Meningitis-Associated Genes in *Mycobacterium* species

Saw S.H.[1,2], Yap S. F.[1] and Ngeow Y.F.[1]

[1] Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, 43000 Bandar Sungai Long Selangor Malaysia;
[2] Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, 31900 Kampar Perak Malaysia.

Abstract

Central nervous system (CNS) involvement in tuberculosis is still prevalent in many parts of the world. It is not clear what promotes CNS invasion and pathology in this disease. Apart from host susceptibility, microbial factors could be involved as well, as specific genetic traits of *Mycobacterium tuberculosis* (*Mtb*) have been reported in isolates from patients with TB meningitis. In this study, we used whole genome sequences (WGS) to search for homologs of genes reported previously by other workers to be associated with meningitis in mycobacterial, meningococcal, pneumococcal and *E. coli* K1 infections. In eight *Mtb* isolates from the cerebrospinal fluid of Malaysian patients, we found homologs of various meningitis-associated genes reported for *Mtb*, *M. leprae*, *M. bovis*, *M. lepromatosis*, *M. ilatzerense* and *M. immunogenum* as well as *Streptococcus pneumoniae* and *Neisseria meningitidis*. Representative putative genes were verified with PCR-Sanger sequencing and found to be related to the PPE protein family known to comprise immune-related proteins that play important roles in mycobacterial antigen variation, the host immune suppression system, and the synthesis of bacterial walls with high degrees of hydrophobicity. The detection of common meningitis-associated genes in mycobacterial and non-mycobacterial neuro-pathogens raised speculations on the existence of a pan-bacterial mechanism of CNS infection. However, a wider search of NCBI genome databases revealed the presence of these apparently neurotropic genes in many respiratory *Mtb* isolates. The relevance of these genes to CNS disease needs to be further evaluated by gene expression and gene functionality studies.

Keywords: *Mycobacterium tuberculosis, central nervous system, meningitis, whole genome sequencing, meningitis-associated gene, PPE proteins*

1. Introduction

Tuberculosis (TB) is one of the oldest documented communicable diseases and its incidence has been on the rise since the early 1980s. The major portal of entry for *Mycobacterium tuberculosis* (*Mtb*) is the respiratory tract. Following inhalation into the pulmonary alveoli, TB bacilli are phagocytosed by alveolar macrophages, but they can survive the hostile intracellular environment to cause local lesions or be disseminated to extra-pulmonary sites (Nicholas A Be, Kim, Bishai, & Jain, 2009; Bini Estela & Hernandez Pando, 2014; Donald, Schaaf, & Schoeman, 2005). It is still unclear what factors (microbial or host) determine a confined pulmonary lesion (usual presentation) or dissemination to extra-pulmonary sites, including the central nervous system (CNS). However, the exact mechanisms of infection of the CNS are poorly understood (Sundaram, Shankar, Thong, & Pardo-Villamizar, 2011).

Both host susceptibility factors and specific mycobacterial genetic traits have been implicated in CNS infection. The host association has been studied extensively and numerous clinical studies have reported the greater risk of CNS infection in immune-compromised individuals (Yang et al. 2004; Vinnard & MacGregor 2009; Elmas et al. 2011; Sáenz et al. 2017). In addition, various polymorphisms in human genes have been identified to be associated specifically with susceptibility to *Mtb* meningeal infection (Campo et al., 2015; Hawn et al., 2006; Hoal-Van Helden et al., 1999). With respect to the mycobacterium, five *Mtb* genes (Rv0311, Rv0805, Rv0931c, Rv0986, MT3280) have been identified to be associated with invasion or survival in the CNS but not in lung tissues (N A Be et al., 2008). In particular, the sensor domain of *Mtb* pknD (Rv0931c) was reported to be able to trigger the invasion of brain endothelia but not the lung epithelia (N. A. Be, Bishai, & Jain, 2012). The genetics of neuro-tropism has also been described in other meningitis-causing agents such as HIV (Dunfee et al. 2006; Eugenin et al. 2006), *Escherichia coli* (Kim, 2006), *Neisseria meningitidis* (Coureuil et al., 2012; Tinsley & Nassif, 1996), *Mycobacterium leprae* (Shimoji, Ng, Matsumura, Fischetti, & Rambukkana, 1999; Singh & Cole, 2011), fungal and parasitic

Proceedings of RSU Research Conference (2019)

pathogens (Brown et al., 2014; Matsuura et al., 2000). However, data on the factors and mechanisms underlying neuro-tropism in mycobacterial infections is still lacking. New knowledge is needed to augment current understanding of TB meningitis and the *Mtb* strain-specific traits that are related to this pathology. Uncovering microbial genetic factors that are associated with neuro-tropism in *M. tuberculosis* might also lead to the future development of new diagnostics, improved therapeutics and novel vaccines against TB.

2. Objectives

In this study, it is hypothesized that certain microbial genetic factors play a role in the predilection of certain *Mtb* strains for neuro-tropism. In order to look into this, the whole genome sequences of CSF-derived and sputum-derived *Mtb* were subjected to homologue analysis comparing the genes reported previously by other scientists to be associated with meningitis in mycobacterial, meningococcal, pneumococcal and *E. coli* K1 infections. Verification of six putative genes derived from the next generation sequencing (NGS) was performed.

