

Genotyping of *Mycobacterium leprae* from Brazilian leprosy patients suggests the occurrence of reinfection or of bacterial population shift during disease relapse

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We performed genotyping of *Mycobacterium leprae* present in skin biopsy samples that were collected during the first and the second disease occurrences from eight leprosy patients, seven of whom were diagnosed as suffering from disease relapse. Sequence analysis of part of the *M. leprae* *rpoB*, *folP1*, *gyrB* and *gyrA* genes did not show genetic change that supported the presence of drug-resistant bacilli. However, we observed a synonymous nucleotide change at position 297 of *gyrA* among five of these patients, one presenting C to T (CgyrAT) and four presenting T to C (TgyrAC) at this position. Additional genotyping by analysis of the four short tandem repeats GAA, GTA9, AT17 and TA18 showed that the *gyrA* single nucleotide polymorphism change was accompanied by a change in short tandem repeat genotype. Our data suggest that leprosy relapse in these patients, living in an area endemic for leprosy, could be caused by *M. leprae* with a genotype different from the one that caused initial disease.

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INTRODUCTION

Since the introduction of standardized multi-drug therapy (MDT) schemes as established by the WHO in 1981 (Grosset *et al.*, 1989), a considerable reduction in the prevalence of leprosy in many endemic countries has been observed. Nonetheless, a decline of disease incidence rate has been observed only recently in some regions. As new cases are considered a major indicator of the maintenance of disease transmission (Rinaldi, 2005), this shows that

there is still a need for better measures to control leprosy (WHO, 2008). Relapsed cases could be a new source of disease transmission, but differentiating the diagnoses of disease relapse and of reactional state poses some difficulties in the field, contributing to continuing disease transmission in some situations (Linder *et al.*, 2008; Shetty *et al.*, 2005) or overdiagnosis of relapse in others.

Leprosy relapse is due to incorrect patient handling or inadequate drug intake, leading to the reappearance of drug-susceptible and/or -resistant bacilli, to persistent bacteria or to reinfection with *Mycobacterium leprae* after cure (dos Santos Damasco *et al.*, 1986; Oliveira *et al.*, 2002; Reddy & Cherian, 1991). These peculiarities of leprosy have important implications for the case management and

Abbreviations: BL, borderline lepromatous leprosy; LL, lepromatous leprosy; MB, multibacillary; MDT, multi-drug therapy; SNP, single nucleotide polymorphism; STR, short tandem repeat; VNTR, variable-number tandem repeat.

control of multibacillary (MB) leprosy, as examination and skin smears are not sensitive enough to monitor short- or long-term efficacy of chemotherapy. A reliable determination of the relapse rate is the single most important parameter to determine the efficacy of MDT (Linder *et al.*, 2008; Oskam *et al.*, 2008).

On a global level, hardly any data are available on the relative contribution of these factors to disease relapse; here, we present data obtained by genotyping *M. leprae* isolates obtained from a selection of patients that are part of a larger study for determination of the importance of relapse among leprosy patients in Brazil.

The recent discovery of genetic variability among isolates of *M. leprae*, based mainly on differences of copy numbers of a set of short tandem repeats (STRs) and a set of single nucleotide polymorphisms (SNPs), allowed recognition of bacterial strains, a better definition of species phylogeny and differentiation between relapse due to drug resistance and that due to reinfection (Cambau *et al.*, 2002; Matsuoka *et al.*, 2000; Monot *et al.*, 2008; Ramasoota *et al.*, 2000; You *et al.*, 2005). Combining genetic analysis of the genes *rpoB*, *folP1*, *gyrA* and *gyrB* with variable-number tandem repeat (VNTR) typing in samples from the first and second occurrences of disease in eight leprosy patients, we propose that reinfection may provoke relapse.

METHODS

Patients. The present study was based on evaluation of samples from eight leprosy patients as part of a project that was designed for more accurate determination of the frequency of relapse among Brazilian leprosy patients (Oliveira *et al.*, 2006–2007). Among these relapse cases, we were able to collect skin biopsy samples during both the first and the second disease episodes from eight patients, all residents of Rio de Janeiro and diagnosed at the 'Ambulatório Souza de Araújo' of the Leprosy Laboratory at the Oswaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil). These patients were not selected for any particular reason except that they were the only ones confirmed as suffering from relapse and who had clinical samples available that were taken during diagnosis of both initial disease and relapse.

