Molecular Diagnosis of Cutaneous Non-tuberculous Mycobacterial Infection Compared to Conventional Culture Method at Institute of Dermatology, Bangkok

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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 using 16S-23S intergenic spacer region (ITS region) and 16S rRNA using 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, regarding fresh tissue specimen, there were significantly more cases of positive culture than the molecular test when analyzed by Fisher's Exact test at $p < 0.05$. 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.

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

1. Introduction

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. 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)

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

In recent years, biomolecular identification of NTM strains by polymerase chain reaction (PCR) and gene sequencing is another method to identify pathogens (Kothavade et al., 2013). Application of novel molecular techniques has facilitated a more rapid and accurate identification of the NTM species 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.

2. Objectives

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

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.

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

3. Materials and Methods

This study was conducted as a descriptive retrospective chart review. The medical record of all cases with confirmed diagnosis of cutaneous NTM infection during the time period of (2016 - 2021) was collected from the molecular biology department of Institute of Dermatology, Thailand.

3.1 Selection Criteria

Inclusion Criteria

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

Exclusion Criteria

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

3.2 Data Collection and Data Analysis

The medical record of all cases with confirmed diagnosis of cutaneous NTM infection during the time period of 2016 - 2020, with the results of both culture 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.

For culture method, tissue sample of patients were sent for histopathological investigations including staining with hematoxylin-eosin and Ziehl-Neelsen stain. Culture was performed with blood agar, Lowenstein-Jensen agar, and BD MGIT Mycobacteria Growth Indicator Tube and incubated at 37℃ and 30℃ to favor the growth *Mycobacterium haemophilum* and *Mycobacterium marinum* species. The 'No Growth' within 2 months was 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℃ for 5 minutes followed by temperature 94℃ for 40 cycles at various temperatures for 1 minute and then at 72℃ 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 (ThermoFisher 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. Along with the conventional method, direct PCR and sequencing from tissue samples had also been performed in aforementioned cases.

In addition, 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.

Data analysis and statistics used in data analysis: Clinical and demographic characteristic of the patients are collected and analyzed by Chi-square test, Fisher Exact 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)

4. Results and Discussion

4.1 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.

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. 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%). 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 immunocompromised cases such as malignancy, post-organ transplantation, or patients with Acquired Immune Deficiency Syndrome. Others (27.5%) had underlying medical conditions such as dyslipidemia, thyroid disease and psoriasis, etc. (Table 1)

Table 1 Dermographic data of patients with confirmed diagnosis of non-tuberculous mycobacterial infection at Institute of Dermatology, Bangkok during 2016-2021

Variables	Result
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)*	

Variables	Result
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%)
Acquatic-related injury	6 (11.8%)
Other	14 (27.5%)

[*]1 case had missing data on underlying diseases.
[+]1 case had missing data on history of skin injury.

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. Papules, nodules, patches and plaques 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.

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 2)

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. (Table 3)

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. 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 3)

Table 2 Acid-fast bacilli test, histopathology, culture and PCR from colony, and molecular diagnosis with fresh tissue or FFPE

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%)

Variables	Results
Foreign body type granuloma	4 (8%)
Histiocytic granuloma	2 (4%)
Non-specific histological picture	31 (62%)
Cultural Diagnosis & PCR from colony (n=52)	
No growth	8 (15.4%)
M. abscessus	25 (48.1%)
M. marinum	17 (32.7%)
M.fortuitum	1 (1.9%)
Other	1 (1.9%)
Molecular diagnosis (PCR and sequencing from fresh tissue or FFPE) (n=52)	
Negative	36 (69.2%)
M. abscessus	9 (17.3%)
M. haemophilium	3 (5.8%)
M. marinum	4 (7.7%)

*Only 29 cases had data on AFB test.

Table 3 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 fresh tissue or FFPE)	Negative	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

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. The morphology of the lesions did not correlate with the molecular result.

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 commonly 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.

4.2 Discussion

During the last few decades, there has been an increasing trend in the NTM infection worldwide Similar to a previous research in Thailand (Chirasuthat et al., 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. 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).

NTM infection has a wide range of clinical manifestations, including papules, nodules, plaque, patch, ulcers, abscess, sporotrichoid lesions and cellulitis-like lesions 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 similar results with plaques and nodules being the predominant clinical manifestations and 50% of cases presented with a solitary lesion (Chairatchaneeboon et al., 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). However, there were no immunocompromised patients detected in our study, which might be the cause of lack of systemic involvement and disseminated infection in our study.

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, followed by mixed cell granuloma. 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)

Due to limited number of cases, we were not able to calculate the sensitivity and specificity of the molecular diagnosis of PCR from fresh tissue or FFPE compare to the gold standard culture method. However, the results of the gold standard cultural method and molecular method using fresh tissue or FFPE show statistically significant difference (44 cases versus 16 cases) when analyzed by Fisher's exact test at p-value < 0.05. 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 multilocus sequence analysis (MLSA) (Kim & Shin, 2017). 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 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. 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 quinolones, and macrolides. 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 et al., 2013).

The incidence of cutaneous NTM infection in this study might be under-reported due to our criteria requiring both culture and PCR from fresh tissue sample or FFPE. In addition, the results of our study might be limited by the retrospective nature and collection of data from a single center. Therefore, further researches with larger population with multi-center data collection and longer research period is recommended to detect the efficacy of molecular technique comparing to the gold standard culture method.

5. 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 showing less sensitivity. *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.

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