DLSU Research Congress 2025
De La Salle University, Manila, Philippines
June 26 to 28, 2025

Exploring *Mycobacterium tuberculosis* Virulence Factor Interactions through *In Silico* Methods: Contributions to Sustainable Disease Control in the Philippines

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Abstract: Tuberculosis (TB) is a disease that has pervasively plagued the Philippines, which is further exacerbated by the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis*. Virulence factor genes (VFG) are responsible for proteins that contribute to pathogenicity. The protein-protein interaction (PPI) networks of their gene products are of interest as they may provide avenues for TB treatment development. The VFGs of 15 randomly selected *M. tuberculosis* samples from the Second National Drug Resistance Survey on Tuberculosis in the Philippines were determined using Reads-based Virulence Factors' Scanner (RVFscan). The PPI networks were created and analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). The PPI were found to mainly enable the bacteria to perform iron sequestration and to modulate host immune responses through its peripheral cellular components, and therefore, confirming the aforementioned as prime targets for novel TB therapies.

Key Words: *Mycobacterium tuberculosis*; virulence factor genes; protein-protein interaction networks

1. INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) was the leading infectious disease killer in 2023 globally, surpassing COVID-19, as 8.2 million people were newly diagnosed with TB worldwide—the highest number ever recorded. Moreover, the Philippines was identified as the fourth-highest contributing country to the number of cases (World Health Organization, 2024). Flores et al. (2022) reported that approximately one million Filipinos have been diagnosed with active TB. Socioeconomic factors and underfunding for public health have impeded TB response in the country (Flores et al., 2022; Dychiao et al., 2022). Globally, there is still no effective vaccine for TB (Wang et al., 2023). *Mycobacterium tuberculosis*, the causal agent of TB, can survive various physiological conditions inside its host (Sengupta et al., 2023). *M. tuberculosis* invades through the host's airway, destabilizes the host's immune system, establishes its survival niche, and starts a new round of infection in another host (Rahlwes et al., 2023).

The virulence of *M. tuberculosis* and its properties for immune evasion and antibiotic resistance can be closely associated with its genetic makeup, particularly with its virulence factor genes (VFGs). VFGs encode for proteins in pathogens that improve their chances of infecting hosts, such as by helping them proliferate rapidly, evade host protection mechanisms, destroy their hosts, and adapt to their environment (Alkatheri et al., 2023). However, studies have shown that the proteins from

VFGs are not in isolation from each other; several virulence factors interact with each other to further improve upon their pathogenicity (Liao et al., 2022; Pinto et al., 2023). Both studies have underscored the importance of protein-protein interaction (PPI) network analysis in understanding the interplay between the virulence factors of pathogenic bacteria to provide insights regarding their immunity and virulence, and ultimately, to identify means to control their spread.

Given that virulence factor interactions provide pathogens better capacity to infect their hosts, analyzing said interactions between *M. tuberculosis* virulence factors may provide proteomic insights into the mechanisms that underlie the pervasiveness of TB in the Philippines. In pursuit of this, the following shall be addressed: 1) Collect and perform VFG profiling of *M. tuberculosis*; 2) determine the PPI among virulence factors of *M. tuberculosis;* and 3) identify the most relevant PPI in *M. tuberculosis* pathogenicity.

2. METHODOLOGY

2.1 Sourcing Whole Genome Sequenced (WGS) M.tuberculosis Samples from the Philippines

The study randomly sampled 15 of the *M. tuberculosis* samples from the study of Phelan et al. (2019). Phelan et al. (2019) performed whole genome sequencing on 178 *M. tuberculosis* short-term cultured sputum sample isolates collected by the Second National Drug Resistance Survey on Tuberculosis in the Philippines. The raw sequenced data of the aforementioned study was acquired from the European Nucleotide Archive (ENA) with the study accession number ERP114520.

Table 1. Fifteen (15) randomly selected sequences from the whole genome sequenced *M. tuberculosis* clinical isolates of Phelan et al. (2019)

Sequence Number	Run Accession Number	Read Count (bp)
1	ERR3256237	640,475
2	ERR3256276	938,843
3	ERR3256129	641,128
4	ERR3256217	854,737
5	ERR3256283	790,967
6	ERR3256211	908,506
7	ERR3256199	759,016
8	ERR3256205	874,401
9	ERR3256265	766,861
10	ERR3256160	975,659
11	ERR3256191	3,410
12	ERR3256262	926,148
13	ERR3256282	1,090,861
14	ERR3256244	873,056
15	ERR3256246	740,603

2.2 Detection of Virulence Genes

The study used RVFScan developed by Jiang et al. (2023) in order to detect VFGs from the *M. tuberculosis* sequences. RVFScan is available at https://rvfscan.hugobiotech.com/. RVFScan is capable of accurately detecting and annotating VFGs from submitted sequences by coalescing a comprehensive VFG database with a similarity matrix-based criteria such that assembly will not be required (Jiang et al., 2023).

