Molecular epidemiology of *Mycobacterium abscessus* infections in a subtropical chronic ventilatory setting

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The aim of this study was to investigate the high level of pulmonary *Mycobacterium abscessus* infections and implement a surveillance programme among 43 ventilator-dependent patients, 15 with pulmonary *M. abscessus* infections, in a hospital long-term respiratory care ward (RCW) in central Taiwan. *M. abscessus* isolates were obtained from 35 patients in the RCW of hospital A, 6 patients in the RCWs of another three hospitals (B, C and D), and from 4 water sources in two of the hospitals (A and B). Strains were characterized by methods including *hsp65* PCR–RFLP and PFGE. The patients were followed-up by chest X-ray for 1 year. All clinical isolates were type I and II, and belonged to ten distinct clusters of PFGE patterns. Five clinical strains in two hospitals belonged to a single cluster, whilst four clinical strains in the other two hospitals belonged to a single unique cluster. The strains from hospital A fell into nine clusters and were distinct from the strains isolated from the water supply. Patients infected with type I strains showed a significantly more rapid progression of disease. The number of different strains involved suggested either that there had been a polyclonal outbreak or that a high level of endemic infections was present in the RCW of hospital A. This and the lack of homology between the clinical and environmental isolates from hospital A raised the possibility that pulmonary *M. abscessus* infections may have been spread by the movement of patients between RCWs, a routine practice in Taiwan's integrated delivery system.

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INTRODUCTION

Reports of nosocomial outbreaks of non-tuberculous mycobacteria (NTM), many of which have been linked to contaminated hospital water systems (Wallace *et al.*, 1998), have been increasing (Phillips & von Reyn, 2001). *Mycobacterium abscessus* is a rapidly growing NTM species that can cause a variety of conditions ranging from infection of surgical wounds to chronic pulmonary disease (Fox *et al.*, 2004; Koh *et al.*, 2002; Phillips & von Reyn, 2001; Scholze *et al.*, 2005). Although patients with chronic lung diseases are known to be more susceptible to infection by *M. abscessus*, few outbreaks have been reported among patients with chronic ventilator dependency in respiratory care wards (RCWs).

Abbreviations: AFB, acid-fast bacilli; ATS, American Thoracic Society; ICU, intensive care unit; IDS, integrated delivery system; NTM, non-tuberculous mycobacteria; RCC, respiratory care centre; RCW, respiratory care ward.

In March 2004, a patient in the RCW of hospital A developed a productive cough and a persistent fever. Sputum specimens from this patient and the two patients in adjacent beds yielded *M. abscessus*. To determine the extent and source of these infections, and establish a surveillance programme, we performed molecular typing of clinical isolates from the 38 patients in the ward, from 5 patients who were admitted to the ward after the beginning of the study, and from patients of 3 other hospitals within our integrated delivery system (IDS) that also had apparent outbreaks. We also analysed specimens of the tap water and from a drinking water machine from two of the hospitals.

METHODS

Patients and clinical features. All 38 patients in the long-term RCW of hospital A who had been admitted before 15 May 2004 (initial patients) and 5 patients who were admitted from May 2004 to

March 2005 (newly admitted patients) were included in this study. Patients were considered to have *M. abscessus* pulmonary disease if they met the American Thoracic Society (ATS) criteria for NTM pulmonary disease (Griffith *et al.*, 2007). Briefly, the patients had to be symptomatic, have abnormalities visible on chest radiography or high-resolution computed tomography, and fulfil at least one of the following three microbiological criteria: positive culture results for *M. abscessus* from at least two sputum specimens; positive culture results for *M. abscessus* from at least one bronchial wash or lavage specimen; or acid-fast bacilli (AFB) or granulomas and positive cultures for *M. abscessus* from lung histopathological specimens or at least one sputum sample or bronchial washing that was culture positive for *M. abscessus*.

