SHORT COMMUNICATION

Multiple Isolates from Aids Patients: Aspects of an Analysis by a Genotypic Marker and Antimicrobial Susceptibilities Variations

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Twenty-one Mycobacterium avium *multisolates, from ten human immunodeficiency virus-infected patients, were typed by restriction fragment length polymorphism using as marker the IS1245 and characterized by minimum inhibitory concentration for nine different antibiotics. Two out of four patients harboring multisolates with different fingerprint profile, were therefore considered as having a polyclonal infection, since their isolates were taken from sterile site. This result confirms that polyclonal infection caused by* M. avium *occurs with a nonnegligenciable frequency. Analyzing the multisolates susceptibility profile of each patient it was observed that most of them were infected with strains having appreciably different antimicrobial susceptibility patterns, no matter what the genotypic pattern of the strains was. These results have strong implication for the treatment of the patients.*

Key words: *Mycobacterium avium* - polyclonal infection - susceptibility test

Disseminated disease caused by *Mycobacterium avium* complex (MAC) is the most common opportunistic infection among patients with the acquired immunodeficiency syndrome (Aids), affecting their life quality and shortening their survival (Gordin & Masur 1994). It occurs in a range of 15 to 40% of human immunodeficiency virus (HIV) infected patients in developed countries and 95% of the isolates are represented by *M. avium* (Inderlied et al. 1993). However, in developing countries such as Brazil, this opportunistic infection was not considered a major problem for Aids patients until recently, when it was demonstrated that MAC infection are beginning to emerge associated with Aids (Barreto et al. 1993, Landgraf at al. 1994, Fandinho at al. 1994). One of the reasons for the emergence of this pathogen may be related with better survival of Aids patients in places like São Paulo and Rio de Janeiro, because Aids treatment has greatly improved (Barreto et al. 1993).

Management and prevention of *M. avium* infections in HIV infected patients in Brazil are becoming an important health issue. The disease by this organism, usually found in soil and water, has been suggested to be due to the reactivation of a subclinical endogenous infection, presumably acquired through the oral route from the environment.

Recently, we conducted the first DNA fingerprint analysis in more than 100 strains of *M. avium* isolated from Brazilian Aids patients (Saad at al. 1999*)* and two interesting results came up from this study: like *M. tuberculosis*, *M. avium* appears to show geographic clustering and in contrast to tuberculosis this opportunistic microorganism is involved in polyclonal infections.

Polyclonal infections are related with simultaneous infections of two genetically distinct strains and this may be a problem if these strains show different antimicrobial susceptibility patterns. Sometimes, to treat MAC infection becomes an inglorious battle because of its diversified drug resistance profile.

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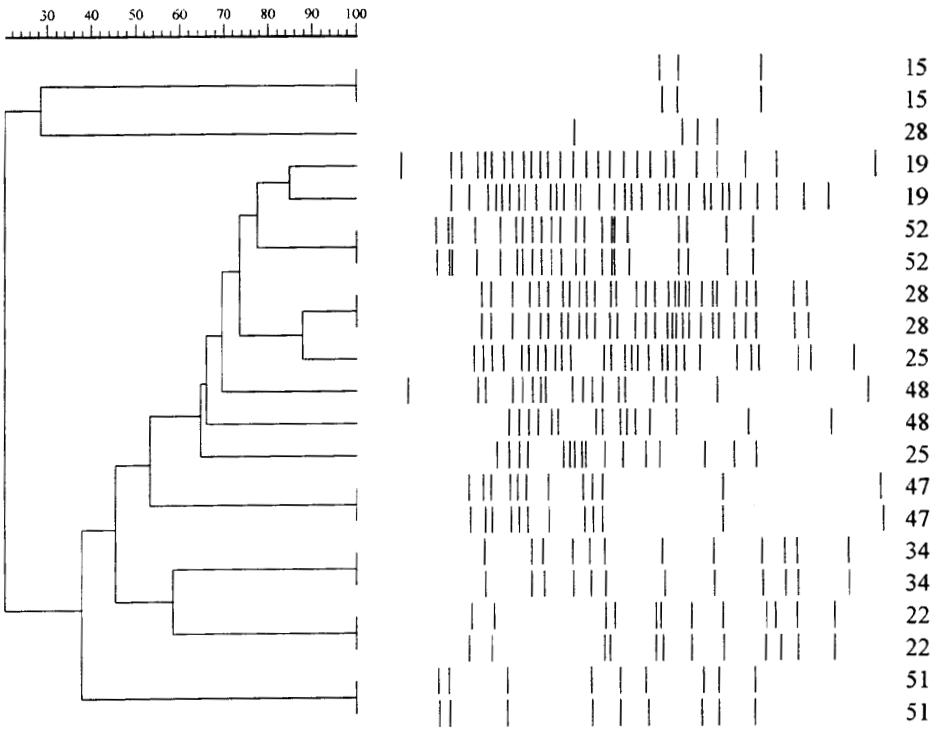
In the present study we analyzed 21 *M. avium* multiple isolate strains from ten Aids inpatients from Hospital Emílio Ribas and Reference Center DST/Aids, São Paulo, Brazil, by genotypic and phenotypic markers: the IS1245 using a restriction fragment length polymorphism (RFLP) technique (Guerrero et al. 1995) and minimum inhibitory concentration (MIC) for nine antibiotics to determine the susceptibility variations among polyclonal and monoclonal infections. The strains were isolated from a single patient at different or the same clinical site, at different or the same time periods. Most of the patients had CD4 counts <100/ mm^3. All strains were isolated and identified at the Mycobacteriology Laboratory of the Instituto Adolfo Lutz, São Paulo. Polyclonal infection was defined if patient with disseminated infection harbored multiple isolates with different fingerprinting patterns.

