資料 1. ハンセン病診断確定のための検査

A. 皮膚組織液の抗酸菌塗抹検査[95)]

1. 皮膚組織液の採取方法

・皮膚をアルコール綿で拭いてから、周囲をつまんで持ち上げ、中心部を長さ約 5 mm, 深さ約 2 mm にメスで切開する。血液が出来るだけ混合しないように組織液をメスですくい上げ、ガラス板（プレパラート）の上に均一に円形に（直径約 5-7 mm）塗りつける。

・1 枚のプレパラートに、等間隔（できれば円形のマーク付のプレパラート）に 6 カ所分まで塗抹できる。

・塗抹後は空気乾燥させ、出来るだけ早く固定・染色を行う。

・染色まで時間がかかる場合には、火焔固定を行っておく。あるいは、空気乾燥させたプレパラートを、底に新しいホルマリン原液（37% ホルムアルデヒド）が少量入った蓋付きの小型染色ビン（Coplin jar）の中に入れて、ホルマリン蒸気で 3 分間固定後、約 80 度で 2 ～ 3 分間加熱する。

2. 採取部位

多菌型（MB）が疑われる場合	活動性病変を含む複数個所。耳朶を含むことが望ましい。
少菌型（PB）が疑われる場合	活動性病変。環状紅斑や斑の場合は、その辺縁部から採取

治療効果を確認するために定期的に菌検査を行う場合には、前回と同じ部位から採取すること。

3. 固定・染色方法

固定	軽く火焔固定
フクシン染色	石炭酸フクシン染色液 * をかけて、室温で 30 分染色
水洗	スライドグラスの裏面を流水で洗う
脱色（分別）	塩酸アルコール（70% エタノールに塩酸を 1% の割合で加える）で、過剰な染色を脱色（約 30 秒）
水洗	スライドグラスの裏面を流水で洗う
対比染色	メチレンブルーで 30 ～ 60 秒染色
水洗・乾燥・検鏡	

* 国内市販の染色液：片山化学 Code No.37-0060 チール石炭酸フクシン溶液など

4. 菌指数（Bacterial Index, BI）の算出方法

100x の対物レンズ視野中の抗酸菌数	BI
0/100 視野	0
1-10/100 視野	1+
1-10/10 視野	2+
1-10/ 視野	3+
10-100/ 視野	4+
100-1000/ 視野	5+
1000/ 視野	6+

・BI は、採取部位の中の最大値と、採取部位全体の平均値で表す方法がある。

・6 カ所採取して、各々の部位で BI が 0、3、3、2、4、2 の場合には、最大値は 4、平均値は 14/6=2.3 となる。

・治療開始時に治療方針を決める場合には最大値を用いるが、治療効果を判定する場合（BI の減少を見る）には、平均値のほうが適している。

B. 皮膚病理組織学的検査

（国立感染症研究所ハンセン病研究センターに行政検査として依頼可能）

・皮膚病変の生検によって、ハンセン病の確定診断と病型の決定を行う場合には、病変部と正常皮膚の境界部から検体を採取することが望ましい。パンチ生検でも良いが、皮内神経と皮下組織が含まれる必要がある。また、次に述べる PCR の目的のために、組織の一部を 70% エタノール液中もしくは凍結して保存することが望ましい。

・組織の固定にはバッファーホルマリンを用いる。

・病理組織診断の詳細は、文献 96, 97 を参照のこと。

C. 皮膚組織からの *M. leprae* 特異的 DNA の遺伝子増幅法（PCR）による検出

（国立感染症研究所ハンセン病研究センターに行政検査として依頼可能）

・現在、*M. leprae* に特異的でほかの抗酸菌と交差しない DNA 配列を標的とした、遺伝子増幅法（PCR）がいくつかの施設で施行されている[98)]。

・いずれの標的の場合にも高い特異性が得られており、陽性の場合には *M. leprae* が存在すると考えて良い。また高感度法として、ゲノム中に 37 コピー存在する RLEP 配列を標的に用いる方法[99)]、

リアルタイムPCR法等[100]が開発されている。
・一方、PCRの感度については、未固定新鮮組織や凍結組織の場合には抗酸菌数として10個以下であっても検出可能であり、充分に高い。凍結が出来ない場合には、生検組織の一部を純70%エタノールで固定して送付することが望ましい。
・生検組織のパラフィン切片からもPCRは実施可能であるが、一般的にはホルマリン固定・包埋の過程でDNAは細断化されやすく、200 base pairあるいは場合によっては100 base pair程度まで断片化されることも稀ではない。そのために、パラフィン切片からの場合には、長い塩基配列を増幅するPCRでは偽陰性になることもあるので、注意が必要である。

D. 薬剤耐性検査

(国立感染症研究所ハンセン病研究センターに行政検査として依頼可能)
・古典的な方法として、マウスの足蹠で検査対象の菌を増殖させた後に、一定量の菌をマウスに接種し、同時にマウスに抗菌薬を投与して菌の増殖が抑制されるかどうかを見る方法があるが、結果を得るまでに長期間を要する。
・菌のパルミチン酸代謝をアイソトープを用いて比較的短期間に測定する方法(BACTEC法[101]、Buddemeyer法[102])が開発されているが、放射線取扱いなどの問題から、実施できる機関は限られている。
・菌の遺伝子の特定部位が突然変異することによって抗菌薬耐性が生ずることが明らかになり、この変異を塩基配列決定(シーケンス)法により比較的簡単に検出することが可能となった。この検査のためには、生検材料からのDNA抽出及びPCR法による各標的遺伝子上の薬剤耐性決定領域(耐性変異箇所が集中する部位)を増幅しシーケンスして耐性変異の有無を調べる[103]。

E. 血清検査

(国立感染症研究所ハンセン病研究センターに行政検査として依頼可能)
・糖脂質であるPGL-Iの末端3糖構造は、らい菌に特徴的である。ハンセン病患者の血清には抗PGL-I抗体が含まれており、血中抗PGL-I抗体価はらい菌特異ゼラチン粒子凝集反応やELISAによって測定できる[104]。
・多菌型患者では75-100%が陽性であるが、少菌型での陽性率は15-40%程度である[105]。
・測定キット(商品名:セロディア・レプラ)は、富士レビオより発売されている。

※ハンセン病の行政検査

ハンセン病の検査は検体数が少ないため、一般検査機関では実施していない。ハンセン病研究センターでは、病理検査(作成、染色、診断)、PCR検査(組織、血液等)、血清PGL-I抗体検査、薬剤耐性遺伝子変異検査などを行政検査として無料で実施している。検査依頼は都道府県衛生主管課を通して行い、検体はハンセン病研究センターに送付する。

(問い合わせ先:国立感染症研究所ハンセン病研究センター、〒189-0002 東京都東村山市青葉町4-2-1　電話042-391-8211、http://www0.nih.go.jp/niid/lrc/)。

資料2. 皮膚科医向けハンセン病マニュアル

新規患者に対する外来診療の現状

ハンセン病は保険診療になり、新規患者の殆どは大学病院や一般病院、クリニックなどの皮膚科を受診して保険診療が行われている。現在の課題としては、①医師がハンセン病に対する知識がないために、初診から診断までに長期間を必要とする。②日本人患者の場合は、本人及び家族などに時として病気に対するいわゆる「偏見」があり、病名の告知やカルテへの病名記載等に十分な配慮が必要である。③在日外国人患者の場合は、言葉の問題(意志の疎通が不十分)、診療代金(高価で払えない)、雇用主との関係(解雇や帰国の可能性)などがある。各医療機関では患者の診療を行って頂き、検査や治療等の不明点などについて日本ハンセン病学会がサポートすることで、一般医療へのハンセン病の定着をお願いしたい[106]。

