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Evaluation of a PCR-Based Universal Heteroduplex Generator Assay as a Tool for Rapid Detection of Multidrug-Resistant *Mycobacterium tuberculosis* in Peru

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Multidrug-resistant tuberculosis is an increasing health problem worldwide, especially in developing countries. The PCR-UHG-Rif assay, which detects mutations within the *rpoB* gene associated with rifampin resistance, was evaluated for its ability and reliability to detect and identify drug-resistant *Mycobacterium tuberculosis* in a developing country where tuberculosis is highly endemic.

Mycobacterium tuberculosis infection continues to be the major infectious cause of human morbidity and mortality in the world (28). The synergistic interaction between human immunodeficiency virus and tuberculosis (TB) infection has increased the prevalence of multidrug-resistant tuberculosis (MDR-TB) (1, 12, 19), which constitutes a significant obstacle in the control of TB (15).к

Peru has the highest prevalence of drug-resistant TB in South America (4.5%) (17, 27). Active TB among young adults (ages 15 to 44) is as high as 800 per 100,000 in parts of the general population (8), with MDR-TB as high as 94% among patients with treatment failures (2).

Assays for rapid detection and drug susceptibility testing of *M. tuberculosis* are necessary for the effective control and prevention of TB and MDR-TB. Rapid detection of TB and MDR-TB could be accelerated by using DNA amplification techniques based on PCR and mutation detection assays (5, 9, 13, 22). Targeting rifampin resistance is a good strategy, since rifampin resistance is conferred by mutations within a short sequence in the *rpoB* gene of *M. tuberculosis* (20, 24). In addition, most of the rifampin-resistant strains are also resistant to isoniazid and, hence, are MDR-TB (6, 27).

The PCR-UHG-Rif assay, based on the amplification and detection of mutations in the *rpoB* gene, has been developed and evaluated in the United States for the rapid and sensitive detection of *M. tuberculosis* and its rifampin genotype directly from sputum specimens (23, 25). The sensitivity of this assay for the detection of *M. tuberculosis* and rifampin susceptibility in smear-negative sputum samples has not been established. The goals of the present study were (i) to confirm the ability of the PCR-UHG-Rif assay to determine rifampin resistance accurately by testing a sizable number of drug-resistant specimens, (ii) to determine its sensitivity for the detection of smear-negative TB, and (iii) to determine how well the assay performed in a laboratory in Peru, a developing country where TB and drug-resistant TB are highly endemic.

A total of 1,892 sputum samples were analyzed. Of these samples, 1,390 were obtained from 288 patients attending the Pulmonary Clinic at Maria Auxiliadora Hospital and 502 samples were from 106 patients attending the Infectious Diseases Clinic at Dos de Mayo Hospital in Lima, Peru. Two consecutive sputum samples were collected per patient before treatment was started (month 0) and at 1 month (month 1), 2 months (month 2), and 4 months (month 4) after starting treatment. Patients were treated for 2 months with isoniazid, rifampin, pyrazinamide, and ethambutol six times a week followed by 4 months of treatment with isoniazid and rifampin twice weekly, as recommended by the World Health Organization (26).

TABLE 1. Comparison of the PCR-UHG-Rif assay to culture (gold standard) for detection of *M. tuberculosis* directly from sputum sediments

PCR-UHG-Rif	Culture[a]		% Predictive value	
	Positive[b]	Negative	Positive	Negative
Positive[c]	695	117	85.6	
Negative	133	947		87.7
% Sensitivity-specificity	83.9	89.0		

[a] NALC-OH-treated sputum sediments were cultured by using L/J and MODS.
[b] Culture-positive samples were positive for growth in one or both culture systems.
[c] Positive specimens were positive for the 193-bp *M. tuberculosis*-specific band on heteroduplex gel.

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TABLE 2. Comparison of the PCR-UHG-Rif assay to sputum smears (gold standard) for detection of *M. tuberculosis* directly from sputum sediments

PCR-UHG-Rif	Smears[a]		% Predictive value	
	Positive	Negative	Positive	Negative
Positive[b]	590	222	72.7	
Negative	1	1079		99.9
% Sensitivity-specificity	99.8	82.9		

[a] Smears of sputum samples were prepared by using Auramine O staining (7).
[b] The positive specimens were positive for the 193-bp *M. tuberculosis*-specific *rpoB* fragment band on heteroduplex gel.