For the antimycobacterial susceptibility testing, stock solution of amikacin (AMK), ethionamide (ETH), rifampin (RIF), rifabutin (RIB), ciprofloxacin (CPR), clofazimine (CLF), clarithromycin (CLR), streptomycin, (SM) and ethambutol (EMB) were prepared as described previously (Telles & Yates 1994). The stock solution of the drugs was diluted in Middlebrook 7H9 medium and 200 µl of each drug were added to wells 2-10 of the first row of the microtitre plates. Two fold dilutions were then made with the transfer of 100 µl amounts. Each drug was diluted in the following range: 0.5 to 32 µg/ml for SM, AM, ET and CLR, 0.12 to 8 µg/ml for RIF and CLF; 0.25 to 16 µg/ml CPR and EMB; 0.06 to 4 µg/ml for RIB. The MIC was defined as the lowest concentration of the antibiotic that inhibited visible growth.

The results with RFLP showed that six out of ten patients (15, 22, 34, 47, 51 and 52) had multiple isolates with an identical RFLP pattern (100% matching patterns). In two other patients (25 and 48) the fingerprinting pattern strains, isolated from non-sterile site, were distinct from the strain that was causing disseminated infection (Figure). These findings may suggest that some strains are more virulent than others and may cause disseminated disease while others remain restricted to the pulmonary area. Despite the fact that these patients harbored two genetically different strains, they were not considered to have polyclonal infection, because one of the strains was recovered from non-sterile site and this may reflect just colonization. The patients 19 and 28, with two strains of *M. avium* isolated from sterile sites, had disseminated



Schematic representation of IS1245 restriction fragment lenght polymorphism fingerprint of multiple *Mycobacterium avium* strains isolated from patients 19 and 28 harboring polyclonal infection and patients 25 and 48, with isolates from sterile and non-sterile sites, showed different genotypic patterns; patients 15, 22, 34, 47, 51, 52 harboring multiple isolates showing the same fingerprint patterns. Scale depicts similarity coefficient.

polyclonal infection as the fingerprinting patterns of the strains were quite different, although patient 28 had also disseminated infection with the same genetic pattern strain found in his respiratory tract (Figure). In each of these patients, both strains were cultured 10 and 60 days apart, respectively. The presence of genetically distinct multiple isolates from a single patient is not a rare event as reported in France (Picardeau at al. 1997).

