Amplified fragment length polymorphism analysis of *Mycobacterium avium* complex isolates recovered from southern California

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Fine-scale genotyping methods are necessary in order to identify possible sources of human exposure to opportunistic pathogens belonging to the *Mycobacterium avium* complex (MAC). In this study, amplified fragment length polymorphism (AFLP) analysis was evaluated for fingerprinting 159 patient and environmental MAC isolates from southern California. AFLP analysis accurately identified strains belonging to *M. avium* and *Mycobacterium intracellulare* and differentiated between strains within each species. The method was also able to differentiate strains that were presumed to be genetically identical in two previous studies using large RFLP analysis with PFGE, or PCR-amplification of DNA segments located between insertion sequences IS1245 and IS1311. For *M. avium*, drinking-water isolates clustered more closely with each other than with patient or food isolates. Patient isolates were more genetically diverse. None of the environmental isolates shared identical AFLP patterns with patient isolates for either species. There were, however, environmental isolates that shared identical patterns, and patient isolates that shared identical patterns. A subset of the isolates, which are referred to as MX isolates due to their ambiguous identification with the Gen-Probe system, produced AFLP patterns similar to those obtained from *M. intracellulare* isolates. Sequence analysis of 16S rDNA obtained from the MX isolates suggests that they are strains of *M. intracellulare* that were not correctly identified by the *M. intracellulare* AccuProbe from Gen-Probe.

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INTRODUCTION

Mycobacterium avium and *Mycobacterium intracellulare* are slow-growing species of mycobacteria that are ubiquitous in the environment, phylogenetically closely related, and clinically significant. Together they, and possibly other species of mycobacteria, comprise the *M. avium* complex (MAC). *M. avium* and *M. intracellulare* are the most frequently isolated mycobacterial species in clinical laboratories in the United States, causing a wide range of

opportunistic infections (Evans *et al.*, 1998; Horsburgh *et al.*, 1991; Wallace *et al.*, 1998; Woods *et al.*, 2003). There is recent evidence to suggest that the rate of MAC infections in immunocompetent individuals is on the rise in the United States (Cappelluti *et al.*, 2003; Iseman, 1995; Lumb *et al.*, 2004; Myojo *et al.*, 2003; Prince *et al.*, 1989; Wallace, 1994).

Wolinsky (1979) suggested that person-to-person transmission of MAC infections was likely rare and the majority of infections are environmentally acquired. Numerous studies have attempted to establish the link between MAC infection and source by typing patient, animal and environmental isolates. Drinking water has long been suspected as a source and has been intensively studied (Aronson *et al.*, 1999; Covert *et al.*, 1999; Horsburgh *et al.*, 1991; Tobin-D'Angelo *et al.*, 2004; von Reyn *et al.*, 1994, 2002). Raw vegetables routinely washed with water, as well as soils, have been screened as possible sources of MAC exposure (Bauer *et al.*, 1999; Dvorska *et al.*, 2002; Yaiko

Abbreviations: AFLP, amplified fragment length polymorphism; IWGMT, International Working Group on Mycobacterial Taxonomy; LRF, large RFLP; MAC, *Mycobacterium avium* complex; RFU, relative fluorescent unit.

The GenBank/EMBL/DBJ accession nos for the 16S rDNA sequences of the MX isolates are AY648863–AY648870 and AY652954–AY652961.

Tables of strains and peak data are available as supplementary material with the online version of this paper.

et al., 1995; Yoder *et al.*, 1999; De Groote *et al.*, 2006). Several studies have also implicated animals as sources of MAC infection by discovering similar strains in humans and animals (Motiwala *et al.*, 2003; Biet *et al.*, 2005; Johansen *et al.*, 2007). However, inadequate sampling, recovery and typing methods continue to be impediments in our efforts to elucidate the source of MAC infections. Though several fingerprinting methods exist, many are labour-intensive, costly, or not reproducible or sensitive for typing MAC at the subspecies level (Aronson *et al.*, 1999; Cangelosi *et al.*, 2004; Gaafar *et al.*, 2003; Roiz *et al.*, 1995; Smole *et al.*, 2002; Yoder *et al.*, 1999; Garriga *et al.*, 2000).

