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Molecular epidemiology of *Mycobacterium avium* complex isolated from patients with and without AIDS in Brazil and England

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SUMMARY

Mycobacterium avium complex (MAC) is ubiquitous throughout the world. It is an opportunistic pathogen in AIDS patients but the number of cases in HIV negative patients is also increasing. The aim of this study was to determine whether patients were being infected with different MAC strains or whether one strain was dominant. DNA obtained from isolates in Brazil and England were compared using pulsed field gel electrophoresis (PFGE). Strains from 22 Brazilian patients clustered into 7 groups but 68/90 patients had a unique strain. In all patients, Brazilian and English, the same strain was isolated repeatedly over time, some over several years. This study shows that it is most likely that Man is infected from the environment and that one strain can survive without change for many years both in the environment and in Man.

INTRODUCTION

Mycobacteria of the *Mycobacterium avium* complex (MAC) are ubiquitous in nature and are widely distributed in soil and water especially in surface waters [1]. MAC have been isolated from aerosols generated over streams, estuaries and ocean waters and have also been found in aerosols produced by fountains [2]. They have been isolated from a variety of environmental sources throughout the world [3]. It is unclear how patients become infected with these organisms but inhalation of aerosols containing the bacilli has been implicated as a source of infection in AIDS patients [4]. There has also been an increase in the number of MAC infections in non-immuno-compromised patients [5], especially children with lymphadenopathy [6].

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Du Moulin and colleagues [7] found MAC in 11 of 16 hot water taps and showerheads examined and in 3 of 18 cold water taps in a hospital in Boston, Massachusetts. The concentration of bacilli was found to be highest in the hot water taps and showerheads. The MAC were predominantly of serotype 4, the most prevalent serotype causing disease in AIDS patients in that area. Von Reyn and colleagues [4], using Pulsed Field Gel Electrophoresis (PFGE), examined strains of MAC isolated from AIDS patients, environmental sources and the water systems in patients' homes and hospitals in Boston, Massachusetts. They were able to identify the same strain in three patients and the hot water system of the hospital that they had attended, and also a second strain from two patients and the hot water system of a second hospital in the area that they had attended. No MAC was isolated from the patients' home environments.

The purpose of this investigation was to study the strains of MAC isolated from HIV infected (HIV^+) and non-infected (HIV^-) patients in São Paulo in order to determine whether different patients were being infected with the same strain or if patients were being infected with multiple MAC strains particularly if MAC were repeatedly isolated over time. A comparative analysis was also made with strains isolated from HIV^+ and HIV^- patients in London.

MATERIALS AND METHODS

Mycobacterial strains and growth conditions

The isolates of MAC used were cultured and identified from clinical specimens received at the Setor de Micobacterias, Instituto Adolfo Lutz (IAL), São Paulo, Brazil and the Regional Tuberculosis Centre (now the Mycobacterium Reference Unit), London, England. The identification methods used a combination of microscopic, cultural, growth and biochemical characteristics [8] and, where appropriate, molecular hybridization analysis (GenProbe, San Diego, CA, USA). In general MAC are slowly growing, non-photochromogenic mycobacteria which grow at 25 °C, do not hydrolyse Tween 80 and are resistant to ciprofloxacin at a concentration of 5 μg/ml in Lowenstein-Jensen medium. For a more complete identification see Collins and colleagues [8]. There were 170 isolates from 90 Brazilian patients (82 HIV^+, 8 HIV^-) and 44 from 8 English patients (6 HIV^+, 2 HIV^-) (Tables 1, 2).

The English patients comprised 5 males and 3 females age range 32–81 years. The HIV^- patients were both female and European Caucasian and both presented with pulmonary disease. Of the 6 HIV^+ patients, 3 males were black, 2 from sub-Saharan Africa and 1 from the West Indies, the other 2 males and 1 female were European Caucasian. All the HIV^+ patients had AIDS by definition of MAC dissemination.