TABLE 3. Comparison of the PCR-UHG-Rif assay to the *IS*6110 PCR assay (gold standard) for the detection of *M. tuberculosis* directly from sputum sediments

PCR-UHG-Rif	*IS*6110 PCR[a]		% Predictive value	
	Positive	Negative	Positive	Negative
Positive[b]	754	58	92.9	
Negative	322	758		70.2
% Sensitivity-specificity	70.1	92.9		

[a] The positive samples for the hemi-nested *IS*6110 PCR contained a 337-bp product on ethidium bromide-stained 2% agarose gel.
[b] The positive specimens were positive for the 193-bp *M. tuberculosis*-specific *rpoB* fragment band on heteroduplex gel.

Sputum specimens were processed at the Infectious Diseases Laboratory, Universidad Peruana Cayetano Heredia, Lima, Perú. All specimens were coded and processed simultaneously by our routine technicians in a blinded fashion.

The specimens were homogenized, decontaminated, and concentrated by the *N*-acetyl-L-cysteine-NaOH method (14). From each decontaminated sputum, one smear was prepared, stained with Auramine O, and then graded (21); one slant of Lowenstein-Jensen (L/J) (Difco, Detroit, Mich.) was inoculated; the microscopic observation broth drug susceptibility assay (MODS) was carried out to detect the presence of *M. tuberculosis* (4, 18); a hemi-nested PCR assay, targeting insertion element *IS*6110, was performed to identify the presence of *M. tuberculosis* (4, 7, 10, 16); and the PCR-UHG-Rif assay was performed as previously described (23, 25).

M. tuberculosis isolates were tested for their susceptibility to rifampin and isoniazid by using a well-described colorimetric susceptibility test, the microplate Alamar Blue assay (MABA) (4, 11).

Data were analyzed using STATA package version 7.0 (Stata Corporation, College Station, Tex.).

A positive TB culture was considered the "gold standard" test. A total of 43.8% (828 of 1,892) of the samples were culture-positive by either broth (MODS) or L/J slope solid culture (Table 1). Of the culture-positive samples, 90.2% (747 of 828) were positive on L/J and 94.9% (786 of 828) were positive with MODS. The *IS*6110 PCR assay detected *M. tuberculosis* in 56.9% (1,076 of 1,892) of the samples, while the PCR-UHG-Rif assay detected the bacterium in 42.9% (812 of 1,892) of the samples. The sensitivity, specificity, and predictive values of the PCR-UHG-Rif assay compared to those of culture, auramine staining, and *IS*6110 PCR are shown in Tables 1, 2, and 3, respectively.

The PCR-UHG-Rif assay detected 99% of the smear-positive samples. However, it detected only 49% of the smear-negative, culture-positive samples. The sensitivity of the PCR-UHG-Rif (compared to that of culture as the gold standard) was significantly higher for smear-negative culture-positive samples collected 4 months after treatment than for samples collected before treatment (odds ratio [OR] = 16.47, P = 0.008) or 1 (OR = 13.0, P = 0.016) or 2 months after treatment (OR = 9.6, P = 0.037) (Fig. 1). In smear-negative cases, sensitivity of the PCR-UHG-Rif increased as the time after treatment increased, presumably because PCR-UHG-Rif detected nonviable *M. tuberculosis* killed by treatment (Fig. 2).

