Genetic Diversity among *Mycobacterium avium* Complex Strains Recovered from Children with and without Human Immunodeficiency Virus Infection

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The genetic diversity and molecular epidemiology of *Mycobacterium avium* complex (MAC) infections in children with and without human immunodeficiency virus (HIV) infection were evaluated. Isolates recovered from 136 children were subtyped by sequence analysis of a 360-bp region of the gene (*hsp65*) encoding a 65-kDa heat-shock protein. Twenty-one distinct *hsp65* alleles were identified. On the basis of *hsp65* genotype, 6 isolates were not MAC organisms. Of the remaining 130 samples, 61% were *M. avium*, 37% were *Mycobacterium intracellulare*, and 2% were species nonspecific MAC. Eighty-eight percent of the isolates obtained from HIV-infected children were *M. avium*. In contrast, only 38% of the isolates obtained from children without HIV infection were *M. avium* (*χ² test, P < .001*). *M. avium* isolates were further subtyped by Southern blot analysis with insertion element IS1245. Taken together, no evidence for a single clonal *M. avium* strain causing infection was detected.

The *Mycobacterium avium* complex (MAC) is composed of *M. avium*, *Mycobacterium intracellulare*, and strains not formally assigned to either species [1, 2]. Bacteria of this complex cause cervical lymphadenitis in children, pulmonary infections in patients with chronic respiratory disease [1, 3, 4], and disseminated disease and death in AIDS patients [5, 6]. Although it is well known that 25%–50% of adults with AIDS in the United States are infected with MAC [7–9], it is not widely appreciated that 10% of children with AIDS also are infected with MAC [10–12]. As defined by hybridization and species-specific nucleotide probes, *M. avium* are responsible for 80%–95% of disseminated MAC disease in adult AIDS patients, whereas *M. intracellulare* cause ~40% of MAC pulmonary infections in immunocompetent patients [5, 13]. The genetic diversity of MAC isolates infecting pediatric patients has not been investigated. Restriction fragment length polymorphism (RFLP) analysis identified species-specific allelic variation in the gene (*hsp65*) encoding a 65-kDa heat-shock protein expressed by mycobacteria [14, 15]. Recent studies have shown that automated DNA sequence analysis of a 360-bp region of the *hsp65* gene is a rapid and unambiguous method for species and subspecies differentiation of MAC isolates [16–18]. Twenty-five *hsp65* alleles have been reported from MAC isolates [16–18], and >50 *hsp65* alleles have been identified from isolates of other *Mycobacterium* species [16, 17, 19]. To improve our understanding of the molecular epidemiology and genetic diversity of pediatric MAC infections, we characterized *hsp65* allelic polymorphism in 136 MAC isolates infecting human immunodeficiency virus (HIV)–negative and –positive children living in various regions of the United States. Representative isolates determined to be *M. avium* were further subtyped by Southern blot hybridization analysis with the species-specific insertion element, IS1245 [20, 21].

**Materials And Methods**

**Bacterial Isolates**

A sample of 136 isolates of MAC cultured from children in 19 states and the District of Columbia was studied. All isolates were identified as MAC by the referring laboratories with conventional...
diagnostic laboratory techniques or mycobacteria culture identification DNA probe analysis (Accu- Probe; Gen-Probe, San Diego). Twenty-six of the isolates were obtained from a collection held by R. Wallace, Jr. (University of Texas Health Center at Tyler), 4 from a collection held by C. Incheder (University of California at Los Angeles), and 31 from a collection held by E. Wolinsky (MetroHealth Medical Center, Cleveland); the remainder were collected from seven pediatric AIDS centers around the United States.

**Automated DNA Sequencing of hsp65**

**Isolation of DNA.** Isolates were cultured on slants of Lowenstein-Jensen or Middlebrook 7H10 solid media for 2–4 weeks. All of the following procedures were performed in a biosafety cabinet in a biosafety level 3 laboratory. A small sample of the culture was collected on the end of a sterile wooden applicator and emulsified in an Eppendorf tube containing 500 μL of STE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0], 1% Triton X-100); the bacteria were harvested by centrifugation at 20,000 g for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 100 μL of 95% ethanol. After overnight incubation at 4°C, the suspension was centrifuged at 20,000 g for 10 min and the supernatant was discarded. Following the addition of 0.1 mL of STE buffer and ~50 μg of 0.1-mm zirconium oxide beads, the bacteria were lysed by agitation at 5000 rpm for 100 s with a Mini-BeadBeater (Biospec Products, Bartlesville, OK). The supernatant containing genomic DNA was used for analysis.