3. Materials and Methods

Genomic DNA Extraction and Purification

Bacterial DNAs of *Mtb* were isolated using Phenol-Chloroform-Isopropanol (PCI) method to obtain a high yield of DNA. Lysis of *Mtb* was performed by overnight incubation with 10.0 mg/ml lysozyme at 37°C, following by the addition of 10% SDS. It was then purified using 5.0 M of sodium chloride, followed by the addition of phenol/chloroform/isoamyl alcohol (25:24:1) (ROTI, Germany) to remove all proteins. Finally, nucleic acids were recovered from the aqueous solution with ethanol precipitation using 3.0 M of sodium acetate and ice-cold isopropanol, and overnight incubation of the mixture at - 20°C. The pellet was washed with 80 % ethanol and dried at room temperature. The required DNA precipitate was dissolved and diluted with autoclaved distilled water.

MiSeq® Sequencing

The extracted genomic DNA of *Mtb* was then quantified using Qubit® fluorometer (Invitrogen, USA) and Qubit dsDNA High sensitivity kit. DNA (6 pM) was loaded into the pre-filled reagent cartridge and the sequencing was performed on the Illumina MiSeq platform (Illumina, USA).

Read Quality Assessment and Assembly

The quality of raw sequences generated from MiSeq was checked using FastQC. Raw reads were trimmed at Phred probability score of 30 and were *de novo* assembled using CLC Genomic Workbench 5.1 (Qiagen Inc., Netherlands). Trimmed sequences were assembled with length fraction of 0.8 and similarity fraction of 0.8. All assemblies were evaluated based on statistical assessment, focusing on genome size, sequence continuity and number of contigs. The genomes were further screened for contamination against common contaminants databases and then used for downstream analyses. To decrease the possibility of inaccurate assembly, the assembly and scaffolding of the genomes in IDBA-UD, a *de novo* assembler of NGS data (Peng, Leung, Yiu, & Chin, 2012) and SSPACE, a stand-alone program for scaffolding pre-assembled contigs using next generation sequencing (NGS) paired-read data (Boetzer & Pirovano, 2014), were repeated, respectively.

Amino Acid Comparisons

The assembled genomes of the eight CSF strains were annotated using the self-training annotation algorithm in GeneMarkS (Besemer, Lomsadze, & Borodovsky, 2001). Orthologous protein sequences were identified in the ProteinOrtho program, with e-value of $1x10^{-5}$ (Lechner et al., 2011). The effect of amino acid substitution was evaluated using the I-mutant webserver (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi) (Capriotti, Fariselli, & Casadio, 2005) for change in protein stability, the GlobPlot standalone python script (https://globplot.embl.de/) (Linding et al., 2003) for globularity and the ProTherm database for the calculation of Gibbs free energy change (https://www.abren.net/protherm/).

Identification of Meningitis-Associated Genes

Scientific literatures that have been reported for meningitis-associated genes in *Mtb* were extensively reviewed and collected. 63 putative genes were revealed in articles from Av-Gay and Everett (2000), Pethe et al. (2001), Tsenova et al. (2005), Jain et al. (2006), Be et al. (2008), Be et al. (2012) and Haldar et al. (2012). The amino acids of these 63 putative genes were compiled and homology studies were performed in five other mycobacteria associated with neuropathology, which consists of *M. leprae*, *M. lepromatosis*, *M. bovis*, *M. llatzerense* and *M. immunogenum*. Homology analysis was performed in the proteins reported to be associated with *Streptococcus pneumoniae*, *Escherichia coli* K-1 and *Neisseria meningitidis*. 141 proteins have been reported to be associated with *Streptococcus pneumoniae* by Orihuela et al. (2004), Molzen et al. (2011) and Mahdi et al. (2012). On the other hand, 164 virulence genes derived from *Neisseria meningitis* were reported by Hao et al (2011). Finally, the neurotropic genes reported by Pouttu et al. (1999), Huang et al. 2001 and Yao et al. (2006) which confer tissue tropism in *Neisseria meningitis* and *E. coli* K1 were reviewed and tabulated.

Verification of Putative Findings from WGS analysis

Six putative genes (Rv3425, Rv1141c, Rv0311, Rv3344c, Rv2606c and Rv2397c) identified in CSF strains were randomly selected to be verified using PCR-sequencing. The PCR primers used in this study are listed in **Table 1**. These primers were designed based on the sequence of the reference H37Rv genome retrieved from the TubercuList website (https://tuberculist.epfl.ch/). PCR primer pairs were designed with similar melting temperatures (Tm ± 2 °C), low probability of forming thermo-stable secondary structures and homo-dimers in particular, under PCR annealing conditions as determined by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and synthesized by 1st BASE Laboratories (Singapore).

Table 1 Oligonucleotide primers used to verify 6 putative meningitis-associated genes found in the *in silico* study.