Demographic and clinical information, such as age, sex, clinical symptoms/signs, chest radiographs, and the presence of co-morbidities, were collected from the patients' medical records. None of the initial and newly admitted patients had open wounds or surgical sites. During the 12 month follow-up period after May 2004, chest radiography was performed every 3 months, the films were examined and any deaths were recorded. Serial changes in the chest radiographs were graded as 'no change', 'improved' or 'progressed'. The study was reviewed and approved by the institutional review board of Taichung Veterans General Hospital.

Clinical mycobacterial isolates. Three or four transbronchial aspirates were collected from each of the 38 initial patients and the 5 newly admitted patients in the RCW of hospital A. Acid-fast staining and mycobacterial culturing were performed on each of the aspirate specimens as follows. Transbronchial aspirates were digested using a standard *N*-acetyl-L-cysteine/NaOH technique (Thornton *et al.*, 1998). The digests were streaked onto Lowenstein-Jensen (L-J) slants, incubated at 37 °C with 5 % CO₂ and inoculated into BACTEC MGIT tubes (BD Diagnostic Systems) containing medium selective for mycobacteria; the slants were incubated for up to 2 months. The bacterial suspensions that grew from any MGIT tubes were streaked onto L-J slants. The colonies that grew on the L-J slants were collected and further examined with an AccuProbe test (Gen-Probe) specific for *Mycobacterium tuberculosis* (Sloutsky *et al.*, 2004) as well as with standard biochemical methods for identifying NTM (Witebsky & Kruzak-Filipov, 1996).

In addition, because three RCWs in hospitals B, C and D in central Taiwan reported having nosocomial mycobacterial outbreaks between 2004 and 2005, two AFB-positive clinical isolates were also obtained from each of these RCWs and examined.

Environmental mycobacterial isolates. At the time of collection of the transbronchial aspirates from the enrolled patients, three repeated isolations of environmental mycobacterial specimens were performed. Isolation of mycobacteria from each of two sources of tap water and the one drinking water machine in the RCWs in hospitals A and B was performed according to the method of Falkinham *et al.* (2001). Briefly, 300 ml water samples were centrifuged (5000 *g* for 20 min at 25 °C). The pelleted cells were suspended in 1 ml sterile distilled water, and 0.1 ml samples were spread on the surface of Middlebrook 7H10 agar medium (BBL Microbiology Systems) containing 0.5 % (v/v) glycerol and 10 % oleic acid/albumin enrichment. Sodium thiosulfate was used to neutralize free disinfectant in the tap water, cetylpyridinium chloride was used to kill non-mycobacterial microorganisms and the specimens were plated on Middlebrook 7H10 agar plates. The plates were incubated at 37 °C with 5 % CO₂ for 2 months. Middlebrook 7H10 agar plates were placed beside the patients for detection of organisms in the room. Swabs for culture were also taken from the ventilator and other inanimate surfaces. Mycobacterial colonies were identified by AFB staining, standard biochemical methods and sequencing of the 16S rRNA gene (Goldblatt & Ribes, 2002; Kirschner *et al.*, 1992).

Heat-shock protein 65 encoding gene (*hsp65*) PCR-RFLP analysis. Mycobacterial strains were classified into subtypes on the basis of the restriction patterns of the *Telenti* fragment (441 bp) from the gene encoding the 65 kDa heat-shock protein (Hsp65) during analysis by RFLP (Brunello *et al.*, 2001). Mycobacterial genomic DNA was extracted using the cetyltrimethyl ammonium bromide method recommended by Ollar & Connell (1999). The PCR was performed with primer pairs Tb11 (5'-ACCAACGATGGTGTGCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCT-3') according to the method of Telenti *et al.* (1993) under the following conditions: 1 cycle at 95 °C for 5 min; 35 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. The PCR products were analysed by 2 % agarose gel electrophoresis.

