Rapid detection of resistance to rifampicin in *Mycobacterium leprae*

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Rifampicin resistance of *Mycobacterium leprae* is a potential threat to the success of multidrug therapy (MDT). However, the prevalence of leprosy caused by rifampicin resistant organisms is unknown. The mouse footpad assay of the drug susceptibility of *M. leprae* is not generally available, and a complete survey employing this method is not possible, because the need for fresh biopsy specimens containing large numbers of organisms for inoculation of the footpad means that only a small proportion of all patients can be tested.

The genetic basis of rifampicin resistance in mycobacteria has been described in the past few years. Early methods for detecting rifampicin resistance-associated mutations relied upon sequencing or single-strand conformation polymorphisms, and were not readily usable in the field laboratory. In 1999, a line-probe assay was developed in the Institute Pasteur, in which PCR product of the *M. leprae rpoB*-gene could be directly probed for common rifampicin resistance mutations. In August 1999, we tried this method for the first time in Kathmandu, Nepal.

Sixty biopsy specimens from six centres in India and Nepal were collected from patients who relapsed after multidrug therapy (MDT), defaulters from MDT who were re-starting treatment, and patients with an apparent failure to respond to MDT (evidenced by no decline of the bacterial index). Punch biopsy specimens (4 mm) obtained from active lesions were fixed in 70% ethanol and posted to Kathmandu. One to 18 months later, the specimens were re-hydrated and the DNA extracted by three rounds of freezing in liquid N_2 and boiling. The *M. leprae rpoB*-gene was amplified by PCR, using biotinylated primers, and the 388 base-pair product was detected on agarose gel. Eleven oligonucleotide probes were fixed onto a nylon membrane, and denatured PCR product was incubated on the membrane for 60 min. After washing, bound PCR product was detected by incubation of the membrane with luminol and detection of chemiluminescence by exposure on X-ray film. Each membrane-strip contained oligo-probes for wild type and mutated sequences. Plasmids containing mutations were used as positive controls.

Only eight of the 60 samples yielded a PCR product of the required size, of which five could be used in the line-probe assay. Four strains of *M. leprae* from mouse footpad experiments were also tested in the line-probe assay. Of these nine strains, three were found to be wild-type, and six were found to contain mutations (five serine to phenylalanine mutations and one apparent serine to phenylalanine plus serine to methionine double mutation). The line-probe assay results were confirmed by sequencing, except in the case

of the double mutation. Two of the five strains that had been obtained as biopsy specimens demonstrated resistance-associated mutations; these had been obtained from patients whose bacterial index had not fallen after 24 doses of MDT. Four strains obtained as isolates in the mouse footpad, with serine to phenylalanine mutations, were three strains that had been passaged 5–14 times over the past 5–10 years, and one primary isolate from a new, untreated LL patient. All of the strains that had been obtained from mice had earlier been demonstrated to be susceptible to rifampicin administered in a dosage of 10 mg per kg body weight.

The line-probe assay proved to be a successful method for detecting rifampicin resistance in a field setting in Nepal. Of concern, however, was the finding that mutations had occurred in apparently susceptible strains. We have extended these observations by testing isolates both by the line-probe assay, and in mice administered rifampicin in dosages of 5 mg and 10 mg per kg. It is possible that this powerful genetic technique is capable of detecting rifampicin-resistant *M. leprae* that are susceptible to clinical doses of rifampicin.

DISCUSSION

Dr Colston: To your knowledge, are there any reports of this mutation not conferring resistance to rifampicin on *M. tuberculosis*?

Dr Roche: In the case of *M. tuberculosis*, mutations in various portions of the *rpoB*-gene confer different levels of resistance, and mutations at this position in the *M. tuberculosis* gene produce high levels of resistance.

Professor Grosset: The important issue is the presence of mutations in the *rpoB*-gene without clinical evidence of resistance to rifampicin. Have you encountered any such instances?

Dr Roche: What we have shown are strains of *M. leprae* in which mutations are present that do not demonstrate resistance in mice. We believe the explanation of the highly resistant strains that you have described to be the presence of additional mutations in other portions of the gene that we have not probed.

Professor Jarlier (Bactériologie et Hygiène, Faculté de Médecine Pitié-Salpêtrière, 91 boulevard de l'Hôpital, 75634 Paris Cedex 13, France): We have studied 83 biopsy specimens obtained from 77 patients. All were patients with MB leprosy; 43 were patients in relapse, and 34 were newly diagnosed. For all but three specimens, which arrived in our laboratory 2 weeks after the biopsy had been performed, the susceptibility to rifampicin of the strains of *M. leprae* was tested in mice by the weekly administration of 10 mg rifampicin per kg body weight by gavage. *M. leprae* from all of the specimens were subjected to the molecular test, which consisted of sequencing a 45-codon segment of the *rpoB*-gene. Between 50 and 60% of the strains multiplied in mice, whereas the molecular test was carried out for more than 90% of the strains. Results from 45 patients were obtained both in mice and from PCR; 34 strains, 18 from relapses, and 16 from newly diagnosed patients, were determined to be susceptible to rifampicin in mice, whereas 11 strains, all from relapses, were found in mice to be resistant. Mutations in the *rpoB* gene were found in the PCR products of all of the resistant and none of the susceptible strains.