No	Primer	Sequences	Size (bp)	Tm (°C)	Amplicon size (bp)
1	Rv3425 Fwd	ATGCATCCAATGATACCAGCG	21	58.8	531
2	Rv3425 Rev	CTACCCGCCCCTGTAGATC	19	58.7	
3	Rv1141c Fwd	ATGCCAGATTCCGGGATTGC	20	60.8	807
4	Rv1141c Rev	TCAGGAACCGGTGAAGTTGG	20	59.9	
5	Rv3344c Fwd	GCACAGGCCAGTCCGGCG	18	66.4	1455
6	Rv3344c Rev	TCAGGGTGTTGCGCCGGC	18	65.9	
7	Rv0311 Fwd	AGCTGGCAGTTCTGTTACCC	20	57.4	556
8	Rv0311 Rev	CAGGTGGCAGCTTTGGTTTC	20	57.1	
9	Rv2606c Fwd	ATGGATCCTGCAGGTAACCC	20	56.8	900
10	Rv2606c Rev	TCACCAGCCGCGCTGGGCGA	20	69.6	
11	Rv2397c Fwd	TCGGATTCGTCTTCCAGCAC	20	57.2	471
12	Rv2397c Rev	AGAAGGACATCACGAAGGCG	20	57.2	

Each PCR reaction consisted of 12.5 µL master mix (GoTaq Green Master Mix; Promega), 2 µL of each forward and reverse primers, 1 µL DNA template and distilled water in a total volume of 25 µL. The thermal cycle procedure was based on a three-step cycling procedure: initiated with pre-denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 45 s, primer annealing temperature,

56.1°C to 60.2°C for 30 s, and extension at 72°C for 2 min. Finally, the PCR process was ended by a final extension at 72°C for 5 min. When the reaction was completed, 5 µL of the PCR amplicons were analysed by agarose gel electrophoresis. The amplicons were then purified and subjected to DNA sequencing using forward and reverse primers.

4. Results and Discussion

Whole genome sequence (WGS) analysis gained popularity owing to the availability of next generation DNA sequencing platforms, large datasets easily accessible from public databases and advanced bioinformatics tools (Hui, 2014; MacLean, Jones, & Studholme, 2009; Metzker, 2010; Morozova & Marra, 2008). Genetic information from WGS analysis is often used to assess population natural variation and predict host-pathogen relationships including virulence, immune modulation and response to therapy (Ford et al., 2012). Primary analysis such as filtering and trimming the sequences was performed to improve the data quality. Data metrics were generated to measure the data quality. These metrics were included in the secondary analysis using CLC Genomics Workbench (Qiagen Inc., Netherlands).

Genome Data Trimming and Assembly

The genomes of eight CSF strains showed approximately 55X to 92X sequencing coverage. The detailed statistical measurements of the genomes after assembly are shown in **Table 2**.

Table 2 Statistical measurements of the UM-CSF genomes.

Strain	N50	No. contigs	No. scaffolds	Reads used (%)	Scaffold genome Size (bp)	No. protein CDS
CSF01	36,496	315	234	96.67	4,282,569	4271
CSF04	98,670	140	136	93.09	4,392,768	4323
CSF05	74,553	182	138	97.56	4,338,921	4190
CSF06	83,364	135	127	87.01	4,371,105	4310
CSF08	91,928	174	156	93.77	4,352,163	4353
CSF09	122,404	133	103	89.71	4,355,856	4291
CSF15	91,928	129	126	94.05	4,374,121	4313
CSF17	122,408	131	111	90.99	4,356,783	4308

Proteins Reported to be Associated with Meningitis in Mtb

Of 63 proteins reported for *Mtb* (Av-Gay & Everett, 2000; N. A. Be et al., 2012; N A Be et al., 2008; Jain, Paul-Satyaseela, Lamichhane, Kim, & Bishai, 2006; Pethe et al., 2001; Tsenova et al., 2005), homologs of 56–60 were found in CSF strains but only two, Rv0311 encoding a hypothetical protein and Rv0619 encoding a probable galactose-1-phosphateuridylyltransferase GalTb), were found in all eight CSF strains. This suggests that these possibly neurotropic genes are not universally present in *Mtb* causing CNS disease. On the other hand, a similar search was performed in five other mycobacteria that are associated with neuropathology. These comprised *M. leprae* and *M. lepromatosis* that cause different forms of leprosy, *M. bovis* that is usually linked with extra-pulmonary TB, and two rapid-growers *M. llatzerense* and *M. immunogenum* that had been isolated from a case of brain abscess (Greninger et al., 2015). Fifty-six homologs of the 63 meningitis related genes from *Mtb* were identified in *M. bovis*, followed by 16 in *M. leprae*, 15 in *M. lepromatosis*, 14 in *M. llatzerense*, and 11 in *M. immunogenum*. The CSF strains shared four of the 63 meningitis-related genes (Rv0014c, Rv1837c, Rv2176 and Rv0984) with all five of these mycobacterial species and five other genes (Rv1273c, Rv2318, Rv0983, Rv0966c and Rv0805) with the three slow-growing mycobacteria. The Rv2947c (*pks* 15/1) gene was found in the CSF strains, *M. leprae* and *M. ilatzerense*. In the Beijing genotype of *Mtb*, an intact *pks* 15/1 is believed to be responsible for virulence and extra-pulmonary disease (Reed et al., 2004). Consistent with their extra-pulmonary (CNS) location in the host, five of the eight UM-CSF strains were genotyped as Beijing ST1 and each carried an

intact *pks* 15/1 gene. This seems to suggest that there might be common genetic traits in mycobacteria that are responsible for diseases of the nervous system (central and peripheral) in human.