ハンセン病の診断

日本では時間をかけて患者を診察でき、検査も十分行えるため、図5のように皮疹、神経、らい菌、病理組織の4項目を総合して診断する。ハンセン病研究センターでは、病理検査、PCR検査、血清

PGL-I 抗体検査、薬剤耐性遺伝子変異検査などを無料で実施している（上記の資料 1 を参照）。

知覚異常を伴う皮疹、治りにくい皮疹、末梢神経障害などの主訴で皮膚科や、神経内科に受診し、主治医が日常見かけない皮疹、はっきりしない症状などで、疑診、診断名不明のもとに病院・大学の皮膚科へ紹介する事が多いので、これらの症状の場合は必ずハンセン病を鑑別に入れる。

皮膚症状からは、乾癬、白癬、癜風、単純性粃糠疹、脂漏性皮膚炎、尋常性白斑、サルコイドーシス、環状肉芽腫、環状紅斑、結節性紅斑、梅毒、皮膚結核、皮膚非結核性抗酸菌症などが鑑別にあがる。神経症状からは糖尿病性ニューロパチー、末梢神経炎などがあがる。

医療機関で注意して頂きたい点

未だに一部に病気に対する偏見があるため、患者に病名を告げる時には家族、職場などを含めて十二分な説明を要する。また、カルテに病名を記載する場合にも患者に説明する。

外国人においては、言語の問題もあり、意を十分に尽くせたかを繰り返し確かめる必要がある。長期間の治療のため医療費負担も増加するが、特に外国人（オーバーステイもいる）には医療費負担増、交通費、通院のための休暇などのために治療継続が不可能にならぬように工夫や支援を要する。

ハンセン病回復者に対する外来診療の現状

療養所退所者、当初から外来通院している元患者などのハンセン病回復者は、過去の「ハンセン病」歴が、他人に知られることを避ける場合がある。その理由として、①一般市民のハンセン病に対する偏見・差別、②医師・医療関係者のハンセン病あるいは後遺症、社会的背景等についての知識・経験の不十分さ、③回復者の過去の経験等から「ハンセン病」既往歴を秘匿すること、などが挙げられる。しかし回復者は高齢に近づき、再発や後遺症、さらに一般の病気に対して大きな不安を抱いている。回復者は病気の場合、以前治療の場であった療養所、ハンセン病専門診療所、特定の大学病院などに遠路であっても通院することもある。しかし、すでにハンセン病は「普通」の病気であり、一般社会で障害を持ちながら普通の生活ができるノーマライゼーション（normalization）を実現すべきであり、診療を一般医療へ統合（integration）し、安心して診療できる体制作りに取り組んでいただきたい[107]。

資料 3. 治癒を告げるときに患者に渡す指導票として、治癒後の生活への説明書を例示

治療を終えた患者さんへ

あなたの病気は治りましたので、ここで治療は終了します。

治ったあとも、治癒を確実にしていくために、しばらくの間は定期的に診察を受けてください。あとに書かれた注意事項のような症状に気づいたら、すぐに診察をうけてください。そうすることで、起こりうる障害を少なくして治していくことができます。すでに障害のある方は、これ以上悪化しないよう、注意事項の自己点検を心がけてください。

何か心配なことがあったとき、あるいは体に何か変化があったときに相談できる医師や看護師、または病院や療養所を必ず決めておいてください。家族や知り合いに、あなたが治る前のような症状の人がいたら、診察を受けるよう勧めてください。

注意事項

1. 皮膚の症状

発病したときのような症状がいつのまにか出てくるときと、急に赤い斑点やしこりが出てくるときがあります。新しい皮膚の変化に気づいたら、ハンセン病の症状なのかどうかをなるべく早く確かめてもらってください。

2. 神経の症状

顔や手足に、ピリピリ、ムズムズ、チカチカなどの変わった感じが続いたり、じんじんするしびれ感、痛み、感じが鈍くなるといった変化が出てきたら、麻痺が起こっていく可能性があり、診察を受ける必要があります。

目や口が閉じにくい、食べ物が口からこぼれやすい、しゃべりにくい、手足に力が入りにくい、物をつかみにくい、足を引きずる、タタミにつまずく、といった症状が出たらすぐに診察をうけてください。

3. 現在の麻痺

現在(　　)

の部分で感じが鈍くなっていますので、やけどや傷ができていないかどうか、毎日確かめてください。とくに足の趾、足の裏に注意してください。

(　　)

の部位で、筋肉を動かす力が低下しています。これ以上悪くならないよう、運動療法を続けてください。装具を使っている方は、傷をこしらえていないかどうかを調べてください。

4. 目の症状

目に異常を感じたら、すぐに診察を受けてください。たとえば、目が痛む、まぶしい、涙が多い、涙がこぼれやすい、視力の低下、まぶたが閉じにくい、目が赤い、目やにが出る、さかまつげ、など。

まぶたが閉じない方は、目に傷をつくらないよう保護メガネを使用し、医師の指示に従って目薬(点眼薬)や目の軟膏を使ってください。

資料4. サリドマイド

ENLに保険適応になっているサリドマイドはサレド®カプセル(藤本製薬)のみである[12,108]。50 mgカプセルと100 mgカプセルがある。

炎症を起こすサイトカインというタンパク質の産生を抑えたり[109,110]、好中球(白血球の一つ)の血管内皮細胞への接着を抑える[111]。また、抗体の産生を抑えることにより、免疫複合体(抗体、抗原、補体)が誘起する炎症反応を抑制する作用があると考えられている[112]。これらの作用でENLに対して効果が出現するとされている。

1) サリドマイド使用にあたって

サリドマイド製剤安全管理手順(Thalidomide Education and Risk Management System: TERMS®)への登録が必要である。サリドマイドは、過去に妊娠中の女性が服用することにより胎児に重大な障害又は死産、流産を引き起こした。胎児への暴露を避けるため、本剤の使用についてはTERMS®を遵守する必要がある。TERMS®を遵守できない場合には本剤を使用することはできない。

TERMSは処方医師や責任薬剤師などの規定があり、産科婦人科医師との連携など、サリドマイド使用にあたっての遵守が定められている。詳しくはTERMSについてのホームページ(http://www.fujimoto-pharm.co.jp/jp/iyakuhin/thalido/TERMS-3.pdf)を参照されたい。

2) ENLに対してサリドマイドが使用できる患者の要件

a. サリドマイドの催奇形性及びTERMS®に関する教育を受け、理解度が確認されていること。

b. TERMS®の遵守に同意が得られていること。

c. 原則として、薬剤管理者よりTERMS®の遵守に同意が得られていること。

d. 妊娠する可能性のある女性患者は、本剤服用開始予定日の4週間前及び2週間前の妊娠検査が陰性であること、又は同意日の4週間前から性交渉をしていないことの確認がされていること。

