HCV co-infection seemed to have no influence over the CD4 cell decline or plasma HIV viral load in HIV-infected patients undergoing HAART interruption. On the other hand, the reported benefit of HGV co-infection, probably caused by HIV and HGV competitive replication, which may influence the lower plasma HIV viral load and the higher CD4 cell count found in patients receiving HAART [6], was not found during treatment discontinuation. This latter finding has to be treated with caution, since the populations studied were not balanced (six and 16 patients, respectively), and larger studies are desirable.

In summary, neither HCV (including genotype 1) nor HGV co-infection had any influence on CD4 cell count decline or plasma HIV viral load rebound in HIV-infected patients undergoing HAART interruption. However, larger numbers of patients should be analysed to confirm these results.

## ACKNOWLEDGEMENTS

This work was partially supported by Red de Investigaciones en Sida (RIS) from the Ministerio de Sanidad y Consumo, Fondo de Investigaciones Sanitarias (FIS 04 /0272). We thank M. M. Gonzalez, and A. Gayoso for their technical support.

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# RESEARCH NOTE

## Comparison of redox and D29 phage methods for detection of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis*

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## ABSTRACT

Rapid, accurate and inexpensive methods are essential to detect drug-resistant *Mycobacterium tuberculosis* and allow timely application of effective treatment and precautions to prevent transmission. The proportion method, the MTT and Alamar Blue redox methods, and the D29 mycobacteriophage assay, were compared for their ability to detect resistance to isoniazid and rifampicin. When tested against a panel of known *M. tuberculosis* strains, the redox methods and the D29 assay showed good sensitivity and specificity compared to the proportion method, suggesting that they could be useful alternatives for identifying multidrug resistance in *M. tuberculosis*.

**Keywords** Antibiotic resistance, D29 mycobacteriophage, detection, drug susceptibility testing, *Mycobacterium tuberculosis*, redox methods

**Original Submission:** 17 February 2005; **Revised Submission:** 13 August 2005; **Accepted:** 20 September 2005

*Clin Microbiol Infect* 2006; 12: 293–296
10.1111/j.1469-0691.2005.01355.x

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Resistance to anti-tuberculosis drugs is becoming ubiquitous [1,2]. Rapid and accurate detection is essential for controlling the spread of drug-resistant strains of *Mycobacterium tuberculosis*, but among the conventional methods for drug susceptibility testing, the proportion method is too slow, and automated systems are expensive [3–5]. Several molecular methods for the rapid detection of drug resistance have been described [6–8], but cost implications have precluded their routine implementation in tuberculosis-endemic countries, creating an urgent need for simple, rapid and inexpensive methods. Two promising alternative techniques are colourimetric assays, which use oxidation–reduction indicators (redox methods), and the mycobacteriophage assay [9–15]. In the present study, two redox indicators, Alamar Blue and 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT), were tested against an in-house D29 phage assay for their ability to detect resistance to isoniazid and rifampicin in *M. tuberculosis*.

The study was performed with a panel of 18 coded *M. tuberculosis* strains with known drug susceptibility patterns. Isoniazid and rifampicin were prepared at concentrations of 1 mg/mL in distilled water and 10 mg/mL in dimethylsulphoxide, filter-sterilised, and kept frozen at −20°C until use. To prepare the inoculum, a loopful (10 μL) of bacterial growth on Löwenstein–Jensen medium was placed in a vial with 3 mL of 7H9 broth and about ten 1-mm glass beads. The vial was vortexed until a fairly turbid suspension was obtained, and then left to stand for 15 min to minimise the possibility of aerosols. The supernatant was then transferred to a second tube and 7H9 broth was added until the turbidity was equal to a 1× McFarland standard. The resulting suspension was diluted 1:20 with 7H9 broth and used for inoculation. The redox assays were performed in microtitre plates as described previously [16–18], with drug concentrations of 0.03–1.0 mg/L for isoniazid and 0.06–2.0 mg/L for rifampicin. After incubation for 5 days at 37°C, 25 μL of Alamar Blue or 10 μL of MTT solution were added to each well. Following a further overnight incubation at 37°C, the plates were assessed for colour development. The breakpoint drug concentrations defining resistance were isoniazid 0.2 mg/L and rifampicin 0.5 mg/L.