Meningitis-Associated Genes from Other Bacterial Pathogens

Streptococcus pneumoniae, *Escherichia coli* K-1 and *Neisseria meningitidis* are pathogens known to cause meningitis in humans. Of 141 proteins reported to be associated with *S. pneumoniae* meningitis (Mahdi, Wang, Van Der Hoek, Paton, & Ogunniyi, 2012; Molzen et al., 2011; Orihuela et al., 2004), three, Rv1699 (CTP synthase PyrG), Rv2606c (pyridoxine biosynthesis protein SnzP) and Rv0357c (adenylosuccinate synthetase PurA) were found in the CSF strains. These genes showed 51 – 68 % sequence similarity with their homologs in *S. pneumoniae* but were identical in all CSF strains and H37Rv, in protein sequence as well as globularity. When compared against 164 *N. meningitidis* virulence genes reported by Hao et al. (2011), CSF strains shared two virulence homologs with this neuro-pathogen: Rv2457c, encoding ATP-dependent CLP protease ATP-binding subunit clpX and Rv2397c, encoding sulfate-transport ATP-binding protein ABC transporter CysA1 (Hao et al., 2011). The genes for cell surface outer membrane Opa and Opc proteins that were previously reported to confer tissue tropism in *N. meningitides* (Virji, Makepeace, Ferguson, Achtman, & Moxon, 1993) were not found, neither were homologs of previously reported *E. coli* K1 neurotropic genes such as IbeA, IbeB, AslA, YijP, and OmpA (Huang & Jong, 2001; Pouttu et al., 1999; Yao, Xie, & Kim, 2006), in CSF strains.

However, the five genes associated with *S. pneumoniae* and *N. meningitidis* meningitis was also found in many respiratory *Mtb*. The detection of common homologs in neuro-pathogens from different bacterial taxa raises speculations on the existence of a pan-bacterial mechanism of CNS infection. Although it was disappointing to find that many of the possibly neurotropic traits in the CSF strains were also found in respiratory *Mtb*, this finding is consistent with the observation by other workers that many virulence genes are conserved in non-pathogenic bacteria. For instance, all four *mce* operons in the genome of *Mtb* (Kumar, Bose, & Brahmachari, 2003) have been found in both pathogenic and non-pathogenic mycobacteria (Chitale et al., 2001; Haile, Caugant, Bjune, & Wiker, 2002). CLP proteases on the whole, are common in many bacterial spp. (De Mot, Nagy, Walz, & Baumeister, 1999). The ABC transporter complex involved in sulfate/thiosulfate import is found in pathogens as well as environmental bacteria (Szklarczyk et al., 2015). Many designated virulence genes in *N. meningitidis* were also found to be present in non-pathogenic species such as *N. lactamica* (Snyder & Saunders, 2006). All these observations suggest that pathogenic bacteria have adapted their genomes from a free lifestyle to the intracellular environment with minimal acquisition of exclusive virulence genes (Forrellad et al., 2013).

Figure 1 summarizes the next generation sequencing (NGS) findings of 17 meningitis-associated genes that are found in the eight CSF *Mtb* strains, *M. leprae* and *M. lepromatosis*, *M. bovis* and two rapid growers *M. Ilatzerense* and *M. immunogenum*, together with pathogens known to cause meningitis in humans, *Streptococcus pneumoniae* and *Neisseria meningitidis*.

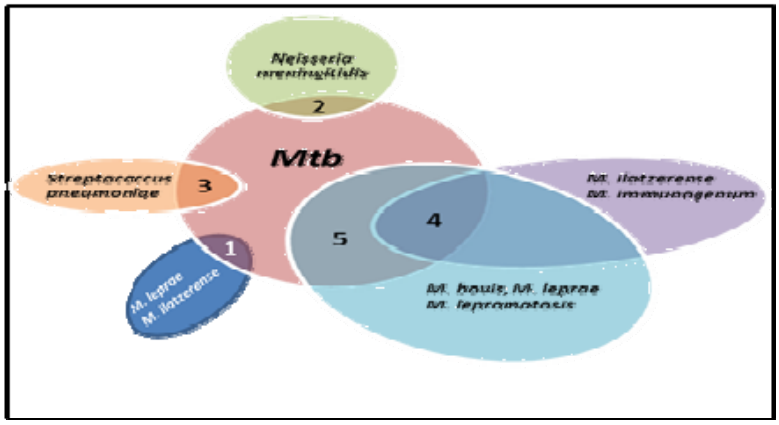

Figure 1 Venn diagram showing the number of meningitis-associated genes that are shared between the CSF *Mtb* strains, mycobacterial and non-mycobacterial neuro-pathogens.

Verification of Putative Findings in UM-CSF Strains

While next-generation sequencing technologies have brought many advantages to genomic research, the chain-termination method of DNA sequencing remains widely used, especially for the validation of WGS findings. Sanger sequencing is preferred for the sequencing of single genes as it is cost-effective. It is used to verify sequences for site-directed mutagenesis or the presence of cloned inserts and it is less error-prone than NGS (Biotech, 2018; Thermo Fisher Scientific, 2014). Thus, PCR-sequencing assays were used to verify the existence of six putative genes identified in the *Mtb* genomes from CSF. These genes represent different types of sequence variations predicted. They were three genes (Rv3425, Rv1141c, Rv3344c) with putative deletions, one gene (Rv0311) with a non-synonymous (ns)-SNP, and two homologues genes (Rv2606c, Rv2397c) reported to be associated with *S. pneumoniae* and *N. meningitides* meningitis. Of the six genes studied, the verification of Rv3344c and Rv0311 variations in the CSF samples was successful (**Figure 2**). The sequence obtained for Rv3344c indicated that the smaller than predicted PCR product size (825 bp instead of 1455bp) was probably due to multiple deletions. The result of the Rv0311 verification showed the converse. The G-T SNP in the gene was predicted in all eight CSF strains used but was confirmed only in five of them, indicating a possible sequencing error in the other three strains (**Figure 2**). BLASTN and BLASTX analysis (**Table 3**) shows the 825 bp sequence to be a putative PPE family protein similar to a sequence reported from a *Mtb* isolate from the human brain (Husain et al., 2017).

