ORIGINAL ARTICLE

Optimized Preparation of Urine Samples from Acute Melioidosis Patients for In-Solution Proteomic Studies using LCMS QTOF or MALDI TOF MS

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ABSTRACT

INTRODUCTION: Investigation of urine proteome in patients with acute melioidosis may reveal potential disease markers, from either bacterial or human proteins. We used an in-solution gel-free method instead of 2-DE to detect human and Burkholderia pseudomallei proteins in urine of patients with acute melioidosis. Here, we propose a simpler, economical method for preparing urine samples directly from melioidosis patients, for in-solution proteomic analysis using LCMS-QTOF MS/MS or MALDI-TOF MS/MS. **MATERIAL AND METHODS**: We adapted an acetone-TCA based protein precipitation method with LCMS-QTOF MS to detect the B. pseudomallei proteins directly from urine of acute melioidosis patients (culture positive and negative). This process involves protein precipitation, desalting, trypsin digestion, and optimization for the mass spectrometry. **RESULTS**: A total of 3,866 human peptides were detected across 11 urine samples from clinically suspected acute melioidosis patients. Among these, were three Burkholderia specific proteins detected in 75% of culture positive samples. Large amounts of acute phase proteins, cell mediated immunity proteins, complement pathway proteins and inflammatory mediators were seen upon gene ontology (GO) annotation and GO enrichment analysis. **CONCLUSIONS**: This simple in-solution sample preparation method can be replicated easily for LCMS/MS-QTOF and MALDI-TOF proteomic analyses, avoiding tedious optimization steps in 2-DE. This method is cost effective and can be done in centres without specialized 2-DE or MS equipment and elutes can be easily transported for analysis and bioinformatics. This is the first study to analyse urine samples directly for B. pseudomallei proteins. Discovery of the entire proteome as a whole is important in leading to biomarker discovery.

Keywords
Burkholderia pseudomallei, biomarker, melioidosis, urine sample, proteomics

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INTRODUCTION

Melioidosis is an infection caused by the soil dwelling, Gram-negative bacteria *Burkholderia pseudomallei*, and it is known to be endemic in Malaysia, Thailand, Northern Australia, and many other tropical and sub-tropical countries. Melioidosis typically causes febrile illness with respiratory symptoms and septicaemia, but also known to cause fulminant fever and severe septicaemia in diabetics, elderly and immunocompromised patients.[1] The gold standard of diagnosis of melioidosis is still conventional culturing methods.[2] Polymerase chain reaction (PCR) method is available, however limited to tertiary referral hospitals.

Urine is a complex and diverse source of potential protein biomarker candidates, where the desirability lies with its collection by non-invasive techniques. Urine is the glomerular filtrate of plasma, excretion of cells from the renal tract, and urogenital secretions. They give a dynamic picture of a person's physiologic, pathologic and metabolic state at any given time.[3] The idea of a urine biomarker to determine severity of disease, prognosis, or efficacy of therapeutics is highly desirable.[4]

Two-dimensional electrophoresis (2-DE) (gel-based proteomics) is a well-established technique; however, it suffers from some ongoing concerns regarding

quantitative reproducibility and limitations on its ability to study certain class of proteins. With the appearance of MS-based proteomics, new avenues have emerged for quantitative analysis.[5] In shotgun proteomics (bottom up strategy) complex peptides fractions, generated after protein proteolytic digestion, can be resolved using different fractionation strategies, which offer high-throughput analyses of the proteome of an organelle or a cell type, and provide a snapshot of the major protein constituents.[6]

Previous research on biomarkers of *B. pseudomallei* involved mostly testing of suitable antigens on culture plate grown bacteria instead of bacterial components derived directly from human samples.[7,8] Mariappan et al. (2010) studied different cultured isolates of *Burkholderia* sp. through 2-DE and Western blot analysis and found different immunogenic proteins differently regulated.[9] Young et al. (2014) have studied urinary bacterial proteins in tuberculosis (TB) patients, and found a panel of 20 human proteins that were significantly different for with and without TB infection.[4]

Thus, this study was aimed to formulate an easier, reproducible method to prepare urine samples from acute melioidosis patients for bottom-up proteomic studies, using either MALDI TOF MS/MS or LCMS QTOF MS/MS. This method can be performed in less equipped laboratories, to then transported to specialized centres for proteomic studies and bioinformatic analysis.