Dr Daumerie: Did the relapses occur after treatment with standard MDT?

Professor Jarlier: Virtually all of the relapses occurred after treatment with other regimens.

Dr Roche: What was the nature of the other regimens?

Professor Grosset: We have not observed a single instance of rifampicin resistance among patients who received standard MDT. All of the relapses occurred after what was really monotherapy with rifampicin.

Dr Roche: The apparent contradiction between the results of my study and your results probably lies in the treatment of the patients. I suggest that rifampicin monotherapy selects mutants with a high level of resistance, whereas MDT selects those with only a clinically insignificant level.

Professor Jarlier: I agree that the way in which the resistant mutants were selected in the patients may have been different in our two studies. However, shouldn't one expect a strain carrying the mutation most frequently observed in rifampicin resistant strains of both *M. leprae* and *M. tuberculosis* be accompanied by clinical evidence of resistance?

Professor Ji: The available data appear to demonstrate that resistance to rifampicin is single-step resistance. How, then, is it possible that you find varying levels of resistance in Nepal? On another matter, how good is the evidence that, on the basis of demonstrating mutations in the *rpoB* gene, we can diagnose rifampicin resistance in the field?

Dr Roche: Until we have validated our observations in Nepal among patients receiving standard MDT, we cannot be certain that our tools are adequate. Our present method appears to demonstrate resistance among patients who respond quite well to the MDT.

Dr Colston: Is it possible that, in Nepal, you are dealing with mixtures of resistant and susceptible strains of *M. leprae*?

Dr Cole: I find it difficult to understand Dr Roche's observations. All of the previously encountered strains of *M. leprae* that carry the serine to phenylalanine substitution at the 531 position showed resistance at 10 mg per kg. Also, in all other bacterial species in which this mutation has been described, the mutant strains are highly resistant. The mouse work should probably be repeated. And the possibility of a mixed population of organisms should be investigated.

Professor Grosset: A relapse during treatment with rifampicin occurs because the great majority, if not all, of the organisms are resistant. Rifampicin is so actively bactericidal that all of the susceptible individuals in a population of *M. leprae* are killed. This is indeed the case in tuberculosis. Therefore, a mixture of susceptible and resistant strains appears unlikely indeed.

Dr Daumerie: Many experts have identified monitoring of rifampicin as a high priority for research. Can the experts here tell us whether the technique is reliable, or is more time required for development? And, if so, how much time?

Professor Grosset: I cannot qualify as an expert in molecular biology. However, from the work at the Pasteur Institute and that described by Professor Jarlier at the Pitie-Salpetriere, and also from that in Nepal, I believe we now have enough information to proceed to implement a programme based on these molecular techniques that is designed to detect rifampicin resistance. What technique should be employed?

Dr Noordeen: I think the problem lies in having two rather different situations—one, in which patients have been treated in a rather artificial setting by a variety of regimens, and the other, in which patients have been treated with standard MDT in a natural setting. Our objective is to mount surveillance of rifampicin resistance in the framework of leprosy control programmes. Dr Roche's data, resulting from a study carried out in such a natural setting, cast some doubt on the specificity of the test. Can the test be improved? Or can we try not to dilute its specificity by restricting the variety of cases chosen for screening? I suggest, as the criterion for screening, a patient who has relapsed after standard MDT, and who has failed to respond to retreatment.

Professor Grosset: I agree with Dr Noordeen in part. If we wish to demonstrate that treatment by MDT does not create resistance to rifampicin, a phenomenon that has yet to be reported, we should undertake a study designed to confirm this. I think that this would be important to WHO.

Professor Ji: The first signs of rifampicin resistance would be cases of acquired resistance. Therefore, we should screen only MB patients who had completed MDT at least four or five years earlier, and whose skin smears show a BI ≥3+.

Dr Noordeen: I think it important to apply the technique only to the few patients likely to harbour rifampicin resistant strains of *M. leprae*, and not to dilute the study by testing the very large numbers of patients at very low risk of resistance. In the field, the experience is that 0·1–0·2% of patients relapse at some time after completing MDT. Although these proportions are small, the absolute numbers of relapses may be quite large in some countries. The vast majority of these patients have been reported to respond to retreatment by the same regimen. Therefore, they represent problems of microbial persistence rather than drug resistance. I believe the test should be applied only to those patients who fail to respond to retreatment, thereby avoiding dilution of the cohort at greatest risk and avoiding the problem of false positives.

Dr Klatser: Dr Roche, did you exclude false positives as the result of amplicon amplification in the cases in which you detected mutations in the face of susceptibility to rifampicin in the mouse?

Dr Roche: These cases occurred early in our study. Since then, we have examined a large number of strains, without encountering any more of these cases. If we had been dealing with amplicons amplification, we should have continued to find new cases.

Dr Dockrell: Surely, the chance to obtain false-positive results in a PCR in which one loses hybridization to one product and gains it to another is really quite small.