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Carbon utilization phenome of *Leptospira interrogans* serovar Manilae strain K64

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ABSTRACT

Background: Leptospirosis, an acute febrile disease caused by the pathogenic species of genus *Leptospira,* is one of the neglected emerging zoonoses that is of global public health concern. The recent genus-wide sequencing of *Leptospira* isolates led to the need for better understanding of the complex metabolic mechanisms of this organism. However, majority of the published studies on *Leptospira* metabolism were still the pioneering works of Baseman and Cox in the 60's and their contemporaries. Knowledge on the carbon sources that supports the growth of a *Leptospira* species will not only contribute to the limited metabolic studies but will further support the reported genes and metabolic pathways of this organism. **Objective:** Thus, this study aimed to describe the carbon utilization phenome of *Leptospira interrogans* serovar Manilae strain K64, one of the dominantly circulating pathogenic *Leptospira* in the Philippines. **Methods:** A previously optimized Biolog™ Gen III sole carbon utilization phenotype microarray assay protocol for leptospires was adapted. **Results:** *L. interrogans* serovar Manilae strain K64 showed utilization of 29 carbon sources belonging to sugars and sugar derivatives, amino acids, methyl ester, carboxylic acid and fatty acids. These were N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, D-fructose, D-galactose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-fructose-6-phosphate, D-gluconic, D-glucuronic acid, glucuronamide, D-saccharic acid, D-aspartic acid, D-serine, L-alanine, L-arginine, L-histidine, L-pyroglutamic acid, L-serine, D-lactic acid methyl ester, citric acid, D-malic acid, L-malic acid, alpha ketoglutaric acid, alpha ketobutyric acid, and acetoacetic acid. **Discussion and Conclusion**: The carbon sources utilized by *L. interrogans* serovar Manilae strain K64 agreed well with the identified genes and metabolic pathways among *Leptospira* species. Moreover, these 29 carbon sources have been previously reported to be associated in the biosynthesis of peptidoglycan, lipopolysaccharide, histidine, sulfur, amino acids, and isoleucine and in other metabolic pathways such as glycolysis, pentose-phosphate, pyruvate and fatty acid in *Leptospira* spp.

Keywords: *Leptospirosis; Leptospira interrogans; phenotype microarray; carbon utilization phenome*

INTRODUCTION

Leptospirosis is a preventable disease in animals and humans. In majority of the developing countries in the Asia Pacific region and in the Philippines, poor living conditions along with frequent typhoons, being largely a water-borne zoonoses, contribute to the risk of infection (Victoriano et al., 2009). It is now an emerging global disease due to the re-emergence of the disease in non-endemic areas and becoming an urban problem in highly endemic areas (Kariv et al., 2001; Picardeau, 2013). To elucidate the current disease burden of leptospirosis, studies are focused on the need for continued monitoring of the prevailing serovars in a given geographical area and improvement of diagnostic capabilities (Gloriani et al., 2016a; Gloriani et al., 2016b; Saito et al., 2017; Tabo et al., 2018; Villanueva et al., 2010; Villanueva et al., 2014; Villanueva et al., 2016; Villanueva et al., 2018; Yanagihara et al., 2007; Zamora & Gloriani, 2015).

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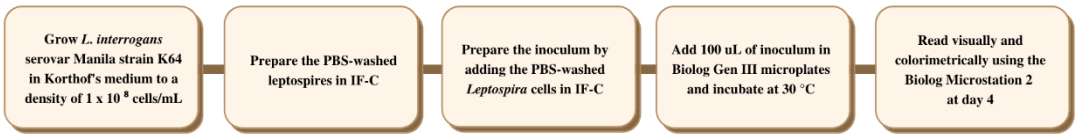

Figure 1: Biolog™ Gen III sole carbon utilization phenotype microarray assay for *Leptospira*