For restriction fragment analysis, 20 µl PCR product was digested with *Bst*EII or *Hae*III. The reaction mixture was analysed by 6 % PAGE and visualized by ethidium bromide staining under UV irradiation (Gaafar *et al.*, 2003; Taillard *et al.*, 2003; Zhang *et al.*, 2004a). A DNA ladder (20 bp marker; Bertec) was used as a size marker. Subtype assignments were made on the basis of polymorphisms in the length of the PCR restriction fragments according to the PRASITE Identification of Mycobacteria website (<http://app.chuv.ch/prasite/index.html>).

PFGE. Analysis of large restriction fragment profiles by PFGE was performed by a modification of the methods of Wallace *et al.* (1993) and Sperner *et al.* (1999). Briefly, isolates of *M. abscessus* were cultured for 3–5 days at 37 °C in L-J medium. The bacteria were collected and resuspended in SEM buffer (75 mM NaCl, 25 mM EDTA) and the bacterial concentration was adjusted to an OD₆₀₀ of 1.6. Cell suspensions were mixed in a 1:1 ratio with 1 % SeaKem Gold agarose (Cambrex) and pipetted into disposable plug moulds (Bio-Rad). The plugs were lysed with 20 µl lysozyme (20 mg ml⁻¹; Sigma-Aldrich) and 20 µl lysostaphin (500 µg ml⁻¹; Sigma-Aldrich) and incubated at 37 °C for 2 h. The lysis solution was replaced with 2 ml cell lysis buffer [50 mM Tris/HCl (pH 8.0), 50 mM EDTA, 1 % sarcosine] and 20 µl proteinase K (20 mg ml⁻¹; Sigma) and incubated at 56 °C for 2 h with shaking (160 r.p.m.). The plugs were washed twice with ddH₂O in a shaking water bath at 56 °C for 15 min each and then four times with Tris (10 mM)/EDTA (1 mM) buffer (pH 8) for 15 min each.

The plugs containing the genomic DNA were placed into 200 µl solution containing 20 U *Dra*I (New England Biolabs) and incubated at 37 °C for 4 h, washed with 200 µl Tris (10 mM)/EDTA (1 mM) buffer (pH 8) and embedded in 1 % SeaKem Gold agarose gel. PFGE was carried out on a CHEF DRIII apparatus (Bio-Rad) at 14 °C for 20 h in 0.5 × TBE buffer [1 M Tris/HCl (pH 8), 0.9 M boric acid, 10 mM Na₂ EDTA] with or without 100 µM thiourea (Zhang *et al.*, 2004b) at 200 V. The pulse time was ramped from 20 to 40 s for 14 h and from 150 to 180 s for 6 h over a total run time of 20 h. The gel photographs were then scanned and analysed using Bionumerics software version 3.3 (Bio-Image Systems). Genomic DNA from *Salmonella enterica* serovar Braenderup H9812 (Chang *et al.*, 2005) and *Staphylococcus aureus* NCTC 8325 (Singh *et al.*, 1999) were used as DNA size standards. DNA bands were visualized by ethidium bromide staining under UV light and photographed.

PFGE pattern analysis. PFGE patterns were analysed by computerized band analysis with Bionumerics software version 3.3 and classified on the basis of the criteria of Luna *et al.* (2000). When a pattern differed from the others by four or more bands, the pattern was designated a main type or cluster and given a name with the first letter of the restriction enzyme and an assigned number (e.g. D1–D10 for *Dra*I). When there were three or fewer band differences, the PFGE patterns were designated subtypes and given a subscripted number (e.g. D1₁, D1₂). The subscript 1 indicated one band added to the pattern, whereas a subscript followed by a letter in parentheses [e.g.

D1_{1(A)}] denoted the addition of one different band to the pattern. Likewise, the subscript 2 (e.g. D1₂) indicated that one band was deleted from the pattern; the subscript 3 (e.g. D1₃) indicated that two bands were different and the subscript 4 (e.g. D1₄) indicated that three bands were different from the main pattern. Isolates that had identical or related (with three or fewer band differences) *Dra*I patterns during PFGE were considered to be genetically related, whereas isolates with four or more different bands were considered to be genetically unrelated. To avoid confusion, the patterns from environmental mycobacterial isolates were named with the letter E and an assigned number (e.g. E1) and the standard *M. abscessus* ATCC 19977 strain was classified as S1.

