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Mycobacterium ulcerans and *Mycobacterium marinum*: Pathogenesis, Diagnosis and Treatment

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Abstract: Skin and soft tissue infections are common presentations for non-tuberculous mycobacteria (NTM). The cutaneous infections caused by NTM may cause localized or diffuse lesions. *M. ulcerans* is one of the most identified pathogens that involves in the skin and soft tissue mycobacterial infections. Meanwhile, *M. marinum*, as an NTM has also become important emerging causal agents of cutaneous disease in various geographical regions. Although having common ancestry and highly similar in genetic makeup, *M. ulcerans* and *M. marinum* have differential impacts on the host innate immune system. In term pathogenesis, prolonged cell exposure to exotoxin mycolactone produced by *M. ulcerans* could lead to Buruli ulcer. Meanwhile, like most pathogenic mycobacteria, *M. marinum* evades the host immune responses by invading and replicating inside host cells and it is capable of modulating host immune responses. This article aims to provide a general overview and comparisons between the pathogenesis, diagnosis, prevention and therapeutic strategies for *M. ulcerans* and *M. marinum*.

Keywords: *Mycobacterium ulcerans*; *Mycobacterium marinum*; diagnosis; pathogenesis; treatment

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INTRODUCTION

Mycobacteria are a group of aerobic, acid-fast bacteria which are slender, non-flagellated and rod in shape. They possess waxy cell wall composed of mycolic acid, enabling them to be resistant to decolorization even with the use of acidified alcohol. The acid-fastness property of mycobacteria can be demonstrated by the Ziehl-Neelsen stain[1]. In general, the genus *Mycobacterium* is broadly categorized into three main groups which include the *Mycobacterium tuberculosis* complex (MTC), *Mycobacterium leprae* and non-tuberculous mycobacteria (NTM). Being the two major human pathogens, *M. tuberculosis* and *M. leprae* cause tuberculosis and leprosy, respectively.

Unlike the other two groups which are pathogenic to human, NTM, or atypical mycobacteria, encompasses a variety species which commonly inhabit the aquatic and terrestrial environments. More than 170 species of NTM has been discovered and the list keeps increasing. These mycobacteria form biofilm that contributes to their survival in diverse ecological niches[2], including soil, water (such as household water) plants, animals and food products. Although NTM disease is not notifiable in most countries, the rise in the prevalence of NTM disease has become a growing health concern in the recent years. The reasons include the aging of the population, the increasing number of immunocompromised patients and the increased awareness of the disease. The NTM can cause pulmonary as well as extrapulmonary diseases (lymphadenitis, cutaneous disease, disseminated disease), that often inflicting immunocompromised individual and patients with pre-existing conditions. Generally, NTM infections are acquired from environmental exposures via inhalation (e.g. aerosol) or inoculation (e.g. trauma, plastic surgery, acupuncture) [3,4].

Skin and soft tissue infection is one of the common

presentations for NTM. Mycobacteria responsible for most skin disease include *M. ulcerans*, *M. marinum*, *M. chelonae*, *M. fortuitum*, *M. avium-intracellulare, M. tuberculosis* and *M. leprae*. The cutaneous diseases caused by mycobacteria usually manifest as nodules with characteristics of crusting, ulcers and hypo- and hyperpigmentation. Furthermore, cutaneous infections associated with these mycobacteria may cause localized or diffuse lesions. *M. ulcerans* is one of the most identified pathogens that involves in the skin and soft tissue mycobacterial infections. Meanwhile, *M. marinum*, has also become the emerging pathogens causing cutaneous diseases in people from various countries. Although having a common ancestry and they are highly similar in terms of genetic makeup, *M. marinum* and *M. ulcerans* exhibit differential impacts on the innate host immune system. The production of mycolactone plays a main role of *M. ulcerans* in the pathogenesis of Buruli ulcer disease. Meanwhile, *M. marinum* is similar to most pathogenic mycobacteria where the bacteria evade the host immune responses by invading and replicating inside host cells and are capable to modulate host immune responses (Figure 1).