All patients were initially considered as suffering from leprosy relapse based on standardized and optimized procedures for diagnosis and epidemiological criteria for definition of relapse, including diagnosis of active clinical leprosy by an expert professional and confirmation by bacteriological analysis of slit skin smears and histopathological examinations; all this after having been considered cured from the first disease occurrence after Brazilian Leprosy Program treatment regimens. For this project in particular, additional criteria as well as those traditionally used for the definition of relapse were used, including more stringent exclusion criteria, such as lack of biopsy samples and not having completed treatment. In addition, patients were diagnosed only in leprosy reference centres with diagnosis quality control and uniform procedures (Oliveira *et al.*, 2006–2007). Also, we also made sure that patients had been treated only by official treatment regimens as adopted by the National Leprosy Program. Note that, in Brazil, a particular MDT scheme ('DNDS') was used that included daily 600 mg doses of rifampicin during the initial 3 months; this was replaced by the MDT scheme as recommended by the WHO since 1986.

Clinical samples. As part of the diagnostic procedure, a slit skin smear sample was collected from four sites. A skin biopsy was collected by using a 6 mm punch and cut in half, submitting one part to fixation in 10% neutral-buffered formalin and paraffin embedding for histopathology examination, and the other to snap-freezing or immersion in 70% ethanol for PCR analysis.

Histopathology of skin biopsies. The formalin-fixed specimens were cut serially into 5 µm sections and stained with haematoxylin/eosin and Wade's stain, for visualization and counting of acid-fast bacilli using the logarithmic index (Ridley & Hilsen, 1967) and disease classification according to Ridley & Jopling (1966). Sections were viewed and captured on a Nikon Eclipse E400 microscope equipped with a Cool Snap Pro camera (Media Cybernetics) and Image Pro Plus 4.0 software.

Among the eight patients, six received MDT for MB disease during both disease stages, whilst two patients who were diagnosed in 1987 received a common treatment scheme used in Brazil before the introduction of the WHO-recommended MDT (Table 1).

Extraction of DNA. Frozen or ethanol-fixed samples [the latter after being rehydrated by immersing for 10 min in deionized water (Milli-Q; Millipore)] were cut into small pieces and macerated by using disposable sterile sticks (Scienceware; Bel-Art Products) in 1.5 ml microcentrifuge tubes. After addition of 500 µl deionized water and 100 µl zirconium beads (0.1 mm; BioSpec Products), 150–200 µl Sephadex (FlexiPrep; GE Healthcare) was added and the suspension was mixed vigorously for 10 s, centrifuged at 12 400 g for 3 min and the supernatant was removed. The tissue/beads/Sephadex/DNA-containing pellet was suspended in 200 µl washing buffer [20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 200 mM NaCl] and absolute ethanol was added to a final concentration of 60%. The mixture was mixed vigorously for 3 min and, after sedimentation by centrifugation at 14 000 r.p.m. for 3 min (rotor, Sigma 80301) and removal of the supernatant, the pellet was washed with 70% ethanol and air-dried at room temperature. To obtain DNA, 50–150 µl deionized water (depending on the pellet size) was added and, after mixing and incubation for 5 min at room temperature, centrifuged at 13 000 r.p.m. (rotor, Sigma 80301) for 1 min. The supernatant was transferred to another tube and stored frozen.

Amplification and sequencing analysis of part of *rpoB*, *folP1*, *gyrB* and *gyrA* genes. Partial *rpoB*, *folP1*, *gyrB* and *gyrA* genes were analysed by direct sequencing of PCR products generated using conditions described previously, including the use of amplification primers *MrpoBF* (5'-GGTGGTCCGCTATCAAG-3') and *MrpoBR* (5'-TTTGGGTACGGTGTTCG-3') (Ramasoota *et al.*, 2000), *folPF* (5'-TACTTACTGTAATCCCCTGTGCTG-3') and *folPR* (5'-TTGATCCTGACGATGCTGTC-3') (You *et al.*, 2005), *gyrBF* (5'-ACTGATCCTCGAAGTCTGAACTG-3') and *gyrBR* (5'-CAATGCCGT-AATAATTGCTTGAA-3'), and *gyrAR* (5'-CATCGCTGCCGGTGG-GTCATTA-3') and *gyrAF* (5'-CCCGGACCGTAGCCACGCTAA-GTC-3') (Cambau *et al.*, 2002), generating PCR products of respectively 289, 173, 187 and 178 bp.

