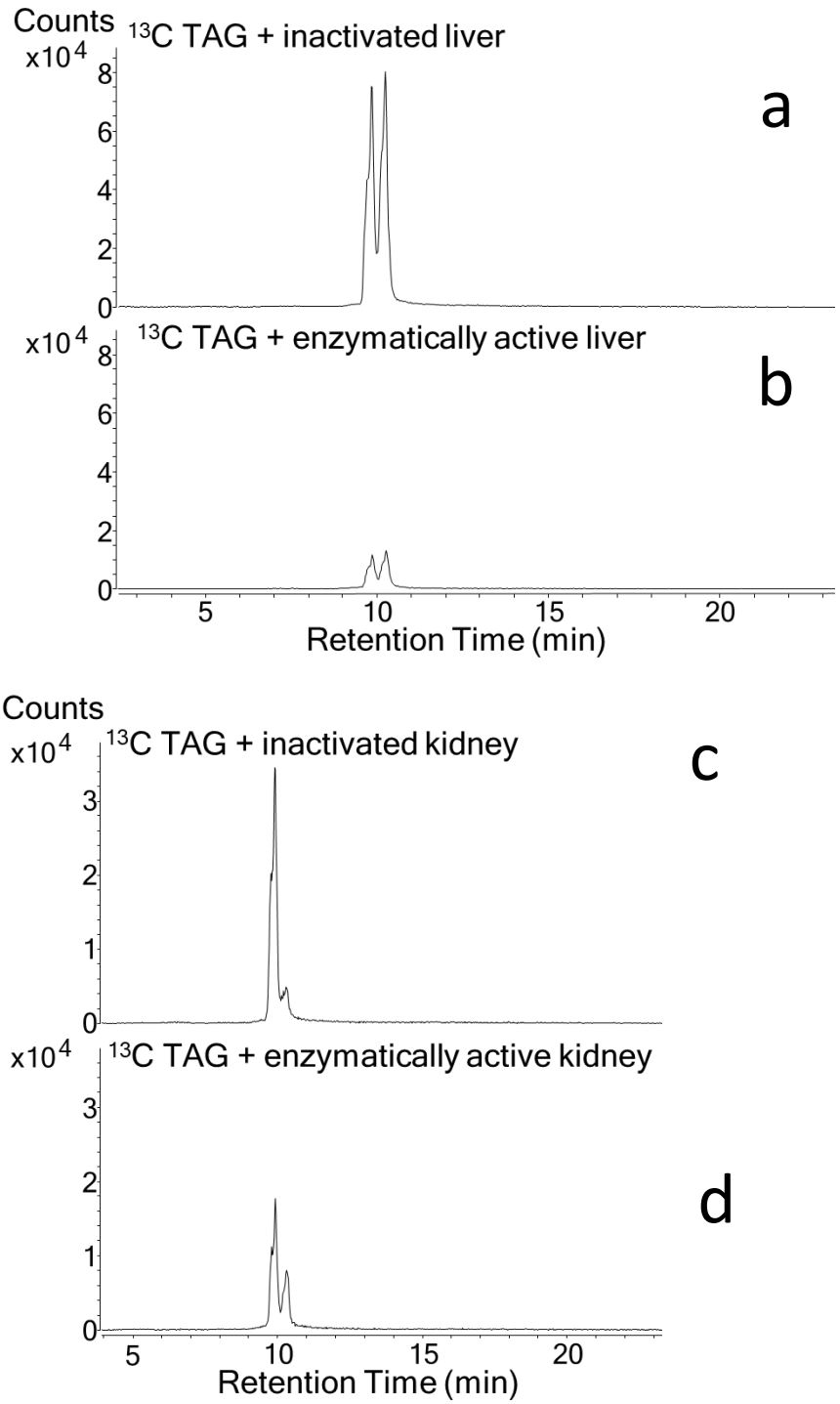


Figure 4.8: LC-MS of a [^{13}C] TAG standard treated with inactivated bovine liver supernatant (a), active liver supernatant (b), inactivated bovine kidney supernatant (c), and active kidney supernatant (d). The extracted ion chromatograms are for *m/z* 905.82 representing the ^{13}C isotopic mass of the TAG standard. As assessed by counts, TAG standard is reduced by 81.25% in active bovine liver. When treated with bovine kidney, TAG is reduced by 47.06%

