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Association between 16S-23S Internal Transcribed Spacer Sequence Groups of *Mycobacterium avium* Complex and Pulmonary Disease▽†

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Organisms within the *Mycobacterium avium* complex (MAC) may have differential virulence. We compared 33 subjects with MAC pulmonary disease to 75 subjects with a single positive culture without disease. *M. avium* isolates were significantly more likely to be associated with MAC pulmonary disease (odds ratio = 5.14, 95% confidence interval = 1.25 to 22.73) than *M. intracellulare*.

Exposure to aerosols containing organisms in the *Mycobacterium avium* complex (MAC) likely occurs often (11, 12), but resultant pulmonary disease is uncommon (13, 16). The MAC group of organisms consists of two relatively common named species, *M. avium* and *M. intracellulare*, and a third group of organisms that until recently were not denoted by species names. Whether some species of MAC are more likely to cause pulmonary disease than others is unknown.

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We performed a case-control study comparing persons with MAC pulmonary disease to persons with a single positive respiratory culture but no evidence of disease. Persons who had a respiratory specimen that grew MAC between 1 January 1993 and 30 June 1999 and available medical records were considered for inclusion ($n = 600$). Case subjects were considered for inclusion if they met the 1997 American Thoracic Society criteria for MAC pulmonary disease (1). The following criteria were used to exclude both potential cases and controls: (i) human immunodeficiency virus infection, (ii) cystic fibrosis, (iii) age under 18 years, (iv) recipient of organ or bone marrow transplant, (v) pulmonary alveolar proteinosis, and (iv) active tuberculosis. The control group was randomly selected from patients with a positive respiratory MAC culture who did not meet American Thoracic Society criteria and had a low likelihood of having MAC pulmonary disease, including no radiographic evidence of MAC pulmonary disease. All controls had (i) a single positive culture for a MAC organism from a respiratory site (as described above), (ii) negative acid-fast smears on the same specimen and all other respiratory specimens collected, and (iii) at least two mycobacterial cultures obtained from the respiratory tract. Patients were excluded from the control group if they had (i) a diagnosis of MAC pulmonary disease by an infectious disease specialist or a pulmonologist, (ii) received a specific therapy directed at MAC pulmonary disease (as defined above under inclusion criteria for cases), or (iii) granulomatous inflammation in any biopsy specimens taken from the respiratory tract.

Clinical isolates were identified by the Duke Clinical Microbiology Laboratory as MAC based on growth characteristics, lack of pigmentation, biochemical testing, and confirmation with the MAC AccuProbe (Gen-Probe, San Diego, CA). Duplicated samples comprising 10% of the isolates were analyzed as a quality control.

The internal transcribed spacer region between the 16S and 23S rRNA genes was amplified from bacterial lysates using 30 cycles of PCR with the primers CCL (5′-TTGTA

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TABLE 1. Characteristics of case and control subjects

Parameter	No. (%) of subjects[a]		P
	Case (n = 33)	Control (n = 75)	
Female	30 (91)	29 (39)	<0.0001
Mean age (SD) in yr	65.6 (11.1)	60.1 (15.2)	0.039
Race/ethnicity:			<0.0001
White	30 (91)	47 (63)	
Black	1 (3)	26 (35)	
Other/unknown	2 (6)	2 (3)	
Diabetic	1 (3)	13 (7)	0.043
Chronic obstructive pulmonary disease	6 (18)	30 (40)	0.027
Gastroesophageal reflux	5 (15)	10 (13)	0.80
Malignancy	1 (3)	13 (17)	0.060
Fibrotic lung disease	0 (0)	3 (4)	0.55
Collagen vascular disease	4 (12)	8 (11)	1.0

[a] Values are expressed as indicated except as noted for mean age in column 1.