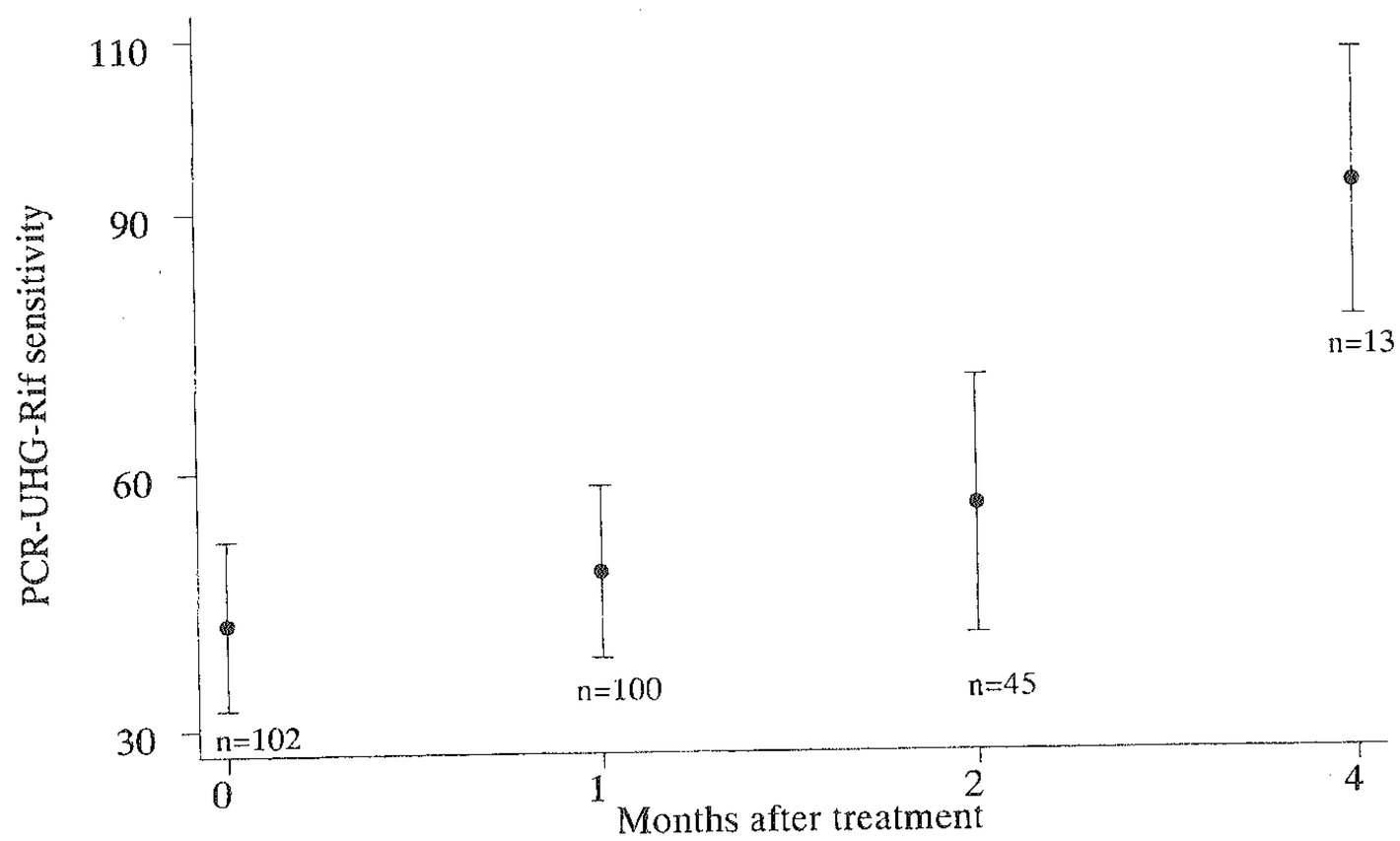

FIG. 1. Results sensitivity of the PCR-UHG-Rif assay compared to that for culture (by either L/J or MODS or both) among smear-negative sputum specimens. Each bar represents the 95% confidence interval for the sensitivity of the PCR-UHG-Rif test compared to that for culture.

