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In-House Phage Amplification Assay Is a Sound Alternative for Detecting Rifampin-Resistant *Mycobacterium tuberculosis* in Low-Resource Settings

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An in-house mycobacteriophage amplification assay for detecting rifampin-resistant *Mycobacterium tuberculosis* showed 100% sensitivity, 97.7% specificity, and 95.2% predictive value for resistance in a test of 129 isolates from a hot spot area of multidrug-resistant *M. tuberculosis*. The applicability of the test was demonstrated in the routine work flow of a low-resource reference laboratory.

Even in countries with excellent tuberculosis (TB) control programs, 8 of 10 patients with treatment failure harbor multidrug-resistant (MDR) *Mycobacterium tuberculosis*. Delays in identifying drug resistance lead to prolongation of ineffective first-line drug regimens, poor treatment outcome, and transmission of resistant strains, which amplifies the epidemiological problem (4, 12). In practice, early laboratory diagnosis is rarely achieved where it is most crucial: in countries with limited resources and high burden of MDR *M. tuberculosis* (14). It is precisely in these settings that effective tools able to anticipate failure of first-line chemotherapy would have a considerable impact on TB control (16).

Rifampin (RIF) is a key agent against *M. tuberculosis*, and the effectiveness of the standard TB treatment is significantly reduced by RIF resistance, regardless of the in vitro susceptibility of the bacilli to other drugs (3). Furthermore, in settings with high burden of MDR *M. tuberculosis*, RIF resistance becomes a predictor of multidrug resistance (19). This association has been seen in the past decade in metropolitan areas of Argentina, where a few MDR *M. tuberculosis* strains disseminated among hospitalized AIDS patients (15).

The chronic economic crisis afflicting many Latin American countries restricts their access to imported diagnostic methods, such as *M. tuberculosis* RIF resistance detection systems. These limitations, from which Argentina has not been spared, have stimulated the development and testing of low-cost alternative techniques.

One affordable option for drug resistance detection is the mycobacteriophage amplification assay, which makes use of phage infection to explore bacterial viability after exposure to antibiotics (9, 17). This ingenious method, which requires neither highly skilled personnel nor expensive reagents, is available as a commercial diagnostic kit but also appears to offer robust detection of RIF resistance as an in-house version (1, 5, 6). The present study was designed to test the performance of an in-house phage D29 assay for RIF resistance detection in the routine work flow of a reference laboratory with limited resources receiving isolates from a hot spot area for MDR *M. tuberculosis*.

Eleven RIF-susceptible and 9 RIF-resistant strains (characterized previously) were tested in a blind manner in duplicate (8). The phage test was evaluated in 129 *M. tuberculosis* complex isolates referred to our laboratory for drug susceptibility testing between April and December 2003. The isolates had been obtained from the same number of patients who were either at risk of drug-resistant *M. tuberculosis* ($n = 106$) or receiving treatment for MDR *M. tuberculosis* ($n = 23$). *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG strains served as pansusceptible controls.

Susceptibility testing to first-line drugs was performed by the proportion method on Lowenstein-Jensen slants (18) and served as the "gold standard" for this study.

The D29 mycobacteriophage assay (9) was standardized and adapted to our laboratory conditions. To reduce costs, a modified Luria broth (Difco) with 2% glucose and 1 mM calcium chloride was used instead of enriched Middlebrook 7H9 medium in all steps. The assay was performed either in screw-cap 2-ml vials or in microtiter plates. Microtiter plates were more practical when five or more isolates were being tested at one time, but special precautions had to be taken to avoid cross-contamination. The biohazard was similar in both options. Suspensions of bacilli were incubated overnight with RIF at 0.2, 3.0, and 10.0 mg/liter and in the absence of drug. Results were read 24 h after the bacilli were plated onto a *Mycobacterium smegmatis* lawn. Ten or more plaques were considered indicative of RIF resistance.