Analyzing the Table, it is clear that clarithromycin is the most effective drug to the tested strains (85.7% of sensitive), followed by clofazimine and ethionamide (70% and 61.9%, respectively) and by ciprofloxacin, rifabutin and amikacin that showed moderate activity (42.8%, 47.6% and 57.1%, respectively). Poor *in vitro* activity to the *M. avium* strains was performed by ethambutol, rifampicin and streptomycin (15%, 19% and 23.8%, respectively). As whole, there was not significant difference among the number of resistant strains isolated from sterile and non-sterile sites (p=0.91). However, strains isolate from sterile sites (8/13) were significantly more sensitive to ETH than those from non-sterile sites (1/8, p=0.03). Correlation was not found between clonal pattern and the exhibited susceptibility activity since significant MIC variation (≥ 4-fold dilution) in one to four antibiotics was detected, no matter what the genotypic pattern of the strains was. CFZ was the antibiotic that showed the most significant MIC variations between strains from a single patient (highlighted MICs, Table). In this study, two patients (22 and 51) that were treated with multidrug therapy had a better survival (seven months). These patients received five and seven different drugs, respectively, and the strains were resistant to three of them. On the other hand, two patients (28 and 47) treated only with tuberculosis therapeutic schema (rifampicin, isoniazid and pyrazinamide) only survived two months after *M. avium* infection. The treatment implications for the patients with multiple isolates need further investigation. Prospective study with *M. avium* polyinfected patients must be done to correlate the MIC variation with the treatment evolution. The knowledge of susceptibility patterns of the infecting strains may

TABLE

Restriction fragment lenght polymorphism (RFLP) and minimum inhibitory concentration (MIC) patterns of multisolates from acquired immunodeficiency syndrome patients with and without polyclonal *Mycobacterium avium* infection

Patient number	Clinical specimen	RFLP pattern	MIC (mcg/ml)[a]								
			AMK	ETH	RIF	RIB	CIP	CFZ	CLR	SM	EMB
15	Blood	A	4	8	8	0.5	2	1	4	16	8
	Blood	A	8	4	4	1	4	2	4	8	8
19	Blood	T	**4**	**>32**	>8	1	4	0.5	>32	4	8
	Blood	DDD	**>32**	**0.5**	8	0.5	4	0.5	>32	4	8
22	Blood	T	2	4	8	0.5	4	**0.25**	8	16	8
	Sputum	T	2	8	4	0.5	4	**2**	4	8	8
25	Blood	NNN	2	1	8	0.5	0.25	**0.25**	2	16	8
	Sputum	Sl	2	2	8	0.25	0.25	**2**	4	32	8
28	Blood	NNN	4	**1**	>8	**2**	**2**	**0.25**	4	8	4
	Sputum	NNN	4	**8**	8	1	**0.5**	**2**	4	8	8
	CSF	Bl	4	8	>8	**0.5**	**4**	**0.5**	4	16	8
34	Blood	DD	**>32**	0.5	>8	1	**8**	**0.12**	2	16	8
	Bone Marrow	DD	**4**	1	>8	0.5	**2**	**0.5**	2	8	4
47	Lympho node	EE	8	1	4	1	4	**2**	4	**32**	8
	Blood	EE	4	1	2	1	2	**0.5**	4	**8**	8
48	Blood	BBBB	**8**	**0.5**	8	2	8	**4**	8	**32**	16
	Sputum	RRRR	**>32**	**16**	>8	1	4	**0.25**	8	**4**	16
51	Sputum	Hl	>32	2	8	0.5	4	0.12	**1**	8	>16
	Feces	Hl	>32	4	8	1	8	0.25	**8**	8	8
52	Sputum	SSS	**>32**	2	>8	0.5	**0.5**	1	1	4	16
	Urine	SSS	**4**	1	>8	0.5	**4**	ND	1	4	ND

AMK: amikacin; ETH: ethambutol; RIF: rifampicin; RIB: rifabutin; CIP: ciprofoxacin; CFZ: clofazimine; CLR: clarithromycin; SM: streptomycin; EMB: ethionamide; *a*: MIC(mcg/ml) key reading: AMK 8 (resistante=R), 4 (moderate sensitive=MS), 2 (sensitive=S); ETH 4 (R), 2 (MS), 1 (S); RIF 8 (R), 4 to 1 (MS), 0.5 (S); RIB 1 (R), (S); RIB 1 (R), 0.5 to 0.2 (SM), 0.12 (S); CIP 4 (R), 2 (MS), 1 (S); CFZ 1 (R), 0.5 (MS), 0.25 (S); CLR 16 (R), 8 (MS), 4 (S); SM 8 (R), 4 (MS), 2 (S); EMB 8 (R), 4 (MS), 2 (S); MIC≤4 fold; ND: not done.

improve the right choice of the antibiotic combination therapy in order to extend the patient survival.