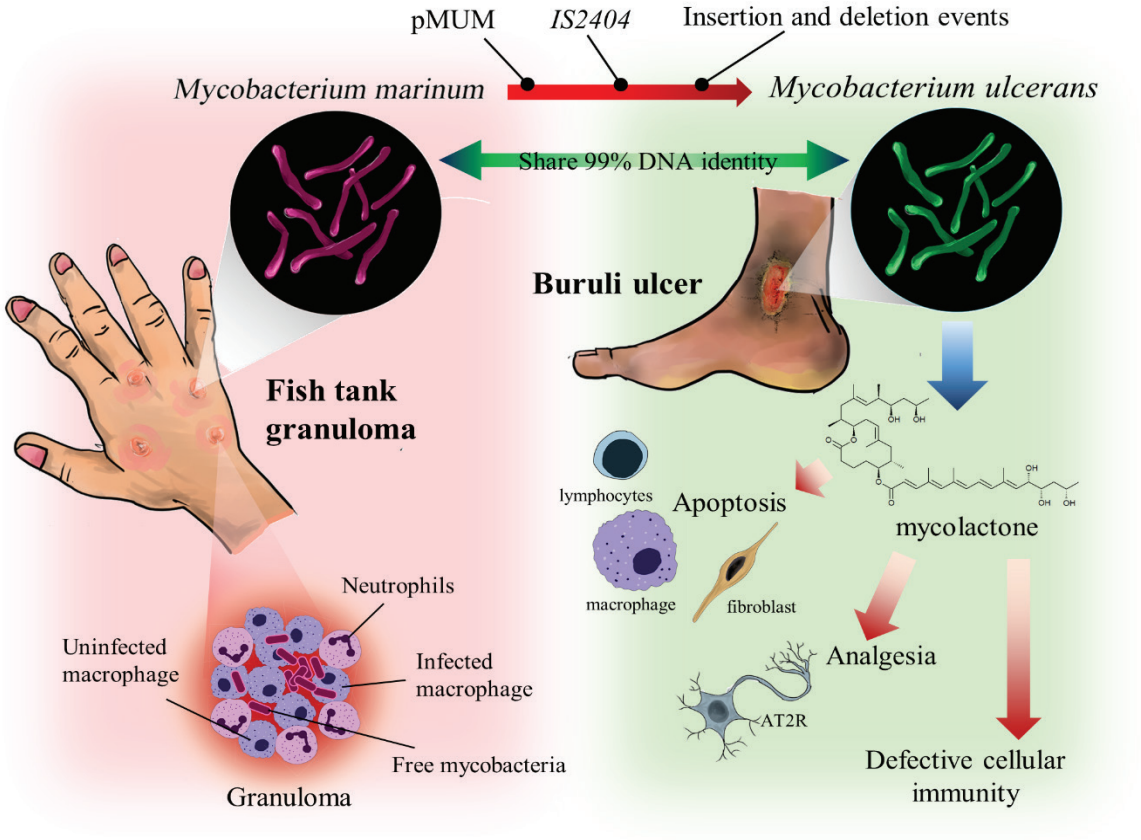

Figure 1. The differential clinical presentations and the pathogenesis between *M. marinum* and *M. ulcerans* despite they share high genomic similarity.

The aim of this review is to provide an overview on both *M. ulcerans* and *M. marinum* as the two major human pathogenic mycobacteria species commonly implicated in cutaneous diseases. Besides that, the pathogenesis and diagnosis of *M. ulcerans* and *M. marinum* infections are summarized and compared. The prevention and ideal strategies to control the diseases are also discussed in this review.

MYCOBACTERIUM MARINUM VERSUS *MYCOBACTERIUM ULCERANS*

Both *M. marinum* and *M. ulcerans* are known to be the two opportunistic mycobacterial pathogens, also named as the mycolactone-producing mycobacteria (MPM), that secrete plasmid-encoded mycolactone exotoxins[5]. Mycolactone is a cytotoxic polyketide metabolite produced by MPM, essential for bacterial virulence, to induce Buruli ulcer-like lesions characterized by extensive necrosis and void of inflammation in intradermal administered animal models[6]. The mycolactone molecule is known to cause apoptosis of mammalian cells, especially more toxic toward the anchorage-dependent cells leading to cytoskeletal rearrangements and detachment in *in vitro* experiments. Interestingly, studies also indicate that mycolactone alters the primary role of innate immunity, including immune cells trafficking and TLR-induced cytokine production.

