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Mycobacterium marinum infection in Taiwan

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Mycobacterium marinum often causes skin infections, tenosynovitis, arthritis, and osteomyelitis, and occasionally results in severe disseminated infections in immunocompromised patients. In this study, the clinical features of 14 cases of *M. marinum* infection were retrospectively analyzed. One patient had septic arthritis, the other 13 had skin infections and/or tenosynovitis. It usually took 2 months or longer for a definite diagnosis to be made in these patients. Three of the 14 patients were cured using clarithromycin alone or in combination with rifampin plus ethambutol. Most patients did not respond to conventional antituberculosis agents. Pulsed-field gel electrophoresis and infrequent-restriction-site polymerase chain reaction are efficient tools for the molecular typing of *M. marinum*. Both methods yielded a concordant result, and 4 of 12 isolates were genetically closely related to each other based on their banding patterns. This study indicates that these isolates were derived from the same clone. Because *M. marinum* infection is curable, early diagnosis is important. Poor healing of wounds after exposure to aquatic animals appears to be the most important clinical clue indicating the need for culture and inclusion of *M. marinum* infection in the differential diagnosis.

Key words: Infrequent-restriction-site polymerase chain reaction, *Mycobacterium marinum*, pulsed-field gel electrophoresis

Mycobacterium marinum is an atypical mycobacterium belonging to Runyon's classification group I, which is photochromogenic and slow-growing. It is well known as a cutaneous pathogen, which causes sporotrichosis-like lesions [1-3], tenosynovitis [4], bursitis [5], arthritis [6], and even osteomyelitis [6,7]. *M. marinum* may occasionally cause disseminated infections in immunocompromised hosts [8-11]. Because of its rarity, most physicians are not familiar with this infection and optimal treatment remains to be established [12,13].

To clarify the epidemiology, treatment, and prognosis of *M. marinum* infections, the medical records of 14 patients with *M. marinum* infection treated at the Chang Gung Memorial Hospital during a 1.5-year period were reviewed. Both pulsed-field gel electrophoresis (PFGE) and infrequent-restriction-site polymerase chain reaction (IRS-PCR) were used to type the isolates to explore the genetic relatedness of *M. marinum* strains in causing infections in this series of patients.

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Materials and Methods

Clinical data

From June 1999 through November 2000, a total of 14 clinical isolates of *M. marinum* were collected from the clinical microbiology laboratory of the Chang Gung Memorial Hospital, Taoyuan. The isolates were collected individually from 14 patients. The medical charts of these patients were reviewed retrospectively. Data on the following clinical characteristics were collected: demographic data, site of lesions, underlying diseases, initial impression, results of biopsy, treatment, and outcome. Outcome was defined as cure (no signs of infection), improvement (diminution or disappearance of lesions without follow-up visits), failure (progression of infections or no improvement during treatment), or lost to follow-up (less than 1 month of treatment and without further outpatient clinic follow-up).

Bacterial strains

Twelve isolates were available for further molecular epidemiological study and were identified according to standard methods [14]. The criteria included growth rate, colony morphology, pigmentation, niacin test, nitrate

reduction test, semiquantitative catalase test, tween 80 hydrolysis test, arylsulfatase test, urease test, and pyrazinamidase test. A specific polymerase chain reaction-restriction fragment length polymorphism method was used to further differentiate *M. marinum* from other mycobacterial species [15]. The isolates were frozen at –70°C in skim milk with 50% glycerol until use.

Pulsed-field gel electrophoresis

The procedure of PFGE was performed according to previously published protocols with some modification [9,16]. Bacterial colonies grown on 7H11 agar plates (Difco Laboratories, Detroit, MI, US) for 2 weeks were harvested in 1 mL of 10 mM Tris-1 mM ethylenedinitrilotetraacetic acid disodium (EDTA) (10 mM Tris, Merck, Darmstadt, Germany; 1 mM EDTA, Ferak, West Berlin, Germany). Before DNA extraction, ethambutol, a cell-wall-active agent, was added to previous bacterial suspensions (final concentration was 1 μg/mL, a concentration expected to be subinhibitory) and they were incubated at 30°C for 16 h. Cell suspensions were mixed with 1.6% agarose and cast into plugs. The plugs were treated with lysis solution (1 M NaCl, 100 mM Na_2EDTA [pH 7.5], 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, and lysozyme 5 mg/mL; Roche, Mannheim, Germany) overnight at 37°C. Lysis solution was replaced with ESP reagent (0.5 M EDTA, 1% sodium lauroyl sarcosine, proteinase K 1 mg/mL; Merck) overnight at 55°C. The plugs were then washed once in sterile distilled water at 55°C for 30 min and 4 times in 10 mM Tris-0.1 mM EDTA buffer at 55°C for 30 min each time. The genomic DNA contained in the plugs was digested overnight with 40 U of *Xba*I (Biolabs, Beverly, MA, US) in 1 x NE buffer 2 (Biolabs) at 37°C. The plugs were washed in 10 mM Tris-0.1 mM EDTA buffer at 37°C for 1 h and loaded into a 1% agarose gel prepared and run in TBE buffer (0.0445 M Tris, 0.0445 M boric acid, 0.001 M EDTA; Amresco, Solon, OH, US) at 14°C. A plug containing lambda DNA ladder (Bio-Rad Laboratories, Hercules, CA, US) was stored at 14°C. An auto-algorithm mode was chosen with the running molecular weights ranging from 20 kb to 380 kb. The gel was stained with ethidium bromide and photographed under UV illumination before it is ready for analysis.