In this study we report the use of amplified fragment length polymorphism (AFLP) analysis for typing patient and environmental MAC isolates to determine possible sources of human exposure. AFLP is a random amplified polymorphic DNA technique in which genomic DNA is digested with restriction enzymes and fragments are randomly amplified to generate a pattern of bands. For a detailed description of AFLP, the reader is encouraged to consult publications by Blears *et al.* (1998), Jones *et al.* (2005) and Vos *et al.* (1995). AFLP is highly sensitive for discriminating between closely related strains of bacteria (Arnold *et al.*, 1999; Burke *et al.*, 2004; Janssen & Dijkshoorn, 1996; Keim *et al.*, 1997; Lindstedt *et al.*, 2000; Moreno *et al.*, 2002; O'Shea *et al.*, 2004; Valsangiacomo *et al.*, 1995; Picardeau *et al.*, 1997). We were interested in determining if AFLP could differentiate strains within and between species of MAC, and if the sensitivity, speed and reproducibility surpass that of other typing methods. The strains included MAC isolates from AIDS and non-AIDS patients, as well as from drinking-water and food sources recovered from southern California. A portion of the isolates were typed previously using large RFLP (LRF) analysis with PFGE or PCR amplification of the region between insertion sequences IS1245 and IS1311 (Aronson *et al.*, 1999; Yoder *et al.*, 1999), and were included to compare the discriminatory power of the methods. Fourteen isolates referred to as MX (due to their ambiguous identification with the Gen-Probe system) were included to determine if they are strains of *M. avium* or *M. intracellulare*, or if they constitute a separate, monophyletic group within MAC.

METHODS

Bacterial strains and culture conditions. MAC isolates were provided by Olive View – UCLA Medical Center. One hundred and sixty-three isolates were obtained from patient and environmental sources, and identified as *M. avium*, *M. intracellulare* or MX using AccuProbe (Gen-Probe) for MAC, or SNAP (Syngene) as described previously (Aronson *et al.*, 1999; Yoder *et al.*, 1999). The isolates are described in Table 1 and additional information is given in Supplementary Table S1 available with the online journal. Isolates were grown for 4 weeks in Middlebrook 7H9 broth with ADC enrichment (Difco Laboratories) at 37 °C with 10% CO₂, prior to DNA extraction.

DNA purification. DNA was prepared from each of the isolates using a freeze-thaw technique (Reischl *et al.*, 1994) to lyse cells, and the Wizard genomic DNA purification kit (Promega) to purify DNA. Cell

Table 1. Number and source of *M. avium*, *M. intracellulare* and MX isolates

AccuProbe ID	No. of isolates from each source				Total
	Patient*	Water†	Food‡	Unknown	
<i>M. avium</i>	70	22	12	0	104
<i>M. intracellulare</i>	20	19	0	2	41
MX§	1	8	0	5	14

*AIDS patients and non-AIDS patients with no prior history of immunodeficiency.

†Home, hospital, commercial buildings, reservoirs.

‡Unprocessed and processed vegetables.

§MX isolates produced a positive reaction with the general MAC AccuProbe, and a negative reaction with the *M. avium* and *M. intracellulare* species-specific probes.

pellets were resuspended in 600 µl nuclei lysis buffer supplied with the Wizard kit. The cell suspension was subjected to liquid nitrogen for 3 min, followed by boiling for 1 min 15 s for five cycles. DNA was purified from the lysate following the Wizard genomic DNA purification kit protocol for Gram-positive bacteria beginning with the RNase treatment step. DNA was quantified using the PicoGreen dsDNA quantification kit (Molecular Probes).