For the D29 mycobacteriophage assay, high-titre lysates of the phage were prepared as described previously [12] and diluted with 7H9-RSS broth (5 g albumin, 2 g glucose, 0.85 g NaCl and 100 mL $H_2O$) containing 1 mM $CaCl_2$ to a working titre of $10^7$ PFU/mL. The final drug concentrations in the plates were rifampicin 5 mg/L and isoniazid 1.0 mg/L. For the assay, 75-μL aliquots of *M. tuberculosis* suspension were added to the wells of microtitre plates containing 75 μL of 7H9-RSS $CaCl_2$ with rifampicin or isoniazid. After incubation for 48 h at 37°C, 50 μL of D29 suspension were added to each well, and the plates were incubated at 37°C for 90 min. Extracellular phages were inactivated with 100 μL of 30 mM ferrous ammonium sulphate. To determine drug resistance, 10 μL of the incubation mixture was spotted on to Luria–Bertani indicator plates. Phage plaques were observed after overnight incubation at 37°C and compared with uninoculated negative controls. Positive controls without antibiotics, and negative controls without phage, were also included. The strains were considered drug-resistant if lytic plaques appeared on the indicator plates, with none seen on the negative controls. The proportion method was used as a standard method for comparison purposes and was performed according to standard procedures [3].

The results obtained for the 18 coded strains with the proportion method and with Alamar Blue were 100% concordant with those deter-

**Table 1.** Resistance profile of 18 coded strains of *Mycobacterium tuberculosis*, as determined by four different susceptibility testing methods

| | MTT | | Alamar Blue | | D29 assay | | Proportion method | |
|---|---|---|---|---|---|---|---|---|
| Strain no. | INH | RMP | INH | RMP | INH | RMP | INH | RMP |
| 001 | S | S | S | S | S | S | S | S |
| 002 | R | S | R | S | R | S | R | S |
| 003 | R | S | R | S | S | S | R | S |
| 004 | R | R | R | R | R | R | R | R |
| 005 | R | R | R | R | R | R | R | R |
| 006 | R | R | R | R | R | S | R | R |
| 007 | R | R | R | R | R | R | R | R |
| 008 | S | S | S | S | S | S | S | S |
| 009 | R | S | R | S | R | S | R | S |
| 010 | R | R | R | R | R | R | R | R |
| 011 | S | S | S | S | S | S | S | S |
| 012 | S | R | S | R | S | R | S | R |
| 013 | R | S | R | S | R | S | R | S |
| 014 | S | R | S | R | S | R | S | R |
| 015 | R | S | R | S | R | S | R | S |
| 016 | R | R | S | R | R | R | S | R |
| 017 | S | S | S | S | S | S | S | S |
| 018 | S | S | S | S | S | S | S | S |

R, resistant; S, sensitive; INH, isoniazid; RMP, rifampicin, MTT, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide.

mined by the reference laboratory that distributed the strain panel. The level of agreement between the two redox methods, as determined by the kappa test, was 0.886 for isoniazid and 1.0 for rifampicin. Compared with the proportion method, all three alternative methods showed good specificity and sensitivity (Table 1). One strain (no. 16), was isoniazid-sensitive according to the proportion method and the Alamar Blue assay, but was resistant according to the D29 phage assay and the MTT assay. Another strain (no. 3) was isoniazid-sensitive only by the D29 phage assay. Sensitivity and specificity in detecting isoniazid resistance were thus 100% and 88%, respectively, with the MTT assay, and 90% and 88%, respectively, with the D29 phage assay.