CACACCGCCCGTC-3′) and 23S (5′-TCTCGATGCCAA GGCATCCACC-3′) (5). Purified PCR products were sequenced directly using the internal primer P5B (5′-GACG AAGTCTAACAAGGTAGC-3′) at the Duke University Medical Center's DNA Sequencing Facility. Sequences were aligned by using CLUSTAL W (version 1.83; ftp://ftp.ebi.ac.uk /pub/software/dos/clustalw/clustalw1.83.XP.zip), and isolates were assigned to known sequevars based on exact matching. A phylogenetic tree was constructed based on maximum likelihood using PHYLIP 3.65, (http://evolution.gs.washington.edu/phylip.html). The data were resampled with 100 bootstrap replications. Groupings found on more than 70% of the bootstrap replications are marked on the phylogenetic tree. The relationship between sequevar group and case status was assessed by using odds ratios and exact 95% confidence intervals with SAS version 9.1 (SAS Systems, Cary, NC).

Forty case subjects were selected, but isolates were only available for thirty-three. Eighty control subjects were selected, but four did not have isolates and one had a dual/ambiguous sequence, leaving seventy-five subjects with analyzable sequence data (Table 1). Sequencing of the 16S-23S internal transcribed spacer from the 108 isolates revealed 22 distinct sequevars (see the supplemental material), whose phylogenetic relationships are demonstrated in the Fig. 1. Six isolates (5.6%) were genetically distant from the rest of the *M. avium* complex ("Non-MAC" sequevar group). Sixty-nine (63.9%) isolates clustered with *M. intracellulare*, thirteen (12.0%) clustered with *M. avium*, and twenty (18.5%) clustered with neither species ("MAC, other"). Compared to *M. intracellulare* group isolates, *M. avium* group isolates were significantly more likely to be associated with true MAC pulmonary disease (Table 2).

We found a significant association between *M. avium* sequevar group MAC isolates and MAC pulmonary disease, with a >5-fold increase in the odds of true MAC pulmonary disease in the *M. avium* sequevar group compared to the *M. intracellulare* sequevar group. However, consistent with other studies (3, 4, 8, 18, 25), the majority of MAC isolates (63.9%) among study subjects with or without MAC pulmonary disease belonged to the *M. intracellulare* sequevar group. *M. intracellulare* organisms are preferentially aerosolized (compared to *M. avium*) (17), resulting in relatively greater potential for respiratory exposure to *M. intracellulare*. Our data suggest the hypothesis that when respiratory exposure occurs, *M. avium* may be more likely to cause pulmonary disease than *M. intracellulare*; in other words, *M. avium* might be more virulent after respiratory exposure, all other factors being equal. *M. avium* possesses specific virulence factors not found in *M. intracellu-*