In conclusion, the results showed that, in agreement with others (Arbeit et al. 1993, von Reyn et al. 1996), disseminated polyclonal infection is not an infrequent occurrence. Our findings also suggest that different strains of MAC may have different clinical site tropism; this issue requires further studies. The finding of multiple isolates from a single patient harboring different DNA fingerprint patterns seems to indicate that exposure to multiple environmental sources is relatively frequent and (or) polyclonal environmental colonization is a common event (von Reyn et al. 1994). However, 60% of the studied patients had monoclonal infection and the question is: were they exposed to a monoclonal source? Von Reyn et al. (1996) hypothesized that monoclonal infection might also result from exposure to a polyclonal source. However, some strains might carry virulence factors that may lack in other strains present in the same environmental source, so studies must be done on the pathogenesis of different strains of this opportunistic microorganism. Our results also claim for discussion on the susceptibility profiles of *M. avium* multiple isolates that may be critical in the development of effective treatment and prevention.

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REFERENCES

Arbeit RD, Slutsky A, Barber TW, Maslow JN, Niemczyk S, Falkinham III JO, O'Connor GT, von Reyn CF 1993. Genetic diversity among strains of *M. avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. *J Infect Dis 167:* 1384-1390.

Barreto JA, Palaci M, Ferrazoli L, Martins MC, Suleiman J, Lourenço R 1993. Isolation of *Mycobacterium avium* complex from bonemarrow aspirates of AIDS patients in Brasil. *J Infect Dis 168*: 777-779.

Fandinho FCO, Grinsztejn B, Veloso VG, Lourenço MCS, Werneck-Baroso E, João E, Nogueira AS, Fonseca LS 1994. Diagnosis of disseminated mycobacterial infection: testing a simple and inexpensive method for use in developing countries. *WHO Bull 74*: 361-366.

Gordin F, Masur H 1994. Prophylaxis of *Mycobacterium avium* complex bacteremia in patients with AIDS. *Clin Infect Dis 18*: S223-S226.

Guerrero C, Barnasconi D, Burki T, Bodmer T, Telenti A 1995. A novel insertion element from *Myco-bacterium avium*, IS1245, is a specific target for analysis of strain relatedness. *J Clin Microbiol 33:* 304-307.

Inderlied CD, Kemper CA, Bermudez LEM 1993. The *M. avium* complex. *Clin Microbiol Rev 6*: 266-310.

Landgraf IM, Palaci M, Vieira MFP, Mizuka SY, Martins MC, Sato DN, Ferrazoli L, Telles MA, Gorla COM, Vaz TMI, Lourenço R, Marques LRM 1994. Bacterial agents isolated from cerospinal fluid of patients with acquired immunodeficiency syndrome and neurological complications. *Rev Inst Med Trop São Paulo 36*: 491-496.

Picardeau M, Varnerot A, Lecompte T, Brel F, May T, Vincent V 1997. Use of different molecular typing techniques for bacteriological follow-up in a clinical trial with AIDS patients with *Mycobacterium avium* bacteremia. *J Clin Microbiol 55:* 2503-2510.

Saad MHF, Fonseca LS, Ferrazoli L, Fandinho F, Palaci M, Grinsztejn B, Kritski A, Werneck A, Poltoratskaia N, Johnson Jr WD, Riley LW 1999. IS1245 genotypic analyze of *Mycobacterium avium* isolates from patients in Brazil. *Int J Infect Dis 1*: 192-196.

Telles MA, Yates M 1994. Single and double drug susceptibility testing of *Mycobacterium avium* complex and mycobacteria other than tubercle (MOTT) bacilli by a micro-dilution broth minimum inhibitory concentration (MIC) method. *Tuberccle Lung Dis 75*: 286-290.

Von Reyn CF, Maslow JN, Barber TW, Falkinham III JO, Arbeit RD 1994. Persistent colonization of potable water as a source of *Mycobacterium avium* infection on AIDS. *Lancet 343:* 1137-1141.

Von Reyn CF, Pastel M, Arbeit RD 1996 Clinical and epidemiologic implications of polyclonal infection due to *Mycobacterium avium* complex. *Res Microbiol 147*: 24-29.