Metabolomics Reveals Deregulation of Lipid and Amino Acid Metabolisms in the Lungs of Rats Post-*K. pneumoniae* Infection

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Abstract. The host's metabolic response towards pathogenic infection was investigated using a *Klebsiella pneumoniae* infected rat model via metabolomics approaches. The lungs of rats serve as control and post-*K. pnaumoniae* infections were subjected to metabolomics analysis to assess the sensitivity of metabolomics in revealing metabolic changes and perturbations associated with the infection. 35 metabolites were significantly altered between the control and infected groups. Most of the detected metabolites were derived from lipid and amino acid metabolisms, suggesting that the host is under severe inflammation and oxidative stress even at post-*K. pneumoniae* infection. Our findings provide insight into the potential of metabolomics as a powerful tool for unravelling the pathogenesis of bacteremia.

Keywords: *Klebsiella pneumonia*, Metabolomics, Metabolisms.

1.0 Introduction

Infections are among the top three leading causes of death worldwide [1]. According to Sahiledengle et al. [2], in the United States alone, approximately, 2 million patients developed healthcare-associated infections and nearly 100,000 of these patients were estimated to die annually. Even in developed countries, with appropriate intensive care and antibiotic treatment, severe bacteremia is still the leading cause of death in critically ill patients [3]. Severe bacteremia is fatal with mortality rates over 50% as observed in developing countries [4]. Infections remain the leading cause of death in developing countries, even with the advancement of diagnostic, critical care medicine,

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decision-making aids, progress in antibiotics development and clinical practice guidelines to assist clinicians with empirical therapy [5]. This is mainly due to the delay in the prompt identification of the pathogen. In addition, current treatments for bacteremia are limited to correcting its immediate manifestations and outcomes [6].

Metabolomics, a cutting-edge field in systems biology, provides a comprehensive view of small molecules and metabolites within biological systems. In the context of infectious diseases, metabolomics allows for the exploration of host-pathogen interactions at a molecular level, shedding light on how infections perturb the host's metabolic pathways and influence disease outcomes. The ability of the pathogens to adapt to the changes in the metabolic landscape of the host will determine the survival of the pathogens and may cause diseases to the host [7]. However, our knowledge of the metabolic host responses to bacterial pathogens during infection is still limited.

Thus, in this study, we employ a rat experimental model to simulate *K. pneumoniae* infection, providing a controlled environment to investigate the host response to infection at the metabolomic level. By analyzing the metabolites present in lung tissues at post-*K. pneumoniae* infection, we aim to evaluate the sensitivity of metabolomics as a tool to uncover the metabolic changes in the host and to determine the metabolic perturbation associated with *K. pneumoniae* in the host. The data obtained will provide the basis for a new drug target design associated with bacteremia or a useful nutritional guide for patients with bacteremia, which could be important for the improvement of a patient's survival.

2.0 Literature Review

Klebsiella pneumoniae is a gram-negative, encapsulated, non-motile bacterium that is commonly found in the environment and can cause pneumonia in patients. It is known to display high degrees of virulence and antibiotic resistance, making it a significant threat to human health [8]. Most of the spectrum beta-lactamase and carbapenem are extremely resistant to *K. pneumoniae*, leading to an increasing number of multidrug-resistant strains. This is because *K. pneumoniae* produces the enzyme carbapenemase, which degrades broad-spectrum-lactam drugs such as carbapenems, penicillin, and cephalosporin [9]. Due to the high prevalence of antibiotic-resistant bacteria, understanding the intricate interplay between the host's metabolome and the pathogen during infection is a critical aspect of infectious disease research.

Mass-spectrometry (MS) based metabolomics has appeared as a tool for evaluating the small molecule components of cellular metabolism [10]. Cutting-edge technology makes it possible to physically separate thousands of different metabolites, and as a result, it offers a more comprehensive view of the metabolome. MS-based metabolomics gives better resolution and sensitivity when compared to NMR-based metabolomics [11]. Thousands of metabolites found in biological samples are separated with a chromatographic system based on their unique chromatographic properties. This allows for a greater throughput of metabolites to be

analyzed by the MS system while retaining its sensitivity [10].