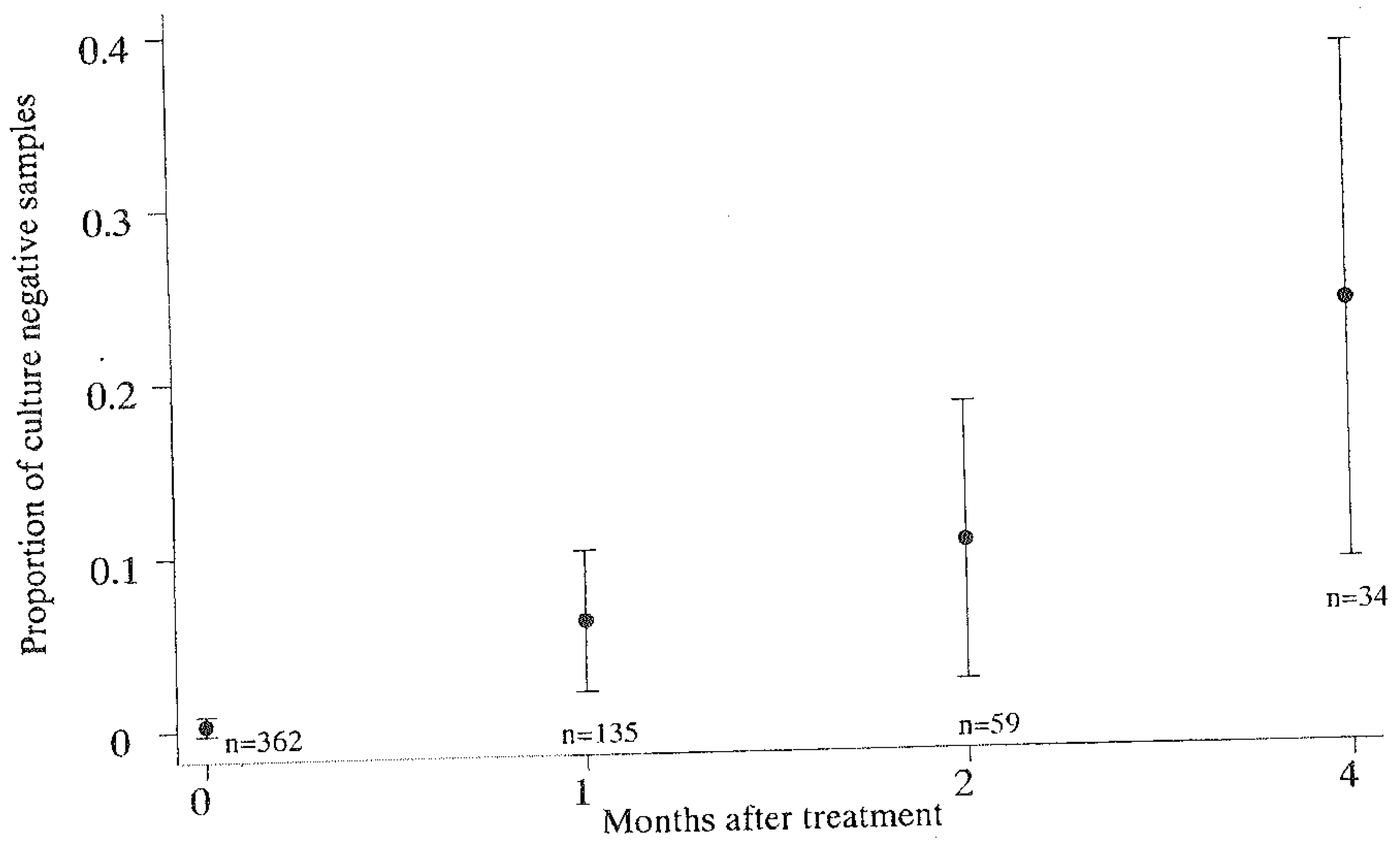

FIG. 2. Proportion of culture-negative samples among PCR-UHG-Rif-positive samples by time of collection of samples after treatment. Each bar represents the 95% confidence interval for the proportion of samples that were PCR-UHG-Rif-positive and culture-negative.

Note that smear-negative cases are less likely to transmit TB (3).

There was no significant difference in sensitivity or specificity of the PCR-UHG-Rif when the two hospital populations were examined separately ($P = 0.79$). No difference was observed when PCR-UHG-Rif sensitivity or specificity was analyzed according to patient HIV status ($P = 0.16$).

When the statistical analysis was performed in a cohort of 73 patients with all eight samples collected, no difference was observed in the performance of the PCR-UHG-Rif assay compared to that of the statistical analysis performed by using the complete set of data. In the cohort, in smear-negative samples the same trend of increased sensitivity of the PCR-UHG-Rif assay was maintained as the duration of treatment increased but was no longer statistically significant (data not shown).

When *M. tuberculosis* was detected, there was a high percentage of agreement in rifampin susceptibility between the MABA and the PCR-UHG-Rif assay (Table 4). Among samples positive by PCR-UHG-Rif, there was no difference in the proportion of sensitive and resistant results between culture-negative and culture-positive samples (data not shown).