MATERIAL & METHOD

Ethics declaration and sample recruitment

This study was undertaken with ethical approval from Ministry of Health Malaysia's Medical Research and Ethics Committee (MREC), the National Medical Research Register (NMRR) ID*:* NMRR-16-2699-33554. Informed written consent was obtained from all the 11 melioidosis patients recruited for this study. The patients were diagnosed by clinicians at medical wards Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang (a tertiary teaching hospital). Of the 11 samples tested, eight were culture positive cases (MEL1–MEL8) and the remaining were culture negative cases (NM1–NM3). Some of the patients were recruited upon admission after PCR testing, and some were recruited after being reported as blood culture positive for *B. pseudomallei*. Urine culturing and sensitivity was done for patients in HTAA as part of septic workup, all had no significant findings. Urine culture would not be as sensitive in detecting systemic *B. pseudomallei* infection unless it was a urinary tract infection.

Clinically suspected cases were recruited using inclusion criteria by physicians in HTAA Kuantan. These were patients who presented with high grade fever with or without respiratory symptoms, with evidence of pneumonic X-ray findings or localized infections, especially those with diabetes and other comorbidities. Mid-stream urine samples of these patients were collected upon admission or upon recruitment into study and the samples were kept cool (-20 °C) until further use. Samples chosen were based on culture result and matching age groups, gender and comorbidities (Table 1). After completion of sample collection, cases were regrouped based on clinical presentation (pneumonic/septicaemic) for analysis purpose only. Grouping was made based on presence or absence of X-ray changes.

Urine sample preparation

Figure 1 shows the step-by-step workflow of methodology in processing urine samples for LCMS/ MALDI TOF MS. ReadyPrep 2-D® clean-up kit, an acetone-trichloroacetic acid (TCA) based clean-up kit was used in this study (BioRad, USA), its ability to concentrate proteins from dilute samples allow higher protein loads for mass spectrometry. The proteins are quantitatively precipitated and concentrated, washing away ionic detergents, salts, nucleic acids, lipids that interfere with protein detection via mass spectrometry.

The protein concentration was quantified using nano-spectrophotometer, as the limit of protein concentration for the liquid chromatography mass spectrometry (LCMS) sample preparation was limited to 5 μg/ 5 ml. The list of protein concentration for each of the above samples is shown in Table 1. Normal protein concentrations can be up to 20mg/ dL. The specific amount of sample to be used was calculated for standardization.

Table 1: The protein concentrations in urine supernatants of recruited melioidosis patients

Sample	Age	Gender	Diabetes	Kidney disease	Culture for *B.pseudomallei*	PCR for *B.pseudomallei*	Protein concentration (mg/dl)	Amount used for sample preparation (µl)
MEL 1	42	Male	Yes	No	Positive	Negative	26.53 *	18.9
MEL 2	65	Male	Yes	Yes	Positive	Positive	18.12	27.6
MEL 3	52	Male	Yes	No	Positive	Positive	21.33*	23.5
MEL 4	47	Male	No	No	Positive	Positive	20.51	24.4
MEL 5	44	Male	Yes	No	Positive	Positive	21.52*	23.3
MEL 6	50	Male	Yes	Yes	Positive	Negative	22.73*	22.0
MEL 7	59	Male	No	Yes	Positive	Positive	34.08*	14.7
MEL 8	54	Female	Yes	Yes	Positive	Positive	25.84*	19.4
NM 1	65	Male	Yes	No	Negative	Negative	29.97*	16.7
NM 2	68	Female	Yes	Yes	Negative	Negative	41.67*	12.1
NM 3	51	Female	Yes	Yes	Negative	Negative	33.39*	15.0

* above normal protein levels in urine

The urine preparation method according to Kim et al. (2006) was adapted for digesting a complicated protein mixture in absence of denaturants, because LCMS QTOF systems do not allow for detergent (urea) presence in the samples.[5] Dithiothreitol (DTT) in 50mM Tris-HCl (pH 8.0) and iodoacetamide (IAA) in 50mM Tris-HCl (pH 8.0) were used for the breaking of protein bonds for digestion with trypsin. Prior to adding trypsin, 50mM Tris-HCl was added to reduce the concentration of urea to 0.6 mM. Then MS grade trypsin was added to the mixture giving a final ratio of 1:50 (w/w trypsin:protein). The mixture was incubated at 37°C for 18 hours. The digestion was terminated by adding formic acid to a final concentration of 5% (v/v).