Cluster analysis was carried out and a dendrogram was constructed using the UPGMA. A band position tolerance of 2% was used to compare fingerprint patterns.

Statistical analysis. Means $\pm$ SD or frequency (%) were calculated for each subtype and analysed. Wilcoxon's rank-sum test or Fisher's exact test were used to compare the co-morbidities and clinical outcomes of the initial patients from whom *M. abscessus* type I was isolated with those from whom *M. abscessus* type II was isolated. Data were analysed with SAS 9.0 (SAS Institute). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Enrolled patients and clinical isolates

From May 2004 to March 2005, the medical records of the 43 patients in the 39-bed RCW of hospital A were reviewed. All patients were dependent on ventilators, and had chronic respiratory symptoms and abnormalities observed on chest radiographs. Among the 38 initial patients enrolled before 15 May 2004, 23 had AFB in smears and mycobacterial growth in cultures from at least three transbronchial aspirates, whilst 5 had AFB in smears and mycobacterial growth in cultures from two transbronchial aspirates (Table 1). Cultures of all of these specimens grew organisms that were identified as *M. abscessus*. Fifteen of the twenty-eight initial patients who had AFB in their smear samples and mycobacterial growth in cultures from at least two transbronchial aspirates had disease progression observed by serial chest radiography and were symptomatic. Therefore,

there were 15 initial patients who met the ATS criteria for *M. abscessus* pulmonary disease. Among the remaining ten patients, one had AFB and mycobacterial growth from the culture of one transbronchial aspirate. Another initial patient had negative smear results for AFB in three transbronchial aspirates but had mycobacterial growth from the culture of one transbronchial aspirate. The cultured specimens from these two patients were also identified as *M. abscessus*. The first clinical isolate obtained from culture of the transbronchial aspirate specimens from each of these 30 patients was collected for further study; thus, a total of 30 independent clinical isolates was obtained. Transbronchial aspirate specimens from the remaining eight patients grew no mycobacteria (Table 1).

Among the five newly admitted patients in the RCW of hospital A (admitted between 15 May 2004 and March 2005), all had AFB in the smear and mycobacterial growth in the culture from one transbronchial aspirate. The cultured specimens from these five newly admitted patients were also identified as *M. abscessus* (Table 1). Two clinical isolates obtained from each of hospitals B, C and D were also identified as *M. abscessus* (data not shown).

Epidemiology assessment

The epidemiological curve describing the infections in hospital A is shown in Fig. 1. Most patients had four specimens sent for AFB testing and culture. A total of 14 patients had at least one specimen positive by AFB stain and mycobacterial culture in May 2004, giving a prevalence of *M. abscessus* infection of 36.8% (14/38). Between May and September 2004, 2 of the initial patients died, 2 more patients were admitted and a total of 12 more patients with at least 1 specimen positive for AFB and mycobacterial culture were found as of September 2004. Thus, there was a prevalence of 63.2% (24/38). Between September 2004 and March 2005, seven of the initial patients died, three more patients were admitted and nine more patients with at least one specimen positive by AFB stain and mycobacterial culture were found in March 2005, giving a prevalence of 76.5% (26/34).

Table 1. AFB staining and mycobacterial culture of transbronchial aspirates from 43 patients in hospital A

AFB stain and mycobacterial culture results*	No. of patients
Four specimens positive by AFB stain and mycobacterial culture	16
Three specimens positive by AFB stain and mycobacterial culture	7
Two specimens positive by AFB stain and mycobacterial culture	5
One specimen positive by AFB stain and mycobacterial culture	1 + 5†
No specimens positive by AFB stain and one specimen positive by culture	1
No specimens positive by AFB stain and no specimens positive by culture	8

*All organisms isolated from positive mycobacterial cultures were identified as *M. abscessus*.