Neither lipid is sensitive to degradation by kidney or liver homogenates, but Ac_2PIM_2 signal is ion suppressed when treated with homogenates and assessed by LC-ESI-TOF-MS detection

It is possible that enzyme activity with specificity for Ac_2PIM_2 or PGL was present in cell membranes and the use of tissue supernatants may not have represented this potential pool of enzymes. Thus, enzymatic degradation of the lipids was also assessed using tissue homogenates, rather than supernatants. When tissue homogenates were used to treat lipids, PGL was not degraded (data not shown). Ac_2PIM_2 treated with organ homogenates possessed no signal via LC-ESI-TOF-MS (Figure 4.9), which initially appeared to be indicative of degradation. However, when Ac_2PIM_2 was spiked into kidney and liver homogenates and visualized on a TLC, it was evident that the PIMs were still intact and had not been noticeably degraded (Figures 4.10 and 4.11). This indicated that LC-ESI-TOF-MS detection of PIM was inaccurate, which may a result of ion suppression mediated by a matrix effect.

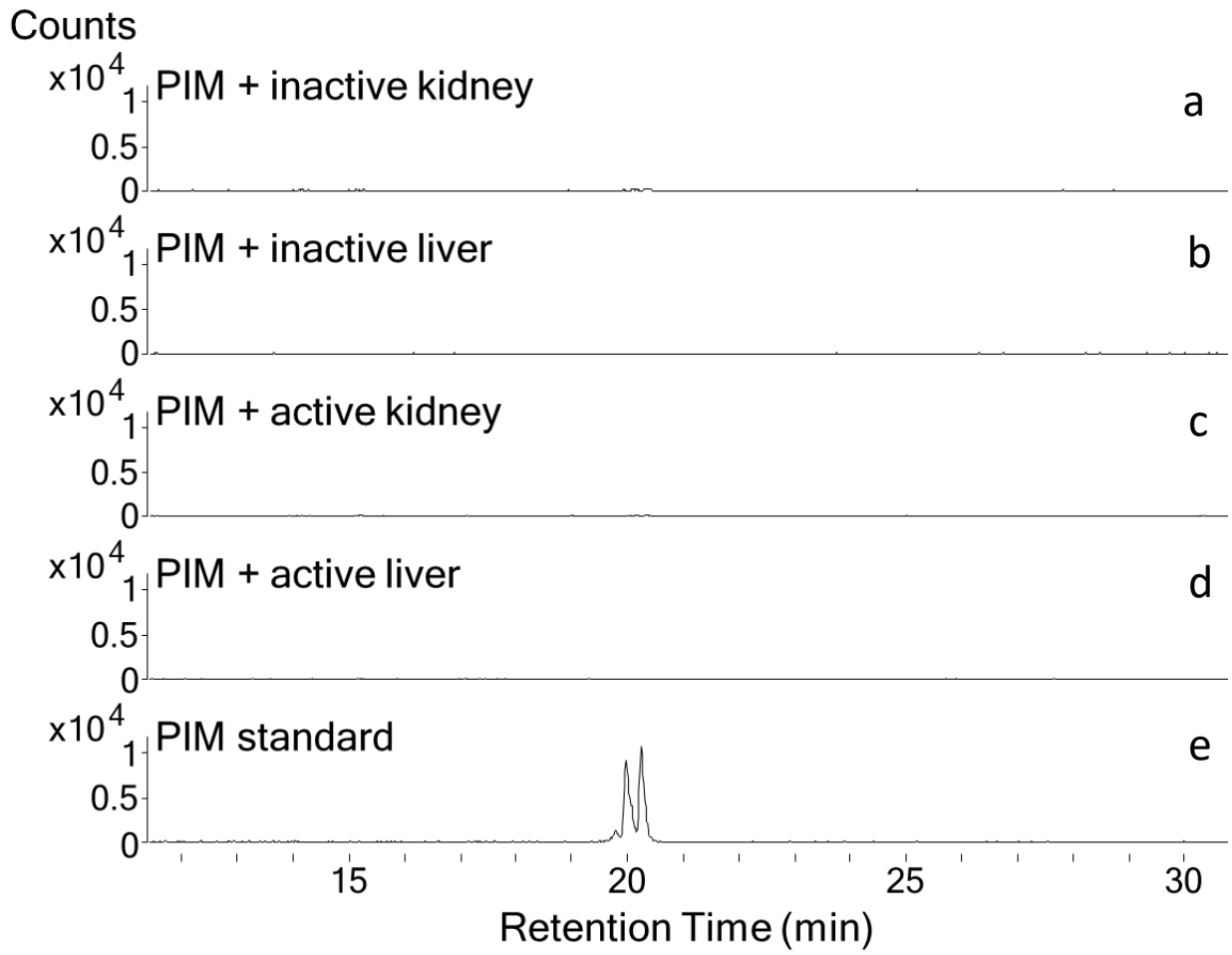
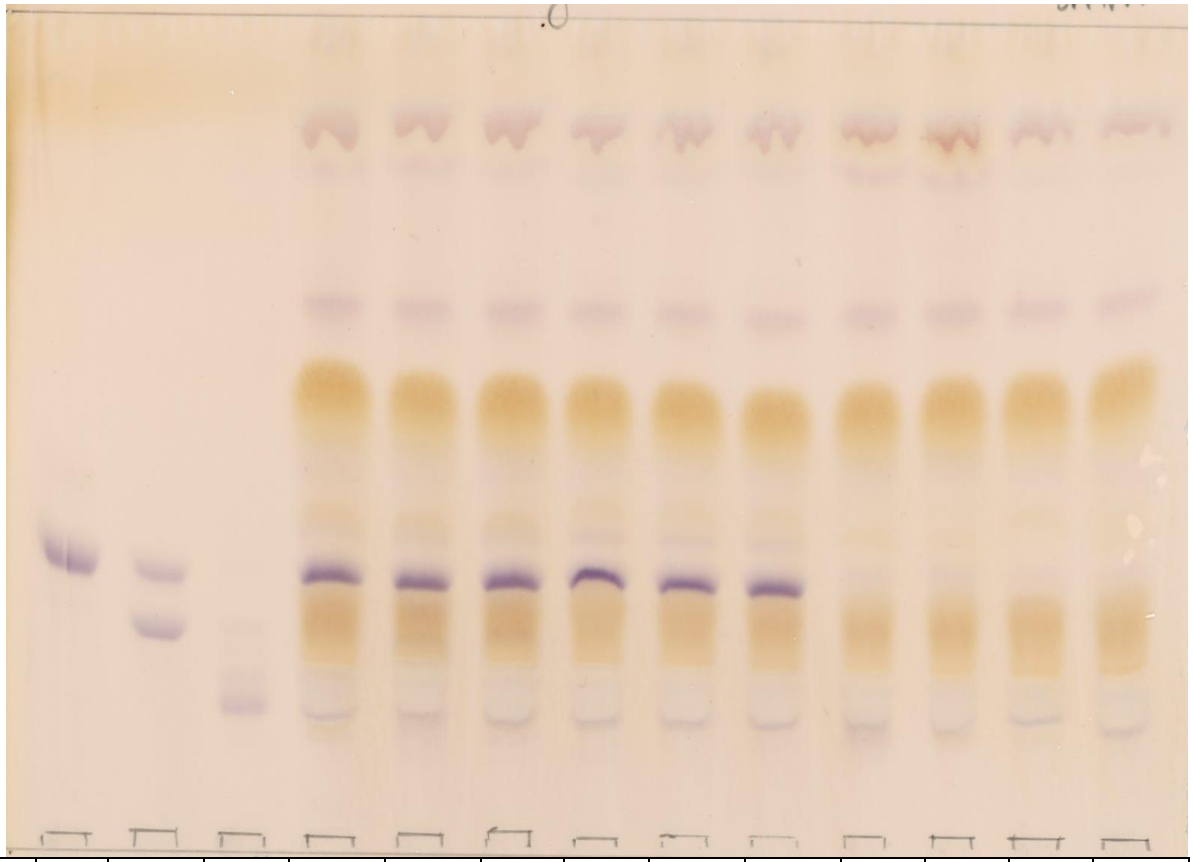
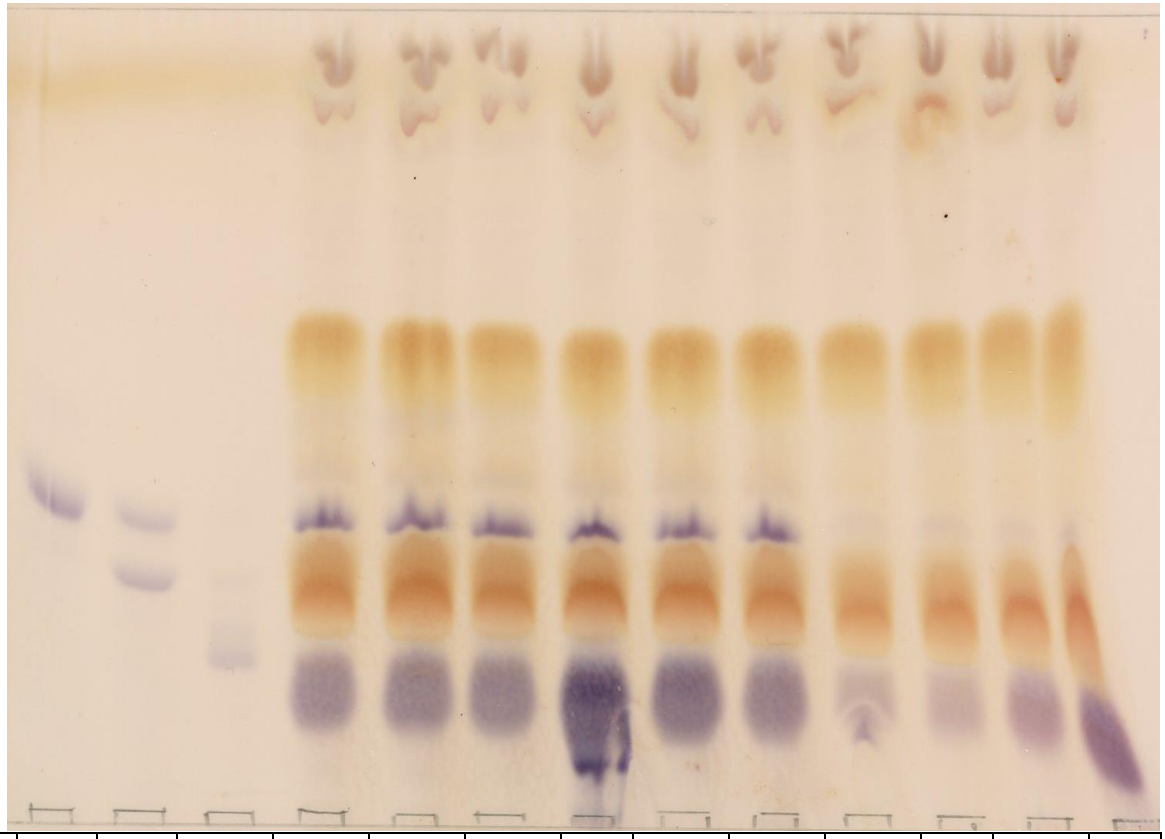