Infrequent-restriction-site polymerase chain reaction

Infrequent-restriction-site polymerase chain reaction method was used to perform molecular typing [17,18]. Oligonucleotides, namely AH1, AH2, AX1, AX2, and PX-G, were purchased from Quality Systems, (Taipei, Taiwan. Bacterial colonies grown on 7H11 agar plates at 30°C for 2 weeks were harvested and washed in 1 mL of sterile distilled water. Cells were digested for 2 h with 80 μL (1 mg/mL) of proteinase K (Worthington, Biochemical Corporation, Lakewood, NJ, US) at 56°C, followed by sonication for 40 min. The crude DNA was further digested by *Xba*I and *Hha*I (Biolabs, Beverly, MA, US), and the subsequent amplifications were performed as previously described [17,18]. The PCR products (5 μL) were loaded into wells of a 12.5% polyacrylamide gel. A DNA ladder (Bayou Biolabs, Harahan, LA, US) in 20 base-pair increments was incorporated as the molecular weight marker. After electrophoresis for 50 min at 600 V, the gel was stained with silver stain and photographed. The gels, staining solutions, and electrophoresis-related instruments were purchased from Pharmacia Biotech (Uppsala, Sweden). To ensure the reproducibility of the method, each isolate was examined at least twice.

Interpretation of banding patterns

The criteria suggested by Tenover *et al* [19] were employed to analyze the fingerprints generated by PFGE. Strains with banding patterns that differed by 3 or fewer bands were considered closely related. There are currently no standardized criteria for analyzing IRS-PCR patterns because the method was newly proposed in 1996. In this study, the guidelines described above for PFGE were employed to analyze the IRS-PCR patterns.

Results

Infection by *M. marinum* was diagnosed in 14 patients in the Chang Gung Memorial Hospital between June 1999 and November 2000. The clinical characteristics of these patients are summarized in Table 1. Their ages ranged from 9 to 72 years (mean, 49 ± 20.9 years). There were 8 males and 6 females. The most common clinical manifestations were poorly healing wounds, pus discharge, and/or subcutaneous nodules (Fig. 1). The upper extremities were involved in 93% (13/14) of these patients. One patient had septic arthritis of the right knee. None of the patients had disseminated infections. Fifty percent of the patients had contact with either fish or shrimp. Two patients had a history of local steroid injection. One had a history of trauma.

The mean time from onset of symptoms to confirmatory diagnosis was around 2 months. These infections were often initially misdiagnosed clinically as sporotrichosis, fungal infection, nocardiosis, tuberculosis, or bacterial cellulitis. Pus discharges from all patients were sent for Ziehl-Neelsen and rhodamine-