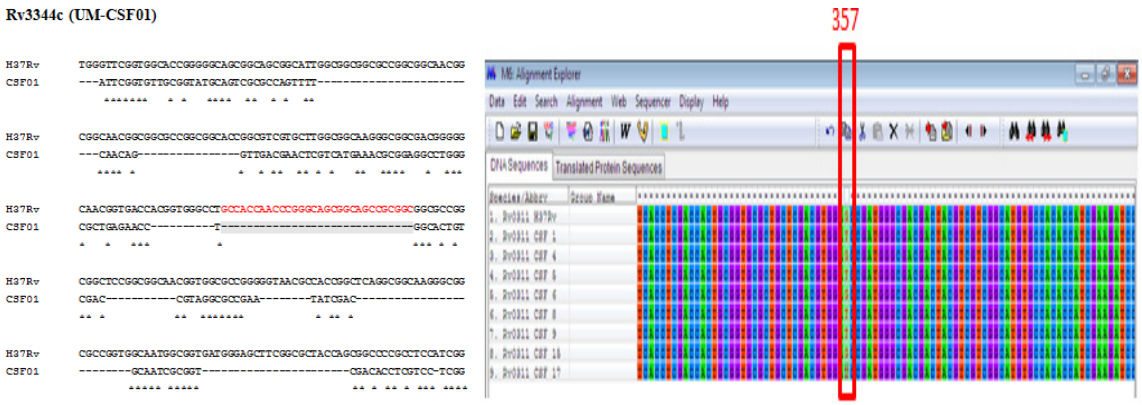

Figure 2 Nucleic acid alignment of: (left) Rv3344v in CSF01. The red letters in H37Rv (position 490 to 529) were predicted to be deleted in UM-CSF01; (right) Rv0311 in eight CSF samples. G to T SNP was observed in nucleotide position 357 in 5 samples: UM-CSF01, 05, 08, 09 and 17.

Table 3 BLASTN and BLASTX results of Rv3344c gene amplified from CSF01 sample.

Gene/BLAST variants	BLASTN			BLASTX		
	Description	E-value	Identity	Description	E-value	Identity
Rv3344c in CSF01	*Mtb* strain C3 (Brain: India)	0.0	99 %	PPE family protein	3e-87	100 %

The meningitis-associated genes in *S. pneumoniae* and *H. influenzae*, Rv2397c and Rv2606c, were verified in all UM-CSF isolates and H37Rv. All these genes and sequences are related to the PPE protein family known to comprise important immune-related proteins that play important roles in mycobacterial antigen variation, the host immune suppression system, and the synthesis of bacterial walls with high degrees of hydrophobicity. Further investigations are necessary with larger numbers of *Mtb* genomes from CNS isolates to be compared with respiratory isolates, and functional studies of these genes are necessary to explore the effect of the genetic differences identified in CNS but not respiratory strains of *Mtb*.

5. Conclusion

The detection of common meningitis-associated genes in mycobacterial and non-mycobacterial neuro-pathogens raises speculations on the existence of a pan-bacterial mechanism of CNS infection. However, all the genes shared by our CSF *Mtb* strains and other neuro-pathogenic bacterial spp. were also found to be common in the respiratory *Mtb* genomes we examined. This finding suggests that CNS infection in TB is more likely to be directed by the expression of multiple virulence factors selected by the interaction between pathogen and host immune responses, rather than the presence of specific genetic traits.

6. Acknowledgements

I offer my sincere gratitude to my advisor Professor Ngeow Yun Fong, for her continuous support of my PhD study and research, for her patience, motivation, enthusiasm, and immense knowledge whilst allowing me the room to work in my own way. Sincere thanks to my co-supervisor, Professor Dr. Yap Sook Fan, for her advices and words of wisdom for the completion of my research study. Extra-large thank you to my family, especially to my twin sister, Seow Hui, who has been a motherly figure, nurturing my needs over these years, and thank you for being such a great and caring sister cum friend to me; To my dearest mom, who has suffered from Alzheimer's disease, thank you is just too small a word to describe how much I appreciate you; your love and reassuring smile has made my life worthwhile. Finally, this work is heartily dedicated to my dad who left for heaven before the completion of this work. May you rest in peace, dad. You will be missed.

7. References

Av-Gay, Y., & Everett, M. (2000). The eukaryotic-like Ser/Thr protein kinases of Mycobacterium tuberculosis. *Trends in Microbiology*, *8*(5), 238–244. Retrieved from https://doi.org/10.1016/S0966-842X(00)01734-0

Be, N. A., Bishai, W. R., & Jain, S. K. (2012). Role of Mycobacterium tuberculosis pknD in the pathogenesis of central nervous system tuberculosis. *BMC Microbiology*, *12*(7), 1–12. https://doi.org/10.1186/1471-2180-12-7

Be, N. A., Kim, K. S., Bishai, W. R., & Jain, S. K. (2009). Pathogenesis of central nervous system tuberculosis. *Current Molecular Medicine*, *9*(2), 94–99. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/19275620

Be, N. A., Lamichhane, G., Grosset, J., Tyagi, S., Cheng, Q. J., Kim, K. S., … Jain, S. K. (2008). Murine model to study the invasion and survival of Mycobacterium tuberculosis in the central nervous system. *The Journal of Infectious Diseases*, *198*(10), 1520–1528. https://doi.org/10.1086/592447

Besemer, J., Lomsadze, A., & Borodovsky, M. (2001). GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions.