Metabolites is the product of metabolism which include sugars (or carbohydrate), fats (or lipid), amino acids and their derivative. Understanding the characteristics of the metabolome (collection of metabolites within a cell) will lead to a better understanding of certain diseases and their associated mechanism. Thus, metabolomics approach in infectious diseases can help researchers understand the metabolism of microorganisms and metabolic changes in the infected host, which leads to a better understanding of the disease risk and thereby provides objective information about disease progression. In addition, the development of bacteraemia-specific biomarkers and molecular diagnostics for the assessment of the host response is expected to foster both drug development and improve the clinical management of bacteraemia [12].

3.0 Methodology

3.1 Bacteria

Klebsiella pneumoniae, isolated from a patient who suffered septicemia was cultured in Mueller Hilton Broth (MIB) (Oxoid Limited, Basingstoke, Hampshire, England) for 11 h to stationary phase, producing a concentration of 2 X 1010 colony forming unit per mL (CFU/mL). The bacteria were also cultured on the Mueller-Hinton (MHA) agar plates, and colonies were counted after 24 hours. Bacterial suspensions were centrifuged at 10,000 g for 10 min, and the resultant pellet was washed twice with sterile normal saline solution for subsequent experiments.

3.2 Research Ethics Approval

Ethical approval was obtained from the local Research Ethics Committee on the use of animals in research (UiTM CARE). All animal experiments were performed based on the Organization for Economic Cooperation and Development (OECD) guidelines.

3.3 Sample Collection

A total of 14 Sprague-Dawley male rats weighed 200-250 g (5 to 7 weeks old) were randomly divided into two groups after one week of acclimatization. The animals were kept in individually ventilated caging (IVC) system with corn cobs, fed with standard pellet and water ad libitum and maintained at room temperature (27 ± 2˚C) with 70-80% humidity and 12-hour light/dark cycle for at least 48 hours before the study started.

The rats were divided into a control group (n=7) and an infected group (n=7). The control group was intravenously injected with 0.3 mL of sterile saline and the treatment group was injected with 0.3 mL of *K pneumoniae* (2 x 1010 CFU/mL), respectively via the tail.

All the rats were closely monitored for signs of infections throughout the experimental period. Body weight of all rats was recorded throughout the

experimental periods. The rats were sacrificed at the end of the experiment (day 9). The lungs of the rats were collected and frozen in liquid nitrogen to preserve the metabolites before being stored at -80°C for further use.

3.4 Organ Metabolite Extraction

The procedure was carried out according to [13] with slight modifications. Two hundred (200) mg of organ (lung) was weighed and ground using a mortar and pestle in liquid nitrogen. Metabolites were extracted using the mixture of chloroform: methanol: deionized water with a ratio of 2:2:1 (v/v). Three (3) mL of chloroform was added to each sample together with 3 mL of methanol and 1.5 mL of deionized water. Samples were vortexed briefly before centrifuged at 10,000 x g for 10 minutes at 4˚C. A portion of the aqueous layer and a portion of the organic layer were removed and pooled together in a new microcentrifuge tube. Supernatants were dried using a vacuum concentrator (Appendorf, Concentrator Plus, Germany). All extracted samples were stored at -80˚C until required.

3.5 Chromatography

Chromatography was performed on a Liquid Chromatography of 1200 Rapid Resolution Series (Agilent Technologies, Santa Clara, CA, USA) consisting of a binary pump, degasser, well plate autosampler with thermostat, thermostat column compartment and 6520 QTOF mass spectrometers equipped with a dual-ESI source. Separation was done using a Zorbax Eclipse Plus C18 with a column ID of 1.8 µm particle size and 2.1x100 mm column dimensions (Agilent Technologies, Santa Clara, CA, USA). The temperature was maintained at 40°C during the run. The mobile phase (A) consisted of 0.1% formic acid (Supelco, Inc) in ultrapure water of 18 MΩ and (B) 0.1% formic acid in acetonitrile (LiChrosolv, Darmstadt, Germany).

The samples were run in batches. Each batch consisted of 6 samples (4 samples, 1 quality control (pooled samples) and 1 blank). Total run time was set at 48 minutes for each analysis with four (4) replicates for each sample. The gradient was developed based on percentage solvent B over time. A linear gradient was developed over 36 minutes from 5% to 95% of mobile phase (B), followed by washing for 5 minutes and equilibration of the column for 7 minutes. The flow rate was 0.25 mL/min and the injection volume was 2 µL.

