A RETROSPECTIVE STUDY ON THE MOLECULAR DIAGNOSIS OF CUTANEOUS NON-TUBERCULOUS MYCOBACTERIAL INFECTION COMPARED TO CONVENTIONAL CULTURE METHOD AT INSTITUTE OF DERMATOLOGY, BANGKOK

BY
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Abstract

With increasing prevalence of cutaneous non-tuberculous mycobacterial (NTM) infection in Thailand, molecular diagnostic methods such as PCR and gene sequencing have played an important role in diagnosis and management of the disease. Although culture is still the gold standard for diagnosis, molecular diagnosis provides faster results enabling prospect of better clinical outcome. We aimed to compare the efficacy of Polymerase Chain Reaction (PCR) and sequencing technique using direct tissue sample to conventional culture method, and also to study the clinical characteristics of cutaneous NTM infections at the Institute of Dermatology. The medical records of all cases with confirmed diagnosis of cutaneous NTM infection during 2016 - 2020, with the results of both culture and PCR/gene sequencing of fresh tissue, formalin-fixed, paraffin-embedded tissue (FFPE) sample were collected. Of all medical records, only 52 cases met our criteria, where only 9 cases had both positive culture and PCR from fresh tissue. Notably, 7 cases had negative culture but positive PCR from fresh tissue while in 36 cases, culture was positive with negative PCR from fresh tissue ($p<0.05$). Neither the duration of lesions (≤4 weeks or >4 weeks) nor the morphology had any correlation with the molecular result. M. abscessus was the most common pathogen followed by M. marinum. Of note, 32 cases (64%) showed non-specific histological features. Most patients had combination therapy with 2 or 3 antibiotics. Combination of clarithromycin with either ciprofloxacin or co-trimoxazole was commonly used.

In conclusion, conventional culture is the gold standard for diagnosis. Negative PCR results in most cases may result from inadequate tissue sampling showing less sensitivity. In addition, our study was limited by the small number of cases during the period of research.

(Total 37 pages)

Keywords: Molecular Diagnosis, Sequencing, Cutaneous Non-tuberculous Mycobacterial Infection, Polymerase Chain Reaction

Student's Signature …... Thesis Advisor's Signature ....

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Chapter 1

Introduction

1.1 Background

Non-tuberculous mycobacteria (NTM) are generally defined as the group of mycobacteria apart from *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* (Nogueira et al., 2021). These are ubiquitous organisms present in environmental sources including wet soil, dust, water, cold-blooded animals and human sewage (Porvaznik, Solovič, & Mokrý, 2017). Over 170 species of non-tuberculous mycobacteria have been identified. The NTM are generally categorized into two major groups according to their ability to grow on solid culture media, i.e., rapidly growing mycobacteria (RGM) and slow growing mycobacteria (SGM) (Gonzalez-Santiago & Drage, 2015).

Transmission of cutaneous NTM infection occur directly via inoculation through skin breaks, for example, during trauma, surgical procedures, injections, tattoos, and body piercings. In addition, they present with a wide range of clinical features of cutaneous NTM infections include erythema, papular lesions, erosions, draining sinuses, abscesses, subcutaneous nodules, ulcerations, and cellulitis-like lesions and sporotrichoid lesions. Usually, the patient has only a single cutaneous lesion; however, when the infection is spread via lymphatics, multiple skin lesions may be present. Common sites of infection are exposed body parts such as face, hands or feet (Franco-Paredes et al., 2018).

As different NTM species require specific treatment and therefore vary in outcomes, correct identification of NTM species is clinically essential. Due to a broad spectrum of clinical manifestations, laboratory procedures are mandatory to reach a rapid and definitive diagnosis of cutaneous non-tuberculous mycobacterial infection.

The diagnosis of cutaneous non-tuberculous mycobacterial infection by conventional methods has several limitations. For example, pathological examination sometimes is unable to identify NTM species, and standard cultural method can take up to 2-4 weeks for inoculation with occasional unsuccessful culture of the organisms (Kromer et al., 2019).

In recent years, biomolecular identification of NTM strains by polymerase chain reaction (PCR) and gene sequencing is another method to identify pathogens (Kothavade, Dhurat, Mishra, & Kothavade, 2013). Application of novel molecular techniques has facilitated a more rapid and accurate identification of the NTM species (Yeo et al., 2019). Diagnosis and management a cutaneous NTM infection has been challenging due to a wide range of clinical manifestations and sensitivity to antimycobacterial agents varies with species. One study reported a maximum delay of up to 52 weeks before initiation of the targeted treatment for NTM, with almost 50% of cases lost to follow-up (Yeo et al., 2019). Conventional diagnostic methods are time-consuming and require multiple culture media and incubation temperatures. In addition, even some lesions characteristic of NTM infection may give rise to negative culture result. This highlights the importance of clinicians to have better understanding about pros and cons of these novel molecular methods using fresh tissue sample or FFPE to have better clinical outcomes.

1.2 Objectives

By completing this retrospective study, we aim to achieve the following objectives:

1.2.1 To study about PCR and sequencing technique using the 16S-23S intergenic spacer region (ITS region) and 16S rRNA using direct tissue sample from lesions, by comparing this to conventional culture method, including subgroup analysis of formalin-fixed, paraffin-embedded tissue samples.

1.2.2 To study the clinical characteristics of cutaneous non-tuberculous mycobacterial (NTM) infections including patient demographic data, diagnostic techniques, treatment, and outcomes at the Institute of Dermatology.

1.3 Research Question

Is molecular diagnosis of cutaneous non-tuberculous mycobacteria using fresh tissue sample superior to conventional cultural method?