Nucleic Acids Research, *29*(12), 2607–2618. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC55746/

Bini Estela, I., & Hernandez Pando, R. (2014). Pathogenesis and immune response in Tuberculous Meningitis. *Malaysian Journal of Medical Sciences*, *21*(1), 4–10. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3952336/

Biotech, G. (2018). Sanger Sequencing. Retrieved March 21, 2018, from https://www.gatc-biotech.com/en/expertise/sanger-sequencing.html

Boetzer, M., & Pirovano, W. (2014). SSPACE-LongRead: scaffolding bacterial draft genomes using long read sequence information. *BMC Bioinformatics*, *15*(211), 1–9. https://doi.org/10.1186/1471-2105-15-211

Brown, J. C. S., Nelson, J., VanderSluis, B., Deshpande, R., Butts, A., Kagan, S., … Madhani, H. D. (2014). Unraveling the biology of a fungal meningitis pathogen using chemical genetics. *Cell*, *159*(5), 1168–1187. https://doi.org/10.1016/j.cell.2014.10.044

Campo, M., Randhawa, A. K., Dunstan, S., Farrar, J., Caws, M., Bang, N. D., … Hawn, T. R. (2015). Common polymorphisms in the CD43 gene region are associated with tuberculosis disease and mortality. *American Journal of Respiratory Cell and Molecular Biology*, *52*(3), 342–348. https://doi.org/10.1165/rcmb.2014-0114OC

Capriotti, E., Fariselli, P., & Casadio, R. (2005). I-Mutant2.0: predicting stability changes upon mutation from the protein sequence or structure. *Nucleic Acids Research*, *33*(Web Server issue), W306–W310. https://doi.org/10.1093/nar/gki375

Chitale, S., Ehrt, S., Kawamura, I., Fujimura, T., Shimono, N., Anand, N., … Riley, L. W. (2001). Recombinant Mycobacterium tuberculosis protein associated with mammalian cell entry. *Cellular Microbiology*, *3*(4), 247–254. Retrieved from https://doi.org/10.1046/j.1462-5822.2001.00110.x

Coureuil, M., Join-Lambert, O., Lécuyer, H., Bourdoulous, S., Marullo, S., & Nassif, X. (2012). Mechanism of meningeal invasion by Neisseria meningitidis. *Virulence*, *3*(2), 164–172. https://doi.org/10.4161/viru.18639

De Mot, R., Nagy, I., Walz, J., & Baumeister, W. (1999). Proteasomes and other self-compartmentalizing proteases in prokaryotes. *Trends in Microbiology*, *7*(2), 88–92. Retrieved from https://doi.org/10.1016/S0966-842X(98)01432-2

Donald, P. R., Schaaf, H. S., & Schoeman, J. F. (2005). Tuberculous meningitis and miliary tuberculosis: the Rich focus revisited. *Journal of Infection*, *50*(3), 193–195. https://doi.org/http://dx.doi.org/10.1016/j.jinf.2004.02.010

Dunfee, R., Thomas, E. R., Gorry, P. R., Wang, J., Ancuta, P., & Gabuzda, D. (2006). Mechanisms of HIV-1 neurotropism. *Current HIV Research*, *4*(3), 267–278. Retrieved from http://www.eurekaselect.com/57601/article

Elmas, Ö. N., Akıncı, A., & Bilir, P. (2011). Tuberculous meningitis associated with diabetic ketoacidosis. *Journal of Clinical Research in Pediatric Endocrinology*, *3*(4), 222–224. https://doi.org/10.4274/jcrpe.373

Eugenin, E. A., Osiecki, K., Lopez, L., Goldstein, H., Calderon, T. M., & Berman, J. W. (2006). CCL2/monocyte chemoattractant protein-1 mediates enhanced transmigration of Human Immunodeficiency Virus (HIV)-infected leukocytes across the blood-brain barrier: a potential mechanism of HIV-CNS invasion and neuroAIDS. *The Journal of Neuroscience*, *26*(4), 1098–1106. Retrieved from https://doi.org/10.1523/JNEUROSCI.3863-05.2006

Ford, C., Yusim, K., Ioerger, T., Feng, S., Chase, M., Greene, M., … Fortune, S. (2012). Mycobacterium tuberculosis - heterogeneity revealed through whole genome sequencing. *Tuberculosis*, *92*(3), 194–201. https://doi.org/10.1016/j.tube.2011.11.003

Forrellad, M. A., Klepp, L. I., Gioffré, A., Sabio García, J., Morbidoni, H. R., de la Paz Santangelo, M., … Bigi, F. (2013). Virulence factors of the Mycobacterium tuberculosis complex. *Virulence*, *4*(1), 3–66. https://doi.org/10.4161/viru.22329

Greninger, A. L., Langelier, C., Cunningham, G., Keh, C., Melgar, M., Chiu, C. Y., & Miller, S. (2015). Two Rapidly Growing Mycobacterial Species Isolated from a Brain Abscess: First Whole-Genome Sequences of Mycobacterium immunogenum and Mycobacterium llatzerense. *Journal of Clinical Microbiology*, *53*(7), 2374–2377. https://doi.org/10.1128/JCM.00402-15