Because of unsuccessful *M. leprae* species-specific generation of PCR products using the PCR conditions for *gyrA*, we designed a new set of primers, *gyrANF* (5'-TAAGTCAGCAGCGTCACTCG-3') and *gyrANR* (5'-GACACACAATAACGCATCGC-3'), that generated a 189 bp fragment only when *M. leprae* DNA was present (data not shown). As an additional control measure, each PCR contained at least one negative control. In addition, sequences obtained from *M. leprae* were different from those of *M. tuberculosis* and other mycobacterial species, potential sources of contamination.

Amplification was performed in a PCR mixture of 50 µl containing 0.25 mM of each dNTP, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris/

Table 1. Patient data

BI, Bacteriological index; BL, borderline lepromatous leprosy; LL, lepromatous leprosy; ?, uninformed; Brazil/MDT, 600 mg rifampicin and 100 mg dapsona day⁻¹ for 90 days; WHO/MDT, 600 mg rifampicin, 300 mg clofazimine and 100 mg dapsona month⁻¹ (supervised) and 100 mg dapsona and 50 mg clofazimine day⁻¹, 12 or 24 monthly doses. Patients B, C and E changed their clinical form; patient H is a control. Patients C, F and G came back with new lesion(s); in the remaining relapse patients (A, B, D and E), relapse reactivated the old lesion. Only patient D had a type 1 reaction; all other patients had type 2 reactions.

Patient	Sex	Age (years)	First disease episode			Treatment regimen	Second disease episode		
			Diagnosis date	Clinical form	BI		Diagnosis date	Clinical form	BI
A	M	51	23 Jul 1992	BL	?	Brazil/MDT	6 Nov 2003	BL	5+
B	M	56	19 Sep 1988	BL	4+	WHO/MDT	23 Apr 2001	LL	6+
C	M	51	16 Sep 1987	LL	6+	Brazil/MDT	17 Sep 1997	BL	4+
D	M	44	27 Aug 1987	BL	5+	Brazil/MDT	5 Jun 2000	BL	3.5+
E	M	32	25 May 1988	BL	4+	WHO/MDT	19 May 2003	LL	6+
F	F	74	17 Jan 1990	LL	5+	WHO/MDT	28 Jan 2002	LL	6+
G	M	59	17 Jan 1990	LL	4+	WHO/MDT	4 Aug 1999	LL	4+
H	M	40	2003 (control)	LL	6+	MDT (no relapse)	2003 (control)	LL	4+

HCl (pH 8.3), 30 pmol of each primer μl^{-1} and 1 U *Taq* DNA polymerase (Invitrogen) and submitting to 94 °C for 5 min, followed by a touch-down procedure consisting of denaturation at 94 °C for 45 s and annealing at 68–63 °C at 45 s, introducing a 1 °C decrease per cycle for the first six cycles. The subsequent 35 cycles were of 94 °C for 45 s, 62 °C for 45 s and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min.

After verification of PCR product quantity and quality on 3% agarose gel, amplicons were purified by using a ChargeSwitch PCR Clean-Up kit (Invitrogen) and sequenced using the same primers as those for generating the PCR fragment of each gene, using an ABI PRISM BigDye Terminator v3.1 Ready Reaction kit (Applied Biosystems).

Sequence data presented here are those obtained after using the forward and reverse primers and, in most cases, having repeated the experiment. In the case of the characterization of the *gyrA* SNP, sequence analysis was also performed after generation of the 189 bp fragment using *Pfu* DNA polymerase (Promega).

Sequences were generated on an ABI 3730 Genetic Analyzer (Applied Biosystems), introduced into SeqScape (Applied Biosystems) and compared with *M. leprae* sequences available in GenBank [NC_002677 and z14314 (*rpoB*), AL023093 (*folP1*), NC_002677 (*gyrB*) and NC_002677 (*gyrA*)]. As a control during PCR amplification and sequencing reactions, we included an aliquot of *M. leprae* DNA prepared from armadillo tissue, kindly donated by Dr Patrick Brennan (Colorado State University, Fort Collins, CO, USA).