1.4 Research Conceptual Framework

The medical record of all cases with confirmed diagnosis of cutaneous NTM infection during the time period of 2016 - 2021, with the results of both cultural method and PCR/gene sequencing of the following samples e.g., fresh tissue, formalin-fixed paraffin embedded sample (FFPE), pus or secretion from lesions, were collected from the Molecular genetics department of Institute of Dermatology, Thailand without being able to identify the patients.

For conventional method, tissue sample of patients were sent for histopathological investigations which include staining with hematoxylin-eosin and Ziehl-Neelsen stain. Culturing was performed with blood agar, Lowenstein-Jensen agar, and BD MGIT Mycobacteria Growth Indicator Tube and incubated at 37°C and 30°C to favor the growth *Mycobacterium haemophilum* and *Mycobacterium marinum* species. The 'No Growth' within 2 months were considered negative culture. In case of positive culture, isolates from the culture were used to undergo polymerase chain reaction (PCR), extraction and amplification of the specific sequence of bacterial DNA coding for 16S rRNA and ITS region using a primer set. DNA replication was performed with TopTaq master mix kit (Qiagen, Germany), with 0.2µg of each primer and 1µL of Genomic DNA. Initial denaturation was initiated at 94°C for 5 minutesfollowed by temperature 94°C for 40 cycles at various temperatures for 1 minute and then at 72°C for 10 minutes. The yield from PCR was measured with agarose gel electrophoresis method. To perform sequencing reactions, Big-dye terminator kit (Life Technologies) was used to sequence the product; 3.5 µl of DNA sample was added to 1.25 µl Big-dye sequence buffer, 0.25 µl BigDye® Terminator v3.1 Ready Reaction Mix and 0.25 µl of forward or reverse primer. Ethanol precipitation of sequencing products was done to purify the product, 20 µl of precipitation solution was added to 5.25 µl of each sample, incubated for 15 minutes at room temperature and then spun at 3000 r.p.m. for 30 minutes. Then the samples were washed again with 100 µl per well of 70% ethanol and spun at 3000 r.p.m. for 10 minutes before being dried. The samples were subsequently re-suspended in 10µl Hi-Di™ Formamide (Thermo Fisher Scientific) and heated at 90°C for 2 minutes. The samples on the 96-well plates were run on the ABI 3730XL Automated sequencer (Applied Biosystems). The sequence of resulting copies was then analyzed and identified to the species level by comparison with reference sequences on BLAST (Basic Local Alignment Search Tool) database.

Although conventional method is the gold standard, it is time consuming and sometimes can yield negative culture. At Institute of Dermatology, fresh tissue samples were also sent directly to do PCR and gene sequence analysis in some patients to fasten the diagnosis and enable better clinical outcomes.

Chapter 2

Literature Review

2.1 Non-tuberculous Mycobacteria

2.1.1 History

It is believed that mycobacteria originated over 150 million years ago (Barberis, Bragazzi, Galluzzo, & Martini, 2017). Even prior to the division of continents, there were records of the presence of Mycobacterial infection in certain areas of the African continent and South America (Franco-Paredes et al., 2018).

In the beginning, the genus *Mycobacterium* included only those causing leprosy and tuberculosis (Nogueira et al., 2021). The domestication of livestock and the invention of water distribution systems had a great impact on the disease distribution of mycobacterial infections (Mostowy & Behr, 2005).

In 1926, *Mycobacterium marinum* was first isolated from a saltwater fish (Aronson, 1926). However, it was not until 1951 that people realized this organism was able to cause disease in people who frequently visited swimming pools. Hence, the disease was named 'swimming pool granuloma' (Norden & Linell, 1951). In 1931, non-tuberculous mycobacteria were isolated by Pinner and it was found out that these bacteria differ from those that cause tuberculosis as they lack virulence for guinea pig and poor susceptibility to anti-tuberculous medicines (Pinner, 1933).

Historically, mycobacteria were classified according to the pigment production and the rate of growth (Runyon, 1959). More than 190 species of NTM have been identified, of which around 40 species have been considered to be pathogenic (Misch, Saddler, & Davis, 2018). The advance in molecular biology in identification with the development of nucleic acid amplification by Kary Banks Mullis lead to better diagnostic methods that have more sensitivity than conventional clinical and histopathological examinations or culture (Nogueira et al., 2021).

2.1.2 Epidemiology

Actual data on the incidence of NTM infection is limited since the disease is not reportable unlike TB. However, there is evidence that the number is increasing rapidly each decade mostly due to advances in diagnostic techniques (Parte, 2014). Another reason for this rising trend might be due to an increase in the vulnerable group who were suffering from immunosuppressive diseases or taking immunosuppressive medications as in cases like malignancy or organ transplantation.

A population-based study carried out in Minnesota, USA revealed almost a 3-fold rise in the local incidence with 0.7 per 100,000 person-years during the years 1980-2009 to 2.0 per 100,000 person-years between 2000-2009 (Wentworth, Drage, Wengenack, Wilson, & Lohse, 2013). Similarly, the annual meeting of Scottish Dermatology Society meeting announced that 67 cases of cutaneous NTM infection were recorded during the 10 years period of 2003-2013, with 23 cases of *Mycobacterium chelonae* infection and 15 cases infected with *Mycobacterium marinum* (Scott-Lang et al., 2014).

2.1.3 Taxonomy, Microbiology and Classification

Taxonomically, mycobacteria are the members of the order *Actinomycetaceae*, and belong to the genus Mycobacterium, which is the only genus within the family *Mycobacteriaceae* (Rastogi, Legrand, & Sola, 2001). More than 190 species of NTM have been identified, many of which have been incriminated in skin and soft tissue infections (SSTI) (Misch et al., 2018).