3.6 Metabolomics Data Analysis

Preprocessing of the LCMS data was conducted using XCMS (https://xcmsonline.scripps.edu/) which includes normalization and annotation of the detected features (m/z-retention time). LCMS data files were first converted to mzML formats using MSConvert (ProteoWizard). The mzML files were then processed by 'xcms' R packages for peak identification and peak table generation.

Data that was filtered and normalized was directed to METLIN Metabolite and Chemical Entity Database (https://metlin.scripps.edu) for compound identification. METLIN is a metabolite mass spectral web-based database developed

to assist the search and identification of metabolites through mass analysis. METLIN database is linked and compared with several databases, HMDB (http://www.hmdb.org/), KEGG (http://www.genome.jp/kegg/), LIPID MAPS (http://dev.lipidmapd.org:25424/) and PUBCHEM (http://pubchem.ncbi.nlm.nih.gov/). Perturbated pathways involved in post-*K. pneumoniae* infections were identified using Metabolomics Pathway Analysis (MetPA) (http://www.metaboanalyst.ca).

4.0 Findings

4.1 Metabolite Profiling

This study aimed to understand how *K. pneumoniae* infection alters the metabolite profiles in the lung tissue of the host. Uncovering the metabolite pathways and processes perturbed during *K. pneumoniae* infection will provide insights into the mechanisms of pathogenesis and the progression of the infection. In this study, a total of 36 lung samples from both control and infected groups underwent metabolomics analysis

Profiling the lung tissue of rats infected with *K. pneumoniae* revealed that 35 metabolites were statistically significant at a p-value of less than 0.05. Table 1 provides a comprehensive listing of the metabolites, including their respective mass, pathways, sub-pathways and metabolites regulation. Among the 35 identified metabolites in the lungs of infected rats, 5 were upregulated, and 30 were downregulated compared to the control group. The metabolites identified in the lungs of infected rats were found to be derived from five primary metabolic pathways and 21 sub-pathways. The perturbed pathways include amino acid metabolism, lipid metabolism, cofactors and vitamins metabolism, carbohydrate metabolism, and nucleotide metabolism. The primary metabolic pathways most affected in the lungs of rats infected with *K. pneumoniae* are amino acid metabolism and lipid metabolism, which exhibit the highest proportion of altered metabolites (Table 1).

Table 1. List of detected metabolites

Pathway	Sub-pathway	Metabolites	Mass	Regulation
Amino acid metabolism	Valine, leucine and isoleucine metabolism	Valine	117.0729	UP
		Leucine	131.0900	DOWN
	Cysteine and methionine metabolism	Methylthioadenosine	297.0834	DOWN
	Lysine degradation	delta1-Piperideine	127.0731	DOWN
	Arginine biosynthesis	Arginine	174.1110	DOWN
		Aspartate	133.0763	UP
	Phenylalanine metabolism	Phenylacetic acid	136.0513	DOWN

	Tyrosine metabolism	4-Hydroxyphenylacetaldehyde	136.0521	DOWN
	Arginine and proline metabolism	2-Oxo-4-hydroxy-5-aminovalerate	147.0565	DOWN
	Glutathione metabolism	Oxiglutatione	612.1598	DOWN
Lipid metabolism	Glycerophospholipid metabolism	2-Lysolecithin	517.3105	DOWN
	Fatty acid biosynthesis	Tetradecanoate	228.2027	DOWN
		Dodecanoate	200.1718	DOWN
	Arachidonic acid metabolism	Thromboxane B2	370.2314	DOWN
		14,15-EET	320.2382	DOWN
		5(S)-HPETE	336.2246	DOWN
		Leukotriene A4	318.2148	DOWN
	Primary bile acid biosynthesis	5-Oxoeicosatetraenoic acid	318.2121	DOWN
		7alpha-Hydroxycholest-4-en-3-one	400.3306	DOWN
		Taurochenodeoxycholate	499.3055	DOWN
	Biosynthesis of unsaturated fatty acid	Icosatrienoic acid	306.2511	DOWN
		Octadecanoic acid	284.2670	DOWN
	Fatty acid degradation	Hexadecano	242.1817	DOWN
		Hexadecanal	240.2396	DOWN
	Linoleic acid metabolism	Linoleic acid	280.2352	DOWN
		9(10)-EpOME	296.2289	DOWN
	alpha-Linoleic acid metabolism	cis-3-Hexenyl acetate	142.0975	DOWN
	Bile secretion	Fexofenadine	501.2795	DOWN
Cofactors and vitamins metabolism	Ubiquinone and terpenoid-quinone biosynthesis	Vitamin K1 2,3-epoxide	466.3508	DOWN
	Pantothenate and CoA biosynthesis	Pantothenate	219.1314	DOWN
	Retinol metabolism	9-cis-Retinoic acid	300.2076	DOWN
		all-trans-5,6-Epoxyretinoic acid	316.2066	DOWN
Carbohydrate and nucleotide	Citrate cycle, inositol phosphate metabolism and purine metabolism	L-Malate	134.0445	UP
		Xanthine	152.0353	UP
		Inosine	268.1060	UP