Genotyping by VNTR analysis. For VNTR analysis, we selected four STRs, GAA, GTA9, AT17 and TA18, described at the time of study to be capable of differentiating between unrelated isolates of *M. leprae* (Truman *et al.*, 2004). For analysis of copy number, we performed direct sequencing of PCR products. In brief, amplification was performed in a 50 μl reaction sample containing 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTP mix, 2.5 U AmpliTaq DNA polymerase (Applied Biosystems) and 100 pmol of each primer for AT17 (5'-ACCCGGAATTCCTCCAAG-3' and 5'-GCCAGAAGCGTCATCTGTTC-3') and TA18 (5'-CCGCGTTA-GCATAGGCAAT-3' and 5'-AACGGTTAGGTGCGAGACCAC-3'), whilst 50 pmol was used for GTA9 (5'-CGCAGATGCAA-CGATCAC-3' and 5'-AATATGCATGCCGGTGGT-3'). After denaturing the DNA at 94 °C for 10 min, PCR was carried out in a thermocycler (Veriti 96 Well Thermal Cycler; Applied Biosystems) by

submitting samples to 40 cycles consisting of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The primer sequences and PCR conditions for GAA were described by Shin *et al.* (2000). For evaluation of PCR yield, gel electrophoresis was performed using 5 μl PCR product and amplicons were purified on QIAquick Spin Columns (Qiagen), followed by sequencing using an ABI PRISM BigDye Terminator v. 3.0 sequencing kit (Applied Biosystems) and analysis on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Sequencing of both DNA strands was performed using forward and reverse primers and sequences were analysed by using MEGA software (v. 4.0; Tamura *et al.*, 2007).

RESULTS

Patients' characteristics and diagnoses

Initially, the eight patients included in this study were considered as leprosy-relapse cases; due to the availability of clinical samples from both the first and second disease stages, these samples were submitted to genotyping. Time for diagnosis of relapse varied between 9 and 15 years, with a mean of 12 years. However, upon more careful verification of the clinical data, patient H was recognized as suffering from the type I reactional state and not from disease relapse; this patient was maintained in our study as a control.

Clinical data available from the eight patients are summarized in Table 1. All were residents of neighbourhoods of Rio de Janeiro that are known to be highly endemic for leprosy and high bacterial loads were observed in the biopsy samples taken during both disease stages, by microscopic analysis of slit skin smear samples from four different body sites, as part of the diagnosis as suffering from MB leprosy.

All of these patients had MB disease confirmed by histological examination, which allowed further differentiation into borderline lepromatous (BL) or lepromatous

(LL) forms of leprosy. Also, all were released at that time as cured, after having completed the official treatment regimen (Brazil/MDT or WHO/MDT).

Sequencing of *rpoB*, *folP1*, *gyrB* and *gyrA*

From each of the eight patients, a skin biopsy sample was available for DNA analysis from the first and second disease episodes and, from all samples, high-quality sequences could be generated for the fragments of *rpoB*, *folP1*, *gyrB* and *gyrA*. The sequences of the fragments of the first three genes were found to be identical to that of the reference DNA or to sequences described for drug-susceptible strains (data not shown), indicating that none of the formerly described drug-resistance-associated SNPs were present (Table 1) (Cambau *et al.*, 2002; Ramasoota *et al.*, 2000; You *et al.*, 2005).

In the case of the *gyrA* fragment sequence, however, we observed a transition from T to C or C to T at position 297 of *gyrA*. The presence of a T at this position was observed in at least one of the samples of the seven relapse cases, whilst both of the samples from control patient H (no relapse) presented *gyrA*-C (Table 2). When concentrating on allele frequency among the samples available, we observed a C in seven of the 16 alleles (44%), whereas T was present in the other nine (56%); a T was present on at least one occasion in seven of the eight patients (88%). Upon analysis and comparison of the SNP composition of each sample from each patient, a transition was observed among five of the seven relapse cases (71%), one (20%) carrying T and the other four (80%) C at position 297 of

gyrA; the other two patients had the allele with T in both of their samples (Table 2).

Genotyping of the four STRs GAA, GTA9, AT17 and TA18

Due to the limited amount of processed sample available for DNA analysis, we were not able to determine the copy number of the four *M. leprae* STRs in all samples from all patients. As presented in Table 2, five of the relapse cases yielded a genotype defined by either four or three of the STRs.

Identical STR copy number in both samples, although defined by copy number of only three of the four STRs, was observed in the two patients (E and F) who also presented identical *gyrA* alleles. On the other hand, a change in at least two or three of the STRs was observed in the three patients that also presented a modification of the *gyrA* SNP (Table 2).