Figure 4.9: LC-MS of Ac_2PIM_2 treated with lysates of bovine kidney and bovine liver. Ac_2PIM_2 was treated with inactivated (a) bovine kidney lysate, inactivated (b) bovine liver lysate, active (c) bovine kidney lysate, and active (d) bovine liver lysate. These treated Ac_2PIM_2 preparations were compared to untreated Ac_2PIM_2 standard (e). The extracted ion chromatograms were generated using the *m/z* values of 1653.13, 1680.15 and 1695.18.



Ac_2PIM_2:	+	+	-	+	+	+	+	+	+	-	-	-	-
Ac_1PIM_2:	-	+	-	-	-	-	-	-	-	-	-	-	-
PIM_2:	-	-	+	-	-	-	-	-	-	-	-	-	-
Time(h):				6	13	24	6	13	24	6	24	6	24
Active?				Y	Y	Y	Y	Y	Y	Y	Y	N	N

Figure 4.10: Ac_2PIM_2 treated with kidney homogenate over 24 hours. By TLC, there was no observable breakdown of PIMs over the course of 24 hours. PIM species directly added to sample or lane denoted by + in chart below. Incubation time of reactions listed in hours in chart. Enzymatic activity of homogenate (active (Y) or inactivated (N)) is listed in final row of chart.