Mycobacteria are aerobic, non-motile acid-fast bacilli with mycolic acid structure. They possess a lipid rich, hydrophobic membrane which serves as a major contribution factor for the bacteria's surface adherence, biofilm formation, aerosolization, and antibiotic/disinfectant resistance. Moreover, mycobacteria are able to replicate at a very slow rate that leads to a poor susceptibility to most antimicrobial agents. In addition, their ability to grow at low carbon levels allows them to be effective competitors in low-nutrient environments (oligotrophs) (Franco-Paredes et al., 2018).

NTM have the ability to grow in natural and artificial reservoirs, such as treated urban water and sewage systems; hot tubs; tattoo inks; swimming pools; pedicure footbaths; showers; fish tanks; or medical equipment like endoscopes and their washing machines and heater-cooler system, freezing devices used to refrigerate surgical solutions, and inadequately sterilized surgical equipment or solutions. Some species of NTM such as *Mycobacterium marinum* or *Mycobacterium avium* are usually isolated from man-made environments, when the natural reservoir of *Mycobacterium xenopi* and *Mycobacterium kansasii* is yet to be discovered (Forbes et al., 2018).

Historically, NTMs have been classified according to their growth rate and production of chromogens (Runyon, 1959). As for growth, they are divided into slow-growers and rapid growers. The slow-growing mycobacteria take 7 days or more to grow, where the rapid growers, less than 7 days. In consideration of pigment formation, mycobacteria are divided into Type I-Photochromogenic: produce yellowish colonies when exposed to light; Type II -Scotochromogenic: regardless of exposure to light, they also produce yellowish colonies; Type III-Achromogenic: produce discreet or no pigmentation. Type IV is classified only because it also represents rapid growth. It is important to emphasize that even fast-growing mycobacteria multiply much more slowly than other types of bacteria (Nogueira et al., 2021).

2.1.4 Transmission and Clinical Presentation

Transmission of cutaneous NTM infections occur via direct inoculation through injury to the skin barrier, which may happen in cases of trauma, surgical procedures, plastic surgery (including liposuction), injections, tattoos, acupuncture, and body piercings. Cosmetic procedures such as mesotherapy (multiple injections of pharmaceutical products, plant extracts, homeopathic substances, vitamins, or other compounds into subcutaneous fat) have been involved in the transmission of rapidly growing mycobacteria (Franco-Paredes et al., 2018). However, the initial exposure or injury may not be recalled since some lesions can take months to develop.

For example, *M. chelonae, M. abscessus*, and others have been associated with infection after tattoos. This is probably due to presence of contaminants in tattoo ink either during the premixed or dilution with tap water. Patients present with localized rash, papules, nodules, or abscess over the tattooed areas (Conaglen et al., 2013).

Resistance to disinfectants such as chlorine, formaldehyde, and glutaraldehyde has led to multiple RGM infection outbreaks related to medical procedures (Wallace, Brown, & Griffith, 1998). Skin and soft tissue infections due to NTM have been reported in various public health and nosocomial settings. Similarly, there has been a report on the association between liposuction and *M. chelonae* and *M. abscessus* infection in a patient who travelled from United States to Dominican Republic for cosmetic liposuction surgery (Furuya et al., 2008). *M. fortuitum* infection has been reported after breast implant surgery (Thomas, D'Silva, Borole, & Chilgar, 2013). More than 1,000 suspected cases of SSTI were seen in patients who underwent video-assisted surgery in Brazil due to a glutaraldehyde-resistant *Mycobacterium massiliense* strain (Duarte et al., 2009).

2.2 Diagnosis of NTM Infection

Diagnosing cutaneous NTM infections: Due to a broad spectrum of clinical manifestations, laboratory procedures are mandatory to reach a definite diagnosis of cutaneous non-tuberculous mycobacterial infection. These include conventional (smear and pathological test, culture test and biochemical analysis) and molecular identification of cutaneous NTM species.

2.2.1 Stained Smear and Microscopy

Smears and pathology specimens are stained with acid-fast stain, Auramine O or Ziehl-Neelsen stain, and then investigated by light microscopy. In this procedure, mycobacterium may occasionally be misidentified as other bacteria exhibiting similar microscopic features (Griffith et al., 2007).

2.2.2 Microbiological Methods

The current gold standard to reach a definitive diagnosis on a clinically suspicious case of NTM infection is culture. Yet, culturing of the mycobacteria is time consuming and requires multiple media types and various temperatures of incubation to yield the optimal growth of the causatic mycobacteria. Generally, the culture results can take up to 2-4 weeks (Kromer et al., 2019).

2.2.3 Molecular Methods for Identification of Cutaneous NTM

2.2.3.1 Differential diagnosis by DNA-DNA hybridization (DDH) assays

A commercial kit containing a panel of 18 major mycobacteria can be used for differential diagnosis (Kusunoki et al., 1991). However, this method has limitations in identifying rare species that are not in the panel and false positive and false identification have also been reported using this kit (Nakanaga et al., 2014).

2.2.3.2 Gene amplification assays

While culturing can take up to several weeks, especially in the case of an SGM infection, identification can be hastened by commercially available genotyping kits for instance 16S rRNA gene sequencing or species-specific PCR (Ichimura et al., 2007).

2.2.3.3 Identification with 16S rRNA gene sequence

Mycobacteria can be identified by sequence analysis of the first one-third of the 16S rRNA gene by comparison with reference sequences in a database (Nakanaga et al., 2014).