The recent genus-wide sequencing of *Leptospira* isolates further elucidated the biodiversity, epidemiology and evolution of this pathogen (Guglielmini et al., 2019). This, however, led to the need for better understanding of the complex metabolic mechanism in *Leptospira* species. Based on published studies, the entire metabolic process in *Leptospira* is not yet clear, even the pentose-phosphate pathway also needs further understanding (Govindaraju et al., 2017). The absence of glycolysis even in the presence of all the genes to produce glycolytic enzymes remains to be further studied (Kefford et al., 1986; Nascimiento et al., 2004; Picardeau et al., 2008; Ren et al., 2003; Zhang et al., 2011). Interestingly, many aspects of *Leptospira* energy metabolism were understood when the genome of two *Leptospira* strains (*L. interrogans* serovar Copenhageni and *L. interrogans* serovar Lai) were completely sequenced (Nascimiento et al., 2004).

To date, majority of the published studies on *Leptospira* metabolism, particularly on the nutritional requirements, were still the pioneering works of Baseman and Cox in the 60's and their contemporaries (Baseman & Cox, 1969; Hennerbery & Cox, 1971). Thus, this study aimed to describe the carbon utilization phenome of *Leptospira* interrogans serovar Manilae strain K64 using the sole carbon utilization phenotype microarray (PM), known to be the third major technology utilized in the omics-driven research and drug development (Bochner et al., 2001; Bochner, 2003; Bochner, 2009). Knowledge on the carbon utilization phenome of one of the four *Leptospira* serovar/serogroups that was previously reported to be predominantly circulating in the Philippines, will not only contribute to the limited metabolic studies, but will further support the reported genes and metabolic pathways of this organism, as well as identify potential drug targets. In addition, similar to the previously reported uses of Biolog™ phenotype microarray technology in the identification of many gram positive and gram negative microorganisms and in mechanism of action studies of novel antimicrobials, its use as a diagnostic tool for leptospirosis by comparing the phenome between pathogenic and non-pathogenic *Leptospira* serovar, as well as, its use as a screening for and elucidating the mechanism/s of potential antimicrobials for leptospirosis could be the future directions of this study.

MATERIALS AND METHODS

Equipment and reagents

The Biolog™ Gen III MicroStation System (Biolog™ Inc., Hayward, CA, USA) was used in the sole carbon source utilization phenotype microarray assay. The Gen III Microplate™ and the inoculating fluid (IF-C) used in the sole carbon source utilization assay were all obtained from Biolog™ (Biolog™ Inc., Hayward, CA, USA). Phosphate buffered saline (PBS), and *Leptospira* culture medium (i.e., Korthof's medium) were of analytical grade.

Leptospira strain and Inoculum preparation

Stock culture of *L. interrogans* serovar Manilae strain K64 was obtained from the Leptospirosis Prevention and Control Laboratory (LepCon), Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. The organism was maintained by continuous culture in Korthof's medium. The inoculum (PBS-washed *Leptospira* cells in IF-C), was prepared using *L. interrogans* serovar Manilae strain K64 grown in Korthof's at 30°C for 4-7 days with bacterial density of approximately 1 x 10 ^{8} cells/mL, as previously described (Manglicmot-Yabes et al., 2020).