**hsp65 sequencing.** A 441-bp segment (nt 396–836) [22] of hsp65 was amplified with a DNA thermal cycler (Perkin-Elmer Cetus) with the following primer, 5'-ACCAACGATGGTGTCCAT-3'; reverse primer, 5'-CTTGTCGAACCGCATACCCT-3'. Unincorporated nucleotides and primers were removed by filtration with microconcentrators (Microcon 100; Amicon, Beverly, MA). Sequencing reactions were performed with a cycle sequencing kit (Prism Ready Reaction DyeDeoxy Terminator; Applied Biosystems, Foster City, CA). Membranes were hybridized overnight with labeled DNA probe and washed under high stringency conditions, and the bound probe was detected (ECL kit; Amersham Life Sciences) with a vacuum blotter (Bio-Rad, La Jolla, CA). Membranes were hybridized overnight with labeled DNA probe and washed under high stringency conditions, and the bound probe was detected (ECL kit; Amersham Life Sciences).

**Analysis of RFLP patterns.** By use of a scanner (Scanmaster 3+, Howtek, Hudson, NH), autoradiographic images were transmitted to a computer (SUN SPARCstation 5; Sun Microsystems, Mountain View, CA) and analyzed (Bioimage Whole Band Analyzer computer program, version 3.2; B. I. Systems, Ann Arbor, MI). The nucleotide sequences that we determined have been deposited in the GenBank database under accession numbers AF031591–AF031606.

**Statistical Analysis**

χ² tests of independence were used to assess whether the distribution of *M. avium* and *M. intracellulare* infections in children with or without HIV infection was significantly different.

**Results**

**Characteristics of patients and isolates.** MAC isolates cultured from 136 pediatric patients from 19 states and the District of Columbia were studied. The states from which isolates were obtained most frequently were Ohio (24%), Texas (16%), and North Carolina (16%). Fifty-nine (43%) of the children were HIV-infected, and their median age was 7.6 years (range, 3 months to 17 years). Of the 59 isolates recovered from children with HIV infection, 53 (88%) were cultured from blood or bone marrow sources, 4 (6.8%) were from stool specimens, 1 (1.6%) was from a tissue biopsy, and 1 (1.6%) was from otitis media drainage. Seventy-seven (57%) of the children were not infected with HIV, and their median age was 4.8 years (range, 7 months to 16 years). Of the 77 isolates recovered from children without HIV infection, 53 (69%) were from head or neck sources, 21 (27%) were from the respiratory tract, and the remaining 3 isolates (4%) were from various other anatomic sites.

**Nucleotide polymorphism.** Characterization of the 360-bp hsp65 fragment from the 136 MAC strains revealed that 21 of
the sequences were unique. All of the nucleotide variation was
due to point mutations. Each distinctive sequence was given
an arbitrary allele designation (e.g., hsp65.1, hsp65.2) (figure
1). Phylogenetic analysis of the 21 alleles compared with
unique hsp65 alleles representing 32 mycobacterial species [16,
18] revealed that the 21 alleles formed 3 distinct genetic clus-
ters (M. avium, M. intracellulare, and other MAC) and 5 lin-
eages that did not align with MAC members (data not shown).

Alleles not clustering with M. avium or M. intracellulare
isolates. Three isolates had allele hsp65.28 or hsp65.29,
which aligned with previously described alleles from MAC
organisms to form the other MAC cluster in the phylogenetic
tree (figure 2). Two isolates were recovered from cervical
lymph nodes of children without HIV infection, and 1 isolate
was from the blood of a child with HIV infection. Six isolates
recovered from children without HIV infection had 1 of 5
alleles that were not assigned to MAC. Three isolates were
Mycobacterium scrofulaceum strains, 1 was Mycobacterium phlei,
and 2 isolates were of unknown mycobacterial species.
These 6 isolates will not be included in further analysis or
calculations.

M. avium cluster. Of the 130 isolates evaluated, 79 (61%)
had 1 of 5 alleles (hsp65.1, hsp65.2, hsp65.20, hsp65.21,
and hsp65.34) which formed the M. avium cluster in the phylo-
genetic tree (figure 2). The majority of the isolates (72%) within
this cluster had allele hsp65.1. Of the 59 isolates recovered
from children with HIV infection, 52 (88%) were assigned to
this cluster, whereas only 27 (38%) of the 71 isolates recovered
from children without HIV infection were allied within this
cluster.

Among the HIV-associated isolates, 47 were cultured from
blood or bone marrow sources, 3 were from stool specimens, 1
was from a tissue biopsy, and 1 was from otitis media drainage.
Seventeen of the isolates recovered from children without HIV
infection were from head or neck sources and 10 were from the
respiratory tract.