AFLP analysis. Typically the AFLP technique employs *EcoRI* and *MseI* restriction enzymes for digestion of genomic DNA prior to PCR amplification. In our study, we chose the restriction enzymes *HhaI* and *PstI* because the recognition sequence of each enzyme has a high G + C content, similar to the 62 to 70 mol% G + C range found in the *Mycobacterium* genome. We also used a single set of primers to amplify fragments within the pool of digested DNA prior to electrophoresis. The forward primer contained a single selective adenosine nucleotide at the 3' end, and the reverse primer contained adenosine and guanine nucleotides at the 5' end to give an even distribution of peaks over a 50 to 500 bp range.

A *PstI* double-stranded oligonucleotide adaptor was prepared by mixing equal volumes of *PstA1* (5'-CTC GTA GAC TGC GTA CAT GCA-3') and *PstA2* (5'-TGT ACG CAG TCT AC-3') single-stranded oligonucleotides at a final concentration of 10 pmol µl⁻¹ in a single microfuge tube, heating to 95 °C for 3 min, and cooling gradually to room temperature. The *HhaI* double-stranded adaptor was prepared in the same manner, by mixing *HhaA1* (5'-ATC AGG ACT CAT CG-3') and *HhaA2* (5'-GAC GAT GAG TCC TGA TCG-3') at a final concentration of 100 pmol µl⁻¹. An adaptor mix was prepared by mixing equal volumes of the annealed *PstI* and *HhaI* adaptors together into a single tube with final concentrations of 5 and 50 pmol µl⁻¹, respectively. Purified DNA was digested with the restriction enzymes *PstI* and *HhaI* (New England BioLabs), and adaptors ligated simultaneously in a digestion/ligation reaction containing 1.5 µl 10 × T4 DNA ligase buffer with 1 mM ATP, 1.5 µl 0.5 M NaCl, 0.75 µl (1 mg ml⁻¹) BSA stock solution, 5 U *PstI*, 5 U *HhaI*, 0.2 µl T4 DNA ligase (New England BioLabs), 3.0 µl adaptor mix, 200 ng template DNA, and dH₂O in a final volume of 15 µl. Reactions were incubated at room temperature overnight, diluted to 150 µl with 135 µl dH₂O, and stored at -20 °C.

A subpopulation of restriction fragments was amplified from each digestion/ligation reaction using the following single set of primers: *PstA* (5'-GAC TGC GTA CAT GCA GA-3') containing a NED dye label (Applied Biosystems) on the 5' end and *HhaAG* (5'-GAT GAG TCC TGA TCG CAG-3'). PCR was performed using GeneAmp PCR

core reagents (Applied Biosystems), in a reaction containing 1 × PCR buffer II, 2 mM MgCl₂, 200 μM each dNTP, 15 ng primer PstA, 30 ng primer HhaAG, 5 μl diluted digestion/ligation reaction, 0.5 U AmpliTaq DNA polymerase and dH₂O in a total volume of 20 μl. PCR was performed in a Perkin Elmer thermal cycler 480 with the following conditions: 1 cycle of 94 °C for 2 min 30 s, 66 °C for 30 s, 72 °C for 2 min; for the next 9 cycles, the annealing temperature decreased by one degree; 20 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min; and 1 cycle of 72 °C for 10 min. PCR products were prepared for analysis on an Applied Biosystems 310 Genetic Analyzer by diluting the product 1 : 10 in dH₂O, and adding 1 μl of the dilution to 15 μl Hi-Di formamide (Applied Biosystems) containing 0.1 μl GeneScan 500 ROX size standard. Before loading, the samples were denatured for 5 min at 95 °C and placed on ice. Electrophoresis was performed according to the PE Applied Biosystems AFLP microbial fingerprinting protocol: revision B (part number 402977B).

Reproducibility experiments. Two different experiments were performed to verify the reproducibility of AFLP patterns generated from MAC isolates. Four MAC strains (CW16, CW32, W289, H08FC8N) were grown in triplicate in Middlebrook 7H9 broth with ADC enrichment and analysed independently. In addition, duplicate dilutions of PCR products were prepared and analysed for all 159 strains.