Biolog™ phenotype microarray (PM) sole carbon utilization technology

The Biolog™ Gen III microplate was used to analyze the ability of *L. interrogans* serovar Manilae strain K64 to metabolize 71 major classes of biochemicals. These biochemicals, pre-coated onto the 96 microplate, include carbon sources belonging to sugars and sugar derivatives (n=35), amino acids (n =11), and esters, carboxylic acids and fatty acids (n =25). A previously described protocol in another study was adapted. The method was a modification of the Biolog™ Gen III sole carbon utilization phenotype microarray standard protocol, optimized for its suitability to evaluate the anti-leptospiral activity of a plant extract and selected antimicrobials against *Leptospira serovars*, was adapted (Manglicmot-Yabes et al., 2020). Briefly, 100 uL of inoculum (PBS-washed *L. interrogans* serovar Manilae strain K64 cells in IF-C), was aseptically dispensed to each well of the Biolog™ Gen III microplates and incubated at 30°C (Figure 1).The procedure was performed in 2 runs in triplicates. Only three replicates per run were used since the manufacturer's claim for reproducibility in the package insert is excellent due to controlled conditions required in the whole procedure and on the basis of the consistent results obtained in the previous study (Manglicmot-Yabes et al., 2020). Those wells with corrected OD of >0.100 and visually comparable with the positive control were considered well utilized and interpreted as positive for carbon source utilization. On the other hand, those wells with corrected ODs of <0.100, although this is above the corrected OD of the negative control, were considered negative or borderline and interpreted as negative for carbon source utilization.

Ethical approvals

This research was registered in the University of the Philippines-Manila (UP) Research Grant and Administration Office (RGAO 2019-1090) and underwent the Institutional Biosafety and Biosecurity Committee (IBBC 2019-014), and Research Ethics Review Board (2020-089-EX) reviews and approvals. All experiments using *Leptospira* cultures were performed in a university-based BSL-II facility.

RESULTS

Twenty nine carbon sources were utilized by *L. interrogans* serovar Manilae strain K64 out of the 71 major classes of biochemicals pre-coated in Biolog™ Gen III microplate (Table 1). These carbon sources belong to sugars and sugar derivatives (n=15), amino acids (n = 7), and carbon sources which belong to esters, carboxylic acids, and fatty acids (n= 7). Results of the carbon utilization phenome or pattern of carbon utilization of *L. interrogans* serovar Manilae strain K64 were consistent within the 3 replicates and between two runs.

DISCUSSION

The carbon sources reported to be utilized by *L. interrogans* serovar Manilae strain K64 in this study had been previously reported in other *Leptospira* species to be associated in biosynthesis of peptidoglycan, lipopolysaccharide, histidine, sulfur, amino acids, isoleucine and other metabolic pathways such as glycolysis, pentose-phosphate, pyruvate and fatty acid (Amineni et al., 2010; Charon et al., 1973; Gerhardt & Ball 1959; Govindaraju, 2017; Nascimento et al., 2004; Ren, 2003; Ricaldi et al., 2012; Vin et al., 1985; Zhang et al., 2011).

Table 1: Carbon sources utilized by *L. interrogans* serovar Manilae strain K64 using the Biolog™ Gen III phenotype microarray

Sugars and sugar derivatives (n=15/35*)	Amino Acids (n=7/11*)	Esters, carboxylic acids and fatty acids (n=7/25*)
1. N-acetyl-β-D-mannosamine	1. D-aspartic acid	1. D-lactic acid methyl ester
2. N-acetyl-D-galactosamine	2. D-serine	2. Citric acid
3. N-acetyl neuraminic acid	3. L-alanine	3. D-malic acid
4. D-fructose	4. L-arginine	4. L-malic acid
5. D-galactose	5. L-histidine	5. Alpha ketoglutaric acid
6. 3-methyl glucose	6. L-pyroglutamic acid	6. Alpha ketobutyric acid
7. D-fucose	7. L-serine	7. Acetoacetic acid
8. L-fucose		
9. L-rhamnose		
10. Inosine		
11. D-fructose-6-phosphate		
12. D-Gluconic		
13. D-glucuronic acid		
14. Glucuronamide		
15. D-saccharic acid		