†Patients admitted after May 2004 (newly admitted patients).

Fig. 1. Epidemiological curve describing the outbreak of *M. abscessus* in hospital A. The prevalence among the patients is shown for May 2004 (initiation of study), September 2004 (midway into the study) and March 2005 (end of study). Grey bars, four specimens positive; white bars, three specimens positive; hatched bars, two specimens positive; black bars, one specimen positive; ▲, prevalence.

Isolation of mycobacteria from environmental water sources (environmental isolates)

One of the two tap water samples collected from the RCW of hospital A grew mycobacterial colonies on Middlebrook 7H10 plates. The colonies were examined, and two strains were observed that were identified as *Mycobacterium mucogenicum* and *Mycobacterium senegalense*. The other water sample produced no growth of mycobacteria. Two tap water samples from the RCW of hospital B were collected, and *M. abscessus* was identified in both samples. No mycobacterial colony growth was found in the specimens from the drinking water machines, room ventilation or inanimate surfaces in the RCWs of hospitals A and B.

hsp65 PCR-RFLP analysis

A total of 39 isolates from the 4 hospitals were processed. In hospital A, two clinical strains isolated from the initial patients were not available for *hsp65* PCR-RFLP analysis (they had been lost). A total of 6 of the clinical isolates from the 28 initial patients (21.4%) had the *M. abscessus* type I restriction pattern showing the 441 bp Telenti fragment derived from the *hsp65* gene sequence. A total of 22 of the clinical isolates from the 28 initial patients (78.6%) were shown to have the *M. abscessus* type II restriction pattern. Among the clinical isolates from the five newly admitted patients, one (20%) was found to be type I and the other four (80%) were type II. Type II was the predominant type in hospital A. The two clinical strains isolated in hospital B were identified as *M. abscessus* type I; each of the two clinical strains in hospitals C and D were *M. abscessus* type II. Of the two environmental strains isolated in hospital B, one was further differentiated as type I and the other as type II (data for isolates from hospitals B, C and D not shown).

PFGE analysis

The clinical isolate from one initial patient was lost during PFGE pattern analysis. PFGE profiles were analysed for a total of 38 clinical strains and 4 environmental strains isolated from the 4 RCWs. PFGE after *DraI* digestion of chromosomal DNA revealed 10–16 fragments ranging from 54 to 664 kb, and 10 distinct cluster patterns among the clinical strains from hospitals A, B, C and D were identified (Fig. 2). The clinical strains isolated from hospital A could be grouped into nine different PFGE clusters. The largest cluster, D1 (a total of 14 isolates), contained six indistinguishable clinical strains, four with one band difference [D1₁ and D1_{1(A)}] and four with three band differences (D1₄). MRG1, the isolate from the first patient who presented with symptoms during the study, and MRG4, the isolate from a patient in the adjacent bed, were in the D1 cluster. The cluster D2 contained seven clinical isolates, including one unique clinical strain (D2) and six strains with two band differences [D2₃ and D2_{3(A)}]. MRG2, the isolate from the other patient adjacent to the first patient, was also in the D2 cluster. The D7 cluster contained five identical clinical isolates, including two isolates (MRG24 and MRG26) from initial patients and one isolate (MRG52) from a newly admitted patient. Two clinical strains from hospital B (CHIN-CHEN1 and CHIN-CHEN2) belonged to the D7 cluster and were identical to those in hospital A. Two clinical strains in hospital C, designated D10, were different from those seen in hospitals A and B but indistinguishable from the two clinical strains in hospital D. In hospital A, one environmental strain belonged to the E1 cluster; and the other environmental strain belonged to the E2 cluster. In hospital B, one environmental strain (CHIN-CHEN20) belonged to the D7 cluster; and the other environmental strain (CHIN-CHEN15) belonged to the D1 cluster (Fig. 2).