2.2.3.4 Identification using other housekeeping gene sequences

The DNA sequences of 16S-23S intergenic spacer region (ITS region), rpoB and the heat shock 65 (hsp65) are also very useful for strain identification (Nakanaga et al., 2014).

A 10-year retrospective study was carried out in 2019 testing 952 samples from 639 patients who had clinical suspicion of NTM infection, during the time period of 2003-2013. This study investigated the reliability and usefulness of smear microscopy, PCR and culture. The time to diagnosis between the direct PCR and conventional culture was also compared. The sensitivity, specificity, positive and negative predictive values of the PCR were 61.6% (53.5-69.1), 99.1% (98.2-99.6), 92.8% (85.8-96.5) and 93.4% (91.6-94.9), respectively. The average time to identification at species level was 35 days (SD, 17.67) for culture and 6 days (SD, 2.67) for the PCR (when positive), which represents a 29-day shorter time to results (Simon et al., 2019).

2.3 Treatment

When there is clinical suspicion for NTM infection, it is recommended to start empirical antibiotic treatment and surgical wound debridement if necessary. Empirical antibiotic treatment is essential because it takes more than 4 weeks to obtain the results of the species identification and drug susceptibility tests (Bae, Yun, Roh, &, 2021). According to the suspected pathogen, a combination of macrolide (azithromycin or clarithromycin) plus fluoroquinolone, doxycycline, or trimethoprim-sulfamethoxazole is often started initially. If the SSTI is severe, injectables such as amikacin and/or meropenem may be added (Wang & Pancholi, 2014).

Based on in vitro DST, the empiric regimen should be tailored down to two to three active drugs. A macrolide, if DST indicates susceptibility, should always be included. Antibiotic resistance or adverse drug effects may require the use of alternative antibiotics that are less cost effective and more toxic. Close monitoring of side effects is needed given that extended course of therapy is often required (Griffith et al., 2007).

The duration of therapy of NTM SSTI in immunocompetent patients is usually 1 to 2 months after symptoms resolve but may be longer for slower healing lesions. Additional workup may be needed if there is no response to therapy after 4 to 6 weeks. Differential diagnosis for NTM SSTI includes sporotrichosis, nocardiosis, cutaneous leishmaniasis, M. tuberculosis, aspergillosis, cryptococcosis, and histoplasmosis (Wang & Pancholi, 2014).

Chapter 3

Research Methodology

3.1 Study Design

This study is conducted as a descriptive retrospective chart review.

3.2 Study Population

The medical record of all cases with confirmed diagnosis of cutaneous NTM infection during the period of (2016 - 2021) were collected from the molecular biology department of Institute of Dermatology, Thailand.

3.3 Selection Criteria

3.3.1 Inclusion Criteria

All cases diagnosed as cutaneous NTM infection with molecular diagnostic results and culture results were included.

3.3.2 Exclusion Criteria

Cases with incomplete data such as PCR and/or culture or insufficient clinical data as specified above were excluded from this study.

3.4 Sample Size

From previous studies, prevalence of cutaneous NTM infection and sensitivity of PCR are 0.25% and 95.23 % respectively. With these figures known, the sample size to determine the sensitivity of the PCR will be:

$$n_{\mathrm{Se}} = \frac{Z^2_{\frac{\alpha}{2}}\widehat{\mathrm{Se}}(1-\widehat{\mathrm{Se}})}{d^2 \times \mathrm{Prev}} \qquad (3\text{-}1)$$

where, n_{se} = Sample size populaton,

Se = Predetermined value of sensitivity of PCR,

d = Precision of estimate or maximum marginal error (5%),

Prev = Prevalence of cutaneous NTM infection,

α = 0.05, Zα/2 inserted by 1.96.

$$n_{\mathrm{Se}} = \frac{1.962 \times 0.95 \times 0.05}{0.052 \times 0.25} = 292$$

3.5 Data Collection and Data Analysis

The medical record of all cases with confirmed diagnosis of cutaneous NTM infection during the period of (2016 - 2020) were collected from the molecular genetics department of Institute of Dermatology, Thailand. Patient's demographic data, underlying diseases, history of previous trauma, cosmetic procedures or surgery, clinical manifestations, laboratory investigations including histopathological studies, microbiological cultures and antimicrobial susceptibility tests, treatment regimens and outcomes were all collected and analyzed. The diagnosis of cutaneous NTM infection was confirmed by clinical findings, histological features, microbiological study and molecular methods.

Data analysis and statistics used in data analysis: Clinical and demographic characteristic of the patients are collected and analysed by Chi-square test and *t* test for the categorical and continuous variables respectively. A p-value of <0.05 was considered statistically significant. All analyses were performed using Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA)

3.6 Location

Institute of Dermatology, Bangkok

Chapter 4

Research Results

Within the period of 2016-2021, there were 411 cases of presumed diagnosis of non-tuberculous mycobacterial infection according to the clinical and pathological examinations. However, there were only 52 cases of confirmed cutaneous non-tuberculous mycobacterium infection that met all our inclusion and exclusion criteria, who had both positive culture and PCR (fresh tissue or FFPE) or either one of them.

4.1 Patient Demographic and Clinical Data

The male and female ratio was 1:1 (26 males and 26 females) with median age of 47 years ranging from 16 to 81 years old. The median duration from the onset of symptoms to seeking medical attention was 35 weeks (1- 480 weeks). Thirty cases (58.8%) did not have any underlying disease. There were no cases of malignancy, post-organ transplantation, or patients with Acquired Immune Deficiency Syndrome. Others (27.5%) had underlying medical condition such as dyslipidemia, thyroid disease and psoriasis, etc. (Table 4.1)

Regarding to history of skin injury, almost half of the cases (49%) denied any form of prior injury or surgery. Around 30 percent had miscellaneous injuries, including insect bites, knife wound, splinter wound, dog scratches, burn injuries and vehicle accidents. Interestingly, all of 6 cases (11.8%) who gave history of aquatic-related accidents had *M. marinum* in the culture result. Some cases had history of cosmetic-related procedures such as injection (2 cases, 3.9%) and tattoo (4 cases, 7.8%) (Table 4.1).

