Improved contamination control for a rapid phage-based rifampicin resistance test for *Mycobacterium tuberculosis*

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A prospective study was conducted of the rapid *FASTPlaque-Response* test for determination of rifampicin resistance in *Mycobacterium tuberculosis* with and without the addition of an antimicrobial supplement containing nystatin, oxacillin and aztreonam (NOA) to control specimen-related contamination. A total of 631 smear-positive sputum specimens was tested. The age of specimens ranged from 0 to 21 days. The NOA antimicrobial was effective at controlling contamination, with 4.1 % of specimens contaminated when the NOA antimicrobial supplement was used compared with 13.9 % contamination without NOA. Overall levels of interpretability of the test with NOA were 87.8 % with specimens of ≤3 days and 79.0 % for all specimens. This compared with 70.1 and 73.8 % readable results, respectively, from conventional culture-based drug susceptibility testing (DST). Sensitivity, specificity and overall accuracy of the *FASTPlaque-Response* test for rifampicin resistance were 98.1, 96.3 and 96.6 %, respectively, for all specimens with NOA, and 93.2, 96.3 and 95.9 % without NOA, when compared with resolved conventional DST results. Inclusion of the NOA supplement reduced contamination, increased the number of interpretable results and did not adversely affect the performance of the *FASTPlaque-Response* test. Thus, the use of NOA improves the robustness of the test, facilitating its wider implementation.

INTRODUCTION

The emerging worldwide problem of drug resistance complicates tuberculosis (TB) control measures. Multi-drug resistance (MDR), defined as resistance to at least rifampicin and isoniazid, is a particular problem in the treatment of TB, as these are the two most effective drugs used in short-course chemotherapy (WHO, 2004). Rifampicin resistance is an effective surrogate marker for MDR TB in many settings (WHO, 2003) and is a useful predictor of treatment failure (Suarez *et al.*, 2002). Recent estimates predict that there were between 376 000 and 620 000 new MDR TB cases in 2004 (Zignol *et al.*, 2006).

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Abbreviations: DST, drug susceptibility testing; MDR, multi-drug resistance; NHLS, National Health Laboratory Service; NOA, nystatin, oxacillin and aztreonam; TB, tuberculosis.

Conventional methods of detecting TB drug resistance rely on culture-based drug susceptibility testing (DST). In solid culture systems, this can take up to several months. This period can be reduced to 3–4 weeks by the use of newer liquid-based culture systems (Heifets & Cangelosi, 1999). New rapid methods of DST directly from clinical specimens will enable earlier detection of resistance, which is expected to improve treatment outcomes and decrease transmission (Fischl *et al.*, 1992).

Several new rapid DST methods have emerged recently (Pai *et al.*, 2006). These include phage-based methods (Jacobs *et al.*, 1993; Wilson *et al.*, 1997) and DNA amplification/hybridization technologies (Morgan *et al.*, 2005). These methods can be applied directly to smear-positive sputum specimens. One of these methods, the *FASTPlaque* technique, uses bacteriophage to report the presence of viable *Mycobacterium tuberculosis* in a specimen (Seaman *et al.*, 2003; Wilson, 1997). The technique has been applied to both the detection of *M. tuberculosis* from clinical specimens (Albert *et al.*, 2002; Muzaffar *et al.*, 2002) and DST of *M. tuberculosis* cultures (Albert *et al.*, 2001; Kisa *et al.*, 2003; Wilson *et al.*, 1997). Recently, it has also been applied to DST directly from smear-positive sputum

(Albert *et al.*, 2004), now commercialized as the *FASTPlaque-Response* test. Portions of decontaminated sputum specimen are incubated in the presence and absence of rifampicin, and the occurrence of plaques (clear zones) reflects the viability of *M. tuberculosis* after incubation with the drug, as bacteriophage require viable *M. tuberculosis* to replicate and lead to plaque formation. However, the proportion of uninterpretable results, either due to overgrowth of contaminating micro-organisms present in sputum or due to insufficient plaques in the control (drug-free) plate, has been identified as a potential limitation to the test's use.

This study evaluated the use of an antimicrobial supplement containing nystatin, oxacillin and aztreonam (NOA) to control contamination in the *FASTPlaque-Response* test and thus increase the proportion of interpretable results. In addition, the effect of the NOA antimicrobial supplement on the performance of the test was determined. The effect of specimen storage times on test performance was also evaluated.