4.2 Clustering Analysis

The Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) were constructed on the metabolites detected in both control and *K. pneumoniae*-infected lung (Figure 2). PCA did not reveal a clear separation between the control and infected groups (Figure 1 (A)), whereas supervised component analysis (PLS-DA) revealed a clear separation among the metabolites identified in both control and infected groups (Figure 1 (B)). This proves that varying expressions or metabolites regulation have been detected between the control and infected groups.

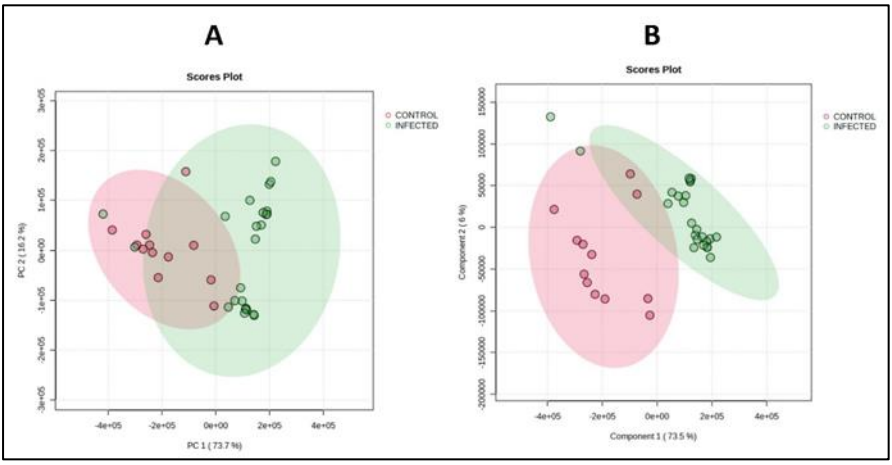

Fig. 1. Differential expression of metabolites in control and infected rat lung analyzed using metaboanalyst's data annotation features. A) The Principal Component Analysis (PCA) of metabolites in positive mode. B) PLS-DA of the metabolite identified in positive mode.

4.3 Network and Pathway Analysis

Metabolomics Pathway Analysis (MetPA) have identified 27 perturbed pathways in the lungs of the rat infected with *K. pneumoniae*. Among the perturbed pathways detected were linoleic acid, pantothenate and CoA biosynthesis, valine, leucine, and isoleucine biosynthesis, arginine biosynthesis, and arachidonic acid metabolism (Figure 2).

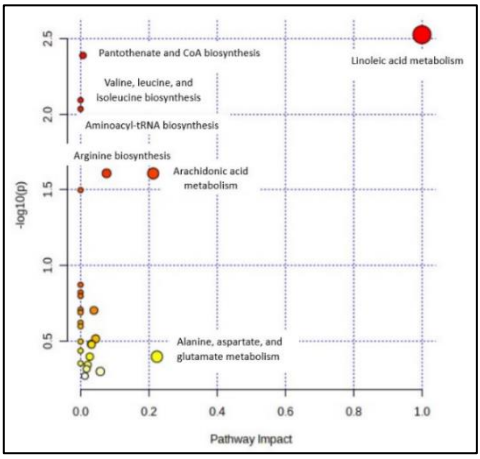

Fig. 2. Perturbated metabolic pathways observed in the lungs of control and infected rats at post-*K. pneumoniae* infections.