DISCUSSION

After the introduction of MDT, occurrence of leprosy relapse is generally low (WHO, 1994). However, after the introduction of WHO surveillance in 2009, many countries informed the WHO not only of relapse cases, but also of drug-resistant cases (WHO, 2009a). The difference in relapse frequency as defined by these studies is probably due to differences in definitions of what should be considered as a relapse case and of 'cured' leprosy, and to modification of diagnostic procedures that did not always yield a clear difference between relapse and reactional states (Opromolla, 1994). According to Pannikar *et al.* (1989), 'Re-infection is one of the causes of relapses: a person who contracts leprosy probably has a larger chance of re-infection than another person, considering the former's susceptibility'. This, however, is not generally accepted, as even LL patients present a more intense immunological response under MDT treatment (Desikan, 1995). Nonetheless, we believe that, in regions that are highly endemic for leprosy, especially in the crowded peripheral areas of metropolitan cities, treatment does not present coverage as good as that in other regions, therefore increasing exposure to disease transmission and the risk of reinfection. Rafi *et al.* (1995) also considered that reinfection could be responsible for relapse.

For Ramu (1995), the incubation period is generally 'bizarre' in reinfection. Skin and nerve lesions do not correspond to the original lesions. Shaw *et al.* (2000) agreed with the long incubation period and suggested that, in high-prevalence areas such as those where the presented patients lived, reinfection should be considered.

The absence of observed genotypes that are indicative of drug resistance also suggests that treatment failure is not a major cause of disease relapse. The contribution of alternative mechanisms, such as reinfection or the

Table 2. Genotyping data

D, Sample collected at first diagnosis; R, sample collected at relapse diagnosis; ND, not determined. All samples were found to be wild-type by *rpoB*, *folP1* and *gyrB* sequencing.

Patient	Sample	SNP	VNTR
		<i>gyrA</i>	GAA/GTA9/AT17/TA18
A	D	C	ND
	R	T	13/10/13/14
B	D	T	10/10/12/ND
	R	C	12/9/13/30
C	D	T	16/11/14/22
	R	C	10/10/14/ND
D	D	T	10/10/13/15
	R	C	12/12/ND/14
E	D	T	13/10/14/15
	R	T	13/10/14/ND
F	D	T	12/ND/11/14
	R	T	12/13/11/14
G	D	T	9/ND/13/14–15
	R	C	ND
H (control)	D	C	ND
	R	C	ND

appearance of latent or minor fractions of the bacterial population, is reasonable. This is supported by our observation of the high frequency of genotype change, as defined by the SNP at position 297 of the *gyrA* gene (Monot *et al.*, 2009; WHO, 2009b). In the present patient set, we observed the presence of the T allele in 56 % of samples and 88 % of patients (only one patient had samples with only the C allele), a frequency that is considerably higher than that observed in the whole study population of relapse patients mentioned before (A. da Silva Rocha, unpublished results). The occurrence of SNPs is considered a rare event in *M. leprae*; Monot *et al.* (2005) identified four populations of *M. leprae*, defined as SNP types 1–4, circulating across continents. Our data on combined analysis of these four genotypes defined by SNPs and the nature of the *gyrA* SNP at position 297 demonstrated that the *gyrA* T allele was always of SNP type 3, whilst the *gyrA* C allele was associated either with SNP type 1 or 4 (unpublished results). This association between the conventional SNP types and the one that we defined in *gyrA* suggests that the latter may serve as an additional phylogenetic marker, representing two major *M. leprae* populations in Brazil.

Also, the high frequency of allele change in the relapse patients observed presently could be representative of a genotype that has been selected from a bacterial population that is composed of multiple isolates with different genotypes during the development of disease relapse. We observed that the genotype data, as defined by the SNP at position 297 of *gyrA*, were supported by the VNTR data (Table 2).

In summary, sequence analysis of the *rpoB*, *folP1*, *gyrB* and *gyrA* genes and of four STRs of *M. leprae* in samples from leprosy patients suggests strongly that selection of a bacterial subpopulation or reinfection, and not development of drug resistance, is mainly responsible for disease relapse. The use of molecular tools to differentiate between relapse and reinfection was suggested by Oskam *et al.* (2008), but without experimental evidence. Our data suggest that, in most cases, there is no need for the use of alternative schemes for the therapeutic treatment of these cases of relapse. However, as this study was performed on patients that were residents of Rio de Janeiro only, further studies are needed to evaluate whether this is an isolated feature of relapse patients from this region. The need for further investigation of leprosy relapse is evidenced by the observation that, in the north-east region of Brazil, 34 % of relapse patients present with multiple leprosy cases within their family (Brito *et al.*, 2005).

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