M. marinum was first isolated from salt water dead fish by Aronson (1926)[7] and was considered as an opportunistic human pathogen after its retrieval from granulomatous skin lesions from Swedish swimmers in the year of 1951[8]. M. marinum is categorized under the Runyon's Group I photochromogenic NTM that are commonly found in non-chlorinated fresh or salt water[9]. Being an opportunistic human pathogen, *M. marinum* causes zoonotic infection in individuals who had exposed through direct-contact with the bacterium from fishes, especially when handling the contaminated aquariums[10]. In general, *M. marinum* infections manifest as superficial skin infections that marked by granuloma and lymphangitis. Thus, the infection of M.

marinum is also termed as the 'fish tank granuloma' or 'aquarium granuloma'. Meanwhile, more severe infections that can spread in a sporotrichoid pattern[11] to deeper tissue inflicting tendinitis, arthritis and osteomyelitis may occur especially in immunocompromised host[12,13].

M. ulcerans is the causative pathogen of a neglected tropical disease, Buruli disease, being one of the most common mycobacterial disease worldwide after tuberculosis and leprosy[14]. Buruli disease is a chronic, necrotizing skin disease with cutaneous tissue destruction and large ulcerations. The Buruli disease cases primarily occur in central Africa, and other regions, including Asia, South America, the western Pacific and Australasia[15]. *M. ulcerans* strains possess a large circular virulence plasmid named pMUM which contains 3 genes encoding polyketide synthases (*mlsA1, mlsA2* and *mlsB*) responsible for the synthesis of the lipid toxin mycolactone[16]. Interestingly, *M. ulcerans* is genetically closely related to the *M. marinum*, thereby they share 99% DNA identity in which *M. ulcerans* exhibit reduced genomes. The comparative whole genomic studies suggest that the emergence of *M. ulcerans* has recently evolved from a *M. marinum* progenitor via acquisition of the virulence plasmid pMUM and subsequent reductive evolution[16]. Acquisition of this plasmid has been considered to be the main contributor for Buruli ulcer in humans[17]. The reduced genome of *M. ulcerans* was subjected to substantial gene loss due to DNA deletion s, DNA rearrangements mediated by insertion of IS*2404* and IS*2606* elements for niche adaptation[18,19].

Both *M. ulcerans* and *M. marinum* strains have optimum growth temperature around 32°C[20], but they grow poorly at 37°C and above, thus reflecting the preference of both strains for the skin and their limited systemic dissemination[21]. Considering both pathogens belonging to the group of slow-growing mycobacteria, *M. marinum* has longer doubling time than *M. ulcerans* when grown in microbial culture medium[22]. In Australia, a mean incubation period of four and half months was identified for *M. ulcerans* infections[23]. Meanwhile, the incubation period of *M. marinum* was approximately ~3 weeks but can be prolonged up to 9 months prior to symptoms onset[24]. Nevertheless, both *M. marinum* and *M. ulcerans* infections may resolve by host immune responses while long-term antibiotic therapy is required on an established infection.

DIAGNOSIS OF *M. ULCERANS* AND *M. MARINUM*

Rapid identification and differentiation of *Mycobacterium* species are crucial to determine the appropriate therapeutic regimens. However, a definitive diagnosis of cutaneous mycobacterial infections can be challenging to make in the clinical routine. There are several diagnostic methods available include histology, microbiological cultures and molecular detection. Histologically, *M. marinum* infections manifest non-specific acute or chronic inflammation as well as positive for tuberculous granulomas and abscesses. However, the detection of acid-fast rod-like bacteria is unusual. Meanwhile, *M. ulcerans* infections are associated with septal subcutaneous necrosis of adipose-rich tissue and positive for acid-fast bacteria detection[25].

The conventional microbiological detection of mycobacteria can be done in specific solid or liquid medium to assist in devising successful antibiotic regimens. The cultivation of NTM is greatly varied depending on the type of pathogen. Incubation of both solid and liquid media at both 30°C and 35°C are usually done to optimally recover the NTM. Different media compositions and conditions according based on specific NTM metabolic needs are also required for their isolation, hence the suspected mycobacterial species could be suggested by the clinicians based on source of exposure and the clinical symptoms to the microbiologists[25].