One in every two patients had only a single lesion and one-fourth of the cases (13 cases) had more than 5 lesions. In all of the cases, the distribution was localized, and no systemic involvement was reported.

Table 4.1 Demographic data of patients with confirmed diagnosis of non-tuberculous mycobacterial infection at Institute of Dermatology, Bangkok during 2016-2021

Variables	Results
Median Age, yr (range)	47 (16-81)
Gender (n=52)	
Male	26 (50%)
Female	26 (50%)
Duration of symptoms, week (ranges)	35 (1-480)
Underlying diseases (n=51)	
None	30 (58.8%)
Diabetes mellitus	5 (9.8%)
Autoimmune disease	2 (3.9%)
Other	14 (27.5%)
History of skin injury (n=51)	
None	25 (49%)
Injection	2 (3.9%)
Tattoo	4 (7.8%)
Aquatic-related injury	6 (11.8%)
Other	14 (27.5%)

Papules, nodules, patch and plaque account for the morphology of lesions in majority of cases (76.5%) followed by sporotrichoid lesions, and ulcers and abscesses with 13.7 percent and 9.8 percent respectively. Most of the lesions existed in the exposed areas of the body. Sixty percent of cases had lesions in upper and lower extremities such as thigh, leg, forearm, hand and feet in the decreasing order of incidence. Other areas include 3 cases of trunk lesion, 1 case each in feet, face and genital areas (Table 4.2).

Table 4.2 Clinical characteristics of patients with confirmed diagnosis of non-tuberculous mycobacterial infection at Institute of Dermatology, Bangkok during 2016-2021

Variables	Results
Number of lesions (n=52)	
1	29 (56.9%)
2	5 (9.8%)
3	1 (1.9%)
4	3(5.9%)
≥5	13(25.5%)
Morphology group (n=52)	
G1 as Papule,Nodule,Patch,Plaque	39 (76.5%)
G2 as Ulcer, Abscess	5 (9.8%)
G3 as Sporotrichoid lesion,other	7 (13.7%)
Site of lesion (n=52)	
Leg	11 (21.2%)
Forearm	8 (15.4%)
Trunk	3 (5.8%)
Face	1 (1.9%)
Hand	8 (15.4%)
Feet	1 (1.9%)
Genital	1 (1.9%)
Other	19 (36.5%)

4.2 Acid-fast Bacilli (AFB) Test and Histological Examination

Of 52 patients, only 24 cases had medical record on whether they had taken any antibiotic treatment before coming to the hospital of which 12 cases had previous antibiotic therapy and 12 cases did not. Acid-fast bacilli (AFB) test was done in 29 patients, of which 11 cases were AFB positive and 18 were negative. Out of 50 cases that had histological examination, 32 cases (64 %) showed non-specific histological features whereas remaining 36 percent had granulomatous histological pictures, including mixed cell granuloma (13 cases), foreign body type granuloma (4 cases) and histiocytic type granuloma (2 cases) (Table 4.3).

Table 4.3 Acid-fast bacilli test and histopathology

Variables	**Results**
Acid-fast bacilli (AFB) (n=29)	
Positive	11 (37.9%)
Negative	18 (62.1%)
Histopathological result (n=50)	
Mixed cell granuloma	13 (26%)
Foreign body type granuloma	4 (8%)
Histiocytic granuloma	2 (4%)
Non-specific histological picture	31 (62%)

4.3 Cultural Diagnosis with PCR from Colony and Molecular Diagnosis with PCR from Fresh Tissue Sample or FFPE

There was no growth from colony in 8 cases. In 44 culture positive patients, *M. abscessus* was the most common pathogen (25 cases; 48.1%), followed by *M. marinum* (17 cases; 32.7%) and *M. fortuitum* (1 case; 1.9%), and *M. Chelonae* (1 case; 1.9%). The PCR results from skin tissue came out negative in 36 cases (69.2%). Here, *M. abscessus* was still the most frequently detected pathogen (9 cases; 17.3%), followed by *M. marinum* and *M. fortuitum* with 4 cases (7.7%) and 3 cases (5.8%) respectively (Figure 4.1).