The MICs were significantly higher ($P < 0.001$, Kruskal-Wallis test) for samples that were rifampin-susceptible by MABA and resistant by PCR-UHG-Rif (mean MIC = 0.317 μg, $n = 15$) than for samples that were susceptible to rifampin both by MABA and PCR-UHG-Rif (mean MIC = 0.080 μg, $n = 508$). Of the 165 samples that were rifampin-resistant with the MABA test, 162 samples were also resistant with PCR-UHG-Rif and, for 160 of these, rifampin MICs were >16 μg/ml. For two of the three samples resistant to rifampin by MABA but showing a sensitive genotype by PCR-UHG-Rif, rifampin MICs were >16 μg/ml; for the other sample, rifampin MICs were equal to 2 μg/ml.

To determine if the PCR-UHG-Rif assay was a good predictor of MDR-TB (resistance to at least isoniazid and rifampin) directly from sputum samples, the samples were also analyzed for isoniazid susceptibility using MABA and compared to rifampin susceptibility results obtained by MABA and PCR-UHG-Rif. There was a strong correlation between the results of these assays, as the agreement between susceptibility to rifampin and to isoniazid was 86% and 85%, respectively (Table 5).

TABLE 4. Comparison of PCR-UHG-Rif and MABA for determination of rifampin susceptibility of *M. tuberculosis* in clinical specimens[c]

PCR-UHG-Rif	MABA[a]	
	Susceptible (%)	Resistant (%)
Susceptible[b]	508 (97.1%)	3 (1.8%)
Resistant	15 (2.9%)	162 (98.2%)

[a] Samples for which refampin MICs were >1.0 μg/ml as determined by MABA were considered resistant to rifampin (5, 19).
[b] The samples showed a rifampin heteroduplex pattern with the PCR-UHG-Rif assay that was similar to that of *M. tuberculosis* H37Rv (ATTC 27294).
[c] Agreement, 97.4%; κ, 0.9294; P, <0.0001.

TABLE 5. Agreement between isoniazid susceptibility as determined by MABA and rifampin susceptibility as determined by MABA and PCR-UHG-Rif, respectively

Isoniazid susceptibility[c]	Rifampin susceptibility as determined by:			
	MABA[a]		PCR-UHG-Rif[b]	
	Susceptible	Resistant	Susceptible	Resistant
Susceptible	451	28	448	31
Resistant	72	137	63	146

[a] Strains were considered resistant to rifampin when rifampin MICs were > 1.0 μg/ml as determined by MABA (5, 19). Agreement, 85.4%; κ, 0.6347; P, <0.0001.
[b] Samples determined susceptible by PCR-UHG-Rif were those presenting the same heteroduplex pattern as *M. tuberculosis* H37Rv on acrylamide gels. Agreement, 86.3%; κ, 0.6624; P, <0.0003.
[c] Strains were considered resistant to isoniazid when isoniazid MICs were > 0.5 μg/ml as determined by MABA (5, 19).

Detection of *M. tuberculosis* by culture and susceptibility testing in Peru is primarily based on L/J slants. More sophisticated techniques are not widely used in most developing countries because of the need for radioactive reagents, high costs, and the special equipment requirements.

This study demonstrated that the PCR-UHG-Rif assay was very effective for the simultaneous detection of smear-positive *M. tuberculosis* and susceptibility to rifampin directly from ethanol-fixed NALC-OH-treated sputum sediments, confirming previously published results (23). It also confirms that the assay is a good predictor of MDR-TB.

In addition, we demonstrated the utility of the PCR-UHG-Rif assay in a laboratory in a developing country where equipment, molecular expertise, and funding are limited. Excluding labor and equipment costs, the PCR-UHG-Rif assay can be performed for approximately $2.75 per specimen with standard PCR and electrophoresis equipment and polyacrylamide minigels. It should be emphasized that PCR equipment costs are similar to the cost of an enzyme-linked immunosorbent assay reader.

Because of its high sensitivity, specificity, and predictive values for smear-positive TB and MDR-TB, the incorporation of this assay as a routine diagnostic tool for testing *M. tuberculosis* susceptibility to rifampin should permit the detection of MDR-TB patients within 24 h of specimen acquisition. When used in a hospital setting, the detection of rifampin resistance by the PCR-UHG-Rif assay should facilitate early isolation and appropriate treatment of MDR-TB that may decrease nosocomial transmission of MDR-TB. Its use as a single test in smear-negative samples, however, is limited by its relatively low sensitivity for detecting tuberculosis in these cases.

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