METHODS

Specimens. From 1 March to 10 May 2005, smear-positive sputum specimens were collected in the National Health Laboratory Service (NHLS) laboratory, a large South African TB referral laboratory. Transport of specimens from clinics to the laboratory was at ambient temperature, with refrigerated overnight storage at the laboratory prior to processing. Specimens received on Friday were stored over the weekend at 2–8 °C.

Smear-positive sputum sediments (1+ or greater; Enarson *et al.*, 2000) of 0.5 ml or larger were included in this study. Specimens were collected consecutively, according to the selection criteria, from those available in the NHLS laboratory, up to a total of approximately 20 samples per day.

Sample processing. Specimens were decontaminated using the NALC-NaOH procedure (Kent & Kubica, 1985) within the NHLS laboratory. Following suspension of the pellet in phosphate buffer (pH 6.8), 0.1 ml was removed for a smear, and approximately 0.5 ml was inoculated into MGIT culture (BD Biosciences). The remainder of the pellet (approximately 0.5 ml) was stored at room temperature for approximately 4 h until smear results were available and selection of smear-positive specimens could be made.

Smear microscopy. Smears of sediments were prepared, stained with auramine O and analysed by NHLS staff. Smear results were classified according to International Union Against Tuberculosis and Lung Disease guidelines (Enarson *et al.*, 2000).

MGIT-960 culture. All specimens were processed for cultivation using the MGIT-960 system according to the manufacturer's recommendations, including the use of PANTA antibiotic supplement (BD Biosciences). Positive cultures were confirmed by acid-fast staining, and cultures free of contamination underwent identification and susceptibility testing.

Culture confirmation and DST. Conventional DST was performed using a modified 7H11 proportion method (Kent & Kubica, 1985), with final concentrations of 1 µg rifampicin ml^{-1} and 0.2 µg isoniazid ml^{-1}. At the same time, isolates were confirmed to be

M. tuberculosis complex by lack of growth on Lowenstein–Jensen medium containing *p*-nitrobenzoic acid (Allen, 1984).

FASTPlaque-Response testing. NOA was added to *FASTPlaque-Response* Medium Plus (Middlebrook 7H9-based medium containing oleic acid/albumin/glucose/catalase-based supplement) to give a final concentration of 50 000 IU nystatin l^{-1}, 2 mg oxacillin l^{-1} and 30 mg aztreonam l^{-1}. Medium Plus, either with or without NOA, was then used throughout the test procedure for *FASTPlaque-Response*.

Sediments (from the decontaminated pellet) of between 0.5 and 1.0 ml were made up to 1 ml with phosphate buffer (pH 6.8) and homogenized using a vortex mixer. Two 0.5 ml portions were removed from each sediment and processed by the *FASTPlaque-Response* method, according to the manufacturer's instructions (Biotec Laboratories), in either the presence or absence of NOA.

Result plates from Saturday were stored at 2–8 °C and read on Monday morning. All other plates were read on the day immediately after the second overnight incubation. Interpretation of test results was according to the manufacturer's instructions. For each specimen, the drug-free (RIF−) sample had to contain 100 plaques or more to be considered valid for interpretation. The specimen was determined to be rifampicin-susceptible if there were ≤49 plaques in the drug-containing (RIF+) sample, or as rifampicin-resistant if ≥50 plaques were observed.

rpoB mutation analysis. Identification of mutations in an 81 bp region of the *rpoB* gene encoding rifampicin resistance was performed as described by Victor *et al.* (1999) using a dot-blot hybridization method, and conventional DST was repeated on the isolated cultures of specimens in which the *FASTPlaque-Response* results and conventional DST disagreed.

RESULTS AND DISCUSSION

Conventional testing results

A total of 631 smear-positive sputum specimens (1+ or greater) was used in the study. The number of specimens that were 1+, 2+, and 3+ smear-positive was 148 (23.5 %), 108 (17.1 %) and 375 (59.4 %), respectively. Specimens were from new cases (8), new suspects (67), retreatment suspects (123) and retreatment cases (63). The remaining specimens (370) were from patients of unknown category (data not provided with specimen examination request forms).

A total of 3 (0.6 %) non-TB mycobacteria were identified from 486 isolated mycobacterial cultures, but no further work was performed to speciate these isolates. Positive MGIT cultures (containing *M. tuberculosis* complex) took an average of 14.5 days (range 5–56 days) to become positive. DST took a further 21 days to yield results. Hence, a total theoretical elapsed time for the culture-based determination of rifampicin resistance was an average of 35.5 days (26–77 days). In contrast, the *FASTPlaque-Response* test yielded results in 2 days (or 4 days if plates were kept over the weekend to be read on the Monday) following initiation of testing.