Table 1. Clinical manifestations of 14 patients with *M. marinum* infection

Case no.	Age	Sex	Infection source	Site of lesions	Underlying disease	Initial impression	Histopathology	Treatment	Outcome
1	11	M	Fish tank	Right index finger	None	Sporotrichosis	Granulomatous inflammation	INH RIF EMB	Improved
2	9	M	Shrimp sting	Right forearm	None	Sporotrichosis	Suppurative granulomatous inflammation	CLR EMB	Improved
3	72	F	Trauma	Right hand	DM Right forearm Colle's fracture	Right hand cellulitis	Necrotizing granulomatous inflammation	INH RIF EMB PZA	Failure
4	69	F	Fish tank	Right wrist	None	Fungal infection	Granulomatous inflammation	DOX CLR RIF CIP	Improved
5	50	M	Fish bone	Left hand	Gout	Extrapulmonary tuberculosis	Suppurative granulomatous inflammation	CLR	Cured
6	25	M	Local steroid injection	Right hand	None	Extrapulmonary tuberculosis	ND	INH RIF EMB PZA	Lost to follow-up
7	51	M	Fish tank	Left hand	IHD DM HTN	Tuberculous tenosynovitis	Granulomatous inflammation	CLR RIF EMB	Cured
8	69	F	Fish tank	Left little finger	CRF DM HTN	Extrapulmonary tuberculosis	ND	INH RIF EMB CLR	Cured
9	39	M	ND	Right hand	None	Atypical mycobacterial infection	Suppurative granulomatous inflammation	DOX	Improved
10	65	M	Fish tank	Right hand	None	Sporotrichosis	Suppurative granulomatous inflammation	DOX RIF	Lost to follow-up
11	57	F	Local steroid injection	Right forearm	DM HTN Chronic hepatitis	Sporotrichosis or atypical mycobacterial infection	Necrotizing granulomatous inflammation	DOX SXT	Improved
12	62	M	ND	Right knee	Pneumoconiosis	Septic arthritis	ND	INH RIF EMB	Failure
13	42	F	ND	Left forearm	RA Osteoporosis HTN	Atypical mycobacterial infection	Granulomatous inflammation	INH RIF EMB CLR CIP	Improved
14	59	F	ND	Left finger	SLE DM HTN Asthma	Nocardiosis	ND	INH RIF EMB PZA	Lost to follow-up

Abbreviations: INH = isoniazid; RIF = rifampin; EMB = ethambutol; CLR = clarithromycin; DM = diabetes mellitus; PZA = pyrazinamide; DOX = doxycycline; CIP = ciprofloxacin; ND = not documented; IHD = ischemic heart disease; HTN = hypertension; CRF = chronic renal failure; SXT = sulfamethoxazole-trimethoprim; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus

auramine stain and mycobacterial culture with Löwenstein-Jensen medium and 7H11 agar plate. Nine biopsy specimens were collected for histological examination. Initially, 64% (9/14) of the clinical specimens were positive for acid-fast stains. Histopathological examination revealed non-caseating

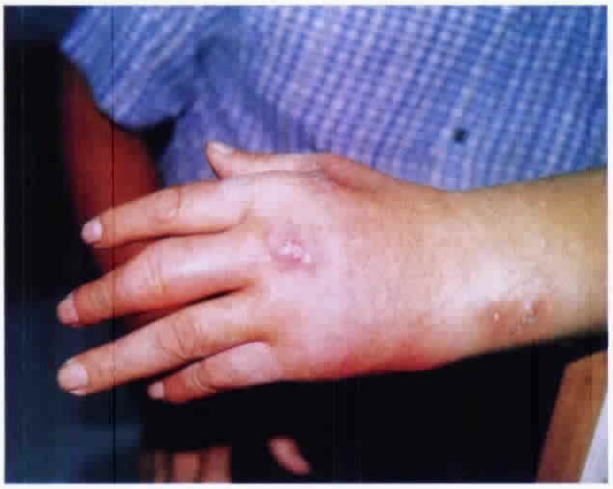

Fig. 1. Tenosynovitis and multiple non-healing wounds with some purulent discharge in a patient with *M. marinum* infection.

granulomatous inflammation in all patients. Five patients were treated with conventional antituberculosis agents, including isoniazid, rifampin, ethambutol, and pyrazinamide. Two patients did not respond to these agents, 2 were lost to follow-up, and one improved after therapy. Another 9 were treated with ciprofloxacin, clarithromycin, doxycycline, or sulfamethoxazole-trimethoprim with or without conventional antituberculosis agents. Three of the 9 patients were cured, 5 showed clinical improvement, and one was lost to follow-up.

The DNA fingerprints of the 12 clinical isolates are shown in Fig. 2. The 2 molecular typing methods yielded concordant results. A total of 9 DNA fingerprints were obtained. Four isolates revealed genetically closely related patterns, whereas diverse patterns were yielded in the remaining 8 isolates (Fig. 2).