M. intracellulare cluster. Of the 130 isolates evaluated, 48
(37%) had 1 of 9 alleles (hsp65.3, hsp65.4, hsp65.5, hsp65.6,
hsp65.7, hsp65.9, hsp65.18, hsp65.22, and hsp65.32) assigned
to the M. intracellulare cluster (figure 2). Most of the isolates
within this cluster had allele hsp65.3 (35%) or hsp65.7 (29%).
Only 6 (10%) of the 59 isolates recovered from children with
HIV infection occurred within this cluster, whereas 42 (59%) of
the 71 isolates recovered from children without HIV infection
occurred within this cluster. Among the HIV-associated iso-
lates, 5 were cultured from blood or bone marrow sources and
1 was from a stool specimen. Thirty-one of the isolates recov-
ered from children without HIV infection were from head or
neck sources, 9 were from the respiratory tract, and 2 were
from other anatomic sites.

IS1245 analysis of isolates within the M. avium phylogenetic
cluster. The majority (92%) of M. avium isolates had allele
hsp65.1 or hsp65.2, which differed by only one nucleotide (a
silent substitution of C→T at position 575). Because of this
restricted allelic diversity, an additional subtyping strategy was
used. RFLP profiling with insertion element IS1245 is useful
for identifying strain variation in M. avium for epidemiologic
studies in this species [20, 21]. The IS1245 insertion element is
found only in M. avium, not M. intracellulare or other mycobac-
terial species. To further differentiate the M. avium isolates,
75 of the 83 isolates were subtyped by RFLP analysis with insertion
sequence IS1245. The copy number of IS1245 ranged from 3
to 25. Each isolate had a unique IS1245 RFLP profile (data not
shown), and hence there was no sharing of RFLP patterns among
the M. avium isolates. There was no correlation between hsp65
allele and IS1245 copy number or band positions.

Figure 1. Polymorphic nucleotide
sites in 360-bp fragment of hsp65
gene sequence from 130 MAC iso-
lates (16 alleles). Nucleotide num-
bers are based on previously pub-
lished sequence for hsp65 of
Mycobacterium tuberculosis [22]
and are indicated vertically at top.
Sequences of 16 alleles are com-
pared with previously published se-
quence of the hsp65 fragment of M.
avium 88-1107 (GenBank accession
no. U17923). Nucleotides at sites
where there was no variation are not
shown.
Figure 2. Phylogenetic tree of 16 hsp65 alleles obtained from 130 M. avium isolates compared with 8 hsp65 alleles representing MAC (int = intracellular) [16, 19]. No. of strains with each allele recovered from either HIV-positive or HIV-negative children is indicated in columns. Horizontal lengths represent nucleotide sequence distances; bar represents 4% genetic difference. Dotted lines are artificial extensions of phylogenetic tree incorporated for clear alignment of allele list and should not be included in calculating genetic distances.

Discussion

**Subtyping of MAC.** Many strategies have been used for differentiating MAC strains. These strategies include serologic typing [23, 24], phage typing [25, 26], multilocus enzyme electrophoresis analysis [27, 28], RFLP profiling of plasmids [29], chromosomal DNA [30–34], a 360-bp fragment of the hsp65 gene [14, 15] and insertion elements [20, 21, 35–40], nucleotide sequence analysis [16–18, 41–49], and analysis with commercial DNA probes [5, 13].

Investigations of MAC isolates with commercially available nucleotide probes suggest that 77%–98% of invasive isolates recovered from adult AIDS patients are due to M. avium, whereas respiratory isolates from non-AIDS patients are frequently M. intracellulare [5, 13]. In addition, several studies have suggested that MAC associated with adult AIDS patients forms a restricted genetic or phenotypic subgroup of M. avium strains, whereas MAC associated with pulmonary disease is more diverse [23, 24, 42, 44, 50]. MAC isolates obtained from AIDS patients are generally assigned to only a few serovars of M. avium and have been associated with a group of similar RFLP patterns [23, 24, 50]. Nucleotide sequencing results of the 16S–23S rDNA internal transcribed spacer confirmed these findings [41, 43]. However, other studies using IS1245 RFLP and/or pulse-field gel electrophoresis analyses have identified considerable genetic diversity among isolates of M. avium causing bacteremia in adult AIDS patients [21, 30, 31, 33]. Thus, while several studies report that MAC infections in adult AIDS patients may be caused by limited subgroups of M. avium, other evidence indicates that they do not represent a single clonal lineage.

Pediatric isolates have not previously been analyzed by contemporary molecular genetic strategies. We subtyped 59 mycobacterial isolates recovered predominately from blood and bone marrow sources in children with HIV infection and 77 isolates recovered primarily from head and neck tissue of children without HIV infection by sequence analysis of a 360-bp region of the hsp65 gene. Our principal finding is that MAC infections in children with AIDS are primarily caused by M. avium organisms, and that MAC infections in children without AIDS are often due to M. intracellulare. Eighty-eight percent of the isolates obtained from children with HIV infection were M. avium and only 10% were M. intracellulare. In contrast, 38% of isolates obtained from children without HIV infection were M. avium and 59% were M. intracellulare ($\chi^2 [1 df] = 34.22, P = .001$). IS1245 RFLP analysis of isolates with hsp65 sequences
assigned to the *M. avium* cluster did not identify shared DNA patterns, indicating that each patient was infected with a unique strain. Thus, our results strongly mirror the adult situation.