MDR was seen in 56 (8.9 %) of the specimens. Rifampicin resistance without isoniazid resistance occurred in only

four specimens (0.6 %). Isoniazid resistance without rifampicin resistance occurred in 83 specimens (13.2 %). A high correlation (92.9 %) of rifampicin resistance to MDR TB was found in this population.

In this study, out of a total of 631 samples, 238 specimens were ≤3 days old, whilst 391 specimens were ≥4 days prior to processing. Two specimens did not have a collection date provided and were not included in further analyses. Almost half (190) of these older specimens were 4 days old and were due mainly to storage over the weekend prior to testing on a Monday. In addition, the occurrence of older specimens was due to their collection from outlying centres and their subsequent delay in transportation to the central testing facility in Cape Town. Specimens up to 21 days old were encountered and included in the study.

Interpretability of results

Table 1 shows the outcomes of the *FASTPlaque-Response* test, with and without NOA, compared with culture and the conventional DST method. When the three methods were compared for specimens up to 3 days old, the percentage of interpretable results was significantly higher in the *FASTPlaque-Response* test with NOA (87.8 %) compared with the *FASTPlaque-Response* test without NOA (69.3 %) and culture-based DST (70.1 %) ($P<0.001$). There was no significant difference in the proportion of interpretable results between the *FASTPlaque-Response* test without NOA and culture-based DST ($P=0.849$).

This improvement in the interpretability of results for the *FASTPlaque-Response* test with NOA is likely to enhance the cost-effectiveness of rapid rifampicin testing, as fewer repeat tests will be required. Furthermore, providing more results quickly is likely to reduce the number of patients not receiving their results because they fail to return for follow-up appointments. For specimens of 4 days or older, the proportion of interpretable results was slightly higher for the conventional method (76.0 %) than for the *FASTPlaque-Response* with NOA (73.7 %) and higher than that for *FASTPlaque-Response* without NOA (69.3 %). When all specimens were considered, *FASTPlaque-Response* had the highest proportion of interpretable results (79.0 %), followed by conventional DST (73.8 %) and then *FASTPlaque-Response* without NOA (69.3 %).

Effective control of contamination in the *FASTPlaque-Response* test with NOA (0.8 % contamination rate in specimens aged ≤3 days) was a significant factor in the higher level of interpretable results observed. A low level of invalid results (RIF− <100 plaques) observed with NOA (6.7 %) compared with without NOA (15.5 %) was also a contributing factor. The reason for the reduced number of

Table 1. Comparison of *FASTPlaque-Response* results, with and without NOA, with conventional DST

	No. (%) of interpretable results from:		
	FASTPlaque-Response with NOA	*FASTPlaque-Response* without NOA	Culture + conventional DST
Specimens aged ≤3 days (n=238)			
Total interpretable results	209 (87.8)	165 (69.3)	167 (70.1)
Contamination	2 (0.8)	34 (14.3)	61 (25.6)
Invalid RIF−	16 (6.7)	38 (16)	NA
Controls out of spec/results not reported	11 (4.6)	1 (0.4)	10 (4.2)*
Specimens ≥4 days (n=391)			
Total interpretable results	288 (73.7)	271 (69.3)	297 (76.0)
Contamination	24 (6.1)	54 (13.8)	66 (16.9)
Invalid RIF−	69 (17.6)	57 (14.6)	NA
Controls out of spec/results not reported	10 (2.6)	9 (2.3)	28 (7.2)
All specimens (n=629)			
Total interpretable results	497 (79.0)	436 (69.3)	464 (73.8)
Contamination	26 (4.1)	88 (13.9)	127 (20.2)
Invalid RIF−	85 (13.5)	95 (15.1)	NA
Controls out of spec/results not reported	21 (3.3)	10 (1.6)	38 (6.0)*

NA, Not applicable.