Data analysis. Peak data were analysed in the form of electropherograms and tables. Tables were constructed using Genotyper version 2.5 software (Applied Biosystems). The threshold for assigning a peak was set at 250 relative fluorescent units (RFU). Peaks that preceded or followed a larger peak within 1 bp were excluded from the analysis. Peak data were converted to presence/absence data (1/0), from which a distance matrix was calculated using the coefficient of Nei & Li (1979) with PAUP* version 4.0b software (Swofford, 2002). Peak data are shown in Supplementary Tables S2 and S3 available with the online journal. Neighbour-joining and maximum-parsimony analyses were performed using PAUP* to generate trees. A single base change can cause a restriction fragment to disappear and two smaller fragments to appear, and vice versa. Therefore, statistical methods, such as bootstrapping, were not applied to trees generated from AFLP data as it cannot be assumed that the restriction fragments evolved independently (Felsenstein, 2004). Support for tree topology was interpreted from the congruence of trees generated from the two methods of analysis.

16S sequencing and phylogenetic analysis. To identify the MX strains, the gene coding for the small subunit of the ribosome (16S rDNA) was PCR amplified using universal bacterial primers. Sequencing reactions were performed using BigDye Chemistry version 3.1 (Applied Biosystems) and sequences were obtained on an MJ BaseStation51 automated sequencer (MJ Research). Manual sequence alignment was performed with the BioEdit sequence alignment editor [version 6.0.7 by Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>)] on the basis of conserved features of primary and secondary structures using sequences from closely related species obtained from GenBank. Phylogenetic analysis was carried out using maximum-likelihood, neighbour-joining, and maximum-parsimony methods as implemented by PAUP*. *Nocardia abscessus* was used as the outgroup.

RESULTS

Reproducibility of AFLP patterns

Selective amplification with PstA and HhaAG primers produced a mean of 27 bands per isolate, ranging from 78

to 208 bp. Ninety-nine loci were used in the analyses. Reproducibility of patterns was tested by growing four isolates in triplicate and analysing the replicates independently. The fingerprint patterns were 100 % reproducible for three of the four isolates (W289, CW32, H08FC8N). However, a single peak was lacking in the fingerprint generated from one of three replicate cultures for isolate CW16. The peak, which was 85 bp, was in the original data for all three replicates, but was below the peak-calling threshold of 250 RFU for one replicate (230 RFU) and above 250 RFU for the other two replicates (261 and 281 RFU). That is, the questionable peak was of low intensity in all three replicates. Because the peak represented a polymorphic locus, it was retained in the analysis.

In the second reproducibility experiment, replicate dilutions of the same PCR product were analysed, and fingerprint patterns for replicate samples were identical for 146 of the 159 isolates (92 %). One to two peaks were missing in the replicate dilution of 13 strains (8 %); therefore, a third replicate dilution of the PCR product was analysed. The presence of the peak(s) was confirmed in all cases. All of the ambiguous peaks were near the peak-calling threshold of 250 RFU.

AFLP analysis of *M. avium* isolates

A total of 104 *M. avium* isolates were analysed by AFLP, including 70 patient isolates, 22 drinking-water isolates and 12 food isolates. Neighbour-joining and maximum-parsimony analyses of AFLP data yielded trees in general agreement. The neighbour-joining tree is presented in Fig. 1. Of 104 *M. avium* isolates analysed, 93 isolates (89 %) generated unique AFLP patterns. Eleven isolates (11 %) produced an AFLP pattern identical to one or more *M. avium* strains in the study, and are described in Table 2. Isolates W200 and W203 produced identical fingerprints and were isolated from different patients staying in the same hospital in 1991. Isolates W126 and W127 had identical fingerprints, and were obtained from the stool and blood of the same patient, suggesting a disseminated infection. Two pairs of isolates, W123 and W124, as well as W348 and W349, were acquired from different body fluids from two different patients and yielded distinct fingerprints, suggesting polyclonal infections. None of the patient isolates shared identical AFLP patterns with either food or drinking-water isolates.

The *M. avium* isolates cluster into three main groups, as indicated in Fig. 1. The greatest amount of diversity was observed in the patient and food isolates, which are represented in each of the three major clusters. In contrast, the least amount of diversity was seen in the drinking-water isolates, which are only found in cluster 2. An exception is isolate D10CN4W, which was isolated from the same hot kitchen tap water as isolate D10CC5, yet had a unique fingerprint relative to all other drinking-water isolates.