Haile, Y., Caugant, D. A., Bjune, G., & Wiker, H. G. (2002). Mycobacterium tuberculosis mammalian cell entry operon (mce) homologs in Mycobacterium other than tuberculosis (MOTT). *FEMS Immunology & Medical Microbiology*, *33*(2), 125–132. Retrieved from https://doi.org/10.1111/j.1574-695X.2002.tb00581.x

Hao, W., Ma, J. H., Warren, K., Tsang, R. S. W., Low, D. E., Jamieson, F. B., & Alexander, D. C. (2011). Extensive genomic variation within clonal complexes of Neisseria meningitidis. *Genome Biology and Evolution*, *3*(January), 1406–1418. https://doi.org/10.1093/gbe/evr119

Hawn, T. R., Dunstan, S. J., Thwaites, G. E., Simmons, C. P., Thuong, N. T., Lan, N. T. N., … Aderem, A. (2006). A Polymorphism in Toll-Interleukin 1 receptor domain containing adaptor protein is associated with susceptibility to meningeal tuberculosis. *Journal of Infectious Diseases*, *194*(8), 1127–1134. https://doi.org/10.1086/507907

Hoal-Van Helden, E. G., Epstein, J., Victor, T. C., Hon, D., Lewis, L. A., Beyers, N., … Van Helden, P. D. (1999). Mannose-binding protein B allele confers protection against tuberculous meningitis. *Pediatric Research*, *45*(4 Pt 1), 459–464. Retrieved from https://www.nature.com/articles/pr199993.pdf

Huang, S. H., & Jong, A. Y. (2001). Cellular mechanisms of microbial proteins contributing to invasion of the blood-brain barrier. *Cellular Microbiology*, *3*, 277–287. https://doi.org/10.1046/j.1462-5822.2001.00116.x

Hui, P. (2014). Next generation sequencing: chemistry, technology and applications. *Top Curr Chem*, *336*, 1–18. https://doi.org/10.1007/128_2012_329

Husain, A., Tikariha, H., Purohit, H. J., Bhullar, S., Daginawala, H., Singh, L., & Kashyap, R. (2017). Study on strain dependent pathogenesis of tuberculosis. *Unpublished*. Environmental Biotechnology and Genomics Division, National Environmental Engineering Research Institute-CSIR, Nehru marg, Nagpur, Maharashtra 440020, India: NCBI. Retrieved from https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

Jain, S. K., Paul-Satyaseela, M., Lamichhane, G., Kim, K. S., & Bishai, W. R. (2006). Mycobacterium tuberculosis invasion and traversal across an in vitro human blood-brain barrier as a pathogenic mechanism for central nervous system tuberculosis. *The Journal of Infectious Diseases*, *193*(9), 1287–1295. Retrieved from https://doi.org/10.1086/502631

Kim, K. S. (2006). Meningitis-associated Escherichia coli. *EcoSal Plus*, *2*(1), 269–286. Retrieved from http://www.asmscience.org/WEB-INF/jsp/journal/referenceworkarticle.jsp

Kumar, A., Bose, M., & Brahmachari, V. (2003). Analysis of expression profile of mammalian cell entry (mce) operons of Mycobacterium tuberculosis. *Infection and Immunity*, *71*(10), 6083–6087. Retrieved from https://iai.asm.org/content/iai/71/10/6083.full.pdf

Lechner, M., Findeiß, S., Steiner, L., Marz, M., Stadler, P. F., & Prohaska, S. J. (2011). Proteinortho: Detection of (Co-)orthologs in large-scale analysis. *BMC Bioinformatics*, *12*(1: 124), 1–13. https://doi.org/10.1186/1471-2105-12-124

Linding, R., Jensen, L. J., Diella, F., Bork, P., Gibson, T. J., & Russell, R. B. (2003). Protein disorder prediction: Implications for structural proteomics. *Structure*, *11*(11), 1453–1459. https://doi.org/10.1016/j.str.2003.10.002

MacLean, D., Jones, J. D. G., & Studholme, D. J. (2009). Application of "next-generation" sequencing technologies to microbial genetics. *Nature Reviews Microbiology*, *7*(4), 287–296. Retrieved from http://dx.doi.org/10.1038/nrmicro2088

Mahdi, L. K., Wang, H., Van Der Hoek, M. B., Paton, J. C., & Ogunniyi, A. D. (2012). Identification of a novel pneumococcal vaccine antigen preferentially expressed during meningitis in mice. *Journal of Clinical Investigation*, *122*(6), 2208–2220. https://doi.org/10.1172/JCI45850

Matsuura, E., Umehara, F., Hashiguchi, T., Fujimoto, N., Okada, Y., & Osame, M. (2000). Marked increase of matrix metalloproteinase 9 in cerebrospinal fluid of patients with funcal or tuberculous meningoencephalitis. *Journal of Neurological Sciences*, *173*(1), 45–52. Retrieved from https://doi.org/10.1016/S0022-510X(99)00303-2

Metzker, M. L. (2010). Sequencing technologies-the next generation. *Nature Reviews Genetics*, *11*(1), 31–46. Retrieved from https://www.nature.com/articles/nrg2626