*Result not reported for culture due to either a negative culture, the presence of non-tuberculous mycobacteria or loss of the specimen in the reporting system.

invalid results with NOA was unknown and unexpected, as the presence of antimicrobials in the medium was not anticipated to improve the viability or detection of *M. tuberculosis* in the specimens. However, when all specimens were considered, this effect on invalid results was less pronounced and was in keeping with previous reports (Albert *et al.*, 2002)

Contamination rates for MGIT culture followed by DST on 7H11 solid medium were higher than the rate seen with *FASTPlaque-Response*, with 20.2 % of specimens lost due to contamination. Specimens were lost primarily due to contamination of the primary MGIT culture, which occurred at a higher rate than reported elsewhere (8.6 %, Cruciani *et al.*, 2004), despite the fact that PANTA antibiotic supplement was used in the primary MGIT culture, as recommended by the manufacturer. Many of the contaminated MGIT cultures were mixed cultures, containing both acid-fast and non-acid-fast bacteria. Additional decontamination of these mixed cultures is likely to increase the number of interpretable results from culture, but would have resulted in increased workload and longer reporting times. This approach was not routinely used in the NHLS laboratory due to very high throughput. This high contamination rate is unlikely to be due to the age of the specimen giving rise to the opportunity for contaminating organisms to grow prior to decontamination, as less contamination was observed overall (20.2 %) compared with specimens aged ⩽3 days (25.6 %). The majority of specimens (57.4 %) were either 2 or 4 days old. There was a significant difference ($P=0.0251$) in contamination between 2- and 4-day-old specimens, with lower contamination in cultures from older specimens.

In the *FASTPlaque-Response* test, contamination can overgrow the results plates and prevent visualization of plaques. The *FASTPlaque-Response* test had less contamination than the culture-based DST in this study, even in the absence of NOA (13.9 % compared with 20.2 %). However, the presence of NOA reduced contamination further (to 4.1 %), permitting more results to be interpreted. The NOA antimicrobial was effective in reducing contamination to acceptable limits.

Invalid results in which there were inadequate plaques in the drug-free plate were the other main cause of loss of interpretability of results in the *FASTPlaque-Response* assay. A drug-free control plate without rifampicin (RIF−), set up for each specimen, was required to produce at least 100 plaques for interpretation of the RIF+ plate to be valid. Although only smear-positive specimens were included in the study, where large numbers of *M. tuberculosis* cells are expected in each specimen, it is known from previous studies that approximately 10–15 % of smear-positive specimens do not support productive phage infection (Albert *et al.*, 2002, 2004; Muzaffar *et al.*, 2002). This is unlikely to be due to the host range of the bacteriophage, as most of the isolated *M. tuberculosis* strains from these specimens can subsequently be phage-infected from the primary culture (Albert *et al.*,

2002). Instead, this is likely to be due to the harsh decontamination procedure damaging the *M. tuberculosis* cells and reducing their ability to support productive phage infection, or from the suggested presence of phage inhibitors in sputum (Albert *et al.*, 2002, 2004; Muzaffar *et al.*, 2002). The level of invalid RIF− results for all specimens appeared to be consistent with these earlier findings.

There were some specimens where the batch control result was invalid (negative control ⩾10). A single batch of specimens in each of the *FASTPlaque-Response* test procedures (on different days) failed and was responsible for this loss of results. The most likely explanation for this effect was operator error.

The beneficial effects of NOA were diminished somewhat when older specimens were included in the analysis (Table 1). The interpretability of the *FASTPlaque-Response* test with NOA declined from 87.8 % with fresher specimens to 79.0 % in the overall analysis due to a larger proportion of invalid results (13.5 %) and contaminated results (4.1 %). The overall frequency of invalid results was similar to that seen without NOA (15.1 %) and to other studies (Albert *et al.*, 2002, 2004; Muzaffar *et al.*, 2002). Contamination was higher than seen in fresher specimens. However, this may be an overestimation of the specimen-related contamination, as 21/26 specimens that were contaminated came from a single day's batch of results and all of the contaminants had similar colony morphology, suggesting that the contaminant had been introduced into the culture medium due to operator error.

FASTPlaque-Response performance

The performance of the *FASTPlaque-Response* test, with and without NOA, was compared with conventional DST, with specimens aged ⩽3 days (Table 2) and aged ⩾4 days (Table 3) where valid results for the *FASTPlaque-Response* and conventional DST methods were available. The performance of the *FASTPlaque-Response* test was similar in the presence or absence of NOA (Table 4). In addition, the performance of the phage-based test compared with conventional DST was similar to other studies (Albert *et al.*, 2004; Guerra *et al.*, 2005). Neither the age of the specimen (⩽3 days old compared with ⩾4 days) nor the smear grading (1+, 2+ or 3+) had a substantial effect on the ability of the *FASTPlaque-Response* test to identify rifampicin resistance correctly (data not shown).