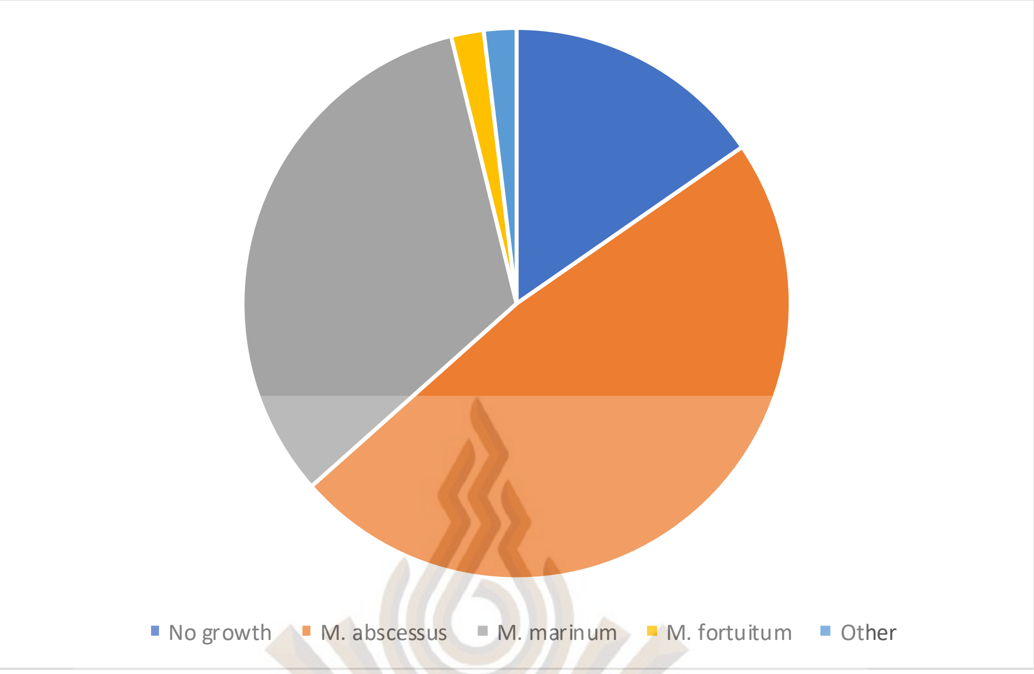

Figure 4.1 Cultural diagnosis & PCR from colony (n=52)

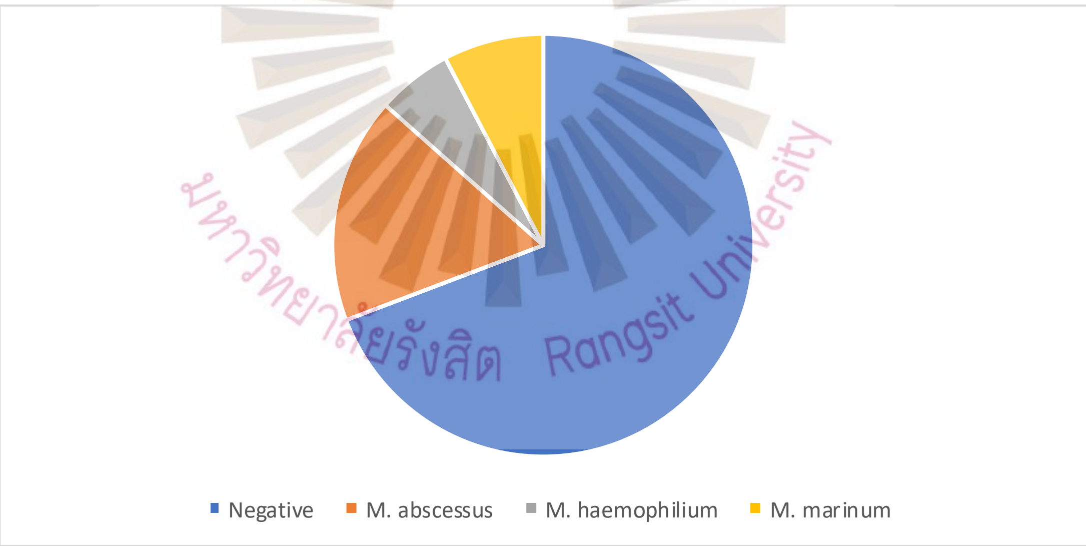

Figure 4.2 Molecular diagnosis (PCR and sequencing from fresh tissue or FFPE) (n=52)

Table 4.4 Comparison of cultural method and PCR with fresh tissue or FFPE according to pathogens

		Cultural diagnosis and PCR from colony (Gold standard)					
		No growth	**M. abscessus**	**M. marinum**	**M. fortuitum**	**Other**	**Total**
Molecular diagnosis (PCR and sequencing from skin tissue)	No growth	0	20	14	1	1	36
	M. abscessus	1	5	3	0	0	9
	M. haemophilium	3	0	0	0	0	3
	M.marinum	4	0	0	0	0	4
Total		8	25	17	1	1	52

Comparing the conventional diagnosis of NTM infection (culture and/or PCR from colony tissue) to PCR and sequencing from fresh tissue sample or FFPE, there were significantly more cases of positive gold standard than the molecular test (44 cases vs 16 cases) when analyzed by Fisher's Exact test at p-value <0.05. There were 8 cases where the gold standard culture was negative but PCR from fresh tissue sample was positive, and 8 out of 44 positive culture patients had positive PCR from tissue also. (Table 4.4) Both tests show low agreement in the opposite direction as Kappa measure of agreement was -0.291. Out of 25 cases of *M. abscessus* infection confirmed by cultural method, 20 cases had negative result in PCR with fresh tissue, while 5 cases presented positive in both. Interestingly, among the 16 cases of culture-positive *M. marinum* infection, 3 cases had *M. abscessus* in PCR sequencing with fresh tissue (Table 4.4).

The duration of lesion at ≤4 weeks and >4 weeks did not show statistical difference in the number of positive or negative mycobacterial infection when tested by the molecular technique. Among the group of fresh tissue specimen, there were significantly more cases of positive gold standard than the molecular test (42 cases vs 14 cases) when analyzed by Fisher's Exact test at p-value <0.05. The morphology of the lesions did not correlate with the molecular result (Table 4.5).