Molzen, T. E., Burghout, P., Bootsma, H. J., Brandt, C. T., Der Gaast-De Jongh, C. E. V., Eleveld, M. J., … Hermans, P. W. M. (2011). Genome-wide identification of Streptococcus pneumoniae genes essential for bacterial replication during experimental meningitis. *Infection and Immunity*, *79*(1), 288–297. https://doi.org/10.1128/IAI.00631-10

Morozova, O., & Marra, M. A. (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics*, *92*(5), 255–264. https://doi.org/10.1016/j.ygeno.2008.07.001

Orihuela, C. J., Radin, J. N., Sublett, J. E., Gao, G., Kaushal, D., & Tuomanen, E. I. (2004). Microarray analysis of pneumococcal gene expression during invasive disease. *Infection and Immunity*, *72*(10), 5582–5596. https://doi.org/10.1128/IAI.72.10.5582-5596.2004

Peng, Y., Leung, H. C. M., Yiu, S. M., & Chin, F. Y. L. (2012). IDBA-UD: A de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics*, *28*(11), 1420–1428. https://doi.org/10.1093/bioinformatics/bts174

Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M. J., Locht, C., & Menozzi, F. D. (2001). The heparin-binding haemagglutinin of Mycobacterium tuberculosis is required for extrapulmonary dissemination. *Nature*, *412*(6843), 190–194.

Pouttu, R., Puustinen, T., Virkola, R., Hacker, J., Klemm, P., & Korhonen, T. K. (1999). Amino acid residue Ala-62 in the FimH fimbrial adhesin is critical for the adhesiveness of meningitis-associated Escherichia coli to collagens. *Molecular Microbiology*, *31*(6), 1747–1757. Retrieved from https://doi.org/10.1046/j.1365-2958.1999.01311.x

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., … Barry, C. E. 3rd. (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature*, *431*(7004), 84–87. https://doi.org/10.1038/nature02837

Sáenz, B., Hernandez-Pando, R., Fragoso, G., Bottasso, O., & Cárdenas, G. (2013). The dual face of central nervous system tuberculosis: A new Janus Bifrons? *Tuberculosis*, *93*(2), 130–135. https://doi.org/10.1016/j.tube.2012.11.011

Shimoji, Y., Ng, V., Matsumura, K., Fischetti, V. A., & Rambukkana, A. (1999). A 21-kDa surface protein of Mycobacterium leprae binds peripheral nerve laminin-2 and mediates Schwann cell invasion. *PNAS*, *96*(17), 9857–9862. Retrieved from https://doi.org/10.1073/pnas.96.17.9857

Singh, P., & Cole, S. T. (2011). Mycobacterium leprae: genes, pseudogenes and genetic diversity. *Future Microbiology*, *6*(1), 57–71. https://doi.org/10.2217/fmb.10.153

Snyder, L. A. S., & Saunders, N. J. (2006). The majority of genes in the pathogenic Neisseria species are present in non-pathogenic Neisseria lactamica, including those designated as "virulence genes." *BMC Genomics*, *7*(1), 128. https://doi.org/10.1186/1471-2164-7-128

Sundaram, C., Shankar, S. K., Thong, W. K., & Pardo-Villamizar, C. A. (2011). Pathology and diagnosis of central nervous system infections. *Pathology Research International*, *2011*(878263), 1–4. https://doi.org/10.4061/2011/878263

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., … von Mering, C. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, *43*(Database issue), D447-452. https://doi.org/10.1093/nar/gku1003

Thermo Fisher Scientific. (2014). Sanger DNA Sequencing. Retrieved July 27, 2014, from http://www.lifetechnologies.com/my/en/home/life-science/sequencing/sanger-sequencing/sanger-dna-sequencing.html

Tinsley, C. R., & Nassif, A. X. (1996). Analysis of the genetic differences between Neisseria meningitidis

and Neisseria gonorrhoeae: two closely related bacteria expressing two different pathogenicities. *PNAS*, *93*(20), 11109–11114. Retrieved from https://www.jstor.org/stable/40291

Tsenova, L., Ellison, E., Harbacheuski, R., Moreira, A. L., Kurepina, N., Reed, M. B., … Kaplan, G. (2005). Virulence of selected Mycobacterium tuberculosis clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. *The Journal of Infectous Diseases*, *192*(1), 98–106. Retrieved from https://doi.org/10.1086/430614

Vinnard, C., & MacGregor, R. R. (2009). Tuberculous meningitis in HIV-infected individuals. *Current HIV/AIDS Reports*, *6*(3), 139–145. https://doi.org/10.1007/s11904-009-0019-7

Virji, M., Makepeace, K., Ferguson, D. J., Achtman, M., & Moxon, E. R. (1993). Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. *Molecular Microbiology*, *10*(3), 499–510. Retrieved from https://doi.org/10.1111/j.1365-2958.1993.tb00922.x

Yang, Z., Kong, Y., Wilson, F., Foxman, B., Fowler, A. H., Marrs, C. F., … Bates, J. H. (2004). Identification of risk factors for extrapulmonary tuberculosis. *Clinical Infectious Diseases*, *38*(2), 199–205. Retrieved from https://doi.org/10.1086/380644

Yao, Y., Xie, Y., & Kim, K. S. (2006). Genomic comparison of Escherichia coli K1 strains isolated from the cerebrospinal fluid of patients with meningitis. *Infection and Immunity*, *74*(4), 2196–2206. https://doi.org/10.1128/IAI.74.4.2196-2206.2006