Fig. 1. Neighbour-joining tree generated from *M. avium* AFLP data. Colours specify the source of isolates. Bar, genetic distance based on the similarity coefficient of Nei & Li (1979).

AFLP analysis of *M. intracellulare* and MX isolates

A total of 41 *M. intracellulare* isolates were analysed by AFLP, including 20 patient isolates, 19 drinking-water

Table 2. Isolates sharing identical AFLP patterns

AccuProbe ID		
<i>M. avium</i>	<i>M. intracellulare</i>	MX*
W200, W203	H15CC1, R12N2	HO3BN4, HO3AN5ST
W126, W127	—	C23BO8A, HO4EN1
W292, D55AC8N	—	—
HO5DN5, HO4AC5, D41EC8N	—	—
F7A, F72B	—	—

*MX isolates produced a positive reaction with the general MAC AccuProbe, and a negative reaction with the *M. avium* and *M. intracellulare* species-specific probes.

isolates and 2 isolates from unknown sources. In addition, 14 MX isolates were analysed by AFLP, including 1 patient isolate, 8 drinking-water isolates and 5 isolates from unknown sources. There were no *M. intracellulare* or MX isolated from food. Fig. 2 depicts the neighbour-joining tree generated from AFLP data for *M. intracellulare*, MX and 10 randomly chosen *M. avium* isolates. Interestingly, all of the MX isolates in this study cluster with *M. intracellulare* isolates, which is distinct from the cluster containing *M. avium* isolates. A closer inspection of the peak data, see Supplementary Tables S2 and S3 available with the online journal, reveals that 8 peaks (8%) are unique to the *M. avium* isolates and 9 peaks (9%) are unique to the *M. intracellulare*/MX isolates. These unique peaks provide the basis for the inter-species separation by AFLP. Therefore, the AFLP identification of the isolates to species level is concordant with the AccuProbe identification of the *M. avium* and *M. intracellulare* isolates. Neighbour-joining analysis also reveals that *M. intracellulare*/MX drinking-water isolates are closely related, patient isolates are closely related, and there is little overlap between the two sources.

Fig. 2. Neighbour-joining tree illustrating the relationship of *M. intracellulare* and MX isolates to *M. avium* isolates. Colours specify the source of isolates. Bar, genetic distance based on the similarity coefficient of Nei & Li (1979). MA, *M. avium*; MI, *M. intracellulare*.

Three groups of *M. intracellulare*/MX drinking-water isolates produced identical AFLP patterns and are described in Table 2. The shared fingerprints of isolates C23B08A and H04EN1, and H15CC1 and R12N2, is surprising in that these pairs of isolates were obtained from different drinking-water sources 2 or more years apart. The other pair sharing a pattern, H03BN4 and H03AN5ST, was obtained from tap water in a patient's hospital room. Isolate H03AN5ST was obtained from the cold-water tap and H03BN4 from the hot-water tap, and the isolates differed morphologically. Three isolates, CW678A, CW678C and CW678D, were obtained from the same fluid sample of a patient yet had distinct fingerprint patterns and indicate a polyclonal infection. As was observed with AFLP analysis of *M. avium* isolates, none of the patient isolates shared identical AFLP patterns with drinking-water isolates.

16S sequence analysis

A sequence alignment containing 16S rDNA data obtained for the MX isolates, in addition to sequences representing rapid- and slow-growing species of mycobacteria obtained

from GenBank, was generated and analysed using neighbour-joining, maximum-parsimony and maximum-likelihood methods. The three methods of analysis produced congruent trees. Fig. 3 depicts the maximum-likelihood tree. The MX isolates are distributed in the *M. intracellulare* node as they were with AFLP data. Therefore, the 16S sequence data are in agreement with the AFLP data, which suggests the MX isolates are strains of *M. intracellulare*. In addition, isolates W85 and W71 were identified as *M. avium* with the *M. avium*-specific AccuProbe but have 16S sequences identical to *M. intracellulare* sequences obtained from GenBank. Sequence data, in addition to AFLP data, support the hypothesis that W85 and W71 are also strains of *M. intracellulare*.