Of six clinical isolates from the four hospitals that were identified as *M. abscessus* type I (strain: MRG3, MRG17, MRG24, MRG26, MRG27 and CHIN-CHEN1), three (MRG24, MRG26 and CHIN-CHEN1) produced smear patterns when PFGE was performed without thiourea; these had clear PFGE profiles after thiourea was added to the running buffer, and were indistinguishable and belonged to the D7 cluster (data not shown).

Clinical isolates, *hsp65* RFLP types and PFGE patterns in hospital A

The largest clusters of D1 and D2 were identified as *M. abscessus* type II. The D4₃, D6, D7, D5 and D8 clusters were found to be *M. abscessus* type I. The clinical isolates of the D1 cluster were distributed widely throughout the RCW, whereas the clinical isolates of the D2 cluster were frequently isolated from one of the three areas in the RCW. Among the strains isolated from the five newly admitted patients, three (MRG51, MRG72 and MRG74) showed D1 patterns (similar to those in the initial patients with strains belonging to the D1 cluster), one (MRG52)

Fig. 2. PFGE patterns of *M. abscessus* digested with *DnaI*. Isolates corresponding to strains D1–D10 were obtained from patient specimens. Isolates corresponding to strains E1 and E2 were obtained from the water supplies of two of the hospitals. The isolate corresponding to strain S1 was *M. abscessus* ATCC 19977.

belonged to the D7 cluster and one (MRG75) belonged to the D9 cluster.

Clinical outcomes after 1 year

The characteristics and outcomes of the 28 initial patients who had AFB in smears and mycobacterial growth in cultures from at least two transbronchial aspirates were analysed according to whether they were infected with *M. abscessus* type I or type II, as determined by the *hsp65* PCR–RFLP results (Table 2). Only 15 patients with disease progression as determined by chest radiography met the

ATS criteria for *M. abscessus* pulmonary disease. Five patients with type I and six with type II received at least 1 month of a drug regimen that included macrolides. There was no statistically significant difference in age, sex or co-morbidities between the patients infected with the two types. The rate of mortality appeared to be higher in the patients infected with *M. abscessus* type I, although this difference did not reach statistical significance. However, the proportion whose disease progressed, as determined by chest radiography, was significantly higher in the patients infected with *M. abscessus* type I ($P=0.0441$; Table 2).

Table 2. Demographics and clinical outcomes of initial patients in hospital A during 12 months of follow-up

Data are presented as means $\pm$ SD or frequency (%) and analysed using Wilcoxon's rank-sum test or Fisher's exact test.

Variable	<i>M. abscessus</i> subtype		<i>P</i> value
	I (<i>n</i> =6)	II (<i>n</i> =22)	
Age (years)	82.17 $\pm$ 7.14	74.59 $\pm$ 11.85	0.0987
Male sex	2 (33.33 %)	13 (59.09 %)	0.3720
Co-morbidity			
Diabetes mellitus	1 (16.67 %)	10 (45.45 %)	0.3547
Renal disease	0 (0.00 %)	1 (4.55 %)	1.0000
Lung disease	4 (66.67 %)	16 (72.73 %)	1.0000
Malignancy	1 (16.67 %)	1 (4.55 %)	0.3889
CNS disorder	6 (100.00 %)	20 (90.91 %)	1.0000
Cardiac disease	4 (66.67 %)	12 (54.55 %)	0.6730
Peptic ulcer	4 (66.67 %)	5 (22.73 %)	0.0638
Surviving	2 (33.33 %)	17 (77.27 %)	0.0638
Serial CXR after 12 months			0.0441*
Progressed	6 (100.00 %)	9 (40.91 %)	
Improved	0 (0.00 %)	4 (18.18 %)	
No change	0 (0.00 %)	9 (40.91 %)	

CNS, Central nervous system; CXR, chest radiography.

*Statistically significant ($P < 0.05$).