3) ENLに対してサリドマイドを処方できる医師、調剤できる薬剤師、医療機関の要件

a. 医師

以下の全てに該当することが必要。

(1) サリドマイドの催奇形性及びTERMS®に関する情報提供を受け、理解度が確認されていること。

(2) TERMS®の遵守に同意が得られていること。

(3) 産科婦人科医師と連携を図ることに同意が得られていること。

(4) 研修医ではない(ただし、日本皮膚科学会認定皮膚科専門医は除く)こと。

(5) 次のいずれかに該当すること。

・日本皮膚科学会認定皮膚科専門医又は国立ハンセン病療養所に勤務する常勤医師

・日本皮膚科学会認定皮膚科専門医又は国立ハンセン病療養所に勤務する常勤医師と連携が可能である医師

・過去にTERMS®に登録の上、本剤のENLに対する処方経験を有する医師

・上記以外にあっては、TERMS委員会にて評価し、藤本製薬株式会社が登録して差し支えないと判断した医師

b. 責任薬剤師

以下の全てに該当することが必要。

(1) サリドマイドの催奇形性及びTERMS®に関する情報提供を受け、理解度が確認されていること。

(2) TERMS®の遵守に同意が得られていること。

c. 対象医療機関

以下の全てに該当することが必要。

(1) 本剤投与に関して、緊急時に十分対応できる設備を有する医療機関。
(2) 本剤を院内にて調剤することが可能である医療機関。

4) サリドマイドの服用参考例（図4、表6）

5) 使用上の注意

a. 次の患者には慎重に投与する

(1) 深部静脈血栓症のリスクを有する患者。サリドマイドにより症状が発現、増悪することがあるため。
(2) HIVに感染している患者。（サリドマイドによりHIVウイルスが増加することがあるため。

b. 重要な基本的注意

(1) サリドマイドには催奇形性（サリドマイド胎芽病）があるので、妊娠する可能性のある女性に投与する際は、少なくとも投与開始予定の4週間前、2週間前及び投与直前に妊娠検査を実施し、検査結果が陰性であることを確認後に投与を開始すること。また、妊娠していないことを定期的に確認するために、間隔が4週間を超えないよう妊娠検査を実施する。
(2) サリドマイドの安全管理を確実に実施するため、1回の最大処方量は12週間分を超えない。
(3) サリドマイド投与開始から投与終了4週間までは、精子・精液の提供をさせないこと。
(4) サリドマイドの抗血管新生作用が創傷の治癒を阻害する可能性があることから、外科手術等を実施した場合、適切な期間サリドマイドの投与を中止すること。
(5) 傾眠、眠気、めまい、徐脈、起立性低血圧が起こることがあるので、サリドマイド投与中の患者には自動車の運転等危険を伴う機械の操作に従事させないように注意すること。
(6) サリドマイドのENLに対する使用にあたっては「らい性結節性紅斑（ENL）に対するサリドマイド診療ガイドライン」を参照の上治療を行うこと。

資料5. 接触者検診と予防服薬について

ハンセン病の新規患者報告の多い国においては、以下に述べる接触者検診と予防服薬が疾病対策のひとつとして議論されており、WHOのテクニカルレポートTRS 968（2012年）[13]にもこの問題が取り上げられている。わが国ではハンセン病の新規発生はほぼゼロとなり、感染・発症のサイクルはなくなっているため、ほとんどの場合にこれらは不要と考えられるが、参考のために紹介することとした。

接触者健診 Contact surveillance

*M. leprae*の宿主となり得るのは、主にヒトである。そのためハンセン病は、ハンセン病患者との直接的・間接的接触があって初めて発症すると考えられている。しかし、感染していても無症候性の場合、長い潜伏期間があることから、感染源を同定するのは容易でない。

これまでの接触者健診は、患者の家族・同居者を中心に行われてきた。同じ屋根の下に住むことで感染そのもののリスクが高まる他、患者と類似した遺伝的要因があれば*M. leprae*に対する抵抗力が低下あるいは欠損している可能性、生活習慣・住宅環境を共有していることで感染機会も高まっていることがその背景としてある。

*M. leprae*の感染リスクは、通常と比べMB患者と一緒に住んでいる場合で約5～10倍、PB患者と一緒に住んでいる場合で約2倍に上昇するといわれている[113,114]。さらに、大人よりも子供の方がそのリスクが高くなる。しかし、一方で、新規患者の約70%には、特に家族歴はないといわれている。まだ診断を受けていない未治療のMB患者や無症候性の菌保有者との接触が、いまだにハンセン病蔓延地域での新規発症がなくならない主な要因と考えられている。

そこで、最近の動向として、ハンセン病の新規発症があった場合、接触者健診を患者の家族・同居者から、患者の家族・同居者との接触者と範囲を拡大して行うといった動きがある。しかし、この場合、どこまでを接触者とするか、どこまで予防服薬をさせるか、また患者のプライバシーの侵害などの複雑な問題をはらむため、未だ一致した見解は得られていない。今後、きちんとした議論がなされ、国際的な基準の確立が待たれる分野である。

予防服薬　Chemoprophylaxis

ハンセン病に対する予防服薬は、長年にわたって試行錯誤が繰り返されてきた。1960年代～1970

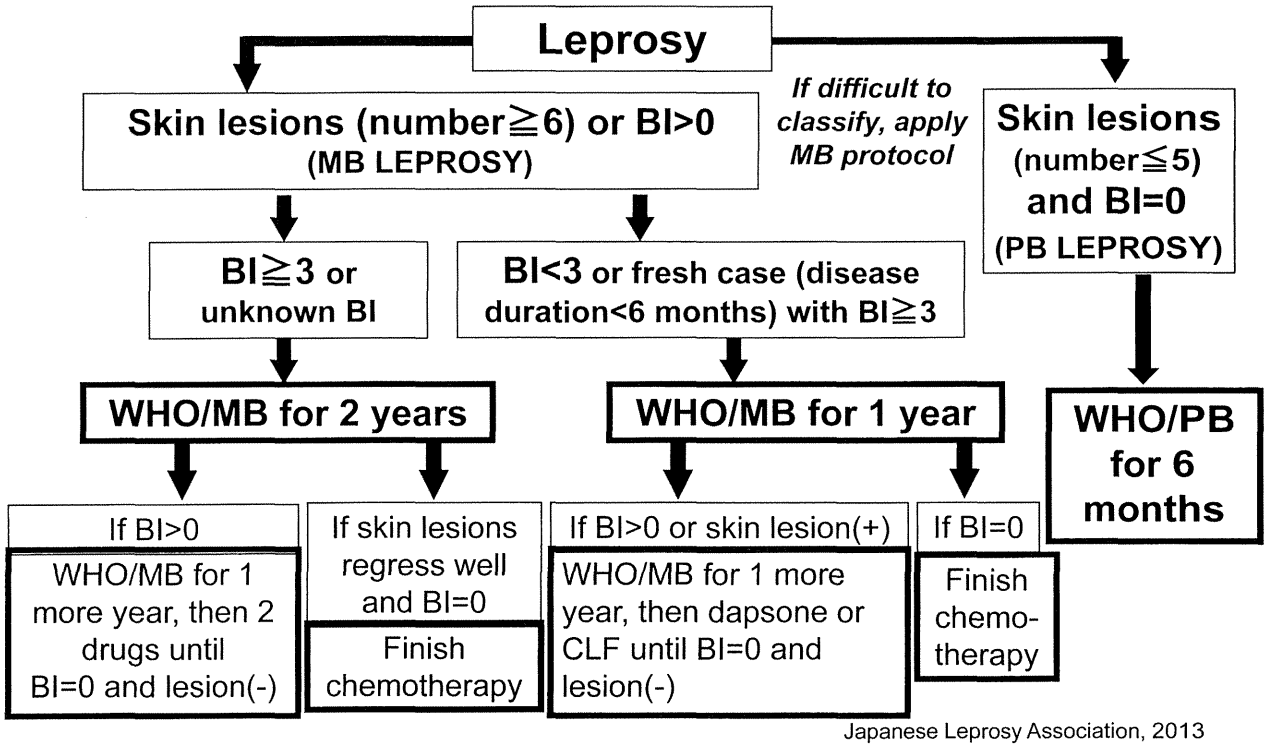
年代まではダプソン dapsone またはアセダプソン acedapsone の投与が試験的にされていたが、耐性菌の出現が懸念されたため、最近ではリファンピシン rifampicin 単剤投与が行われるようになっている。バングラデシュで行われた 2002 年から 4 年間の追跡調査では、リファンピシン単剤 1 回投与で、新規発症は始めの 2 年間は 57% [95% CI: 33-72%] に減少したが、その後 2 年と 4 年では発症に差が認められなかったと報告している[115]。他の調査も総合すると、予防服薬をすることで約 60% 程度の新規発症の減少が認められるが、往々にしてその効果は中程度に留まり、期間も限定的であるとされている[116]。