Ac_2PIM_2:	+	+	-	+	+	+	+	+	+	-	-	-	-
Ac_1PIM_2:	-	+	-	-	-	-	-	-	-	-	-	-	-
PIM_2:	-	-	+	-	-	-	-	-	-	-	-	-	-
Time (h):				6	13	24	6	13	24	6	24	6	24
Active?:				Y	Y	Y	Y	Y	Y	Y	Y	N	N

Figure 4.11: Ac_2PIM_2 treated with liver homogenate over 24 hours. No PIM degradation was observable by TLC. PIM species directly added to sample or lane denoted by + in chart below. Incubation time of reactions listed in hours in chart. Enzymatic activity of homogenate (active (Y) or inactivated (N)) is listed in final row of chart.

4.4 Discussion

In summation, the data yielded here demonstrate that PGL and Ac_2PIM_2 have differing sensitivities to treatment *in vitro* with base, heat, and non-specific esterase activity generated by protease K. This is in line with our hypothesis that PGL is a more stable molecule than those of the PIM family. Unfortunately, a difference in vulnerability to breakdown by endogenous

enzymes in cattle kidney and liver homogenates was not identifiable. TLCs were not sensitive enough to identify breakdown in this context. Mass spectrometry detection of Ac_2PIM_2 was inaccurate when PIMs were incubated with tissue homogenates, as evidenced by the presence of PIMs on a TLC in conjunction with no distinguishable signal generated by mass spectrometry analysis. This inaccuracy may be due to ion suppression as a biological matrix effect[10, 11]. Another possibility is that Ac_2PIM_2 may have bound to macromolecules present in the homogenate, inhibiting ionization and detection on a mass spectrometry platform. We believe the former is more likely, as no shift is seen in PIM migration on a TLC plate. To further investigate the stability of these glycolipids in vivo, an animal study wherein mice are injected with radiolabeled PGL and Ac_2PIM_2 would be informative and trackable on a TLC system. Radiolabeled PIMs and PGL spiked into organ homogenate and detected on a TLC may also yield new information, as radio-labelling improves the sensitivity of detection on a TLC. These future studies would provide additional insight into whether either of these mycobacterial-derived compounds could be a suitable biomarker for diagnostic purposes in terms of survival through the kinetics of clearance.

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CHAPTER FIVE: FINAL DISCUSSION AND FUTURE DIRECTIONS

5.1 Final discussion

Despite a federal program in existence for over a century dedicated to the complete eradication of bTB, outbreaks persist in the United States[1-6]. Worldwide, infection with *Mycobacterium bovis* threatens public health and the livelihood of livestock ranchers[7-11]. Furthermore, *M. bovis* infection also threatens wildlife worldwide[12-17]. Complicating transmission control of this disease, wildlife reservoirs and livestock animals may interact and transmit infection between them. The current diagnostic in use is not sensitive enough to identify all infected animals, perpetuating transmission in herds and to wildlife, as false negatives are not removed from the herd and culled. To address this fundamental gap in bovine tuberculosis control, we investigated a highly abundant, unique cell wall glycolipid of *M. bovis* as a potential biomarker of infection.

M. bovis PGL was established as a detectable molecule in naturally-infected cattle urine in chapter two, which addressed specific aim I. We found that we were able to detect PGL on an LC-ESI-TOF-MS platform at a lower limit of detection than on a MALDI-TOF-MS platform, a previously used type of mass spectrometry that purified PGL has been detected on. PGL of other mycobacterial species has been assessed as a marker of disease status previously, fueling our rationale for this study. Confirmation that intact PGL is detectable in the urine of naturally-infected cattle is a substantial finding that may allow for the development of improved diagnostic methods. Furthermore, we found that pretreating urine via a basic saponification

improved detection of PGL from cattle urine on an LC-ESI-TOF-MS. After using a saponifying pretreatment to reduce interfering signal from host triglycerides and a solid phase extraction to reduce sample complexity and concentrate PGL, we were able to detect 5 positive cows out of 40 total, with an additional 6 cows being equivocal for PGL signal. None of the uninfected cattle samples yielded a signal for PGL, further supporting PGL as a potential biomarker of *M. bovis* infection.