Desalting process

Millipore ZipTips® (Milipore, USA) were used as single step desalting, concentration and purification of samples prior to mass spectrometry. The buffers/solvents used were 100% acetonitrile, 0.1% formic acid, and 60% acetonitrile + 0.1% formic acid. With trial and error while running LCMS, it was observed that the samples had to be eluted four times and then vacuum concentrated to have a good rate of pickup on LCMS QTOF. The sample in quadruplicate was kept at -20°C until ready for analysis using LCMS QTOF MS.

LCMS Run Protocol

Figure 1 shows the workflow methodology for proteomics analysis carried out in this study. As sample amounts were very small, high recovery LCMS vials were used. The Agilent Zorbax Eclipse® C18 (Agilent, USA) column was used, with smaller column capacity. The normal processing of urine sample was observed to be too dilute for adequate MS/MS peaks to form. Thus, after some troubleshooting runs, we found that quadrupling the sample concentration using Ziptips (four times), the samples were then concentrated by

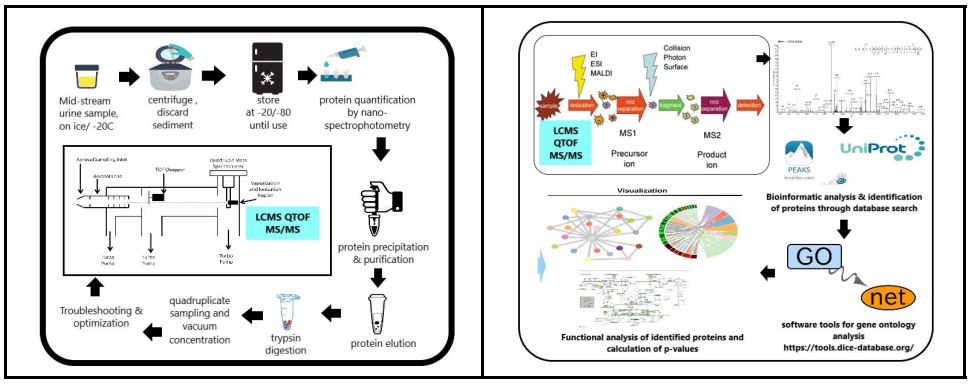

Figure 1: Step by step workflow of processing urine samples for proteomic analysis [image adapted from Young et al. (2014)]. Next is the bioinformatics workflow of proteins detected using LCMS QTOF MS/MS for urine samples. LCMS data was analysed using PEAKSX Studio, using uniport.org and SPIDER database of human and *B. pseudomallei* protein taxonomies. Gene Ontology analysis was done using GO-net.org software tools, as well as generating p-values for identified proteins.

vacuum drying and then run through LCMS. Instrument used was the Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6550 iFunnel Q-TOF LC/MS (Agilent, USA). Vacuum dried samples were reconstituted with 30 µl of Buffer A (water with 0.1 % formic acid).

LCMS settings used in this study were adapted from Gautam et al. (2018) which was optimized for in-solution LCMS QTOF experiments,[10] and also from Juvarajah et al. (2018) using the same LCMS setup locally in Malaysia.[11] The Agilent Large Capacity Chip was used, (300 Ampere, C18, 160 nL enrichment column & 75 um x150 mm analytical column) with a flow rate of 4µL/ min from Agilent 1200 Series Capillary pump and 0.5 µL/ min from Agilent 1200 Series Nano Pump (Agilent, USA).

Solvents used were solvent A (water with 0.1 % formic acid) and solvent B (90 % acetonitrile in water with 0.1 % formic acid.), with the autosampler set at 4°C. Injection volume was set at 1 µl, and gradient was set as in Table 2.