よって、現在のところ、予防服薬は家族・同居者への個人レベルの予防としては適しているといえるが、地域住民にまで行うにはいまだエビデンスに欠ける。低～中蔓延国では、その医学的効果ならびに費用対効果も懸念される。また、家族・同居者に対しても、強制するものではなく、あくまで個人の意志に基づいて行われるべきである。

資料 6. 日本におけるハンセン病の標準的化学療法（図表）の英訳

図 3 と表 3 の英訳を、Suppl. Fig. 1 及び Suppl. Table 1 として示す。

Suppl. Fig. 1. Standard therapy of leprosy in Japan (2013)



Suppl. Table 1. Standard treatment protocol of leprosy in Japan

	MB with BI ≥ 3 or unknown BI		MB with BI < 3 or fresh MB (less than 6 months after the disease onset) with BI ≥ 3		PB	
Therapeutic drugs and administration methods	(WHO/MDT/MB) Rifampicin 600mg/Once a month Clofazimine 300mg/Once a month Dapsone 100mg/Every day Clofazimine 50mg/Every day				(WHO/MDT/PB) Rifampicin 600mg /Once a month Dapsone 100mg /Every day	
Standard duration	2 years		1 year		6 months	
Condition after the completion of standard duration	BI > 0	BI=0	BI > 0	BI=0	Active lesion (+)	Active lesion (-)
Maintenance therapy	MDT/MB for 1 year and follow up with 2 drugs or more until BI=0 and active lesion (-)	No drug, follow up	MDT/MB for 1 year	No drug, follow up	Dapsone or Clofazimine until active lesion (-)	No drug

BI: Bacterial Index, MB: Multibacillary leprosy, PB: Pausibacillary leprosy
Definition of MB and PB cases is based on WHO/MDT (2010).
Japanese Leprosy Association, 2013

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Drug and Multidrug Resistance among *Mycobacterium leprae* Isolates from Brazilian Relapsed Leprosy Patients

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Skin biopsy samples from 145 relapse leprosy cases and from five different regions in Brazil were submitted for sequence analysis of part of the genes associated with *Mycobacterium leprae* drug resistance. Single nucleotide polymorphisms (SNPs) in these genes were observed in *M. leprae* from 4 out of 92 cases with positive amplification (4.3%) and included a case with a mutation in *rpoB* only, another sample with SNPs in both *folP1* and *rpoB*, and two cases showing mutations in *folP1*, *rpoB*, and *gyrA*, suggesting the existence of multidrug resistance (MDR). The nature of the mutations was as reported in earlier studies, being CCC to CGC in codon 55 in *folP* (Pro to Arg), while in the case of *rpoB*, all mutations occurred at codon 531, with two being a transition of TCG to ATG (Ser to Met), one TCG to TTC (Ser to Phe), and one TCG to TTG (Ser to Leu). The two cases with mutations in *gyrA* changed from GCA to GTA (Ala to Val) in codon 91. The median time from cure to relapse diagnosis was 9.45 years but was significantly shorter in patients with mutations (3.26 years; $P = 0.0038$). More than 70% of the relapses were multibacillary, including three of the mutation-carrying cases; one MDR relapse patient was paucibacillary.

There is no doubt about the efficiency of the currently used multidrug therapy (MDT) scheme for treatment of leprosy, as demonstrated by the strong decrease in disease prevalence since its implementation and the low number of reported relapse cases (18). However, there has been a scarcity of in-depth studies of relapse occurrences in recent decades (27). As is known, differentiating diagnosis of relapse and reactional states poses some difficulties in the field, being responsible for under- or overdiagnosis of both disease stages. This is important because undiagnosed relapse cases could contribute to continuing disease transmission. In addition, hardly any data on the contribution of emergence of drug-resistant strains of *Mycobacterium leprae* to leprosy relapses exist.

Diaminodiphenylsulfone (DDS), also called dapsone, was the first drug to be effective against leprosy worldwide, and the first cases of resistance to dapsone were detected in 1964 and involved two single nucleotide polymorphisms (SNPs) in the gene *folP1*, located in codons 53 and 55 (8, 9, 14, 29). Rifampin is the key component of the standard multidrug regimen used for treatment of leprosy, and it has been shown that PCR-based DNA sequence analysis of the *rpoB* gene of *M. leprae* was in full concordance with rifampin susceptibility testing in the mouse footpad system (17, 30). In addition to dapsone and rifampin, ofloxacin is also used for leprosy treatment and is a quinolone with an action mechanism based on interaction with DNA gyrase (2); SNPs in *gyrA* and *gyrB* confer resistance or hypersensitivity to quinolones (15). Although there is not yet an official definition of multidrug resistance (MDR) in leprosy, in parallel with tuberculosis, we adopt this terminology when we encounter resistance to rifampin and one other drug of the standard MDT regimen.

Emerging drug resistance has been observed among many diseases caused by bacteria, and this could pose a challenge for the treatment of leprosy, a neglected disease with a minimal therapeutic arsenal (22). Brazilian studies show relapse rates below 1% (12, 26), and drug resistance does not seem to be an important problem in the country (10, 21). Nonetheless, a pilot project for optimal detection of relapse and the contribution of drug resistance among leprosy patients of five states in Brazil was started in 2006 (26), in parallel with the initiative of the World Health Organization (WHO) to perform global surveillance of drug resistance in leprosy in 2008 (36).

MATERIALS AND METHODS

Study design and patients. A prospective study for detection of relapse in leprosy patients was designed for more accurate determination of the frequency of relapse by drug resistance among Brazilian leprosy patients, based on evaluation of DNA sequencing in samples from 145 leprosy patients, collected during 2006 to 2008, in five states to which leprosy is highly endemic, including Rio de Janeiro, Espírito Santo, Amazonas, Pará, and Ceará (26). All patients were examined by experienced dermatologists in six state reference units in order to guarantee the quality and uniformity of these procedures. Leprosy relapse detection was based on standardized and optimized diagnostic procedures and criteria for definition of relapse (4) and with inclusion criteria being suffering from active clinical lesions of leprosy, as confirmed by smears and histopathological exams, being considered cured from the first disease course after having undergone the

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Brazilian Leprosy Program treatment regimens. Regarding the official treatment regimens from the National Leprosy Program, it is necessary to clarify that Brazil, before adopting the WHO MDT treatment schemes in 1986 (24 doses), used a scheme called DNDS that consisted of 90 daily doses of 600 mg of rifampin, followed by daily doses of 100-mg dapsone monotherapy, up to 5 years and until slit skin smears became acid-fast bacillus (AFB) negative. For each relapse case, a control case, being a new leprosy case of the same sex, clinical form, and municipality of residence and belonging to the same treatment cohort, was selected from the National Information System for Notification of Diseases (SINAN) and enrolled for clinical and laboratory examinations.