With a RIF concentration of 10 mg/liter, the test obtained 100% sensitivity, 100% specificity, and 100% reproducibility with the 20 coded strains. Lower drug concentrations produced a high proportion of ambiguous (1 to 9 plates) or false-positive results among true RIF-susceptible strains and isolates. Of the 129 clinical isolates, 6 showed no plaque formation in the absence of RIF, 2 showed <10 plaques in the presence of RIF,

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TABLE 1. Comparison of the phage D29 assay and the proportion method in detecting RIF-resistant *M. tuberculosis* complex[a]

Proportion method result	No. of isolates with the following phage test result:		Total no. of isolates
	Susceptible	Resistant	
Susceptible	86	1	87[b]
Resistant	0	42	42[c]
Total no.	86	43	129

[a] A total of 129 isolates from 129 patients were tested. The isolates were submitted for routine drug susceptibility testing to a reference laboratory in Buenos Aires, Argentina, 1 April to 31 December, 2003.

[b] Seventy pansusceptible isolates, six isolates were resistant to streptomycin, six isolates were resistant to isoniazid, and five isolates were resistant to isoniazid and streptomycin.

[c] Thirty-nine isolates were also resistant to (at least) isoniazid.

and 1 showed contamination with common microorganisms. Failure could be ascribed to culture ageing, mistimed chemical virus destruction, or experimental error (10). Upon repetition, eight of these nine isolates could be unambiguously classified. For one isolate, the borderline result was reproduced, and the isolate was misidentified as resistant in the third repetition. Interestingly, among the 86 true susceptible isolates, this was the only isolate for which some RIF-resistant clones (0.3%) were detected by the proportion method. When explored by reverse line blot hybridization (11), this isolate showed a wild-type pattern in the hot spot region of the *rpoB* gene. The patient, whose isolate was resistant to isoniazid and streptomycin, was lost to follow-up.

Complete concordance was observed between pairs of isolates from 10 patients. Taking into account the repeat results of the nine initially noninterpretable specimens, the assay correctly classified 128 of the 129 isolates (Table 1), achieving 97.7% specificity, 100% sensitivity, and 99.2% overall efficiency. These values fall within the range of values of internationally validated standard methods (7). When applied to the population at risk of MDR *M. tuberculosis* referred to our laboratory for drug susceptibility testing (prevalence of RIF resistance, 17.9%), the predictive values for RIF resistance and susceptibility were 95.2 and 100%, respectively. The turnaround time is 2 days. Were the test to be performed twice weekly on a routine basis, a realistic schedule in our setting, results would be available within a week.

This study provides further evidence of the proficiency of the in-house version of the phage D29 assay for detecting RIF-resistant *M. tuberculosis* and confirms its feasibility in low-income settings. In previous reports, bacilli to be tested were obtained from log-phase liquid cultures or colonies from fresh subcultures on solid medium (1, 2, 5, 6). In contrast, the present study analyzed the isolates as they arrived, regardless of previous manipulation or culture maintenance conditions. Even though some isolates had been submitted from distant regions of Argentina and could have spent several days in transit, the assay proved to be sufficiently robust, as both its accuracy and the percentage of interpretable results remained quite high.

Recently, the phage D29 assay has been fine-tuned by optimizing the virus inoculum and ascertaining the kinetics of the infection cycle, thus allowing maximal accuracy (10). As the phage system proved capable of detecting a small proportion of resistant bacilli in a given isolate, in principle it might be possible to apply a proportional approach for the interpretation of results (2, 9). In practice, however, most researchers, including us, prefer criteria in which the mere presence of plaques or a fixed minimum number of plaques indicates resistance (1, 5, 9).

In this study, 93% of the patients with RIF-resistant *M. tuberculosis* were also resistant to isoniazid. Thus, RIF resistance can be considered a fairly good predictor of MDR *M. tuberculosis* in the population referred to our laboratory for drug susceptibility testing. This is not surprising, as the laboratory provides reference mycobacterial diagnosis for the metropolitan area of Buenos Aires, Argentina, which is suffering the aftermath of the emergence of MDR *M. tuberculosis* strains in an area where TB is endemic (13).

The phage D29 assay is based on simple microbiological procedures and does not alter the routine work flow of a conventional bacteriology laboratory. Requiring neither imported commercial reagents nor sophisticated equipment, diagnostic tools such as this assay are invaluable in most Latin American countries. In Argentina, an in-house version of the phage assay works out to be 25 to 60 times less expensive than commercial RIF resistance detection systems based on molecular detection or radiometric culture. The assay is an attractive alternative for the detection of RIF resistance in resource-poor settings, because it is robust, economical, rapid, and easy to perform.

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