DISCUSSION

A patient in the RCW of hospital A developed a productive cough and a persistent fever in March 2004. From 1 May to 15 May 2004, we screened the 38 patients in the ward, and 15 of them, including the symptomatic patient and the 2 patients in adjacent beds, had *M. abscessus* pulmonary infections. Fifteen more of these initial patients and five newly admitted patients (who were admitted after 15 May) had specimens that were positive for *M. abscessus*, although they did not fulfil the ATS criteria for infection. Furthermore, in September 2004, a mycobacterial outbreak was reported in hospital B, whilst outbreaks were reported in hospitals C and D in March 2005.

M. abscessus is a rapidly growing NTM species. Nosocomial transmission of this organism can result in a variety of conditions, ranging from surgical wound infections after facial and breast plastic surgery or cardiac surgery to invasive infections such as chronic pulmonary disease (Fox *et al.*, 2004; Koh *et al.*, 2002; Phillips & von Reyn, 2001; Scholze *et al.*, 2005). Since the mid-1970s, the frequency of reported outbreaks in healthcare settings caused by this species has increased (Phillips & von Reyn, 2001). However, *M. abscessus* outbreaks have rarely been reported in patients who are chronically dependent on ventilators.

Because *M. abscessus* is widely distributed in the environment and frequently recovered from water, soil, food and animals, contamination of clinical specimens can occur, resulting in a so-called pseudo-outbreak. Therefore, the diagnosis of NTM lung disease is only made in cases

that conform to strict diagnostic criteria including positive results produced in multiple specimens (Griffith *et al.*, 2007). The cases of 15 of the patients described in this report fulfilled these criteria; therefore, we concluded that true infections were present among these patients in hospital A.

We attempted to identify the sources of the infections by molecular typing of the clinical and environmental isolates. At least one report has described the colonization of patients' respiratory tracts resulting from NTM in the hospital's water supply (Burns *et al.*, 1991), and *M. abscessus* is commonly found in hospital water supplies (Wallace *et al.*, 1998). Therefore, we tested two water sources in the RCWs along with the clinical isolates. The results of analysis by PCR-RFLP showed that all of the clinical isolates belonged to types I (from hospitals A and B) and II (from hospitals A, C and D) as determined by the restriction pattern of the 441 bp *Tel* fragment of the *hsp65* gene sequence (Devallois *et al.*, 1997). PFGE after restriction with *Dra*I revealed that the clinical isolates comprised ten clusters. Nine of these clusters (D1–D9) were found in hospital A, one of the nine found in hospital A was also found in hospital B (D7), and the tenth (D10) was found only in hospitals C and D. In hospital B, both environmental isolates were *M. abscessus*, and at least one environmental isolate (CHIN-CHEN20) belonged to the same cluster as the two clinical specimens (CHIN-CHEN1 and CHIN-CHEN2). The tap water sources, therefore, seemed to be the source of transmission in hospital B. However, in hospital A, both environmental strains

belonged to different species of mycobacteria (clusters E1 and E2) that did not appear in any of the clinical isolates or any isolates from the other hospitals. This result and the observed genetic diversity of the isolates from the patients in hospital A indicated that contamination from the tap water sources was not responsible for the positive clinical specimens and suggested that the patients became infected through contact with multiple other environmental sources or other patients. No instruments were used routinely among the patients in the RCW that could be identified as probable sources of transmission. No mycobacterial colony was found in specimens from drinking water machines, room ventilation or inanimate surfaces. None of the initial patients or newly admitted patients had wounds or surgical sites. Therefore, we speculated that contact with other patients was the probable source of transmission.