This study was approved by the Ethical Committee of Research of the Federal University of Rio de Janeiro (HUCFF/UFRJ) (no. 019/06). Written consent was obtained from individual subjects by having them sign a standard Brazilian form before being admitted in the study. The epidemiologic, clinical, and demographic data collected from each participant center were stored and analyzed at the UFRJ, using the software program Strata 9.0.

Slit skin smear and histopathology of skin biopsy specimens. As part of the diagnostic procedure, slit skin smear samples were collected from four different body sites at the time of diagnosis of disease relapse, and a skin biopsy was done according to standard recommendations (4). After being cut in half, one part of the skin biopsy specimen was prepared for histopathology exam, and the other half was immersed in 70% ethanol for genetic analysis. In order to standardize the histopathology procedure and reporting of results, a consensus meeting was held with the histopathologists from the participating reference centers and a standard protocol was elaborated.

Extraction of nucleic acids. For extraction of nucleic acids, the ethanol was removed from the biopsy specimen, and the latter was rehydrated, cut into small pieces, and subjected to DNA extraction and purification using the Qiagen DNeasy Blood & Tissue kit (Invitrogen do Brasil). In brief, 180 μl of ATL buffer and 20 μl of proteinase K from the kit were added to the biopsy specimen and subjected to vortex mixing, and after overnight incubation at 56°C, DNA was purified using a spin column from the kit as described by the manufacturer.

Amplification and sequencing analysis of part of *rpoB*, *folP1*, *gyrB*, and *gyrA*. Part of the genes *rpoB*, *folP1*, and *gyrA* was analyzed by direct sequencing of PCR products generated using conditions described previously, using the amplification primers M*rpoB*F and M*rpoB*R (31), *folP*1F and *folP*1R (38), and *gyrA*NF and *gyrA*NR (5, 11, 23) and using touchdown amplification conditions described previously (11). Each PCR mixture contained at least one negative control, and after verification of PCR product quantity and quality on a 3% agarose gel, amplicons were purified using the ChargeSwitch PCR clean-up kit (Invitrogen do Brasil) and sequenced using the same primers as those for generating the PCR fragment of each gene, using the ABI BigDye 3.1 Terminator ready reaction kit (Applied Biosystems do Brasil). For characterization of the *gyrA* SNP at position 297, we followed the approaches described previously (11). Sequences were generated on an ABI 3730 genetic analyzer (Applied Biosystems) and compared with the *M. leprae* sequences NC002677 and z14314 (*rpoB*), AL023093 (*folP1*), and NC002677 (*gyrA*), available at GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez/), and for SNP analysis, sequences were introduced into SeqScape. Control DNAs were purified from *M. leprae* NHDP-63 (kindly donated by Patrick Brennan, Colorado State University), and the plasmids *folP*101, -102, and -103 (a gift from Dianna Williams, Louisiana State University). Following the recommendations of the WHO Global Surveillance of Drug Resistance in Leprosy Protocol, samples with mutations suggestive for drug resistance as determined at FIOCRUZ were send for blind sequence evaluation to M. Matsuoka at the Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

In order to verify the presence of inhibitors in the processed biopsy samples, 23 biopsy samples that gave no PCR product in the *gyrA* system were subjected to a reconstitution experiment to verify the presence of eventual PCR inhibitors. For this, these samples were submitted to the PCR using the same conditions as described above, except for the addition of 1.5 ng of NHDP-63 DNA to each PCR mixture. For evaluation of inhibition, the PCR signal for reactions with biopsy sample was compared to that for reconstituted samples without biopsy sample and two positive controls (without reconstitution), as for the earlier PCR experiments. We used three interpretation criteria, with results having either (i) similar or (ii) less signal than the control samples or (iii) no amplification at all.

TABLE 1 Characteristics of relapse patients[a]

Variable	Value for patients
Sex [no. (%) of patients]	
Male	105 (72.4)
Female	40 (27.6)
Clinical form [no. (%) of patients]	
MB	102 (70.3)
PB	43[b] (29.7)
Treatment regimen of first disease course [no. (%) of patients]	
MDT MB 12[b]	22 (15.1)
MDT MB 24	57 (29.3)
MDT PB	31 (21.3)
ROM[c]	2 (1.3)
DNDS + MDT 24	7 (4.8)
DNDS	14 (9.6)
Substitutive regimen[d]	12 (8.2)
Close contact with leprosy case [no. of patients/total (%)]	38/120 (31.6)
Age [median value, yrs (SD)]	47.5 (10.5)
Time from cure to relapse [median value, yrs (SD)]	
All cases	9.45 (4.95)
DR-MDR cases	3.26 (2.62)
Bacillary index [median value (SD)]	2.85 (1.87)

[a] n = 145 relapse cases.
[b] Multidrug therapy with the number of doses between 12 and 24.
[c] ROM, rifampin plus ofloxacin plus minocycline.
[d] Replacement of rifampin by ofloxacin or of dapsone by clofazimine or combined use of rifampin and clofazimine without dapsone. Statistical analysis was performed using Fisher's exact test.

RESULTS

General patient data. Clinical data confirmed that 145 patients suffered from leprosy relapse, and their characteristics are summarized in Table 1. All of these patients presented the inclusion criteria, having been considered cured after completing the official treatment regimen (Brazil/DNDS or WHO/MDT) and having developed a second course of active leprosy disease, as confirmed by bacilloscopic and histopathological examination, also allowing the classification of the clinical form. Most cases (70%) were multibacillary (MB), while the rest were paucibacillary (PB); among the latter, the majority (88%) were borderline tuberculoid. The bacilloscopy index (BI) of the MB cases ranged between 0.25 and 6.0, with an average of 2.85. The average incubation period from cure to relapse diagnosis was 9.45 years, ranging between 1.5 and 25 years, and was significantly shorter in the four resistant cases (3.26 years; $P = 0.0038$), ranging between 1 month and 6.6 years. In addition, two of these cases had been subjected to more than one treatment regimen. Gender analysis showed that males

TABLE 2 Results of DNA sequencing and mutations in the *folP1*, *rpoB*, and *gyrA* genes of *M. leprae*[a]

Gene	No. (%) of samples with conclusive sequencing for IBCAT of[b]: 0	1	2	*P* value[c]	No. (%) of samples with[d]: SNP present	No SNP
folP1	4/60 (6.6)	22/41 (53.7)	31/44 (70.5)	<0.001	3 (5.3)	54 (94.7)
rpoB	5/60 (8.2)	19/41 (46.3)	33/44 (75.0)	<0.001	4 (7)	53 (93)
gyrA	18/60 (29.5)	27/41 (65.9)	32/44 (72.7)	<0.001	2 (2.6)	75 (97.4)

[a] Total no. of relapsed cases: 145.
[b] IBCAT, categorized bacillary index (0, IB = 0; 1, IB > 0 and < 3+; 2, IB > 3+).
[c] Each *P* value is for the three groups as calculated with Fisher's exact test.
[d] SNPs are drug resistance related only.

(72.4%) were more affected than females (26.4%), and the median age of all cases at time of diagnosis of relapse was 47.5, ranging from 13 to 96 years (Table 1).

Upon analyzing treatment regimens, we observed that most of the MB first-disease cases had been treated with the MDT/WHO scheme, having completed either 24 or 12 doses, as adopted by the National Program; some MB cases, however, instead of having received 12 doses, had been subjected to one of the following: (i) a number of doses that varied between 12 and 24, as a consequence of the reduction of MDT treatment from 24 to 12 doses, as recommended by WHO, (ii) the DNDS regimen only, or (iii) the DNDR regimen and 24 doses of the MDT scheme. The last situation occurred in a considerable number of cases (Table 1) and also in three of the cases with drug-associated mutation (see Table 3).