*M. avium* isolates. Disregarding the 6 isolates found not to be MAC, *M. avium* accounted for 88% of isolates recovered from children with AIDS and only 38% of isolates recovered from children without AIDS. Seventy-two percent of these isolates had the same nucleotide sequence (*hsp65.1* allele). Previous sequence analysis of *hsp65* from laboratory reference strains revealed that the majority (66%) of *M. avium* isolates also had this allele [19]. Other methods used to differentiate MAC isolates have also described limited genetic and phenotypic diversity among *M. avium* isolates compared with *M. intracellulare* [41, 43, 44, 49].

Several hypotheses can be invoked to explain the higher prevalence of *M. avium* causing MAC infections in AIDS versus non-AIDS patients. *M. avium* strains may have distinct virulence factors or host-range factors that allow them to cause invasive disease specifically in HIV-infected individuals. It is therefore noteworthy that Reddy et al. [51] demonstrated that most MAC isolates from AIDS patients were more virulent for beige mice than MAC organisms recovered from non-AIDS patients or the environment. Alternatively, AIDS patients may be predisposed to develop infections due to *M. avium* rather than *M. intracellulare*. Bacteria of MAC are environmental organisms; therefore, it is possible that the route of infection or the environmental exposure may be different in AIDS patients and non-AIDS patients. Currently we lack sufficient knowledge about the pathophysiology of these infections to differentiate among the hypotheses.

*M. intracellulare* isolates. Of the isolates assigned to MAC by sequence analysis, 59% of the 71 isolates recovered from children without AIDS and 10% of the 59 isolates recovered from children with AIDS were *M. intracellulare*. The majority (35%) of isolates had the *hsp65.3* allele, results that are similar to findings from a previous study of MAC isolates [18]. Of interest, 14 (29%) of the 48 *M. intracellulare* isolates had the *hsp65.7* allele. This allele was reported in only 1 (4%) of 23 *M. intracellulare* isolates studied previously [18]. There may be several explanations for the marked difference in the prevalences of the *hsp65.7* allele between the two studies. All MAC isolates in this study were recovered from infected children. In contrast, most isolates analyzed in the previous report were derived from animal and environmental sources. Therefore, although isolates with the *hsp65.7* allele were not commonly identified from animals or the environment, they may have a higher predilection to cause disease in children. Alternatively, because 11 of the 14 isolates with the *hsp65.7* allele in the present study were recovered from children living in Cleveland, the difference identified could be an artifact of sample bias.

*Non-MAC isolates.* It is well known that some acid-fast bacteria are incorrectly assigned to MAC. Thus, it is not surprising that several isolates in our study were mistaken for MAC organisms by conventional diagnostic laboratory techniques. These findings add to the concept that species assignment by nucleotide sequence analysis is a more precise method for mycobacterial identification [18, 19, 44, 45, 48].

Utility of pediatric MAC isolates for epidemiologic studies. Additional investigations of pediatric MAC isolates may provide further insight into remaining questions concerning the molecular epidemiology and pathophysiology of MAC infections. For example, it is not known whether most adult patients with AIDS develop MAC disease from a recent infection or by reactivation of an old infection. However, it is generally presumed that childhood MAC infections are recently acquired and not the result of reactivation. Therefore, to identify the environmental source(s) of MAC infections, it may be more informative to study MAC isolates obtained from children rather than adults. These data may also lead to insights concerning the route of MAC infection and possible methods to reduce the exposure to these organisms. In addition, most MAC organisms infecting children without AIDS cause cervical lymphadenitis, which is relatively unambiguous to diagnose clinically. In contrast, MAC isolates cultured from adults without AIDS are usually recovered from respiratory secretions of patients with chronic lung disease. In these instances, it can be very difficult to distinguish clinically between isolates causing pulmonary disease and those colonizing only the respiratory tract.

Summary. The primary goal of this study was to describe the molecular epidemiology and genetic diversity of MAC infections in children. The results demonstrate that *M. avium* is more often the cause of MAC infections in children with AIDS than in children without AIDS. This implies that *M. avium* may have special virulence attributes that permit them to cause disseminated disease in HIV-infected patients compared with *M. intracellulare*. Additional analyses of MAC infections in children may provide further insights into the epidemiology, pathogenesis, and prevention of this disease.

Acknowledgments

We are indebted to E. Wolinsky, R. Wallace, Jr., and C. Inderlied for generously providing MAC strains.

References