Samples with discrepant results were resolved through repeat conventional testing and *rpoB* mutation analysis. In all but 2 cases (of 15 evaluated), the *rpoB* result agreed with the culture-based DST result. In these two strains that were susceptible by conventional DST but resistant by the *FASTPlaque-Response* method, both were found to have mutations at position 511 of the *rpoB* gene and were therefore considered to be resistant for the resolved data analysis. Performance data using the resolved DST results are presented in Table 4.

Table 2. Comparison of *FASTPlaque-Response* results (with and without NOA) with conventional DST results for specimens aged ⩽3 days (resolved data in parentheses)

		Conventional culture DST	
		Resistant	**Susceptible**
FASTPlaque-Response with NOA	Resistant	21 (22)	6 (5)
	Susceptible	0 (0)	126 (126)
FASTPlaque-Response without NOA	Resistant	18 (19)	6 (5)
	Susceptible	0 (0)	97 (97)

Table 3. Comparison of *FASTPlaque-Response* results (with and without NOA) with conventional DST results for specimens aged ⩾4 days (resolved data in parentheses)

		Conventional culture DST	
		Resistant	**Susceptible**
FASTPlaque-Response with NOA	Resistant	30 (31)	8 (7)
	Susceptible	1 (1)	191 (191)
FASTPlaque-Response without NOA	Resistant	21 (22)	7 (6)
	Susceptible	3 (3)	190 (190)

Potential applications of the *FASTPlaque-Response* method

These data demonstrate that the NOA antimicrobial supplement may be used with the *FASTPlaque-Response* test to reduce the number of results lost due to contamination, without any detriment to the performance of the test. Reducing the number of uninterpretable results will result in better service provision, more reliable and rapid results, and improved cost-effectiveness. Use of the NOA supplement with the *FASTPlaque-Response* test resulted in approximately 87 % of specimens (aged ⩽3 days) yielding results, which was a higher level of available results than conventional DST in this study. The *FASTPlaque-Response* test gave results comparable to the conventional DST method, with overall accuracy, sensitivity and specificity all greater than 95 %. However, the *FASTPlaque-Response* test yielded results in 2 days (depending on the working system of the laboratory and whether laboratory staff worked over the weekends) compared with the slower conventional DST result which took an average time of 35.3 days (range 26–77 days).

In addition, these data suggest that the test may be used with older specimens (⩾4 days) with comparable levels of performance to conventional DST. However, slightly reduced levels of interpretation may be encountered compared with fresher specimens. Where there is slow transport from the collection site to the laboratory, or in low-throughput laboratories where batching of specimens is required, the ability to test older specimens may be advantageous.

Application of the *FASTPlaque-Response* method for the screening of TB patients will be influenced by the performance of the test, the incidence of rifampicin resistance in the population and the correlation of rifampicin resistance with MDR TB. The type of patient suitable for testing may vary in different settings depending on the incidence of MDR TB and the correlation of rifampicin resistance with MDR TB. A presumptive diagnosis of MDR TB could be made based on detection of rifampicin resistance by the *FASTPlaque-Response* test within 2 days. Further DST could be carried out to confirm the result and for testing susceptibility to additional drugs for individualized MDR therapy, where appropriate.

Table 4. Resolved performance parameters of the *FASTPlaque-Response* test result (with or without NOA) compared with conventional DST

The 95 % confidence interval is shown in parentheses. PPV, Positive predictive value; NPV, negative predictive value.

Result	With NOA			Without NOA		
	⩽3 days old	**⩾4 days old**	**All specimens**	**⩽3 days old**	**⩾4 days old**	**All specimens**
Overall agreement	96.7 (93.9–99.5)	96.5 (94.1–98.9)	96.6 (94.8–98.4)	95.9 (92.4–99.4)	95.9 (93.3–98.5)	95.9 (93.8–98.0)
Sensitivity	100.0 (83.9–100.0)	96.9 (90.9–100.0)	98.1 (94.5–100.0)	100.0 (81.5–100.0)	88 (75.3–100.0)	93.2 (85.8–100.0)
Specificity	96.2 (91.9–99.5)	96.5 (93.9–99.1)	96.3 (94.3–98.3)	95.1 (90.9–99.3)	96.9 (94.5–99.3)	96.3 (94.2–98.4)
PPV	81.5 (66.9–96.1)	81.6 (69.3–93.9)	81.5 (72.1–90.9)	79.2 (63.0–95.4)	78.6 (63.4–93.8)	78.8 (67.7–89.9)
NPV	100.0 (97.1–100.0)	99.5 (97.1–100.0)	99.7 (99.1–100.0)	100.0 (96.3–100.0)	98.5 (96.8–100.0)	99.0 (97.9–100.0)

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