Table 4.5 Comparison of cultural method and PCR with fresh tissue or FFPE according to pathogens

		Molecular diagnosis		
		Neg	**Pos**	**Total**
Morphology group	G1 as Papule, Nodule, Patch, Plaque	26	13	39
	G2 as Ulcer, Abscess	4	1	5
	G3 as sporotrichoid lesion, Other	6	1	7
	Missing	0	1	1
Total		36	16	52

4.4 Treatment and outcome

Regarding treatment, 48 out of 52 cases received medical treatment in which 1 case had both medical therapy and surgical excision. Thirty-seven patients commenced combination therapy of 2 to 3 medications. The most given combination therapy was clarithromycin with either ciprofloxacin or co-trimoxazole. Other prescribed treatment regimens were clarithromycin with doxycycline, clarithromycin with amikacin, clarithromycin with rifampicin, and clarithromycin with ethambutol.

Half of the cases were improved and still taking treatment at the time of study. Thirty percent of cases achieved clinical remission while 6 cases lost to follow up and 3 cases were referred to another hospital.

Chapter 5

Discussion and Conclusion

5.1 Discussion

During the last few decades, there has been an increasing trend in the NTM infection worldwide (Parte, 2018). Similar to a previous research in Thailand (Chirasuthat, Triyangkulsri, Rutnin, Chanprapaph, & Vachiramon, 2020), the most common pathogen in our study was also *M. abscessus*, followed by *M. marinum*. The transmission of cutaneous NTM infection is conducted directly through skin breaks (Franco-Paredes et al., 2018). However, in this study almost 50% of the patients denied any form of skin injury prior to the lesions. This may be due to the recall bias or prolonged duration between the skin injury and onset of symptoms, median duration of 35 weeks (1-480) in our research, which may have caused the patients failure to recall history of incidents. There were 6 cases with history of cosmetic related procedures (2 cases of injection and 4 cases of tattoo). These cases were caused by rapidly growing mycobacteria (RGM), *M. abscessus* and *M. chelonae*. This finding corresponds to the prior study that rapidly growing mycobacteria are mostly responsible for infections in cosmetic injections and procedures (Cusumano et al., 2017). In this study, we included only true pathogenic mycobacteria, therefore non-pathogens such as *M. cosmeticum* and *M. smegmatis* were excluded.

NTM infection has a wide range of clinical manifestations, including papules, nodules, plaque, patch, ulcers, abscess, sporotrichoid lesions and cellulitis-like lesions (Mougari et al., 2016). Regarding the morphology of lesions, plaque and nodules were the most common forms in this study (19 and 14% respectively). Other forms of lesions included sporotrichoid lesions, papules, patch, ulcer and abscesses. Half of patients had only one single lesion. Another 20-year retrospective study carried out in Thailand demonstrated the same results with plaques and nodules being the predominant clinical manifestations and 50% of cases presented with a solitary lesion (Chairatchaneeboon, Surawan, & Patthamalai, 2018). Increase in incidence of cutaneous NTM infection might be due to rising number of immunocompromised patients such as malignancy, organ-transplantation, and use of immunosuppressive medications (Wentworth et al., 2013). Regarding the underlying medical conditions in our study, there were only 5 diabetic patents in our study and no other immunocompromised diseases detected in our study, which might have led to the lack of systemic involvement and disseminated infection. *2 cases of M. marinum, 1 case each of M. abscessus, M. haemophilum and M. fortuitum had underlying diabetic mellitus.*

The diagnosis of NTM infection cannot be done clinically, therefore need a range of investigations to reach a definitive diagnosis. Routinely, AFB test, histopathological examination and culture are performed when there is suspicion of NTM infection. However, AFB and histopathology results are often inaccurate. Hence, culture is currently the gold standard for definitive diagnosis of NTM infection. Moreover, drug susceptibility test in culture enhances successful antibiotic therapy for respective causative organisms. However, even rapidly growing mycobacteria take more time to grow than other types of bacteria. This leads to delay in treatment and poor outcome in patients. In recent years, molecular diagnostic methods such as PCR and gene sequencing directly from fresh tissue has been enabling clinician to obtain a fast diagnosis and start early treatment. In this study, acid-fast bacilli test was positive in only 37.1% and majority of the patients had chronic non-specific inflammation in histopathological report. This finding is different from a previous study, also carried out in Thailand, in which the most frequent histopathologic findings were mixed cell granuloma and suppurative granuloma (Chairatchaneeboon et al., 2018).

The purpose of this study is to compare the efficacy of conventional culture method with or without PCR from colony to molecular diagnosis of PCR with gene sequencing with fresh tissue or FFPE. However, due to limited number of cases, we were not able calculate the sensitivity and specificity of the molecular diagnosis of PCR from fresh tissue or FFPE compared to the gold standard culture method. The results of the gold standard cultural method and molecular method using fresh tissue or FFPE show statistically significant difference. The small number of positive cases in molecular technique despite positive culture might be a result of inadequate tissue sampling, scarcity of organism in samples sent for PCR or PCR technique itself (Primer problem, laboratory technique). In our study at Institute of Dermatology, polymerase chain reaction (PCR), gene-sequencing was done for only 16S rRNA and ITS region. Some other targets for PCR such as the rpoB gene and hsp65 gene were not identified. A study in 2017 suggested that the rates of species level identification was higher with the utility of multi-locus sequence analysis (MLSA) (Kim & Shin, 2017). Furthermore, despite 16SrRNA is typically used as a housekeeping gene for identification, it shows poor discrimination in species identification of NTM, owing to similar inter-species sequence and identical sequence homology of some NTM species. Notably, sequencing of the *rpoB* and *hsp65* genes allows for faster and more reliable speciation of NTMs, identification of less frequently isolated NTMs that are not included in commercial kits (Opperman et al., 2024).