The *M. tuberculosis* sequences in FASTQ file format were inputted into the *in silico* tool. Afterwhich, *M. tuberculosis* H37Rv was selected from the dropdown menu of species for the VFG detection to run. Results were viewed and downloaded for further analysis using all of the acquired VFGs. Results were further validated through manual cross-checking against the Virulence Factor Database (VFDB), which summarizes the current knowledge of bacterial VFs (Liu et al., 2021).

Table 2. 76 Virulence Factor Genes (VFGs) detected from selected sequences

VFG	Count	VFG	Count	VFG	Count
eccA1	29	*espG3*	28	*mbtF*	29
eccA3	28	*espH*	28	*mbtG*	27
eccA5	27	*espJ*	28	*mbtH*	28
eccB1	27	*espK*	29	*mbtI*	27
eccB3	27	*espL*	27	*mbtJ*	27
eccB5	29	*espR*	27	*mbtK*	29
eccC3	29	*esxA*	27	*mbtL*	27
eccCa1	28	*esxB*	27	*mbtM*	29
eccCa5	27	*esxG*	27	*mbtN*	27
eccCb1	27	*esxH*	27	*mgtC*	29
eccCb5	27	*esxN*	27	*mycP1*	27
eccD1	27	*fbpA*	27	*mycP3*	28
eccD3	27	*fbpB*	27	*mycP5*	28
eccD5	29	*fbpC*	27	*PE35*	27
eccE1	27	*hbhA*	27	*PE5*	27
eccE3	29	*hspX*	27	*phoP*	29
eccE5	27	*ic1*	29	*phoR*	27
erp	28	*ideR*	29	*plcA*	28
espA	28	*irtA*	28	*plcB*	27
espB	27	*irtB*	28	*plcC*	27
espC	29	*lipF*	27	*PPE4*	27
espD	27	*mbtA*	29	*PPE41*	28
espE	29	*mbtB*	29	*PPE68*	27
espF	27	*mbtC*	27	*relA*	29
espG1	28	*mbtD*	29	*Rv1794*	28
espG3	28	*mbtE*	29		

2.3 Construction of protein-protein interaction (PPI) network of M.tuberculosis

To identify the relationship between the virulence factors, their interactions were mapped using STRING v.11.0 by Szklarcyzyk et al. (2023), available at https://string-db.org. The VFGs acquired from RVFscan were inputted into STRING. Then, *M. tuberculosis* H37Rv was selected from the dropdown menu provided. The PPI network and functional enrichment tables were generated. Settings were adjusted such that the edges will only be made between nodes if their interaction has a confidence level equal to or greater than 0.9.

3. RESULTS AND DISCUSSION

3.1 VFG PPI Network of M.tuberculosis

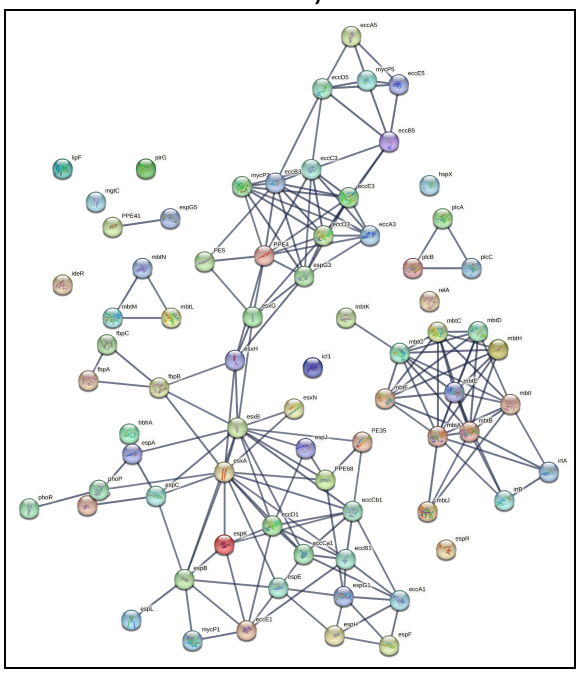

Figure 1. Visualization of the protein-protein interaction (PPI) network of *Mycobacterium tuberculosis* virulence factors.