The isolates were grown in Middlebrook 7H9 medium, containing 10% OADC enrichment, modified by the addition of 0·5 M sucrose, 0·2% D-glucose and 0·05% Tween 80. This modification was a variation of that described by Slutsky and colleagues [9]. After incubation at 35 °C for 4–7 days until an optical density at 600 nm of approximately 0·2 was obtained, 1 ml of 10 × ACT solution (1 mg/ml ampicillin, 10 mg/ml D-cycloserine, 10 mg/ml D-threonine) was added and the mixture incubated for a further 16–20 h. The organisms were harvested by centrifuging at 3000 g for 15 min and washed in TS buffer (50 mM Tris, 0·5 M sucrose, pH 7·6). The resulting pellet was resuspended in 1 ml (0·2%, w/v) sodium azide and left at room temperature for 30 min, then centrifuged at 13000 rpm in a microcentrifuge for 5 min.

The pellet was re-suspended in 0·2 ml TS buffer and 50 μl mixed with 50 μl 2% low melting point agarose in TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0) at 50 °C; 90 μl of this mixture was transferred to a plug mold and chilled at 4 °C for 15 min. All reagents were obtained from Sigma, Poole, Dorset.

Extraction of DNA and restriction endonuclease digestion

Once set the plug was removed from the mold and treated with 2 mg/ml lysozyme in TE buffer for 2 hs at 37 °C, and 0·5 mg/ml proteinase K plus 1% sodium dodecyl sulphate in TE buffer at 50 °C overnight. The plug was washed four times in TE buffer at room temperature for 30–60 min each with the second wash containing 1 mM phenylmethyl sulphonyl fluoride to inactivate the proteinase K.

Half the plug was then transferred to 0·5 ml NEB3 restriction enzyme buffer (New England Biolabs, Hitchin, UK) for 1 h at room temperature after which the buffer was replaced with 0·5 ml NEB3 containing 15 units of *Ase*I (restriction endonuclease from New England Biolabs) and incubated overnight at 37 °C. The enzyme was replaced with 0·5 ml electrophoresis running buffer (0·5 × TBE: 45 mM Tris, 45 mM boric acid, 1 M EDTA, pH 8·0).

Separation of DNA

Pulsed field gel electrophoresis (PFGE) was performed in 1% PFGE agarose in 0·5 × TBE buffer using a CHEF DRII system (Bio-Rad laboratories, Richmond, CA, USA). The running temperature was 14–15 °C, run time 21 h, voltage 6 V/cm with an initial switch time of 20 s and a final switch time of 60 s.

Visualization of DNA

After completion, the gel was stained by placing in 200 ml ethidium bromide solution (0·5 mg/l in dis-

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Table 1. *Number of patients and number of isolates and time scale of isolation of multiple isolates*

Laboratory	Number of isolates/patient tested	Total number of patients	Number HIV$^+$	Time scale of isolation period (months)	Number HIV$^-$	Time scale of isolation period (months)
São Paulo Brazil	1	49	47	—	2	—
	2	24	22	1–22	2*	1–19
	3	9	9†	1–3	0	—
	4	3	2	3–4	1	29
	5	1	1‡	4	0	—
	6	2	1	13	1	14
	7	1	0	—	1	29
	10	1	0	—	1	20
London England	3	3	3	10–26	0	—
	4	1	1	6	0	—
	5	1	1	8	0	—
	6	1	1	10	0	—
	7	1	0	—	1	48
	13	1	0	—	1	72

* 1 patient had 2 different strains isolated in 19 months.
† 2 patients had 2 strains indistinguishable and 1 strain different.
‡ 1 patient had 4 strains indistinguishable and 1 strain different.