*Total number of carbon source belonging to the group

L. interrogans serovar Manilae strain K64 utilized amino sugars believed to be associated with lipopolysaccharides and peptidoglycan synthesis. These were N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine and N-acetyl neuraminic acid. In a study using gas liquid chromatography analysis, N-acetyl-D-galactosamine was also found in leptospiral lipopolysaccharide of *L. interrogans* serovar copenhageni strain L45 (Vin et al., 1985). Moreover, N-acetylneuraminic acid biosynthetic pathway was also reported in *L. interrogans* serovar Copenhageni strain L1-130 (Ricaldi et al., 2012). This was consistent with the finding of the present study wherein *L. interrogans* serovar Manilae strain K64 utilized N-acetyl neuraminic acid. Other sugar and sugar derivatives such as the D-galactose, 3-methyl glucose, D-fucose, L-fucose, D-fructose, L-fucose, inosine, D-fructose-6-phosphate, D-gluconic acid, D-glucuronic acid, glucuronamide, and saccharic acid were also utilized by *L. interrogans* serovar Manilae strain K64. The utilization of these carbon sources was consistent with the previous reports that *Leptospira* has a functional glycolytic metabolism pathway. In the study of Nascimento et al. (2004), the gene LA_1437 of *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain 56601 was reported to be potentially encoding a glucokinase (GLK). Similarly, a gene which is potentially encoding GLK was also reported in *L. biflexa* strain Patoc 1 (Kefford et al., 1986; Picardeau et al., 2013).

Amino acid biosynthesis have been reported in *Leptospira interrogans* serovars Semaranga Tarassovi, and Canicola using radioactive carbon dioxide on growing cells and paper chromatography (Charon et al., 1973; Gerhardt & Ball 1959). Similarly, the need for amino acids to support the growth of *L. interrogans* serovar Manilae strain K64 was also exhibited as evidenced by the utilization of seven amino acids such as D-aspartic acid, D-serine, L-serine, L-alanine, L-arginine, L-histidine, and L-pyroglutamic acid.

β-oxidation of fatty acids was thought to be the only source of energy and carbon in *Leptospira* as reported in the enzymatic analysis of cell-free extracts of the three *Leptospira* strains (Baseman & Cox, 1969; Henneberry & Cox, 1971). Thus, the long chain fatty acids, Tween 80 (polyoxyethylene sorbitan monooleate), Tween 60 (polyoxyethylene sorbitan monostearate) and Tween 40 (polyoxyethylene sorbitan monopalmitate) were usually employed as carbon source in the metabolism of *Leptospira*. Unfortunately, the Biolog™ Gen III panel only includes Tween 40, which apparently, was poorly utilized by *L. interrogans* serovar Manilae strain K64. This result was consistent with the early studies of Cox and Baseman (1969), in which Tween 40 was also unexpectedly, poorly utilized by a B_{16} water strain of *Leptospira*. To fully elucidate the fatty acid utilization of *L. interrogans* serovar Manilae strain K64, other Biolog™ PM panel, which contain more comprehensive fatty acid substrates is highly recommended in future studies. Although the current panel used in this initial study has limited fatty acid substrates, it already screened for 71 carbon sources which are necessary for the growth of mostly gram negative and gram positive bacteria (Bochner et al., 2001; Bochner, 2003; Bochner, 2009).

CONCLUSION

To the knowledge of the authors, this is the first study reporting the carbon utilization phenome of a *Leptospira* strain using the BiologTM sole carbon source utilization phenotype microarray technology. The carbon sources that favor the growth of *L. interrogans* serovar Manilae strain K64 obtained in this study were consistent with the reported genes and metabolic pathways among *Leptospira* species. Moreover, these carbon sources utilized by *L. interrogans* serovar Manilae strain K64 have been previously reported to be associated in the biosynthesis of peptidoglycan, lipopolysaccharide, histidine, sulfur, amino acids, isoleucine and in other metabolic pathways such as glycolysis, pentose-phosphate, pyruvate and fatty acid in *Leptospira* spp.

DISCLOSURES

This paper was presented during the 32nd Faculty Research Forum in the University of the Philippines College of Medicine as a poster presentation. The authors declare no conflicts of interest in this work and the manuscript was approved by all the authors, as well as the funding organization, for publication.

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