There were 7 cases of culture-negative cases that had positive PCR result from fresh tissue or FFPE. Five cases of *M. abscessus* were positive in both culture and PCR from fresh tissue. In these 5 cases, the duration of symptoms was all within 8 weeks. The results of the gold standard cultural method and molecular method using fresh tissue or FFPE show significant difference at p value < 0.05. The molecular technique was not affected by the duration of symptoms since there were no statistical difference in the number of positive or negative mycobacterial infection when divided into 2 groups of duration ≤ 4 weeks and > 4 weeks. In addition, the morphology of the lesion did not have any correlation with the molecular result.

Moreover, 12 patients were reported to have taken antibiotics before acquiring treatment. This might have affected the results of both culture and direct PCR to produce negative yields. The use of antibiotics with a proven mycobactericidal action, such as macrolides and quinolones, can lead to negative test results. Therefore, discontinuation of antibiotic therapy for at least 15 days is recommended before investigations (Nogueira et al., 2021).

As for treatment, a combination of macrolide (azithromycin or clarithromycin) plus fluoroquinolone, doxycycline, or trimethoprim-sulfamethoxazole is often started initially. One study recommended that an initial antimycobacterial combination should ideally include IV amikacin, a quinolone, and a macrolide. Amikacin is generally found to be sensitive for most of the mycobacterial species. Moreover, M. *abscessus* is usually susceptible to macrolides, but resistant to quinolone (El Helou, Viola, Hachem, Han, & Raad, 2013).

5.2 Conclusion

From this study, we can conclude that conventional method of culture with PCR and gene-sequencing from colony is still the gold standard for diagnosis. Negative PCR results in most cases may result from inadequate tissue sampling, or problems in PCR technique, showing less sensitivity. Further researches should be targeted to introduce NTM real-time PCR with higher sensitivity to facilitate the detection rate. *M. abscessus* was the predominant causative organism accountable for most cutaneous NTM infection in Thailand, followed by M. *marinum*. Clinical and laboratory diagnostic methods are mandatory to reach definitive diagnosis of NTM infection. Currently, there is no definitive treatment for cutaneous NTM infection in Thailand. Recommended treatment should include at least 2 active antimicrobial agents for at least 4-8 weeks after clinical clearance.

5.3 Limitation

The limitation of our study is the retrospective nature which may lead to incomplete clinical data in some cases. In addition, fewer than expected cases were collected within the study period since the inclusion criteria must include both culture and/or PCR from colony and PCR from fresh tissue or FFPE. Therefore, the number of NTM infection in this study is underreported. Other limitations include single-center data collection, short study duration and probable recall bias from patients' history, due to retrospective study.

5.4 Suggestion

In order to reach the objectives of this study, further researches with longer study period and larger populations should be carried out in the future. Due to increasing number of cutaneous NTM infection, clinicians should have a high suspicion for these organisms to have better treatment outcomes. In conclusion, we suggest a prospective study should be carried out with proper sampling technique, decontamination and advanced PCR methods to calculate the sensitivity, specificity, positive and negative predictive value of direct PCR from fresh tissue.

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Appendix

Patient Record Form

Case No. ____________________________

Patient Record Form

1. Age : ____________
2. Sex : ☐ Male ☐ Female
3. Underlying disease : ☐ None
   ☐ Diabetes mellitus
   ☐ Post-organ transplantation
   ☐ Malignancy
   ☐ Acquired Immune deficiency syndrome
   ☐ Autoimmune diseases
   ☐ Others__________________________
4. History of Skin Injury:☐ None
   ☐ Surgery
   ☐ Injection
   ☐ Tattoo
   ☐ Aquatic-related injury
   ☐ Others__________________________
5. Duration of symptoms: ___________________________
6. History of previous antibiotic treatment (please specify if any): ___________
7. Acid-fast bacilli (AFB): ☐ Positive
   ☐ Negative
8. Number of Lesions : ☐ 1
   ☐ 2
   ☐ 3
   ☐ 4
   ☐ ≥5
9. Distribution: ☐ Localized
   ☐ Generalized

10. Systemic involvement: ☐ Yes
☐ No
☐ Uncertain

11. Morphology:
☐ Papule
☐ Nodule
☐ Patch
☐ Plaque
☐ Ulcer
☐ Abscess
☐ Cellulitis-like lesion
☐ Sporotrichoid lesion
☐ Others_______________________________

12. Site of lesion:
☐ Leg
☐ Forearm
☐ Trunk
☐ Face
☐ Hand
☐ Feet
☐ Genital
☐ Others________________________________

13. Histological result:
☐ Suppurative granuloma
☐ Mixed cell granuloma
☐ Tuberculoid granuloma
☐ Foreign body type granuloma
☐ Tuberculoid type granuloma
☐ Histiocytic type granuloma
☐ Sarcoidal granuloma
☐ other (specify) ________________________________

14. Cultural diagnosis:
- ☐ M. abscessus
- ☐ M. haemophilium
- ☐ M. marinum
- ☐ M. fortuitum
- ☐ Other ____________________________

15. Molecular diagnosis:
- ☐ M. abscessus
- ☐ M. haemophilium
- ☐ M. marinum
- ☐ M. fortuitum
- ☐ Other ____________________________

16. Treatment received:
- ☐ Clarithromycin
- ☐ Ciprofloxacin
- ☐ Doxycycline
- ☐ Rifampicin
- ☐ Amikacin
- ☐ Combination therapy , specify……..
- ☐ Other ____________________________

17. Outcome:
- ☐ Improved
- ☐ Cured
- ☐ Worse
- ☐ Loss to follow up
- ☐ Other ____________________________

ลงชื่อผู้บันทึกข้อมูล

....

(…………………………………..)

Date…../………………./………………

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