Being one of the most commonly used molecular-based method, polymerase chain reaction (PCR) has been used for rapid detection of pathogens based on specific target DNA sequence[26-28]. Specific polymerase chain reaction (PCR) assays are available and have been established as the gold standard for the identification and discrimination of NTM that representing public health hazard[29]. PCR can be performed on different clinical samples, including swabs, punch biopsies and fine needle aspirates from nodules, plaques as well as edematous lesions that have not ulcerated yet. Several target genes or sequences are commonly used to differentiate *Mycobacterium* sp., including the *16S rRNA*, internal transcribed spacer (ITS), *23S rRNA*, *hsp*-65, *recA* and *rpoB* genes. Among the different genes, PCR targeting the insertion element IS*2404* has shown to be highly sensitive and specific for *M. ulcerans* detection as it is present in high copy numbers (>200 copies) in the *M. ulcerans* genome[30] Timothy. A highly specific real-time qPCR assay was demonstrated to confer enhanced sensitivity of 10-folds higher than the IS*2404* PCR and provide quantitative assessment of *M. ulcerans* dissemination in Buruli ulcer lesions[31]. Meanwhile, molecular detection technique for *M. marinum* is limited due to its high homology with *M. ulcerans.* Typically, molecular identification of *M. marinum* was performed by analysing 16S rRNA or other conserved gene related to a week-growth of photochromogenic colonies[29].

Although PCR has high sensitivity, sophisticated laboratory infrastructure and well-trained personnel are required to obtain reliable PCR assays with strict quality control[32]. Meanwhile, these criteria are not available in endemic communities. Recently, loop-mediated amplification (LAMP), an isothermal amplification technique, has been proposed as an efficient diagnostic tool for detection of *M. ulcerans*. Being a promising alternative to PCR, this technique offers readily readable results within a short turnaround time and without the need of a thermocycler, hence extending molecular diagnosis in fieldwork and at the point of care[33].

PATHOGENESIS OF *M. ULCERANS* AND *M. MARINUM*

M. ulcerans causes an ulcerative skin infection, namely Buruli ulcer, which is a destructive infection of subcutaneous tissues that result in ulcerative lesions in skin, soft tissue and even the bone. *M. ulcerans* differs from many other mycobacteria in term of its implications for pathology and immune response in human. *M. ulcerans* is found to be distributed extracellularly around the coagulative necrosis regions which is different from other mycobacteria that are

intracellular macrophage pathogens. This observation led to early proposal that *M. ulcerans* produces an exotoxin[34]. The cytotoxic molecule 'mycolactone' was successfully isolated and purified from the acetone soluble fraction of lipid extracts of *M. ulcerans* in 1999[35]Kathleen. Principally, the pathogenesis of *M. ulcerans* is mediated by the production of mycolactone which is uncommon among other bacterial exotoxins. Mycolactones consist of a group poorly immunogenic polyketide-derived macrolides that have strong cytotoxic effects against most of the immune cells and skin cells. There are different variants of mycolactone molecules have been identified[36,37]. The typical mycolactone A/B occurs in Africa while mycolactone C is found in Australia. In *in vitro*, mycolactone A/B is more toxic than type C, the clinical significance of these differences remains elusive.

The paramount role of mycolactone in the pathogenesis of *M. ulcerans* was first established via the administration of the purified mycolactone into the skin of experimental animals which resulted in cell death but devoid of acute inflammatory response[38]. The major role of mycolactone in Buruli ulcer pathogenesis was further fortified by the infection of laboratory animals with *M. ulcerans* mutants which lack of mycolactone production. In contrary to the extracellular infection induced by the wild-type *M. ulcerans*, an intracellular inflammatory infection identical to that of *M. marinum*[39] was resulted by the mycolactone-negative mutants[40,41].

Mycolactone has three major adverse implications on the host in mediating the pathogenesis of *M. ulcerans* infection. The chief destructive outcome of mycolactone is its apoptosis and necrosis inducing effects on an array of cells, including the immune cells. Mve-Obiang *et al.* (2003)[42] revealed the potent cytotoxic effect of mycolactone A/B, as low as 0.1 ng/mL was sufficient to induce cell death associated with apoptosis[38] and necrosis[43]. Recently, mycolactone was demonstrated to induce Bim-dependent cell apoptosis via the $mTORC_2$-Akt-$FoxO_3$ axis[44]. Secondly, a down-regulation of overall host immune defence due to the impairments on the production of tumor necrosis factor (TNF) and other secretory proteins via the blockade of Sec61 by mycolactone. Sec61 is a heterotrimeric complex responsible for the transport of all secretory and integral transmembrane proteins into the endoplasmic reticulum in eukaryotic cell. The blockade of Sec61 activity affects the production of interferon-gamma (IFN-γ) and IFN-γ in activated lymphocytes as well as nitric oxide synthase production in macrophages[45]. Thus, an effective immune response is failed to be activated by the host to act upon the underlying mycobacterial infection. Thirdly, mycolactone also causes impairment of pain sensitivity by targeting the type 2 angiotensin II receptors (AT2R) to mediate its analgesic effect. The mycolactone was suggested to induce analgesia by direct cytotoxicity against sensory neurons and Schwann cells, hence resulting in nerve damage[46,47].