DISCUSSION

In this study, AFLP analysis was evaluated for typing MAC organisms. The method is rapid, taking 2 days to complete once DNA is obtained from a pure culture. The automated electrophoresis of samples allows for high throughput. In contrast, the standard LRF-PFGE method takes approximately 8 days to complete and is labour-intensive by comparison. The AFLP method is highly reproducible. The incorporation of an internal standard in every sample allowed for accurate fragment size determination and the ability to compare patterns obtained from different electrophoresis runs. Moreover, using the peak-calling criteria described in Methods, ambiguous bands in replicate fingerprints were rare (1–2 % of bands in replicate runs generated from approximately 10 % of the isolates), and were probably the result of dilution error when preparing samples for analysis. The threshold for assigning peaks can be adjusted to maximize the number of bands and improve reproducibility.

The AFLP method is flexible. For this study, we used two restriction enzymes and a single primer set. However, other restriction enzymes and primer sets can be included to reveal additional polymorphisms, thereby increasing the discriminatory power of the method. Many modifications to the basic AFLP technique have been described and used to type mycobacteria at various taxonomic levels, including species belonging to the *Mycobacterium tuberculosis* complex, *Mycobacterium marinum*, *Mycobacterium kansasii*, *Mycobacterium ulcerans* and *M. avium* subsp. *paratuberculosis*. Huys *et al.* (2000) were able to differentiate *M. ulcerans* from *M. tuberculosis* complex isolates with one primer set, but could not resolve strains belonging to *M. tuberculosis* and *Mycobacterium bovis* with the addition of a second primer set. Chemlal *et al.* (2001) used the same AFLP technique to differentiate strains of *M. marinum* and *M. ulcerans*. AFLP correctly resolved the two species and revealed subgroups within each species. van den Braak *et al.* (2004) expanded the capabilities of AFLP to include three enzyme pairs and eight selective primers to differentiate isolates of *M. tuberculosis* complex. With this strategy, the isolates were correctly identified to species-level, and

Fig. 3. Maximum-likelihood tree generated from analysis of *Mycobacterium* 16S rDNA sequence data. *N. abscessus* is the outgroup. Bar, 1 nt substitution per site.

several unique polymorphisms were identified in *M. tuberculosis* for use in future epidemiological investigations. O'Shea *et al.* (2004) used an AFLP technique incorporating 96 different primer combinations to examine the genomes of 20 *M. avium* subsp. *paratuberculosis* isolates from animals. They discovered extensive genetic polymorphisms in a small number of strains belonging to a relatively monomorphic subspecies. Conversely, Gaafar *et al.* (2003) were able to differentiate strains belonging to one of five subspecies of *M. kansasii* with a simplified AFLP technique using a single enzyme, single adaptor and single primer. This reductionist's approach to AFLP revealed a high level of heterogeneity among a subspecies that was believed to be clonal.

We found AFLP to be sensitive for differentiating within and between species of MAC organisms. The MAC isolates in this study were genetically diverse since few possessed identical patterns. For *M. avium*, the drinking-water isolates were less diverse, while patient and food isolates were more diverse. It has been suggested that harsh decontamination of environmental samples to reduce background organisms may select for a subset of resistant organisms (Aronson *et al.*, 1999). However, lack of diversity was not seen in the food isolates, which underwent the same decontamination process as water isolates. It may be that

a limited number of *M. avium* genotypes are capable of colonizing drinking-water distribution systems. An alternative hypothesis is that patient and food isolates are more genetically diverse due to the fact that people and food come from diverse geographical locations, while organisms colonizing a drinking-water distribution system are more geographically localized. Improved sampling methods, in addition to improved methods for recovering mycobacteria from environmental samples, are critically needed in order to test these hypotheses. The cluster analysis presented in Fig. 1 also reveals that patient isolates comprise the majority of cluster 1 and all of cluster 3, suggesting that specific *M. avium* genotypes may be associated with human infection. Whether these clusters are real or an artefact of the analysis is not known. Additional genotypic or phenotypic data would be helpful in determining the significance of the clusters, as would the inclusion of isolates from geographically diverse locations.