Ampification and sequencing of *rpoB*, *folP1*, and *gyrA*. The results of amplification and DNA sequencing of part of the genes for *folP1*, *rpoB*, and *gyrA* are presented in Table 2. A total of 92 samples (63.4%) yielded sequence results for at least one gene fragment, and informative sequences were obtained for 57 cases (61.9%) for *folP1*, 57 cases (61.9%) for *rpoB*, and 77 cases (83.6%) for *gyrA*. Drug-associated SNPs were detected among 3 of the 57 samples for *folP1* (5.3%), 4 of the 57 samples for *rpoB* (7%), and 2 of the 77 samples for *gyrA* (2.6%). In addition, a statistically significant difference was observed between BI and sequence results (Table 2).

Among the 23 biopsy samples that were tested for the presence of PCR inhibitors, 21 had positive BI and 1 sample had a BI of 0, and for another sample we had no information on the BI. Among these samples, eight (35%) showed a PCR signal similar to that of the positive controls, nine (39%) give weaker signals, and six (26%) gave no PCR product at all (data not shown), meaning that 65% of this sample selection showed some level of PCR inhibition for *gyrA* (data not shown). We did not test PCR inhibition in the PCR systems for *rpoB* and *folP1*.

Regarding the nature of the SNPs, the three changes in *folP1* were always a transition from CCC to CGC in codon 55 (Pro to Arg); in the case of *rpoB*, all occurred at codon 531, with two presenting a change from TCG to ATG (Ser to Met), one from TCG to TTC (Ser to Phe), and one from TCG to TTG (Ser to Leu); the two cases with mutations in *gyrA* presented a transition from GCA to GTA (Ala to Val) in codon 91. On the patient level, mutations suggestive of drug-resistant strains were observed in four cases, including one patient with a mutation in *rpoB* only, suggesting monoresistance to rifampin, one case with SNPs in both *folP1* and *rpoB*, suggestive of multiple drug resistance (MDR) for rifampin and dapsone, and two cases with mutations in *folP1*, *rpoB*, and *gyrA*, strongly suggestive of MDR against the three main antileprosy drugs (Table 2). The sequence results obtained with the four cases that presented SNPs at Fiocruz were confirmed by M. Matsuoka in the Leprosy Research Center, National Institute of Infectious Diseases, Japan.

In addition to the drug resistance-associated SNP in *gyrA*, this gene fragment also presented a synonymous SNP in position 297, and as demonstrated in Table 2, among the 77 samples that were sequenced, 57 (74.03%) presented the C allele, while 20 samples (25.97%) had the T allele. The four cases with drug resistance-associated SNPs presented the C allele.

Characteristics of patients with mutated strains. Table 3 summarizes the data from DNA sequencing and mutations found in the four patients, three being MDR. The first three cases were residents in former colonies for leprosy patients in the Amazon region, and all were subjected to the aforementioned DNDS regimen in their first disease episode. Case one, from the state of Para, presented the most characteristic resistance features, since his treatment failed in a second treatment course (first the DNDS/Brazil regimen and then two courses of MDT/WHO). His last treatment course ended in 2007, while he presented active lepromatous leprosy (LL) disease in the beginning of 2008. The two cases from Amazonas had also undergone two complete treatment schemes before diagnosis of relapse, and their clinical features provoked the suspicion of drug resistance (DR). For the first of these two patients, a mutation on *gyrA* was found, and we discovered that at the end of the

TABLE 3 Summary of drug-resistant relapse cases[a]

Case no. (state[b])	Sex[c]	Age (yrs)	CF	BI	His	Treatment	IP	Result of DNA sequencing[d]: *folP1*	*rpoB*	*gyrA*
1 (PA)	M	49	LL	5	LL	DNDS-MDT 24	1 mo	55, CCC → CGC (Pro → Arg)	531, TCG → ATG (Ser → Met)	No mutation
2 (AM)	M	63	LL	4,5	LL	DNDS-MDT 24	3.2 yrs	55, CCC → CGC (Pro → Arg)	531, TCG → TTC (Ser → Phe)	91, GCA → GTA (Ala → Val)
3 (AM)	M	46	BL	3,5	LL	MDT 24	3.3 yrs	No mutation	531, TCG → TTG (Ser → Leu)	No mutation
4 (ES)	M	38	BT	6,6	BT	MDT 12	6.6 yrs	55, CCC → CGC (Pro → Arg)	531, TCG → TTC (Ser → Phe)	91, GCA → GTA (Ala → Val)

[a] CF, clinical form; BI, bacilloscopic index; His, histopathologic diagnosis; IP, incubation period of relapse; Pro, proline; Arg, arginine; Ser, serine; Phe, phenylalanine; Leu, leucine; Met, methionine; Ala, alanine; Val, valine; MDT/MB 24 (WHO), rifampin (RMP) (600 mg/month) + clofazimine (CLZ) (300 mg/month) (supervised) + DDS (100 mg) + CLZ (50 mg/day), during a period between 12 and 18 months; DNDS (Brazil), rifampin (RMP) (600 mg/day, 90 days) + dapsone (DDS) (100 mg/day up to 5 years until AFB negative). Case one received three treatment courses.
[b] PA, Pará; AM, Amazonas; ES, Espírito Santo.
[c] M, male.
[d] Codon number, mutation.

second scheme (MDT/WHO), this patient also had received ofloxacin but not according to a standard treatment scheme. Finally, the fourth and quite intriguing case from the state of Espírito Santo, southeast Brazil, was diagnosed with borderline lepromatous leprosy (BL) during first disease, presenting positive BI, but was negative in the second disease course, 7 years later, and classified as suffering from the borderline tuberculoid leprosy (BT) form. Although this patient presented a resistance-associated SNP in *gyrA*, we found no history of treatment with ofloxacin, and eventual reinfection by an ofloxacin-resistant strain acquired from his relatives could be possible. Among the 145 patients, 31% informed that they had relatives that were diagnosed for leprosy within 5 years before relapse diagnosis (Tables 1 and 3).

DISCUSSION

The efficiency of the WHO MDT scheme for leprosy treatment is supported by the dramatic decreases in disease prevalence and the low relapse rates in short and medium time frames. Therefore, relapse has not been considered a problem, and organization of studies of this disease characteristic was somewhat neglected, leading to the recent WHO initiative to organize a resistance surveillance project for relapse cases, 26 years after having started MDT. This was possible due to the development and standardization of molecular genotyping procedures of genes associated with drug resistance (5, 16, 23, 24, 38, 39).

After the introduction of relapse surveillance by the WHO, many of the countries of endemicity reported leprosy relapses. In addition, evaluation of the contribution of drug resistance under an international network has been implemented, focusing on MB relapse cases (36). For good-quality data on relapse rates, in addition to laboratory technology, uniformity of clinical criteria relapse diagnosis is important and needs to be standardized within and among countries. Although it not so difficult to diagnose leprosy relapse during the late MB disease form, recognition of relapse is not so easy during early disease, especially in the borderline spectrum cases of disease and under field conditions (19, 20).

In the present study, 29.7% of the relapses were PB cases, 88.3% of these being BT, and this after clinical examination by experienced leprologists and histopathological confirmation by three different pathologists. This was also the case for the BT patient that presented mutations in the *M. leprae* genes *rpoB*, *folP*, and *gyrA*, and possibly this patient, although being MDR, presented this disease form because he was diagnosed very soon after developing relapse, had a better immune host defense response, or had a different strain causing relapse, either by reinfection or strain selection, as observed in a considerable number of relapse cases in another study (11). On the other hand, selection of a particular part of the *M. leprae* population that caused first disease as being responsible for relapse is in accordance with the work of Toman in 1981 (35) and Colston et al. in 1987 (7), raising the possibility that "persistent" *M leprae* could cause relapse in a large proportion of patients, the persistent bacilli presenting a metabolic state that resists the drug without the presence of drug-associated mutations, also suggested by Pattyn (28) and Balagon et al. (1).