Of the eight strains found to be rifampicin-resistant by the redox and proportion methods (100% sensitivity and specificity), the D29 phage assay identified seven as resistant and one (no. 6) as susceptible. The D29 phage assay thus showed sensitivity and specificity of 87.5% and 100%, respectively, for detecting rifampicin resistance. The final concentration of rifampicin in the D29 phage assay was 5.0 mg/L. It is possible that a reduction in the rifampicin concentration used in the phage assay to 2.0 mg/L would have enabled detection of resistance in the one false-negative strain, but such a reduction would also increase the risk of false-positive results.

The redox methods can determine resistance within 7 days, with the advantage of providing MICs. Alamar Blue is relatively expensive, but equivalent results can be obtained using the chemical resazurin, which reduces the cost of this method to *c*. US $3 (2.5 Euros)/strain tested [19]. The D29 phage assay shows good sensitivity and specificity for detecting resistance to isoniazid and rifampicin within only 3 days, is relatively easy to perform and interpret, and is quite inexpensive (US $1; 0.8 Euros). The phage assay could be implemented in clinical laboratories as an initial screening procedure for detecting resistance to isoniazid and rifampicin. Strains showing resistance in the phage assay could then be tested with redox methods for a second-level evaluation of the full susceptibility profile.

## ACKNOWLEDGEMENTS

This study was partially supported by the European Commission under the INCO–DEV Programme (Project No. ICA4-CT-2001–10087) and the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). We thank R. McNerney, London School of Hygiene and Tropical Medicine, for supplying the D29 phage.

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# RESEARCH NOTE

## Changes in macrolide resistance among respiratory pathogens after decreased erythromycin consumption in Taiwan

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### ABSTRACT

Measures to alleviate the growing problem of macrolide resistance in Taiwan resulted in a decrease in macrolide consumption, from 0.629 defined daily doses/1000 inhabitants per day (DIDs) in 1999 to 0.301 DIDs in 2003 (a reduction of 52%). A linear relationship was observed between the decline in erythromycin consumption and the decline in erythromycin resistance in *Streptococcus pyogenes* (46% in 1999 vs. 17% in 2003; $p < 0.001$) and azithromycin resistance in *Haemophilus influenzae* (31% in 2000 vs. 0% in 2003; $p < 0.001$). However, the rate of erythromycin resistance in *Streptococcus pneumoniae* showed a continued increase, from 80.2% in 1999 to 92% in 2003.

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**Keywords** Antibiotic consumption, *Haemophilus influenzae*, macrolides, resistance, *Streptococcus* spp.

**Original Submission:** 31 May 2005; **Revised Submission:** 26 July 2005; **Accepted:** 12 September 2005

*Clin Microbiol Infect* 2006; 12: 296–298
10.1111/j.1469-0691.2005.01348.x

Taiwan has one of the highest levels of antimicrobial resistance in the world [1]. In particular, the resistance of respiratory pathogens (*Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Haemophilus influenzae*) to macrolides is of great concern, since several guidelines recommend macrolides as drugs of choice for community-acquired respiratory tract infections caused by these organisms. Prior to 2000, rates of macrolide resistance in *S. pneumoniae* (80–90%) and *S. pyogenes* (40–70%), and of azithromycin resistance in *H. influenzae* (30–50%), were remarkably high [1–3]. A number of measures were taken to alleviate this growing problem, including rigorous enforcement of prescription completion in pharmacies, and educational programmes for physicians that focused on appropriate antibiotic use. In addition, a restrictive governmental policy was implemented in 2001 to deny reimbursement through the National Health Insurance system for the costs of antibiotics used for the treatment of acute upper respiratory tract infections without evidence of bacterial involvement [4].

To assess the relationship between macrolide consumption and macrolide resistance in the three pathogens mentioned above, data concerning the annual consumption of macrolides (expressed as defined daily doses/1000 inhabitants per day; DIDs) obtained from IMS Health (Intercontinental Marketing Service, Taipei, Taiwan) were evaluated [4]. Pooled disk-diffusion susceptibility data concerning erythromycin resistance in 3677 isolates of *S. pneumoniae* (collected between 1999 and 2003) and 1170 *S. pyogenes*