Chapter three addressed specific aim II, wherein we investigated a new method for detection of PGL. We were able to develop M13 phage that bound with specificity to the PGL of *M. bovis*, but not other mycobacterial PGLs. Using iterative phage display biopanning, we identified thirteen 7mer phage and four 12mer phage that were selected through three rounds of panning and one round of counter selection. Of these, two 7mer phage sequences were found within the 12mer phage sequences, providing more confidence that our selection was successful. None of the selected phage contained sequences known to be nonspecific plastic binders. There was, however, an enrichment of histidine residues noted within the phage sequences. This is in line with the current published literature on binding carbohydrates.

Previous studies have demonstrated the importance of histidine residues in lectins' ability to bind carbohydrates[18-20]. This further elevates our confidence that our phage clones do in fact bind PGL, likely at the rhamnose residue. The six phage with the largest signal to noise ratios were tested for specificity against total lipid fractions (*Mtb* H37Ra and *M. canetti)* and individual lipids of relevance (*M. canetti* PGL, rhamnolipid, and a fatty acid). This analysis confirmed that our phage were binding with specificity to their target (*M. bovis* PGL) and not simply adhering to the plate or to any molecule with a certain hydrophobicity.

When phage were titered out to investigate binding kinetics, we observed that a greater quantity of phage produced the best signal to noise ratio. However, limit of detection (LOD) studies were discouraging, with the LOD greater than 5 μg for all phage clones tested. This was partially due to high background generated in an "empty" well, or a well coated in isopropanol. This leads to two questions that must be addressed in the future. First, although isopropanol is confirmed as compatible with polystyrene (the plastic of the microtiter plate), we do not know if isopropanol drying in the well changes the plate's biochemical surface properties, impacting our assay.

Additionally, what is known as the "coffee ring phenomenon" was noted in this study. During the evaporation of a liquid containing particles, such as isopropanol containing PGL in this study, as evaporation occurs, capillary flow of the liquid occurs as liquid in the periphery evaporates, it is replaced by liquid from the center of the droplet[21]. This edgeward flow results in material deposition in a ring around the perimeter of the droplet. This creates an uneven distribution of dried material (here, PGL). This uneven distribution of PGL resulting from the necessity to dry PGL in the plate wells leads to this phenomenon and is a critical limitation of the platform used to assess (and also possibly within selection of) the phage.

Some work in the physics field suggests that this effect may be overcome via the addition of acetophenone to a volatile solvent[22], electrowetting to counteract the internal flux driven by the evaporating solvent[23], or by adding a surfactant-like polymer, such as polyethylene glycol[24]. Any of these potential solutions would have to be evaluated for their effect on PGL structure, binding, and on the assay itself. In sum, drying urine extracts on a microtiter plate may not be the ideal testing conditions for presence of PGL, and further testing

is required to determine what the best method for assessing for PGL presence is. Prior to our testing, it was not known that the coffee ring phenomenon, otherwise known as the Marangoni effect, would play a role in the ease of detecting PGL.

Nonetheless, when we assayed the top two performing phage against negative pooled urine and spiked positive pooled urine, we found that these two phage (Φ7.2 and Φ7.7) were able to detect PGL in the urine extract with statistical significance ($p = 0.02$ and 5.7×10^{-4}, respectively). Notably, these top two performing phage were placed together in the clustal analysis of all 12mer and 7mer sequences.

Lastly, specific aim III dealt with the stability of molecules through host clearance. We assessed the stability of PGL via *in vivo* modeling contrasted to another unique and abundant mycobacterial cell wall glycolipid, Ac_2PIM_2. This is detailed in chapter four. We contrasted the stability of PGL and Ac_2PIM_2 when treated with heat, base, and proteinase K. In all three assays, PGL was the more stable molecule between the two. Furthermore, we tested these two glycolipids' stability when incubated with enzymatically active bovine kidney and liver homogenates, both supernatants and whole homogenates. Interestingly, we found that when Ac_2PIM_2 was incubated with bovine kidney and liver homogenate, Ac_2PIM_2 detection on an LC-ESI-TOF-MS was lost. Surprisingly, this was not a result of degradation as shown by TLC, but rather more likely the result of a matrix effect generating ion suppression of Ac_2PIM_2 when mixed with organ homogenate. As TLCs are a qualitative assay, we could not assess percentage degradation of Ac_2PIM_2 this way. Furthermore, this begs the question of whether Ac_2PIM_2 has not been detected in clinical samples because it does not survive clearance through the host, or because of ion suppression within a clinical sample matrix.