Suspicion of DR or MDR in leprosy is raised mainly because of maintenance of clinical symptoms, with or without evaluation of the presence of bacilli in skin smears and confirmation by growth in the footpads of mice fed with antibiotics. Bacteriological analysis by smear microscopy is not always reliable, however, and advances in the elucidation of molecular events responsible for drug resistance in mycobacteria have allowed the development of alternative tools for drug resistance screening (6). However, due to the need of technical expertise and specialized equipment, this technique is executed in a limited number of centers in Brazil (38). Nonetheless, SNP detection seems to be more sensitive and is certainly much quicker for detecting DR than the mouse footpad-based technique (23, 33). In two very recent studies, sequence analysis for DR in Latin American leprosy patients was reported, the first report presenting two cases with SNPs in *rpoB* and one case in *gyrA*, suggestive for resistance against rifampin and ofloxacin, respectively, among 38 Mexican cases, suggesting the possible reemergence of DR leprosy in a country where leprosy was considered eliminated (22). The second study included 230 mostly new leprosy cases, two being from Uruguay, 10 from Bolivia, 23 from Brazil,.and 197 from Venezuela. Only two relapse cases presented SNPs in the three genes studied, one from Venezuela in *folP1* and one from Brazil in *folP1* and *rpoB* (34).

The mutations observed presently all have been reported in studies in other countries, including the changes in codon 531 of *rpoB*, causing an amino acid change from Ser to either Met (n = 1), Phe (n = 2), or Leu (n = 1), the SNP observed in *folP1* in codon 55 (n = 3), causing the change of Pro to Arg, and the mutation at codon 91 of *gyrA* (n = 2), leading to a change from Ala to Val. These SNPs had been described earlier in several reports, including those of Honoré and Cole (17), Williams et al. (37), Cambau et al. (6), and Gillis and Williams (14). In addition to the nonsynonymous SNP in *gyrA*, we observed the allele distribution in the relapse cases of a recently observed synonymous SNP at position 297 of *gyrA* (11, 25), showing that 74% of the cases carried *M. leprae* of the SNP type *gyrA* C at position 297. Our own previous data (11) and the recently published data from Singh et al. (34) showed the correlation of the synonymous SNP *gyrA* 297T type with the SNP type 3 and of SNP *gyrA* C with type 1 or 4 defined by Monot et al. (25). Previous data showed the higher frequency of the SNP3 type in southeast Brazil (13) and Latin America (34), and the prevalence of the SNP *gyrA* C could be due to sampling from other regions of Brazil.

We did not obtain PCR products and good-quality sequences from all samples, and this is due partly to the inclusion of samples with low or zero bacterial counts and to the presence of PCR inhibitors, as evidenced by the reconstitution experiment. Indeed the presence of PCR inhibitors in skin biopsy samples has been described before (32).

The significant difference between the period of time between first disease and relapse between resistant and nonresistant cases is in agreement with the work of Pattyn et al. (28), suggesting a difference in the incubation period in these two kinds of relapses. One MDR relapse case, however, showed such a short incubation period (1 month) that we suspect that this patient had not really been cured from his second disease course (Table 3). Our observation that all resistant cases were males is in agreement with findings of other studies (29, 30) and could be associated with the higher prevalence of males in MB leprosy and more frequent irregular self-administered drug intake (including quinolones) in males, causing mainly secondary resistance. This is supported by the recent observation of Singh et al. (34) showing the absence of primary drug resistance as demonstrated by the lack of drug-related mutations in strains from new leprosy patients. Indeed, three

out of four of the DR patients are from leprosy colonies that had received a previous Brazilian treatment regimen before MDT/WHO. Possibly, these cases, despite receiving regular monthly doses of the MDT/WHO scheme, might have been noncompliant regarding the daily self-administered dose of combined dapsone and clofazimine.

Although DR does not seem to be a problem in Brazil, one should note that the three older DR cases had skin lesions typical of leprosy and good access to a health unit and yet suffered from late diagnosis, strongly suggesting the need of inclusion of ex-colony areas as "loci" for epidemiological surveillance for relapse, as per norms defined by the Ministry of Health (4). Also, the observation of two cases of strains of *M. leprae* with MDR against the three most common drugs for treatment is concerning and could become a serious threat for leprosy control. In order to comply with the Global Surveillance of DR in Leprosy, the following had been recommended: (i) to provide a technical guideline from the National Hansen's Disease (Leprosy) Control Programme (4) for establishment of relapse surveillance measures, (ii) to include the study of drug resistance, (iii) to provide recommendations for the management of suspected relapse cases, and (iv) to design a specific investigation form for the cases reported as relapse in the SINAN national information system (3). In addition, we suggest the implementation within the leprosy control program of monitoring of DR and MDR patients and their close contacts and organizing a reference framework.

Our data show that development of DR isolates of *M. leprae* is contributing to leprosy relapse in Brazil but that the following are alternative causes: (i) bacterial persistence, (ii) immunosuppression of the host, (iii) pregnancy, (iv) the presence of advanced leprosy, (v) reinfection, and (vi) factors associated with failures in operational health care, such as late diagnosis, inadequate or irregular treatment of the disease, and misclassification of earlier disease (11, 18, 19, 20). We admit, however, that a limitation of this study is the use of PCR sequencing for SNP detection, with limitations regarding the detection of eventual minor mutant populations. In addition, mutations outside the part of the genes that was sequenced could have been missed.

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PLoS NEGLECTED TROPICAL DISEASES

Detection of Antibiotic Resistance in Leprosy Using GenoType LepraeDR, a Novel Ready-To-Use Molecular Test

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Abstract

Background: Although leprosy is efficiently treated by multidrug therapy, resistance to first-line (dapsone, rifampin) and to second-line drugs (fluoroquinolones) was described worldwide. Since *Mycobacterium leprae* is not growing *in vitro*, phenotypic susceptibility testing requires a one year experiment in the mouse model and this is rarely performed. Genetics on antibiotic resistance provide the basis for molecular tests able to detect for antibiotic resistance in leprosy.

Methodology/Principal Findings: A reverse hybridization DNA strip test was developed as the GenoType LepraeDR test. It includes DNA probes for the wild-type sequence of regions of *rpoB*, *gyrA* and *folP* genes and probes for the prevalent mutations involved in acquired resistance to rifampin, fluoroquinolones and dapsone, respectively. The performances of the GenoType LepraeDR test were evaluated by comparing its results on 120 *M. leprae* strains, previously studied for resistance by the reference drug *in vivo* susceptibility method in the mouse footpad and for mutations in the gene regions described above by PCR-sequencing. The results of the test were 100% concordant with those of PCR sequencing and the mouse footpad test for the resistant strains: 16 strains resistant to rifampin, 22 to dapsone and 4 to ofloxacin with mutations (numbering system of the *M. leprae* genome) in *rpoB* (10 S456L, 1 S456F, 1 S456M + L458V, 1 H451Y, 1 G432S + H451D, 1 T433I + D441Y and 1 Q438V), in *folP1* (8 P55L, 3 P55R, 7 T53I, 3 T53A, 1 T53V) and *gyrA* (4 A91V), respectively. Concordance was 98.3% for the susceptible strains, two strains showing a mutation at the codon 447 that in fact was not conferring resistance as shown by the *in vivo* method.