Table 2: The runtime protocol used for LCMS QTOF MS/MS

Time (min)	Solvent/ Mobile Phase A (%)	Solvent/ Mobile Phase B (%)
0	97	3
45	75	25
50	60	40
51	10	90
53	97	3
60	97	3

Parameters on the Agilent LCMS QTOF MS iFunnel was adapted from Gautam et al. (2018): ion polarity was set to positive, capillary voltage (Vcap) at 1900 V, fragmentor voltage at 360 V, gas temperature 325°C, drying gas flow 5.0 L/ min. LCMS data was analysed using PEAKS X database software SPIDER, searching for *B.pseudomallei* and human taxonomies.[10] Protein database type search used was Uniprot and TrEMBL (https://www.uniprot.org/) with fixed modification; settings for in-solution and post- translational modification set to carbamidomethylation. The proteins from the GO ID terms were then analysed via bioinformatics web tool (https://tools.dice-database.org/GOnet/) to obtain GO enrichment analysis which provides p-values. Database of Immune Cell Expression, Expression quantitative trait loci (eQTLs) and Epigenomics (DICE), a web-based bioinformatics tool was used to construct interactive graphs containing GO terms and genes conveying the hierarchical structure according to available annotations.

RESULTS

Following the protocol for sample preparation and protein precipitation as per published methods, the concentration of proteins allowed per run on LCMS was 5 µg/ml. This led to a small amount of urine sampled from the initial volume. After a run through the LCMS QTOF, it was found to have very poor signal pickup and unconvincing peaks, with no protein database hits either on LCMS QTOF or MALDI TOF. Hence, there was need for a less dilute initial sample concentration so there is more chance for protein discovery.

After preparing each sample and desalting in quadruplicates, pooling them together and vacuum drying them to a smaller quantity, the subsequent sampling for the LCMS QTOF run picked up good peaks and a large number of proteins, as it also did on MALDI TOF MS/MS.

Table 3 shows the common proteins detected in the culture positive samples, and not seen in culture negative group of samples. Specific proteins noted were namely, peptidoglycan recognition protein 1, cold agglutinin FS-1 L-chain, plasma protease C1 inhibitor, vesicular integral-membrane protein VIP36, pigment epithelium-derived factor, mannan-binding lectin serine protease 2, and secreted phosphoprotein 1. The details of these proteins and their functions are as in the Table 4 below.

Three *B. pseudomallei* bacterial proteins that were consistently detected among 75 % of the culture positive cases via LCMS QTOF MS/MS, namely SDR family NAD(P)-dependent oxidoreductase, 3-hydroxyacyl-coA dehydrogenase, and NAD(P)-dependent dehydrogenase (short-subunit alcohol dehydrogenase family). However, MALDI TOF MS/ MS database search revealed many uncharacterized *Burkholderia* sp. proteins.

Table 3: Common human proteins detected in the urine samples of the confirmed melioidosis cases using LCMS QTOF MS/MS

Proteins in common between culture positive and PCR positive cases	Immunoglobulin heavy constant alpha 2	Histone H2A type 2-C
	Alpha-1-acid glycoprotein	Histone H2A type 1
	Immunoglobulin kappa variable 2D-28	IGL@ protein
	CD44 antigen	Lymphatic vessel endothelial hyaluronic acid receptor 1
	Uromodulin	Histone H2A type 1-A
	Peptidoglycan recognition protein 1	Protease serine 2 isoform B
	Immunoglobulin G1 Fab heavy chain variable region	Immunoglobulin kappa variable 2-40
	Histone H2A type 1-B/E	Inter-alpha-trypsin inhibitor heavy chain H4
	Immunoglobulin alpha-2 heavy chain	Histone H2AX
	Histone H2A type 1-C	Mannan-binding lectin serine protease 2
	N-acetylglucosamine-6-sulfatase	IgG H chain
	Basement membrane-specific heparan sulfate proteoglycan core protein	Vesicular integral-membrane protein VIP36
	Plasma protease C1 inhibitor	Serine/cysteine proteinase inhibitor clade G member 1 splice variant 2
	Prothrombin	Histone H2A
	Cold agglutinin FS-1 L-chain	Immunoglobulin kappa variable 2-28
	Carbonic anhydrase 1	Myoglobin
	Secreted phosphoprotein 1 (Osteopontin bone sialoprotein I early T-lymphocyte activation 1) isoform CRA_c	Histone H2A type 1-J
	Epididymis secretory protein Li 51	Prosaposin (Variant Gaucher disease and variant metachromatic leukodystrophy)
	Prosaposin	Pigment epithelium-derived factor
	Aminopeptidase N	Epididymis tissue protein Li 173
	Retinol-binding protein	Cystatin
	Cathepsin D	Angiotensinogen
	Histone H2A type 2-A	Kininogen-1
	CFB	Histone H2A type 1-D
	Immunoglobulin kappa variable 2D-40	Plasma protease C1 inhibitor
	Apolipoprotein C-II	Histone H2A type 1-H
		Profilin-1