As for *M. marinum*, this bacterium causes tuberculosis-like infection in the ectotherms and induces caseating granulomas in zebrafish that are similar to those in humans[48]. *M. marinum* is an opportunistic intracellular pathogen that multiplies in non-acid phagosome of macrophages prior to phagolysosome fusion[49]. Within the cells, *M. marinum* acquires the ability to escape from the phagosome into the cytoplasm to actively stimulate actin-polymerization, resulting to direct spread into adjacent cells via actin-based motility. The translocation of *M. marinum* into the host cell cytosol depends on an intact Region-of-Difference-1-locus (RD1) which encodes a Type-VII secretion system (ESX-1) that plays a role in mycobacterial virulence[50,51]. Thus, this mechanism confers immune evasion for *M. marinum* by spreading from cell to cell, contributing to permanent infection. *M. marinum* was further found to employ the nonlytic spreading mechanism where the mycobacterium is ejected from the cell via the ejectosome, a F-actin based structure, enabling the transmission to naïve host macrophages[52]. Then, the macrophages migrate into deeper tissue, where they start to form the pathological granuloma-like aggregates after phagocytosis of *M. marinum*. Moreover, *M. marinum* was shown to harbour the ESX-5 system of mycobacteria that responsible for the production of various proline-proline-glutamic acid (PPE) and proline-glutamic acid (PE)-polymorphic GC-rich repetitive sequence (PGRS) proteins. These proteins were demonstrated to interact with host immune system and evade the innate immune response via antigenic variation[53], thus contributing to persistent infection[54,55]. For instance, the expression of PPE38 protein on the cell wall of *M. marinum* was shown to involve in bacterial surface properties and pro-inflammatory effects on infected macrophages[56].

In the view of granulomas as host-beneficial protective structures that has long been a tenet of medical and immunology textbooks, studies employing the zebrafish embryo model of *M. marinum* infection have challenged the idea and provided evidence that the granulomas may be harnessed by mycobacteria for their dissemination and proliferation[57,58]. The study revealed that *M. marinum* employs the ESX-1-dependent early macrophage aggregate to promote spread and growth[57]. On top of that, the mature and established granulomas are found to be porous, where newly infecting mycobacteria can infiltrate and persist within[59].

PREVENTION AND TREATMENT STRATEGIES

An utmost priority to curb Buruli ulcer disease is to enhance our knowledge on the transmission pathway of *M. ulcerans* to human that could aid in the preventive measures focusing on early detection and administration of effective treatment. However, the greatest challenge in Buruli ulcer control is that the reservoir and transmission of *M. ulcerans* are unclear. Exposure to water sources near endemic villages has been shown to increase the risk for developing Buruli ulcer, but it is a challenge to reduce the exposure, particularly in children, to such sources in rural West Africa[60]. The development of an effective vaccine to confer protection has enormous significance in areas of high endemicity for Buruli ulcer. However, there is no effective vaccine specifically targeting *M. ulcerans* is available clinically. The Bacillus Calmette-Guérin (BCG)

vaccine is the only licensed vaccine against mycobacterial infections approved clinically that used to prevent tuberculosis. Although the BCG is cross-protective against *M. ulcerans*, it has only been associated with delaying the onset of disease and short-lived protection in small trials. Collectively, outreach programs to educate communities in endemic areas to recognize early stage of Buruli ulcer is extremely crucial for prevention of severe forms of the disease.

Although there are no vaccine and effective protective strategies, antimicrobial therapy has showcased effective treatment of the disease and lowered the recurrence rate. Given that single-drug treatment led to relapse of mycobacterial disease due to the emergence of drug-resistant mycobacterial strains, multi-drug treatment regimens have been employed for mycobacterial infections. It is a common phenomenon that drug-resistant mutants repopulate the lesions following monotherapy, especially in both tuberculosis[61] and leprosy[62]. Therefore, a second companion drug should be combined with the highly active core antimicrobial agent to prevent treatment failure and relapse.

