

Evaluation of the nitrate-based colorimetric method for testing the susceptibility of *Mycobacterium tuberculosis* to streptomycin and ethambutol in liquid cultures

H. Syre¹, H. Valvatne¹, P. Sandven² and H. M. S. Grewal^{1*}

¹Section of Microbiology and Immunology, The Gade Institute, University of Bergen and Haukeland University Hospital, N-5021 Bergen, Norway; ²Division of Infectious Disease Control, Norwegian Institute of Public Health, PO Box 4404 Nydalen, 0403 Oslo, Norway

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Objectives: To evaluate the inexpensive colorimetric nitrate reductase-based antibiotic susceptibility (CONRAS) assay for testing the susceptibility of *Mycobacterium tuberculosis* to streptomycin and ethambutol in liquid cultures, and to compare the CONRAS test with the manual mycobacteria growth indicator tube (MGIT) test, using the radiometric BACTEC 460TB method as reference.

Methods: A total of 89 *M. tuberculosis* isolates were tested for susceptibility to streptomycin and ethambutol using the CONRAS and manual MGIT methods and the results were compared with BACTEC 460TB. Isolates with discrepant results between the CONRAS test and BACTEC 460TB were analysed using the agar proportion method, Etest and mutation analysis of genes involved in resistance to streptomycin and ethambutol.

Results: The agreement between the CONRAS test and BACTEC 460TB was 88% for streptomycin and 84% for ethambutol. The corresponding agreement of the manual MGIT test with BACTEC 460TB was 89 and 80%, respectively. There was good agreement for streptomycin and moderate agreement for ethambutol between the CONRAS and manual MGIT tests on one hand and BACTEC 460TB on the other (CONRAS test, $\kappa_{\text{streptomycin}} 0.74$ and $\kappa_{\text{ethambutol}} 0.59$, $P < 0.001$; manual MGIT test, $\kappa_{\text{streptomycin}} 0.77$ and $\kappa_{\text{ethambutol}} 0.50$, $P < 0.001$).

Conclusions: There is good agreement for the two non-radiometric liquid culture methods (CONRAS and manual MGIT) compared with BACTEC 460TB for the detection of streptomycin resistance. Further standardization is needed for testing of ethambutol resistance using the CONRAS and manual MGIT assays.

Keywords: antibiotic susceptibility tests, rapid methods, nitrate reductase

Introduction

Drug-resistant tuberculosis (TB) represents a threat to TB control programmes. Consequently, laboratories face the challenge of providing rapid antibiotic susceptibility testing (AST) to ensure effective treatment.¹

Current standards for performing AST are the agar proportion method (PM) and the radiometric BACTEC 460TB system (Becton Dickinson, MD, USA).¹ Although both tests are reliable, the PM is hampered by the laborious procedure and the BACTEC 460TB system by the requirement for radioactive material and the high cost. The manual mycobacteria growth indicator tube (MGIT; Becton Dickinson) test is a non-radiometric

approach to the detection of mycobacteria and to AST in liquid medium.²

Recently, we developed a rapid and inexpensive colorimetric nitrate reductase-based antibiotic susceptibility (CONRAS) method for the AST of *Mycobacterium tuberculosis* to isoniazid and rifampicin in liquid cultures.³ The performance of the CONRAS test in detecting the susceptibility of *M. tuberculosis* isolates to rifampicin and isoniazid was excellent. The aim of the present study was to extend the CONRAS test to include streptomycin and ethambutol. The performance of the CONRAS test and that of the manual MGIT test were evaluated against BACTEC 460TB using a panel of *M. tuberculosis* isolates with a high frequency of resistance to streptomycin and ethambutol.

*Corresponding author. Tel: +47-55974631; Fax +47-55974979; E-mail: Harleen.Grewal@cih.uib.no

Table 1. Examination of streptomycin results discordant between the CONRAS test and the BACTEC 460TB method

Isolate	CONRAS ^a	BACTEC ^b	MGIT ^c	Etest ^d (MIC, in mg/L)	PM ^e	Nucleotide mutations in the <i>rrs</i> or <i>rpsL</i> gene ^f
PP13	S	R	S	S (<0.016)	S	wt
6988	S	R	S	S (<0.016)	R	<i>rrs</i> A→T pos 492
78/98	S	R	R	S (<0.016)	R	<i>rrs</i> A→C pos 513
80/98	S	R	S	S (0.032)	R	<i>rrs</i> A→C pos 513
3345	S	R	R	S (0.19)	R	wt
3348	S	R	R	S (0.75)	R	<i>rrs</i> C→T pos 516
3359	S	R	S	S (0.5)	R	wt
7019	S	R	S	S (0.032)	S	wt
3228	S	R	R	R (24)	R	<i>rpsL</i> A→G pos 263
398/98	S	R	R	S (<0.016)	S	wt
6984	S	R	R	R (3)	R	<i>rpsL</i> A→G pos 263

S, susceptible; R, resistant; PM, the agar proportion method; wt, wild-type.