Gene ontology (GO) terms (Figure 2) from the culture positive group showed largest number of proteins groups were involved in immune response, acute phase proteins and defence response proteins. This was consistent with the patients being in the acute phase of infection with flooding of immunological, defence proteins and inflammatory mediators. The next group of proteins were those involved in regulation of normal biological quality and homeostasis, cellular response proteins, transport proteins and structural proteins. The pattern was similar in the culture negative proteins. Even so, using GO enrichment analysis, the statistically significant human proteins gene ontology terms (with p-value <0.05) are lipoprotein particle remodelling, protein -lipid complex remodelling, myeloid leucocyte mediated immunity, phospholipid efflux, complement activation (classical pathway), humoral immune response mediated by circulating immunoglobulin and others.

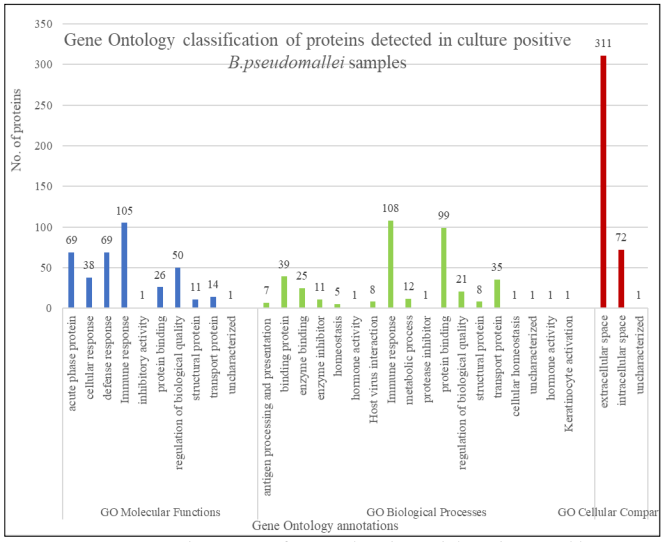

Figure 2: Gene ontology terms for proteins detected in culture positive cases (MEL1-8), divided into molecular functions, biological classes and cellular compartments. (generated using software tools from https://tools.dice-database.org/GOnet/)

P-values were computed using software tools from https://tools.dice-database.org/GOnet/, an online service affiliated to Database of Immune Cell EQTLs, Expression and Epigenomics (DICE) project. The data from PEAKSX was inserted into the pre-programmed software that generated gene ontology analysis and terms.

DISCUSSION

In-solution gel-free method for preparing urine samples for proteomics has allowed us to skip the optimization

process of 2-DE and avoid its limitations. Some troubleshooting was required as protein concentrations were too low for detection. Young et al (2014) did suggest the usage of molecular weight cut-off (MWCO) filters,[4] but due to its unavailability and the concerns on limiting the protein sizes, we opted not to use the MWCO. However, this has caused not only low concentrations of protein, but also masking by high abundance and large sized proteins of smaller proteins

Table 4: Proposed human proteins as biomarkers for acute melioidosis and their functions after comparing between samples from culture positive and culture negative cases