Conclusions/Significance: The GenoType LepraeDR test is a commercially available test that accurately detects for antibiotic resistance in leprosy cases. The test is easy to perform and could be implemented in endemic countries.

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Introduction

Leprosy, the second communicable disease due to mycobacteria after tuberculosis, is still a preoccupying disease as 230 000 new cases have been reported in 2010 (www.who.int/lep/). This disease remains difficult to diagnose and treat in low- and mid-developed countries, especially in rural areas. Global child rate has remained consistently at around 10% of cases for the last years, showing that transmission is still active [1]. Leprosy can be cured if multidrug therapy (MDT) is properly implemented following WHO recommendations: a 6 month regimen for paucibacillary cases and a 12 month regimen (formerly 24-months) for multibacillary (MB) cases both combining rifampin and dapsone, plus clofazimine for MB cases [2]. The relapse rate ranges between 2% and 5% in leprosy depending of the country, and we learned from tuberculosis that relapse cases are at risk of drug resistance [3]. However, in contrast to what we know for tuberculosis, the prevalence of primary and secondary resistance is unknown for leprosy. Consequently, the risk of resistance cannot be assessed and re-treatment regimen cannot be appropriately design.

Mycobacterium leprae is one of the few bacteria that are not growing *in vitro*. It multiplies only in the mouse footpad [4] and in the nine-band armadillo [5]. The in vivo susceptibility testing model, based on footpad inoculation of mice treated with antibiotics, is available in only an handful of highly specialized laboratories and cannot be spread because it requires one year lasting experiment (*M. leprae* doubling time is about 10 to 14 days) and expensive facilities [4,6]. Resistance to anti-leprosy drugs, such as dapsone, rifampin and fluoroquinolones, has been

Author Summary

Although leprosy is a curable disease using a combination of antibiotics for one year, the transmission is still active with 230,000 new cases in 2010. Drug resistance has been described and may prevent eradication of the disease. The infectious agent causing leprosy, *Mycobacterium leprae*, is not growing *in vitro* and antibiotic susceptibility testing is possible only in the mouse footpad model that requires a one year experiment. Consequently this testing is rarely done and antibiotic resistance rates in leprosy are unknown. This is the reason why we endeavored to set a new diagnosis test that detects for antibiotic resistance in *M. leprae*. The test is based on the method of a DNA strip test with a multiplex PCR followed by reverse hybridization. It was developed as an easy-to-use test and it will be available in endemic countries, where these kinds of strip tests are already used for detection of drug resistance in tuberculosis. The results of the new test, Genotype LepraeDR, performed on 120 *M. leprae* strains were concordant with those of the standard PCR sequencing and mouse footpad susceptibility testing.

described since 1967 using this in vivo model [6]. Multi-drug resistance, i.e. resistance to at least two of these drugs, has been described in Africa [7], Asia [8] and South America (unpublished data).

In the late 1990's, thanks to PCR and determination of the *M. leprae* genome [9], molecular methods detecting antibiotic resistance have been set. Rifampin resistance was associated to mutations in the *rpoB* gene encoding the β subunit of RNA polymerase [10], dapsone resistance to mutations in the *folP1* gene encoding the dihydropteroate synthase [11,12] and fluoroquinolone resistance to mutations in the *gyrA* gene encoding the subunit A of DNA gyrase [7]. Various methods have been described to detect the mutations listed above such as PCR- sequencing, heteroduplexes formation, and DNA array [13,14,15,16,17,18]. However, all these methods require specialized laboratories and are not commercially available. No easy-to-use methods are available in the endemic areas.

The DNA strip assay is a methodology widely used for molecular detection of resistance in tuberculosis [19]. The test is based on a classic PCR and reverse hybridization. Because this methodology has been demonstrated to be simple and robust in developing countries, we aimed to develop a new test based on this technology that easily detect for drug resistance in leprosy.

Materials and Methods

M. leprae strains

Hundred and twelve skin biopsies containing *M. leprae* were studied for the evaluation of the test. They have been sent for leprosy diagnosis to the National Reference Center for mycobacteria (NRC-Myc, Paris, France) from 1989 to 2010 and were all smear-positive for acid fast bacilli (AFB) with a minimum amount of 5×10^4 AFB/ml. The samples were anonymized and the collection was used under the IRB approval for diagnosis specimens received at Assistance publique Hôpitaux de Paris, Biology laboratories of Pitie-Salpetriere Hospital. The selected biopsies (54% of the collection) were consecutive biopsies for which mouse culture was performed and for which enough quantity of specimen was available for performing the molecular studies. Skin biopsies were prepared as described previously for mouse inoculation and molecular experiments [17,20].

Eight *M. leprae* strains, which were previously described and propagated in the nude mouse footpad, were taken as reference strains [8,21].

DNA from several mycobacterial strains other than *M. leprae* were tested for analytical specificity: 3 *M. ulcerans*, 5 *M. marinum*, 5 *M. chelonae*, 1 *M. scrofulaceum*, 1 *M. kansasii*, 1 *M. intermedium*, 1 *M. terrae*, 1 *M. malmoense*, 1 *M. fortuitum*. In addition, ten biopsies known to be negative for mycobacteria were also tested for specificity.

GenoType LepraeDR probe description

The design of the mutated (MUT) and wild type (WT) probes were based on the mutations reported in the literature for the resistant strains: in the rifampin resistance determining region (RRDR) in *rpoB* [10,17,22], in the region determining dapsone resistance (DRDR) in *folP1* [11,12,20] and in the quinolone resistance determining region (QRDR) in *gyrA* [7,23]. The probes are listed in Table 1. Wild type probes, one to four according to the gene, were chosen to span the region affected by drug resistance mutations: WT1 to WT4 for *rpoB* (the 430–458 region, numbering system of the *M. leprae* genome TN, GenBank n°NC 002677), WT *folP1* for the 53–55 region and WT *gyrA* for the 89–91 region. Some of the most prevalent mutations in *rpoB* (S456L and H451Y), in *folP1* (P55L) and in *gyrA* (A91V) were included in the strip as specific probes.

GenoType LepraeDR testing

Strips were coated at Hain Lifescience factory (Nehren, Germany) with the different specific oligonucleotides (DNA probes) using the DNA strip technology. Amplification, hybridization and interpretation were performed in a similar procedure as for other GenoType tests [19]. Briefly, 35 µl of 5′-biotinylated primers and nucleotide mix, 5 µl of polymerase buffer, 2 µl of 25 mM $MgCl_2$ stock solution, 3 µl of water and 5 µl of total DNA (20 to 100 ng) were mixed with 1 U of Hot Star Taq polymerase (Qiagen) per reaction. The PCR run was comprised of 35 cycles. After denaturation at 95°C for 15 min, ten cycles at 95°C for 30 sec and at 58°C for 2 min were followed by 25 cycles with a first step at 95°C for 25 sec, a second step at 53°C for 40 sec and a

Table 1. Probes and primers used in the GenoType Leprae DR test for molecular detection of antileprosy resistance.

Antibiotic	Gene	Probe	Targeted codon(s) or mutation*
Rifampin	*rpoB*	WT1	432
		WT2	438–441
		WT3	451
		WT4	456–458
		MUT1	S456L
		MUT2	H451Y
Ofloxacin	*gyrA*	WT	89–91
		MUT	A91V
Dapsone	*folP1*	WT	53–55
		MUT	P55L

na, non applicable.
*numbering system used in the *M. leprae* genome of strain NT (sequence NC 002677 in GenBank).
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