^aPerformed using an initial concentration of 1.0 mg/L streptomycin. If an isolate was resistant, the test was repeated with a higher concentration of streptomycin (6.0 mg/L).

^bDrug concentration: 6.0 mg/L for streptomycin.

^cDrug concentration: 0.8 mg/L for streptomycin.

^dBreakpoint between susceptible and resistant isolates; 2.0 mg/L for streptomycin.

^eBreakpoint between susceptible and resistant isolates; 4.0 mg/L for streptomycin.

^fThe mutations in the *rrs* gene at positions 492 (A→T), 513 (A→C) and 516 (C→T) are all located in the 530 loop. The A→G mutation in position 263 in codon 88 of the *rpsL* gene corresponds to an amino acid substitution from K to R in the encoded protein.

Materials and methods

Isolates

A total of 89 *M. tuberculosis* isolates were included. Of these, 39 were obtained from the Norwegian Institute of Public Health, 8 from Myanmar and 11 from Haukeland University Hospital, Norway. The remaining 30 isolates were obtained from the WHO/International Union against Tuberculosis and Lung Disease (IUATLD) supranational laboratory network.⁴ The *M. tuberculosis* reference strain ATCC 27294 was also included.

CONRAS test

The CONRAS test was performed as described previously.³ AST was performed with an initial concentration of 1.0 mg/L streptomycin and 5.0 mg/L ethambutol. If an isolate was resistant to the antibiotic in question, the test was repeated with a higher concentration of the drug (6.0 mg/L streptomycin and 7.5 mg/L ethambutol).

Manual MGIT test

AST was performed according to the manufacturer's instructions (Becton Dickinson). The critical concentrations were 0.8 mg/L for streptomycin and 3.5 mg/L for ethambutol.

BACTEC 460TB

AST was performed using standard procedures (Becton Dickinson),¹ with critical concentrations of 6.0 mg/L streptomycin and 7.5 mg/L ethambutol.

Etest

The Etest was used according to the manufacturer's instructions (AB BIODISK, Solna, Sweden). For determination of susceptibility categories, breakpoints of 2.0 and 5.0 mg/L for streptomycin and ethambutol, respectively, were used (application sheet; AB BIODISK, 2003).

Agar PM

The PM was performed according to standard procedures.¹ The breakpoints were 4.0 mg/L for streptomycin and 2.0 mg/L for ethambutol.

Mutation analysis of genes involved in resistance

The mutation analysis of genes involved in resistance to streptomycin (*rrs* and *rpsL*) and ethambutol (*embB*) was undertaken using the methodology described by Victor *et al.*⁵

Analysis of isolates with discrepant results

Isolates showing discrepant AST results between the CONRAS test and BACTEC 460TB (Tables 1 and 2) were analysed using Etest and PM and by the analysis of genes involved in resistance to streptomycin and ethambutol. For isolates obtained from the WHO/IUATLD, the WHO expected results⁴ were included in the interpretation of results.

Data analysis

The performance of the CONRAS test and that of the manual MGIT test in comparison with BACTEC 460TB were evaluated in terms of sensitivity, specificity and positive and negative likelihood ratios.^{3,6} The agreement between the CONRAS test, the manual MGIT test and the BACTEC 460TB results was estimated using the kappa statistic. The kappa value was interpreted as follows: <0.2, poor; 0.21–0.4, fair; 0.41–0.6, moderate; 0.61–0.8, good; and ≥0.81, excellent.⁶

Results

For the 89 isolates tested using the CONRAS test, with 1.0 mg/L streptomycin and 5.0 mg/L ethambutol, there were 12 discordant results for streptomycin and 22 for ethambutol compared with

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Table 2. Examination of ethambutol results discordant between the CONRAS test and the BACTEC 460TB method

Isolate	CONRAS ^a	BACTEC ^b	MGIT ^c	Etest ^d (MIC, in mg/L)	PM ^e	Nucleotide mutations in the <i>embB</i> gene ^f
2431	S	R	R	S (2)	S	G→A pos 918
283/96	S	R	S	S (0.064)	R	wt
155/98	S	R	S	S (0.125)	R	G→A pos 918
348/98	S	R	S	R (8)	R	G→A pos 918
5886	S	R	R	S (0.75)	S	A→G pos 916
6984	S	R	R	R (64)	R	A→G pos 916
84/98	S	R	S	S (1)	R	wt
87/98	S	R	S	R (8)	R	A→G pos 916
86/98	S	R	S	R (12)	R	wt
3238	R	S	R	S (1)	R	G→A pos 918
4276	R	S	S	S (0.094)	S	wt
3703	R	S	S	S (0.023)	S	wt
90/97	R	S	S	S (<0.016)	S	wt
6963	R	S	R	S (<0.016)	S	wt

S, susceptible; R, resistant; PM, the agar proportion method; wt, wild-type.