Shown in Figure 1 is a visual representation of the PPI network of the *M. tuberculosis* virulence factors as generated in STRING after the 76 VFGs detected by RVFScan were entered. Only 74 VFGs in total accounted for in the PPI network as *eccCa5* and *eccCb5* were not among the genes recognized by STRING. It is of note that the VFG catalog of Mikheecheva et al. (2017) for *M. Tuberculosis* lists 319 genes VFGs in total. Thus, RVFscan detecting 76 VFGs may be attributed to the tool avoiding false positives and listing only a substantial subset of *M. Tuberculosis* VFGs (Jiang et al., 2023).

The 74 nodes in the PPI network represent the proteins that the VFGs are encoding for. Meanwhile, the 173 edges joining the nodes serve to illustrate the presence of associations between the proteins of the VFGs. The thickness of the edges represent the edge confidence or the likelihood of the represented connections. Figure 1 was made to illustrate relationships between nodes that are at least equal or greater to a confidence level of 0.900 based on the analysis of STRING (Szklarczyk et al., 2023). Notably, the VFGs can be observed to cluster into three distinct clusters mainly made of similar genes, particularly: the *ecc* gene cluster at the top, the *mbt* gene cluster at the right, and the gene cluster mostly with *ecc*, *esp*, and *esx* at the bottom.

3.2 Biological Processes Affected by VFG Interactions

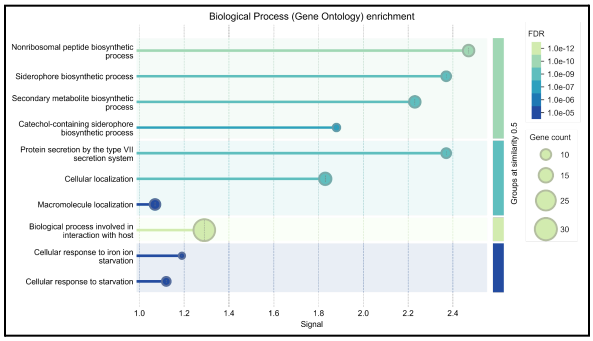

Figure 2. Gene Ontology of the *Mycobacterium tuberculosis* virulence factor genes by their associated biological processes

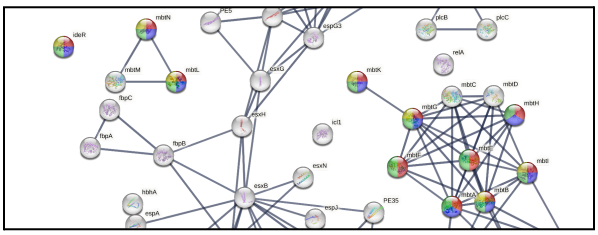

Figure 3. VFGs associated with nonribosomal peptide biosynthetic process (red), siderophore biosynthetic process (blue), secondary metabolite

biosynthetic process (green), and catechol-containing siderophore biosynthetic process (yellow)

As shown in Figure 2, the following are the biological processes affected by the PPI networks of the Philippine samples of *M. tuberculosis* as illustrated by STRING. The biological processes were organized according to their signal values, as the signal balances both strength and false discovery rate metrics (Szklarcyzyk et al., 2023). The VFGs in Figure 3 comprise those responsible for the cluster of biosynthesis processes in Figure 2. Particularly, the majority of these VFGs are *mbt* genes. These genes work complementary to each other for the main goal of synthesizing a siderophore called mycobactin (MBT). Mycobactin is utilized by *M. tuberculosis* in order to acquire iron while they are inside their hosts, as iron is a cofactor that is integral to the metabolic pathways of the bacterium and thus is necessary for their survival and virulence (McMahon et al., 2012; Sritharan, 2016; Zhang et al., 2020).

Nonribosomal peptide biosynthetic process pertains to nonribosomal peptide synthases (NRPS) producing nonribosomal peptides (NRPs). Among these NRPs is isonitrile lipopeptides, which helps the bacterium in transporting metals like iron (Harris et al., 2017). Siderophore biosynthetic process refers to the production of siderophores, such as mycobactin, for iron sequestration (McMahon et al., 2012; Sritharan, 2016; Zhang et al., 2020). Secondary metabolite biosynthetic process encompasses the production of secondary metabolites that, while varied in their contributions to virulence, notably works together with the previously mentioned processes by assisting with the acquisition of iron and for subverting the immune system defenses of the host at large (Alur, 1999). While mycobactin usually makes use of hydroxamate-containing siderophores, Figure 3 reveals that *M. tuberculosis* has interactions responsible for the production of catechol-containing siderophores to the bacterium. Catechol-containing siderophores such as enterobactin serve the same purpose as mycobactin. (Liu et al., 2023).