5.0 Discussion

Organs contribute to the metabolite level observed in the serum and involve highly active and regulated processes that provide metabolites to all tissues of the body, to be utilized in many processes, for example protein synthesis and energy metabolism [14]. Among the organs, lung was the organ that was affected the most by *K. pneumoniae* infection based on histology findings (Data not shown). Metabolomics profiling on lung samples of infected rats revealed the high metabolic impact (even at *K. pneumoniae* post-infection (192-hour) time point), as indicated by the high number and magnitude of variations relative to control. This is consistent with this organ being the major site of *K. pneumoniae* infection as reported by Holden, Breen, Houle, Dozois, & Bachman [15].

5.1 Overview of Perturbed Metabolic Pathway in Response to *K. pneumoniae* Infection.

Metabolomics is becoming increasingly important for the analysis of host-pathogen interactions, particularly in the discovery of biomarkers of diseases. A recent study has shown that metabolomics can be used to identify metabolites that are altered in response to an infection [12]. Furthermore, the metabolomics approach in infectious diseases helps researchers understand the metabolism of microorganisms and metabolic changes in the infected host, leading to a better understanding of disease risk and providing objective information about disease progression and expected patient outcomes [16]. In this study, metabolomics analysis reveals perturbation of lipid and amino acid metabolisms which suggests that the host is

under severe inflammation and oxidative stress even at post-*K. pneumoniae* infection.

5.1.1 Lipid Metabolism. The study conducted by Yan et al. [17] demonstrates that the activation of linoleic acid metabolism in macrophages plays a crucial role in facilitating the elimination of intracellular *K. pneumoniae*. The experimental findings indicate that linoleic acid effectively reduced bacterial load in the organs of the mouse model. As shown by biomarkers analysis, it is proven that linoleic acid and arachidonic acid, from lipid metabolism, have a higher impact on the infection. The linoleic acid in lipid metabolism is highly perturbated in the pathway, which concludes that the metabolites are upregulated in the control group, but downregulated post-infection because linoleic acid is used to fight infection at an early stage of *K. pneumoniae* infection.

5.1.2 Amino Acid Metabolism. The acquisition of amino acids is crucial for cell viability and proliferation [18]. This can be achieved through the utilization of both endogenous and exogenous proteins [19]. Rats have evolved biochemical and metabolic mechanisms to regulate pathogen infection by upregulating the breakdown of amino acids, thereby facilitating immune responses and limiting the availability of nitrogen-containing nutrients to invading *K. pneumoniae*.

The biosynthesis of valine, leucine, and isoleucine plays a significant role in the regulation of metabolism during severe lung infections, enabling the host to survive. Leucine plays a crucial role in the regulation of various cellular processes, including protein synthesis, tissue regeneration, and metabolism [20]. Based on the results, there are significant differences in leucine levels between the control and infected groups, with downregulation in the infected group. This is because most leucines were used during the early stages of infection to combat *K. pneumoniae*. This indicates that the rats are in the phase of surviving the infection. It can be stated that metabolites such as L-Leucine have the ability to stimulate innate immunity mediated by macrophages in the context of lung infection.

6.0 Conclusion & Recommendations

A metabolomic approach was conducted to investigate the response of rat lungs to *K. pneumoniae* at 192 hours post-infection. Principal component analysis (PCA) successfully identified two distinct clusters, representing control and infected populations. MetPA detected several metabolic pathways, including linoleic acid, pantothenate, and CoA biosynthesis, valine, leucine, and isoleucine biosynthesis, arginine biosynthesis, and arachidonic acid metabolism. This metabolomic study has expanded our understanding of the metabolic pathways involved in the host's response to *K. pneumoniae* infection. However, it is necessary to conduct a metabolomic study specific to human populations affected by *K. pneumoniae* to confirm and further validate the biomarkers identified in animal models. Further investigation is required to enhance our understanding of *K. pneumoniae's* intricate antibiotic resistance, especially at the molecular level. This goal is crucial to improve disease diagnosis and

treatment measures against the emergence of antimicrobial resistance (AMR).

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