No	Protein name	Function	References
1	Peptidoglycan recognition protein 1	A pattern receptor that binds murein peptidoglycans of gram-positive and gram-negative bacteria, with bacteriostatic activity, and plays a role in innate immunity	Lu et al., 2006[13]
2	Secreted phosphoprotein 1	Similar to Osteopontin, although mostly functioning in bone remodelling, it also has immune functions. They are also expressed in macrophages, neutrophils, T and B cells, thought to act as immune modulator with chemotactic properties	Wang & Wang, 2008[14]
3	Plasma protease C1 inhibitor	An acute-phase protein, a protease inhibitor whose main function is to inhibit the complement system to prevent spontaneous activation, but also as a major regulator	Cicardi et al., 2005[15]
4	Cold agglutinin FS-1 L-chain	Immunoglobulin-like protein	
5	Vesicular integral-membrane protein VIP36	A transmembrane lectin that shuttles between the endoplasmic reticulum, Golgi apparatus and plasma membrane. This protein binds high mannose type glycoproteins and facilitate their activities.	RefSeq,https://www.ncbi.nlm.nih.gov/gene/10960, Oct 1998
6	Pigment epithelium-derived factor	Belongs to a non-inhibitory group of serpin glycoproteins. Its biological activities include promoting cell survival as well as antiangiogenic, antitumor properties. Has been touted as possible prognostic markers for cancer as well as a potential therapeutic target	Franco-Chuaire et al., 2015[16]
7	Mannan-binding lectin serine protease 2	An enzyme involved in the complement system; it is involved in a pathway that reacts to Ra-reactive (RARF) complement dependent bactericidal factors. This factor binds to Ra and R2 polysaccharides expressed by certain enterobacteria	Dong et al., 2016[17]

A simple reproducible gel-free method is valuable in making proteomics more accessible to centres that are not equipped with the very expensive and elaborate equipment. This method can be reproduced in mid-level laboratories without delay and then couriered for further analysis. It also reduces the time needed for optimization as for 2-DE, missed findings in spot picking, and allows for reproducible experiments.

Problems with 2-DE resolution, such as missing high molecular weight proteins (higher than 250kDa), masking of low abundance or rare proteins, non-detection of hydrophobic or membrane proteins, can be resolved with gel-free in-solution methods.[10]

Most previous studies in describing proteins expressed or proteome of melioidosis, has been on bacterial culture samples. Mariappan et al (2010) studied stationary phase culture supernatant of *B. pseudomallei*, using 2-DE and 113 secreted proteins spots we identified.[9] They included metabolic enzymes, transcription/translation regulators, transport regulators. Our sample however, was a direct sample with more human proteins in the mix.

Young et al (2014) analysed urine proteome between definite TB, latent TB, and non-TB cases using SDS-PAGE, liquid chromatography, and shotgun proteomic analysis mass spectrometry. They reported 10 different mycobacterial proteins observed exclusively in the urine of definitive TB patients, while six mycobacterial proteins were found exclusively in urine of presumed latent TB cases. In addition, using GO enrichment analysis, they identified a panel of 20 human proteins that were significant discriminators for TB disease compared to no TB disease. Seven common human proteins were either differentially over or under expressed in the TB versus non-TB group [4].

Ward et al from US Army Institute of Infectious Diseases undertook in vivo studies with rhesus macaque animal model, to examine host response to infection using proteomic methods. In comparing with Ebola infected and healthy controls, they found the *B. pseudomallei* infected group had expressed 28 unique proteins in altered levels. These proteins were involved in the clotting cascade, immune signalling and complement system activation.[12]

Interestingly our study also showed that a large amount of acute phase proteins, inflammatory proteins, coagulation cascade proteins and immune proteins were detected in the urine along with some bacterial proteins. The urine proteome depicts a dynamic picture of what is happening in the circulation of the body of the patient at the time. This shows a real time

pathophysiological picture of the infectious process of *B. pseudomallei.*

Our urine proteomic studies have confirmed the hypothetical findings of animal model studies and highlighted a few potential urinary human protein biomarkers for future studies. Going forward, investigations on bacterial proteins detectable in the serum and sputum of acute melioidosis patients need to be conducted to further identify candidate biomarkers for rapid testing.

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Ethics declaration

This study was undertaken with ethical approval from Ministry of Health Malaysia's Medical Research and Ethics Committee (MREC), the National Medical Research Register (NMRR) ID: NMRR-16-2699-33554

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