The high signal scores of these iron sequestration related processes are representative of their great influence on the virulence of the Philippine samples of *M. tuberculosis*, and thus, interventions aimed specifically at disrupting their capacity to acquire iron may prove to be ideal for the development of novel approaches against TB. Reshi et al. (2023) suggested an approach combining conventional antitubercular agents with methods that target *M. tuberculosis* iron acquisition. Among the suggested therapies is to directly inhibit the synthesis of siderophores, to destroy the ferritins which are in charge of iron storage, and to increase iron-binding proteins among patients to further starve *M. tuberculosis* from iron.

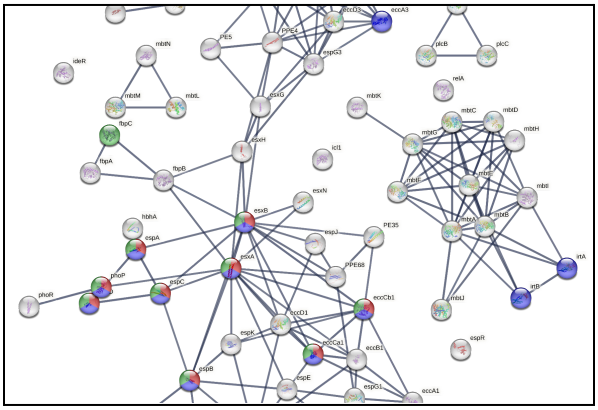

Figure 4. VFGs associated with protein secretion by the Type VII Secretion System (red), cellular localization (blue), and macromolecule localization (green).

The PPI network interactions between the virulence factors under the Type VII Secretion System (T7SS) are next when it comes to having high signals according to STRING (Figure 2). The proteins under the T7SS work hand-in-hand to improve the virulence of *M. tuberculosis* by secreting effector proteins that enable them to affect the immune system defenses of their hosts. Particularly, among the T7SSs of *M. tuberculosis* is the ESX-1 system, which enables the bacteria to evade phagocytosis by macrophages by means of membrane permeabilization. For instance, PtpA is a phosphatase secreted by ESX-1 for the purpose of dephosphorylating proteins of hosts relevant for immune responses (Famelis et al., 2023; Ramon-Luing et al., 2023). Not much studies have delved on the roles of ESX-2 and ESX-4, but they have been found by Pajuelo et al. (2021) to assist ESX-1 in escaping phagocytosis. The ESX-3 and ESX-5 system has also been found to play a role in helping with iron acquisition by secreting mycobactin as well (Famelis et al., 2023). Considering their influences to the survival of *M. tuberculosis*, they have also been suggested to be potential targets for novel TB treatments (Bunduc et al., 2021).

All in all, taking into account the apparent reliance of *M. tuberculosis* samples from the Philippines on VFG PPI interactions for iron sequestration and for the functioning of their T7SS, these are potential targets for TB treatments in the country especially as strains progressively become more resistant to conventional antibiotic treatments.

3.3 Cellular Components Affected by VFG Interactions

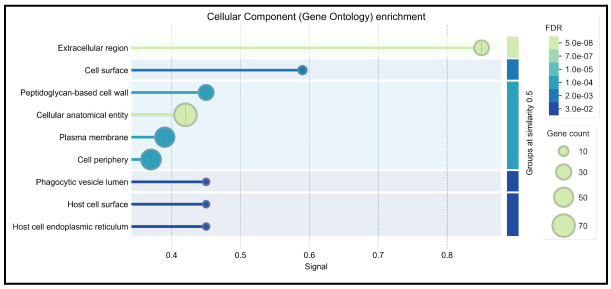

Figure 5. Gene Ontology of the *Mycobacterium tuberculosis* virulence factor genes by their associated cellular components

The cellular component sub-ontology captures the context that gene activities occur at certain locations within or around a cell. To sort the components by enrichment, the components were also organized according to their signal values, as the signal balances both strength and false discovery rate metrics (Szklarczyk et al., 2023).

Figure 5 shows that the VFG PPI interactions of this study highly influence the extracellular region, and mostly at the surface, wall, or membranes of the cell—as evidenced by the high signal scores for extracellular region, cell surface, and peptidoglycan-based cell wall. This suggests that VFG PPI interactions are mainly concerned with transport to and from the cell, and cell to cell interaction, which is consistent with the findings about the biological processes involved.