Table 2. *Site of disease*

Laboratory	Site of disease	Number of patients	Number HIV$^+$	Number HIV$^-$
São Paulo, Brazil	Pulmonary	13	6	7
	Disseminated*	65	65	0
	Skin	1	0	1
	Skin and pericardium	1	1	0
	CSF	10	10	0
London, England	Pulmonary	2	0	2
	Disseminated	6	6	0

* Disseminated includes isolates from more than one internal site, e.g. sputum and urine.

tilled water) for 20–30 min and then washed in distilled water for 1–2 h. The gel was visualized by placing on a UV transilluminator and photographed using a Polaroid camera.

RESULTS

Patient isolates

Of the 90 Brazilian patients 68 (76%) had strains isolated with unique DNA patterns. The isolates of the remaining 22 patients clustered in 7 indistinguishable patterns. All 8 English patients had unique strains.

The same strain (i.e. with the same genotypic and phenotypic characteristics) was consistently isolated over time from individual patients, irrespective of their HIV status. In four patients, however, a genotypically different strain was isolated subsequently (Table 1). Of these patients 3 were HIV positive: the same strain was isolated from 1 patient 4 times and a second unrelated strain isolated once, while 2 patients had 2 isolates of 1 strain and 1 isolate of a second strain. The fourth patient, HIV negative and with a pulmonary infection caused by *M. chelonae*, subsequently had two different strains of MAC isolated 19 months apart.

It was noted that amongst the HIV negative patients, one had the same strain isolated 13 times over a 6-year period without any alteration in its

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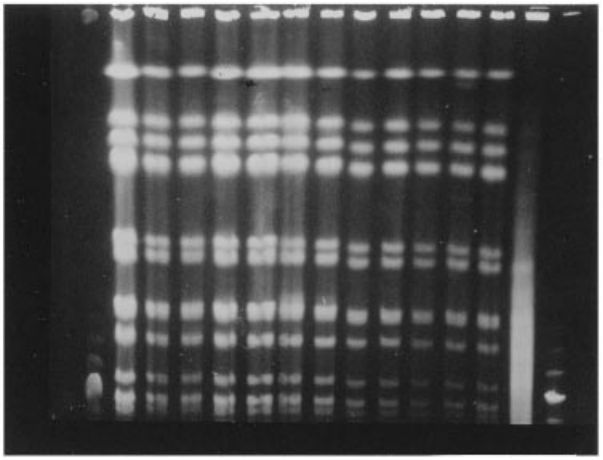

Fig. 1. DNA patterns of strains of *M. avium* complex isolated from a 75-year-old female patient (HIV$^-$) during the period January 1990 to December 1995. Lanes 2–5 are from 1990, lane 6 from 1991, lanes 7–8 from 1992, lanes 9–11 from 1994 and lanes 12–13 from 1995. Lane 14 has degraded DNA. Lanes 1 and 15 are molecular weight markers.

PFGE pattern (Fig. 1). Similarly a second patient had a strain isolated on 10 occasions over a 20-month time period, and 2 patients had a unique strain isolated 7 times over a 30 and 48-month period with no alteration in the PFGE pattern.

Amongst the Brazilian HIV positive patients, 2 had isolates with indistinguishable patterns isolated on 6 occasions over a 10 and 13-month period while another had two isolates of the same strain 22 months apart. Amongst the British HIV positive group, 3 patients had 3 isolates over 10, 26 and 26 months. All strains showed consistent PFGE patterns throughout their respective isolation periods.

Clusters

Amongst the Brazilian patients we found seven clusters (Table 3). There was no similarity amongst the English strains tested nor between the Brazilian and English strains. There was also no clustering between strains from HIV positive and HIV negative patients.

Clusters A–F involved HIV positive patients only while cluster G involved only HIV negative patients.

Cluster A was formed by 20 isolates cultured from 10 patients over a 6-year period, 1989–94. Five patients had multiple isolates from different sites, 1 patient had 2 isolates both from cerebrospinal fluid (CSF), and 4 patients had a single isolate from the CSF.

Cluster B and C consisted of 2 patients each with a single isolate from CSF during 1990–1.