Some of the isolates in our study were typed previously using LRF-PFGE (Aronson *et al.*, 1999). In that study, patient isolates W33, CW15 and CW16, and isolates HO4AC5 and HO5DN5 obtained from hospital drinking-water were identical, suggesting that hospital water was a

source of infection. AFLP analysis was able to differentiate the patient isolates from each other and from the drinking-water isolates, though the drinking-water isolates remained indistinguishable. Moreover, many isolates in the previous LRF-PFGE study could not be typed due to insufficient growth or inability to lyse cells embedded in agarose. We were able to type every isolate in the current study with AFLP.

The PCR-amplification of regions between insertion sequences has also been used to type MAC isolates (Yoder *et al.*, 1999). Insertion sequence PCR was unable to differentiate 16 isolates that were genetically distinct by AFLP analysis, suggesting that insertion sequence PCR is less sensitive. However, the single set of primers we used was unable to differentiate two sets of strains (strains W200 and W203, and W126 and W127), which were distinguishable by insertion sequence PCR. Incorporation of other primer sets into the AFLP analysis might reveal unique polymorphisms in these strains that were not tested. While insertion sequence PCR is rapid and easy to perform, the stability of mycobacterial insertion sequences over time remains poorly characterized, rendering the method potentially unreliable for typing purposes (Keller *et al.*, 2002).

We know little about the rate of genomic evolution of MAC in the environment or human host, and therefore, the stability of the polymorphic loci detected by AFLP. If some loci are unstable over time, strains recently diverged from a common ancestor could appear dissimilar with this method. For instance, *M. avium* patient isolates W123 and W124 were obtained from different body fluids of the same patient, but generated different AFLP patterns. However, the isolates remain close neighbours on the tree in Fig. 1. The different patterns obtained from the isolates may be due to instability in a portion of the loci detected by the primers we utilized. Determining the stability of polymorphic loci over time in strains isolated from humans and the environment may allow us to develop reliable criteria, similar to the 'Tenover criteria', for strain typing of MAC with AFLP (Tenover *et al.*, 1995).

Improved fingerprinting methods may reveal potential sources of infection, and may also suggest how pathogens are related. Because the AFLP data revealed a strong relationship between MX and *M. intracellulare* strains, the small subunit of the ribosome was sequenced to determine if the MX strains are strains of *M. intracellulare* that were not detected with the *M. intracellulare* AccuProbe. Data obtained from phylogenetic analysis of 16S rDNA sequences support the hypothesis that the MX strains in this study are strains of *M. intracellulare*. The International Working Group on Mycobacterial Taxonomy (IWGMT) also identified four MX isolates as strains of *M. intracellulare* using 16S sequence data (Wayne *et al.*, 1996). Our MX strains contain 1 or 2 bp substitutions in the region of the *M. intracellulare* AccuProbe, which may account for the inability of the probe to hybridize to these

strains. While other species of mycobacteria may be misidentified as MX, it is likely that a portion of strains that receive this classification are actually *M. intracellulare*.

To our knowledge, this is the first evaluation of the AFLP technique for differentiating MAC isolates. AFLP revealed that intra- and inter-species differences could be identified using a single primer set with high reproducibility. This study also demonstrated that the identification of MX isolates by AFLP is concordant with phylogenetic analysis of 16S rDNA sequences. Furthermore, AFLP was able to differentiate patient and environmental isolates that were presumed identical using other typing methods. A robust typing method with strain-level resolution is necessary when comparing patient and environmental isolates in order to identify the source of infection and associate a human health risk to that source, especially when the rate of genomic evolution of the organism is not known. Methods with low resolution could lead the investigator to overestimate risk. The AFLP technique has been shown in this study to have excellent discriminatory power, and should be considered for identification and epidemiological investigations of MAC.

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