As noted above, *M. abscessus* outbreaks have rarely been reported in patients who are chronically dependent on ventilators. Therefore, our findings that most of the patients in the RCW of hospital A were infected and that the clinical isolates belonged to a variety of unrelated clusters were contrary to our expectation that the source of the infections would be environmental and that a single clone would be isolated from a only few patients. These findings raise two important questions: first, whether all of the patients had clinically significant pulmonary disease caused by *M. abscessus*, as opposed to airway colonization; and secondly, whether there was a high level of polyclonal yet 'silent' endemic infections over a period of time, of which we were unaware. The ATS states that not enough is known about the pathophysiology of NTM lung disease to be sure that colonization is not, in fact, an indolent or slowly progressive infection (Griffith *et al.*, 2007). Symptoms were present in the patient whose case caused us to initiate the surveillance programme, and 15 of the patients showed signs of disease progression on chest radiography. Therefore, we assumed that, at least among the 15 patients whose diagnoses were confirmed according to the ATS criteria, clinically significant disease was present. We cannot prove whether or not the infections observed in this study represented a polyclonal outbreak or a high level of endemic infections with different clones; however, the number of distinct types and clusters of *M. abscessus* suggests that the latter may have been the case.

We noticed that the isolates from the two patients from hospital B belonged to the D7 cluster, which also contained three clinical strains isolated from patients in hospital A. Furthermore, the isolates from hospitals C and D were identical. This suggested the possibility that these strains could have been acquired by patients who moved back and forth between the RCWs of these hospitals. In Taiwan, RCWs are long-term chronic care facilities that have been developed for managing patients with chronic ventilator dependency. The IDS that was initiated by the Bureau of National Health Insurance in Taiwan (Lin & Zhao, 2002) offers clinical integration of such patients within three types of step-down units: the intensive care unit (ICU), the

respiratory care centre (RCC), a step-down unit of the ICU, and the RCW, a step-down unit of the RCC. These units are integrated both vertically and horizontally (Fig. 3). Therefore, stable patients in one hospital's RCW might be transported to another hospital's RCW (horizontal integration). Similarly, if the clinical condition of a patient in an RCW deteriorates, the patient could be transferred to the ICU of the same or a different hospital (vertical integration). If the patient's condition improves, the patient could be transferred back to the RCC and RCW. Unfortunately, this system could facilitate the spread of nosocomial mycobacterial infections between hospitals if diagnosis is delayed, as is often the case with mycobacterial infection.

We found that the rate of disease progression, as determined by chest radiography, was significantly higher in patients with *M. abscessus* type I isolates than in patients infected with *M. abscessus* type II. Furthermore, there was a slightly higher rate of mortality among these patients. This could indicate that type I is a more virulent form. Molecular typing of clinical isolates from *Mycobacterium kansasii* has shown differences in the pathogenicity among several subtypes (Taillard *et al.*, 2003). However, the clinical significance of the two types of *M. abscessus* found in the clinical isolates from our investigation is unclear; additional studies will be needed to confirm these preliminary results.

Several limitations of this study should be noted. We were not able to undertake sequencing of multiple loci within the isolates. Therefore, we could not differentiate *M. abscessus* from *Mycobacterium massiliense* and *Mycobacterium bolletii* using the methods reported recently by Zelazny *et al.* (2009). The size of the cohort of patients in this study was small, and we do not have data to confirm which of the patients in this study were indeed moved between wards or hospitals during the study period. A case-control study is currently underway to investigate further whether the movement of patients among wards and hospitals within the IDS is a factor in the spread of *M. abscessus* infections.

Fig. 3. Outline of the IDS for patients with chronic ventilator dependency in Taiwan. This system offers clinical integration of such patients within three types of step-down unit: the ICU, the RCC and the RCW. These units are both vertically and horizontally integrated according to the patient's clinical condition.

Conclusions

Fifteen of thirty-eight patients in a long-term RCW for patients with chronic ventilator dependency were found to have pulmonary infections with *M. abscessus* in a single hospital in 2004. Molecular typing of clinical isolates from these and five additional patients who entered the ward after the start of the study revealed that the isolates belonged to nine clusters and comprised types I and II. Clinical and environmental isolates from one other hospital that had an outbreak were similar to several of the isolates from hospital A, and isolates from two other hospitals were similar to each other. Further studies are needed to determine whether these infections may have been acquired by patients who moved back and forth among these hospitals, a routine practice in Taiwan's IDS for patients with chronic ventilatory dependency.

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