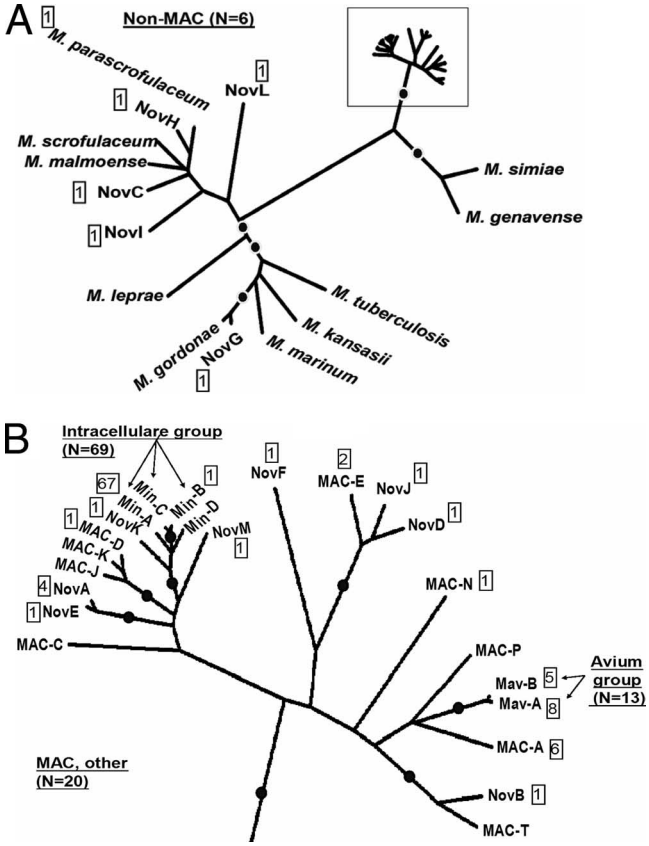

FIG. 1. Phylogenetic tree of *M. avium* complex isolates based on 16S-23S internal transcribed spacer sequences. Line segment lengths represent phylogenetic distances based on maximum-likelihood analysis. The dots represent groupings found on more than 70% of bootstrap replications. "Min" isolates are isolates that cluster with *M. intracellulare*, "Mav" isolates cluster with *M. avium*, and "MAC" isolates cluster with neither species. "Nov" isolates represent novel sequevars (not previously described). Other mycobacterial species are included in the tree for reference. Arrows demonstrate the groups selected for analysis. The box inset in panel A represents organisms belonging to the *Mycobacterium avium* complex; this section of the tree is expanded and represented in panel B. Numbers next to sequevar names represent the total number of subject isolates (case or control) that belonged to this sequevar group.

TABLE 2. Association between case status and MAC sequevar group

Sequevar group	No. of:		OR (95% CI)[a]
	Cases	Controls	
Intracellulare	21	48	1.0 (ref)
Avium	9	4	5.14 (1.42–18.6)
MAC, other	2	18	0.25 (0.05–1.19)
Non-MAC	1	5	0.46 (0.05–4.16)

[a] OR, odds ratio; CI, confidence interval; ref, reference group.

lare (3, 19), is better able to invade and replicate inside macrophages than *M. intracellulare* (14), and has been associated with more invasive forms of MAC disease (3, 10, 21, 27).

Our data differ from the findings of a recent study by Han et al., who found that *M. avium* (identified by 16S rRNA sequencing) isolated from clinical specimens was actually less likely to be associated with pulmonary disease than was *M. intracellulare* (9). In that study, *M. avium* isolation was strongly associated with hematologic malignancy but did not often cause clinical disease (16.1% of isolates), while 63.1% of patients with *M. intracellulare* had clinical disease. Since that study was conducted at a cancer referral hospital, the vast majority of patients had malignancies, so the results are unlikely to reflect what occurs in the general population. Furthermore, the study was cross-sectional, so misclassification of disease status may have occurred. Geographic strain differences resulting in differential exposure and resultant disease may also explain the difference between the Han et al. study and the present study.

Our data have several inherent limitations. Only one mycobacterial isolate per case subject was examined. MAC pulmonary disease is often polyclonal (25), and reinfection with new strains occurs frequently (24). Examining only one isolate may have resulted in misclassification of the infecting MAC sequevar in some case subjects. However, this misclassification would be most likely nondifferential and therefore would reduce the association between any particular sequevar and case status. Some subjects who truly had MAC pulmonary disease may have been inappropriately assigned to the control group. Again, this misclassification would tend to reduce any observed associations between case status and sequevar group. The demographics and comorbidities of the case and control groups were quite different, and observed differences in sequevar distributions may have been a result of confounding. Our study lacked statistical power to thoroughly explore this question, but in an exploratory analysis, no demographic/comorbidity was significantly associated with sequevar group (data not shown). Patients with fibrocavitary MAC pulmonary disease were not well represented in our study, and the sequevars associated with disease among patients with fibrocavitary MAC may well differ from those associated with disease among patients with nodular/bronchiectatic MAC.

The 2007 American Thoracic Society/Infectious Diseases Society of America guidelines for treatment of nontuberculous mycobacterial infections recommend that all clinically significant nontuberculous mycobacteria should be identified to the species level (7). Our data emphasize the importance of species identification in understanding the role of nontuberculous mycobacteria in lung disease. DNA sequencing of mycobacterial isolates is a powerful and increasingly popular method for speciation. The use of the 16S-23S internal transcribed spacer is a well-validated method to divide mycobacteria into species and subspecies groups (5, 6, 15, 26); other commonly used loci are the 16S ribosomal (3), *rpoB* (2), and *hsp65* (20) genes. Use of these techniques has enabled provisional assignment of new species names to at least two previously unspeciated organisms within MAC (22, 23). Further studies using these techniques to study well-defined patient groups will be necessary to better understand the role of MAC in human disease.

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