5.2 Future directions

Through this work, we yielded valuable information pertaining to a potential biomarker for bTB diagnosis that, if utilized, may lead to a more effective diagnostic in cattle. Yet, there are many questions that remain unanswered. In chapter two, PGL was detected on an LC-ESI-TOF-MS platform. PGL was detected in five samples out of forty total clinical samples from infected animals. The urine that was used for this study was filter sterilized prior to receipt by the Belisle lab. In future work, it would likely be advantageous to sterilize the urine via gamma irradiation or begin the process with a Folch extraction, as it is possible to lose material via adsorption on the filter[25]. This was a limitation in our work, in that PGL may have been retained on the filter, decreasing likelihood of detection on any platform and lowering the number of animals whom were classified as positive by PGL detection. Additionally, it is possible that we could improve ionization to improve detection of PGL by MS. This could be achievable via derivatization.

With more information on the clinical status of the infected cattle, additional questions can be probed, such as is the amount of PGL in urine indicative of severity of infection and bacterial load? Can the concentration of PGL in urine be used to assess treatment efficacy? Lastly, because we have established that PGL can be detected in the urine of infected cattle, it would be interesting to expand this work to other species, including humans, as well as wildlife reservoir species. It is reasonable to hypothesize that PGL would also be detectable in the urine of humans infected with *M. bovis.* PGL-I is detectable in urine and sera of human patients infected with *M. leprae*[26-29]. Could the *M. bovis* PGL be detected in the clinical samples of patients infected with *M. bovis*? Similarly, it would be interesting to test other clinical samples, such as milk produced by infected cattle. Could PGL detection be used as a screen to test bulk raw dairy,

making raw dairy safer to consume? The application of PGL detection for evaluation of infection may go much further than evaluation of cattle urine.

Chapter three evaluated phage clones that were selected as binders of PGL. There is much work that can be done to continue this project. First, it may be desirable to repeat the selection process, particularly the counter selection, against the Costar plate used in the chemiluminescent assays. Originally the selection was undergone on Thermo Polysorp© plates; however, we later switched to a chemiluminescent platform, also switching plates. This may have played a role in the high background that was seen in wells with only dried isopropanol. If the phage were counter selected against the Costar plate, this background may decrease, providing greater sensitivity for the phage assays and allowing the limit of detection to drop.

The use of a horseradish peroxidase antibody may also be reconsidered in the future, as the literature has reported that there are unknown compounds in urine that inhibit horseradish peroxidase activity[30]. For this reason, it may be advantageous to switch antibodies to an alkaline phosphatase reporter. Another option would to be circumvent the antibody reporters entirely and to engineer the phage to have a reporter within the phage. One way this has been done was to fuse a GFP reporter gene into the phage genome such that samples with detector phage are probed for fluorescence generated by GFP, completely negating the need for antibodies[31]. Luciferase tagging and addition of luciferin has also been used for this purpose[32, 33]. Creating a phage coupled to a reporter would decrease the complexity of the assay and make it more amenable to point-of-care or field diagnostics. Additionally, it is possible that by fusing the reporter gene to an abundant coat protein (like pVIII, which has 2,800 copies on the virion surface[34]), we may be able to improve sensitivity.

Further work can be done tailoring the phage to potentially improve binding kinetics as well. An example of this would be to reduce the phage to only the variable peptide that binds PGL by generating a synthetic oligopeptide with the consensus sequence we identified as the binder of PGL. This may impact folding dynamics, ablating function; or alternatively, this may improve binding[35].

The detector phage (or a synthetic oligopeptide fused to a reporter) may also be implemented into a lateral flow assay. This would be another step towards a field diagnostic. Additionally, clinical samples should be analyzed with the top performing phage (or the synthetic oligopeptide), as this work stops short of testing clinical samples with the phage clones. We have seen efficacy of the phage in distinguishing spiked from unspiked urine; however, naturally-infected clinical samples must also be assessed when an appropriate LOD is acheived. Lastly, although it is likely that the phage clones bind to the same location on the PGL molecule (the sugar moiety) as suggested by consensus, it may be worthwhile to assess whether signal and sensitivity may improve when multiple clones are used in a mixture versus a single clone being used.