^aPerformed using an initial concentration of 5.0 mg/L for ethambutol. If an isolate was resistant, the test was repeated with a higher concentration of ethambutol (7.5 mg/L).

^bDrug concentration: 7.5 mg/L for ethambutol.

^cDrug concentration: 3.5 mg/L for ethambutol.

^dBreakpoint between susceptible and resistant isolates; 5.0 mg/L for ethambutol.

^eBreakpoint between susceptible and resistant isolates; 2.0 mg/L for ethambutol.

^fThe A→G mutation in position 916 in codon 306 corresponds to an amino acid substitution from M to I in the encoded protein. The G→A mutation in position 918 in codon 306 corresponds to an amino acid substitution from M to V in the encoded protein.

Table 3. Performance of the CONRAS test compared with the BACTEC 460TB test as a reference method for 89 *M. tuberculosis* isolates

Drug	CONRAS ^a results	No. of isolates with the following BACTEC 460TB ^b result		Sensitivity (%)	Specificity (%)	Likelihood ratio ^c	
		susceptible	resistant			positive	negative
STR	susceptible	48	11	73	100	infinite	0.27
	resistant	0	30				
EMB	susceptible	59	9	64	92	8.19	0.39
	resistant	5	16				

^aPerformed with an initial concentration of 1.0 mg/L streptomycin (STR) and 5.0 mg/L ethambutol (EMB). If an isolate was resistant to the drug, the test was repeated with a higher concentration of the drug in question (6.0 mg/L for STR and 7.5 mg/L EMB).

^bDrug concentrations: 6.0 mg/L for STR and 7.5 mg/L for EMB.

^cA positive likelihood ratio above 10 or a negative likelihood ratio below 0.1 indicates an excellent test performance whereas ratios between 5 and 10 (positive likelihood ratio) and between 0.1 and 0.2 (negative likelihood ratio) indicate an adequate performance.⁶

BACTEC 460TB. When isolates resistant to low antibiotic concentrations were retested using high antibiotic concentrations (6.0 mg/L streptomycin and 7.5 mg/L ethambutol), there were 11 (Table 1) and 14 (Table 2) discordant results for streptomycin and ethambutol, respectively. There was good agreement for streptomycin and moderate agreement for ethambutol between the CONRAS test and BACTEC 460TB ($\kappa_{\text{streptomycin}}$ 0.73 and 0.74 and $\kappa_{\text{ethambutol}}$ 0.42 and 0.59, low and high drug concentrations, respectively, $P < 0.001$). There was good agreement between the manual MGIT test and BACTEC 460TB for streptomycin and moderate agreement for ethambutol ($\kappa_{\text{streptomycin}}$ 0.77 and $\kappa_{\text{ethambutol}}$ 0.50, $P < 0.001$). The average time required to obtain a susceptibility result using the CONRAS test was 5.4 (range 3–9) days, compared with 5.1 (range 5–8) days for BACTEC 460TB and 5.2 (range 5–8) days for the manual MGIT test. The results for susceptible

isolates were not more likely to be available before the results for isolates with any resistance (mean 5.3 and 5.5 days, respectively, $P = 0.50$). The performance of the CONRAS and MGIT tests in comparison with BACTEC 460TB is given in Tables 3 and 4.

Discussion

We evaluated the performance of the CONRAS method for the AST of *M. tuberculosis* to streptomycin and ethambutol in liquid cultures. Using BACTEC 460TB as a reference, the sensitivity (true drug resistance) and specificity (true drug susceptibility) of the high-level CONRAS test were 73 and 100% for streptomycin and 64 and 92% for ethambutol, respectively. The sensitivity of the manual MGIT test for streptomycin and ethambutol was

Table 4. Performance of the MGIT test compared with BACTEC 460TB as a reference method for 89 *M. tuberculosis* isolates

Drug	MGIT ^a results	No. of isolates with the following BACTEC 460TB ^b result		Sensitivity (%)	Specificity (%)	Likelihood ratio ^c	
		susceptible	resistant			positive	negative
STR	susceptible	43	5	88	90	8.42	0.14
	resistant	5	36				
EMB	susceptible	55	9	64	86	4.55	0.42
	resistant	9	16				

^aDrug concentrations: 0.8 mg/L for streptomycin (STR) and 3.5 mg/L for ethambutol (EMB).