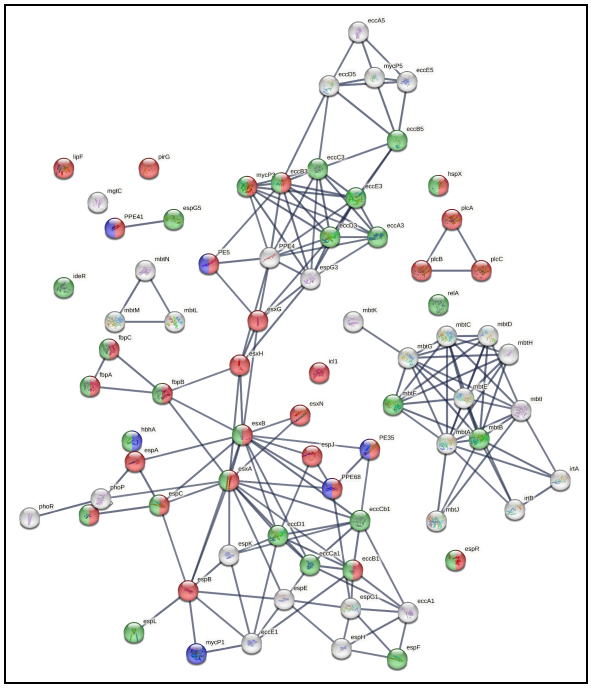

Figure 6. VFGs associated with the extracellular region (red), cell surface (blue), peptidoglycan-based cell wall (green)

As illustrated in Figure 6, the virulence factors responsible for the extracellular region, cell surface, and peptidoglycan-based cell wall comprise the majority of the PPI network, underpinning the role of these cellular components in the pathogenicity of the bacterium. Moreover, studies further corroborate that these cellular components play a vital role with *M. tuberculosis* virulence by not only keeping the bacterium intact, but also by modulating the responses of the host through secretions such as mycobactin and PtpA, as previously discussed (Brennan, 2003; Maitra et al., 2019; Ramon-Luing et al., 2023). Maitra et al. (2019) reasoned that while current first-line drugs such as Isoniazid already target the peptidoglycans of TB, further studies regarding the cellular components remain relevant in finding exploits to circumvent the increasing resistance of *M. tuberculosis* strains to these existing treatments. In line with this, Jacobo-Delgado et al. (2023) suggested the utilization of antimicrobial peptides (AMP), which destroys the structure of the cell wall by creating pores. AMP also affects the intracellular activity of the bacterium, as it is capable of effectively disrupting nucleic acid integrity through binding, and as well as protein and enzyme

synthesis. Furthermore, the wide-scale effect of AMP on *M. tuberculosis* means that the bacterium would be less likely to develop resistances. While this makes it a potent treatment for TB, according to Jacobo-Delgado et al. (2023), further studies are still needed to comprehend all of the effects of AMP as it may pose undesired side effects for patients such as angiogenesis.

While the findings largely corroborated other studies that have looked into virulence factor interactions of *M. tuberculosis*, it highlights the relative prominence of iron sequestration via mycobactin secretion and immune modulation through T7SS among Philippines samples. It should be noted, however, that the sample size, dated samples, and the *in silico* tools utilized limits the findings from being completely reflective of the full complexity and virulence of Philippine *M. tuberculosis* strains. Nevertheless, it still provides insights to local strains that may be of use in developing more sustainable and effective treatments in the archipelago.

4. CONCLUSIONS

The PPI network analysis of the VFGs of the Philippines samples identified potential prime targets for novel TB treatment. In terms of biological processes, it was determined that the virulence factors of *M. tuberculosis* mostly function for iron sequestration by mycobactin secretion and modulating host immune responses through the T7SS. Lastly, it was confirmed that the bacterium rely on their cellular components at and around the cellular surface, wall, and membranes for their virulence. Therefore, in order to formulate possible medications and vaccines in the Philippine context—especially as strains continue to become resistant to the commonly utilized antibiotics—developments against *M. tuberculosis* may focus on the aforementioned targets.

However, further study is needed to better understand virulence factor interactions and their implications for TB and its treatment in the Philippine context. Further studies should increase the sample size, use more recent samples, and conduct the study in the context of different locations. Future undertakings can expand their analyses by the complementary employment of *in silico, in vivo* and *in vitro* methods.

5. ACKNOWLEDGMENTS

The researchers would like to express their gratitude to Phelan et al. (2019) for their publicly accessible sequences of 178 *M. tuberculosis* short-term cultured sputum sample isolates from the Second National Drug Resistance Survey on Tuberculosis in the Philippines.

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