Discussion

In this study, 13 of the 14 patients with *M. marinum* infection had skin infections, manifested as ulcerated and poorly healing wounds with or without subcutaneous nodules. Sporotrichosis was misdiagnosed in 4 of these patients. Adams *et al* [2] and Hernandez-Martin *et al* [3] described *M. marinum* infections that resembled sporotrichosis in their lymphocutaneous nodules,. This study found that most (93%) of *M. marinum* infections involved the upper extremities, a finding compatible with a previous report [20]. These microorganisms grow best in Lowenstein-Jensen media at 30°C, which may explain why they are limited to the superficial and cooler parts of the body [2]. It took as long as 2 months before correct bacteriologic diagnosis was made in this study. This delay may have been partly due to the maintenance of the incubator at 37°C to cultivate *Mycobacterium* spp.. In view of the long time needed to grow this microorganism using conventional culture systems, PCR method can be used to facilitate rapid diagnosis of *M. marinum* infections [21,22].

Five of the 14 patients in this study were treated with conventional antituberculosis agents, including isoniazid, rifampin, ethambutol, and pyrazinamide. Most of these patients did not have a favorable response to this therapy. Although there is no established treatment of choice for *M. marinum* infection, isoniazid and pyrazinamide are generally not suggested because *M. marinum* always produces pyrazinamidase and is intrinsically resistant to isoniazid [5,14]. The other 9 patients were treated with ciprofloxacin, clarithromycin, and doxycycline alone or in combination with antituberculosis agents. Satisfactory results were achieved in this patient group by this treatment. Although routine susceptibility testing of *M. marinum* is not recommended [23], rifampin and rifabutin have been shown to be the most effective drugs against *M.*

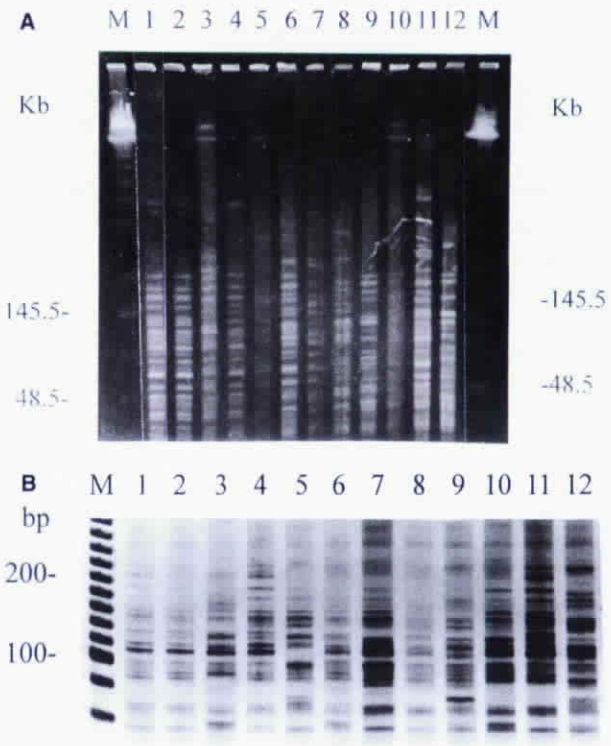

Fig. 2 (A) Fingerprints of *M. marinum* isolates typed by PFGE; lane M is the lambda marker. **(B)** Fingerprints of *M. marinum* isolates typed by IRS-PCR; lane M is the 20-bp DNA ladder. Lanes 1 to 12 are clinical isolates of *M. marinum*. Lanes 1, 2, 4, and 11 are 4 strains with similar banding patterns. The alphabetic order of the isolates corresponds to that in Table 1.

marinum in vitro [24]. Rifampin and ethambutol were suggested by Donta *et al* [13] to be the treatment of choice for *M. marinum* infection, with doxycycline, minocycline, clarithromycin, or sulfamethoxazole-trimethoprim as alternatives [12,13]. Our experience suggests that a minimum of 3 months' therapy with clarithromycin and/or rifampin plus ethambutol is needed. However, because of the small sample size of this study and its retrospective nature, the optimal regimen remains to be determined by other prospective studies.

There are no data regarding the molecular epidemiology of *M. marinum* infections. Holmes *et al* [9] used PFGE to define a case of recurrent, disseminated *M. marinum* infection caused by the same genotypically defined strain. This study is the first to explore the molecular epidemiology of *M. marinum* by using both PFGE and IRS-PCR. In this study, 4 isolates were genetically closely related in their banding patterns, suggesting that one major clone of *M. marinum* is responsible for the majority of infections in Taiwan.

M. marinum infection is a problem in both recognition and therapy. Delayed diagnosis was common in most of the patients in this study, suggesting that most physicians are not familiar with this disease. The importance of early diagnosis of *M. marinum* infection should be stressed because the disease is curable with appropriate antimicrobial agents. Our results suggest that *M. marinum* infection should be included in the differential diagnosis in all patients with poorly-healing wounds after exposure to aquatic animals.

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