^bDrug concentrations: 6.0 mg/L for STR and 7.5 mg/L for EMB.

^cA positive likelihood ratio above 10 or a negative likelihood ratio below 0.1 indicates an excellent test performance, whereas ratios between 5 and 10 (positive likelihood ratio) and between 0.1 and 0.2 (negative likelihood ratio) indicate an adequate performance.⁶

88 and 64%, respectively, and the specificity was 90 and 86%, respectively. Other studies have reported a higher sensitivity, ranging from 75 to 100% for ethambutol and from 82 to 95% for streptomycin.^{2,7,8} However, these studies included fewer resistant isolates than the present study and the precision of the sensitivity estimates was accordingly lower. It is conceivable that other small studies similar to the present study but with lower sensitivity remain unpublished, resulting in bias towards publication of small studies with higher sensitivity.

There were 11 and 14 discordant single-drug results between the CONRAS test using 6 mg/L streptomycin and 7.5 mg/L ethambutol, respectively, and the BACTEC 460TB. Seven of the 11 isolates with discrepant results for streptomycin belonged to the WHO/IUATLD panel of isolates. The expected results from WHO supported the BACTEC 460TB system's resistant outcome in all these seven isolates (data not shown). Five of the 14 discordant isolates for ethambutol were from WHO/IUATLD. All five strains were resistant according to WHO results. The WHO results supported the BACTEC 460TB system's outcome in four of the five isolates. Mutations were detected in 12 of the 20 isolates that tested susceptible using the CONRAS test but tested resistant using BACTEC 460TB (streptomycin 6, ethambutol 6). A mutation in the *embB* gene was detected in one isolate which tested susceptible to ethambutol using BACTEC 460TB but tested resistant using the CONRAS assay. The PM test resolved the result in favour of the CONRAS assay. It is possible that some isolates in which no mutations were detected but which were shown to be resistant using BACTEC 460TB harbour rare mutations in regions other than the *rrs*, *rpsL* and *embB* genes.

In an evaluation of three colorimetric AST methods for *M. tuberculosis*—namely, the nitrate reductase assay performed on LJ (Löwenstein–Jensen medium) slants, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the resazurin microtitre assay (REMA)—Montoro *et al.*⁹ showed with the PM as reference that there was a high level of agreement among these methods in the detection of rifampicin and isoniazid resistance. In contrast, the level of agreement for the liquid-culture-based microtitre plate assays (MTT and REMA) for susceptibility testing for streptomycin and ethambutol was less promising. Testing *M. tuberculosis* for susceptibility to ethambutol using both radiometric and colorimetric tests can be problematic. This may be due to the narrow range between the MICs of susceptible and resistant isolates, reduced activity of the drug in

the culture medium or the bacteriostatic nature of ethambutol.¹⁰ In 1994, the WHO initiated an external quality control for AST of *M. tuberculosis* in 16 laboratories across the world. The specificity values of ethambutol (mean 98%) were significantly higher than the sensitivity values (mean 66%).⁴ Thus, the moderate sensitivity of AST for ethambutol may lead to under-reporting of drug resistance. A recent study² based on 36 *M. tuberculosis* isolates obtained from the WHO, comparing the manual MGIT test with BACTEC 460TB, showed that for ethambutol and streptomycin there was a 78 and 72% agreement, respectively. A possible explanation for the problems observed in testing for ethambutol resistance may be heteroresistance.² As a consequence, the repeatability of AST methods for ethambutol may be low.

Our previous study³ showed that the CONRAS test is rapid, inexpensive and accurate for AST of *M. tuberculosis* to rifampicin and isoniazid. The present study shows that susceptibility data for streptomycin and ethambutol obtained using the CONRAS test are somewhat less reliable. The CONRAS test is comparable to the manual MGIT test but is considerably cheaper. The accurate determination of the susceptibility of *M. tuberculosis* to streptomycin and ethambutol is difficult using colorimetric tests in liquid media and also using conventional methods.^{2,7,9} The good (streptomycin) and moderate (ethambutol) agreement between the CONRAS test and the manual MGIT test in comparison with BACTEC 460TB indicates that the concentrations, especially of ethambutol, in these non-radiometric susceptibility tests need to be better defined.

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Transparency declarations

No declarations were made by the authors of this paper.

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