In 2004, WHO recommended a combination antibiotics alone for small early lesion or as an adjunct to surgical resection for large lesions[63]. A randomised controlled trial reported similar efficacy between the use of either rifampicin and streptomycin (8 weeks) or rifampicin and streptomycin (4 weeks) followed by rifampicin and clarithromycin (4 weeks) which resulted in high recovery rates of exceeding 90% for patients inflicted with early (<6 months) and small lesion (<10 cm)[64]. Recently, a fully oral rifampicin and an extended release formulation of clarithromycin has shown comparable effectiveness for treatment of early and limited Buruli ulcer[65].

According to Center of Disease Control and Prevention, the public water facilities such as swimming pools, spas and hot tub are advised to maintain adequate concentrations of free chlorine, ranging between 0.4 to 1 mg/liter in swimming pool and 2 to 5 mg/liter for spas and hot tub[66,67]. Frequent sanitation and disinfection, and removal of infected fishes are the main control strategies of *M. marinum* infection in fishes. The maintenance personnel for the aquarium should use waterproof gloves to prevent any potential upper limb skin lesions exposure to the pathogen during fish tank-related activities. Proper training is also essential for high-risk populations, such as fishermen and marine-life handlers, to identify signs of *M. marinum* in fish or human in order to facilitate more prompt treatment[68].

To date, there is no clinical trial available which could suggest optimal management of *M. marinum* infections. Furthermore, there is no standardized treatment for cutaneous infections due to *M. marinum*, the therapeutic choice is mainly based on the severity of the infection and the immunocompetency of the patient[9]. Principally, rapid recovery from *M. marinum* in man requires the proper treatment and prevent further progression to deeper tissues. Based on retrospective case studies, single agent antibiotic therapy was shown to successfully treat majority of the limited cutaneous *M. marinum* infections. These single antibiotic agents include minocycline, doxycycline, cotrimoxazole-trimethoprim and clarithromycin have demonstrated positive outcomes in the treatment of *M. marinum* infections[9,69]. Besides that, the combination use of 2 active agents including ethambutol, clarithromycin/azithromycin or rifampicin for 3 to 4 months has been reported to be effective adjunct therapy together with surgical debridement for invasive *M. marinum* infections[70]. Other antimicrobials used for treatment of *M. marinum* infection include ciprofloxacin, moxifloxacin, isoniazid and protionamide[71]. Nevertheless, there were reported cases that yielded negative therapeutic outcomes[72,73]. Several reports also described the worsening of *M. marinum* infection in patients receiving anti-TNF-α therapy[74,75]. Thus, it should be recommended to halt the use of TNF-α inhibitor or other immunosuppressive therapy in *M. marinum* infected patients who are under the course of antibiotics.

Surgical debridement remains a controversial therapy option for *M. marinum* infection and it should be limited to cases that fulfil certain criteria, including cases that associated with poor prognosis involving deep lesions, persistent drainage of sinus and chronic pain[76]. There are also other therapeutic modalities such as local hyperthermic therapy, photodynamic therapy, electrodessication, cryotherapy and X-ray therapy have been recommended to treat *M. marinum* infection[9]. Bacteriophage therapy represents an interesting strategy to be developed for the management of *M. marinum* infection despite only phage therapy using mycobacteriophage D29 for treatment of Buruli ulcer is available at the moment[77].

CONCLUSION

Research on both *M. ulcerans* and *M. marinum* is vital for much needed advancement in the prevention and management of Buruli ulcer and fish tank granuloma, which are both challenging diseases that have been largely neglected. A clearer view of the exact innate and adaptive immune mechanisms leading to protection from *M. ulcerans* and *M. marinum* infections will greatly propel the development of new strategies for effective vaccine design. Moreover, future research focusing on clinical applications and epidemiology is essential to advance our knowledge of mycobacterial pathogens that cause cutaneous infection and improve our capability to control and treat these infections with optimal medical interventions.

Authors Contributions

The literature review and manuscript writing were performed by LT-HT and JW-FL. PR and LC-M provided vital insight and performed proof-reading. The research project is conceptualized by LT-HT and JW-FL.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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