In cluster D there were 2 patients: the first had isolates cultured from sputum and urine in 1992, and the second had a single isolate cultured from blood in 1994. The second patient had attended a hospital in a town 60 km outside São Paulo (hospital III in Table 3) whilst the first had attended a hospital in São Paulo.

Cluster E had 4 isolates from 2 patients attending the same hospital (hospital II in Table 3) in 1994 while cluster F had 3 isolates from 2 patients attending the same hospital in 1993.

Cluster G consisted of one isolate each from a father and daughter, living in the same house, who presented at the same hospital at the same time. A second isolate was cultured from the daughter 19 months later which had a different PFGE pattern to the first isolate. The daughter had 4 isolates of *M. chelonae* over a 2-year period. The first strain of MAC was isolated after the third *M. chelonae* isolate.

DISCUSSION

The consistency of the PFGE pattern amongst individual patient isolates from different sites (both sterile and non-sterile) and over long periods of time suggests that the patients were infected with only one strain of MAC. All the colonial growth was used in determining the PFGE pattern rather than individual colonies and so polyclonal infections cannot be completely excluded. Nevertheless, the consistency of PFGE patterns over many years in some cases indicated that re-infection of these patients by different MAC strains was unlikely.

The majority of the Brazilian patients (76%) had a unique strain of MAC causing disease suggesting an environmental route of infection. All the English patients had a unique strain.

It has been shown in Sweden [10] that BCG can protect against infection by *M. avium* and, as both Brazil (at birth) and England (at age 13 years) have an active BCG vaccination policy, it may be possible that this protects against some strains of MAC and may prevent superinfection. It may also be the reason for the low estimate ($< 1\%$ of 2628 patients) of MAC infections amongst AIDS patients in São Paulo [11] and approximately 10% in England [12].

The two main AIDS hospitals in São Paulo share the same population and patients transfer between them. Thirty-one of 67 (46%) patients attending one

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Table 3. *Details of clusters of strains with indistinguishable PFGE patterns*

Cluster	Number of patients	Number of isolates	Hospital	Time scale of isolation
A	10	20	I+II	1989–94
B	2	2	I+II	1990–1
C	2	2	II	1990–1
D	2	3	II+III	1992–4
E	2	4	I	1994
F	2	3	I	1993
G	2	2	IV	1994

Hospital I, II and IV are in São Paulo City; Hospital III is 60 Km outside. Hospital I and II are the main HIV hospitals in São Paulo and patients transfer between them.

or both of these two hospitals exhibited clustering of isolates (Table 3).

Cluster A involved 20 isolates from 10 patients over a time scale of 6 years. This finding would tend to suggest a common environmental source rather than man-to-man transmission as some of the patients had no common period of time with any other in that group. The fact that the strain had been isolated over a long time period and showed no change in the PFGE pattern would also suggest that this environmental source was free from interference from either man or the elements or that the strain was highly adaptable or persistent. As all patients attended the same hospitals (patients being transferred between them), but were not directly linked in time, the indication was that this source may have been at one of these two sites.

Two of the clusters (B+C) involved patients with only a single isolate from CSF and two other clusters (E+F) involved strains isolated over a short time period of 1 year.

There was some doubt as to the clinical significance of the single isolations from CSF (all identified as *M. avium* by DNA analysis: Genprobe) in the patients in clusters B, C and A (these were the same strains reported by Hadad and colleagues [13]) but whether the strain caused infection or whether it contaminated the specimen at some time during collection, the source remained the same.

Previous studies [2–4, 7] have suggested that water may be the most common source of MAC infection for AIDS patients and investigations are currently focusing on different water supplies and reservoirs at the main hospitals treating HIV patients in São Paulo to identify and ultimately remove this hazard for HIV positive patients.

Pulsed field gel electrophoresis has proved useful in indicating that one or more hospitals could possibly be the source of infection of MAC in HIV positive patients. PFGE analysis has also indicated that Brazilian and English patients are often infected with one strain of MAC which can persist for many years despite treatment and may prove useful in determining whether a course of treatment is effective.

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