Within chapter four, we investigated the relative stability of one potential biomarker versus another (PGL versus Ac_2PIM_2). While PGL was evidenced as the more robust molecule during simple chemical, thermal, and enzymatic testing, assessment of stability with organ homogenates provided additional challenges. Ac_2PIM_2 was not detectable by mass spectrometry after incubation with whole organ lysates (bovine kidney and liver). This poses the question of what element(s) within the homogenate are imposing ion suppression upon Ac_2PIM_2 and is this why Ac_2PIM_2 has yet to be detected intact in clinical samples? Ac_2PIM_2 was a more stable

molecule than originally hypothesized and survived incubation with bovine organ homogenates intact, as shown by TLC. Further separation of the organ homogenate into fractions may reveal which elements in the homogenate affect Ac_2PIM_2 detection by ESI-MS. Additionally, more work may be done to negate and overcome this effect, such as implementing different lipid extraction methods or a treatment method to the lipid/organ homogenate mix to revive detection of Ac_2PIM_2 by degrading the compound causing the ion suppression.

Injecting mice with or feeding mice radiolabeled PGL and Ac_2PIM_2 would allow for more careful tracking of these molecules *in vivo* and also aide in the identification of potential breakdown products. The potential breakdown products, if suitably unique, may also be investigated as possible biomarkers. Mouse studies would greatly contribute to our understanding of how these two abundant mycobacterial glycolipid survive or degrade through the kinetics of clearance of the host. Radiolabeling would also allow for more sensitive detection than the TLCs presented in this work via x-ray film detection, rather visualizing with alpha-naphthol and sulfuric acid.

The results detailed within this dissertation provide support for the use of PGL as a biomarker of *M. bovis* infection. We succeeded in detecting this molecule in the urine of cattle naturally-infected with *M. bovis*, a major development for the field of bovine tuberculosis, and further assessed the possibility of using phage display to develop a sensitive and specific detector of PGL. The phage clones identified in this study hold promise for further refinement and possible implementation into a field diagnostic device. This dissertation provides insight for the scaffolding of a new way to assess for *M. bovis* infection status. Additionally, this work lends support to the notion that unique pathogen-derived molecules may be useful for bTB diagnostic

purposes. Overall, this work looks forward to the possibility of developing diagnostics that do not rely on immune response, which can be a critical source of false negatives and false positives in diagnosis, generating financial loss at best and allowing disease transmission to continue in a population at worst. While developing a sensitive and specific diagnostic is no trivial task, minute steps towards that goal may yield large gains for public health as these developments are expanded upon and hopefully implemented into an improved diagnostic in the future.

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LIST OF ABBREVIATIONS

Ac	Acyl
BCG	Bacillus-Calmette Guerin
bTB	Bovine tuberculosis
CDC	Centers for Disease Control
CE	Collision energy
CFP	Culture filtrate protein
DHB	2,5-dihydroxybenzoic acid
EIC	Extracted ion chromatogram
ELISA	Enzyme-linked immunosorbent assay
ESAT	Early secretory antigenic target
ESI	Electrospray ionization
FA	Formic acid
GC	Gas chromatography
GFP	Green fluorescent protein
GPL	Glycopeptidolipid
HDL	High-density lipoprotein

HIV	Human Immunodeficiency Virus
HM	Homogenate medium
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IFN	Interferon
IGRA	Interferon-γ release assay
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAM	Lipoarabinomannan
LC	Liquid chromatography
LOD	Limit of detection
LPS	Lipopolysaccharide
M	Million; or Molar
MALDI	Matrix assisted laser desorption/ionization
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MDL	Method detection limit
MDR	Multiple drug resistant

Me	Methyl
MHC	Major histocompatibility complex
MS	Mass spectrometry; or multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance
NTM	Non-tuberculous mycobacteria
Φ	Phage
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGL	Phenolic glycolipid
PIM	Phosphatidylinositol mannose
PPD	Purified protein derivative
PPM	Parts per million
QQQ	Triple quadrupole
SPE	Solid phase extraction
Spp	Species

TAG	Triacylglyceride
TLC	Thin layer chromatography
TB	Tuberculosis
TBS	Tris-buffered saline
TOF	Time of flight
TST	Tuberculin skin test