Determination of the susceptibility of *Mycobacterium tuberculosis* to pyrazinamide in liquid and solid media assessed by a colorimetric nitrate reductase assay

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Objectives: The aim of this study was to develop and evaluate two colorimetric nitrate reductase-based antibiotic susceptibility (CONRAS) tests, the CONRAS-liquid test and the CONRAS-LJ test, to enable susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide. To enhance the growth of *M. tuberculosis* in 7H9 broth with acid pH (6.0), the effect of three potential growth-promoting substances (reconstitution fluid, fastidious organism supplement and epidermal growth factor) was evaluated.

Methods: Seventy-five *M. tuberculosis* strains were tested for susceptibility to pyrazinamide in the CONRAS-liquid test performed in Middlebrook 7H9 broth, and 77 *M. tuberculosis* strains were tested for susceptibility to pyrazinamide in the CONRAS-LJ test performed on Löwenstein–Jensen medium. The BACTEC 460TB system, sequencing of the *pncA* gene and Wayne’s assay were used as reference tests. Growth of 10 *M. tuberculosis* strains in conventional 7H9 broth and in acid 7H9 broth with and without growth-promoting substances added was evaluated by the nitrate reductase assay.

Results: By using the BACTEC 460TB system as the reference test, the sensitivity and specificity of the CONRAS-liquid test (100 mg/L pyrazinamide) tested on 75 *M. tuberculosis* strains were 87.5% and 83.7%, respectively. The corresponding sensitivity and specificity of the CONRAS-LJ test (1200 mg/L pyrazinamide) tested on 77 *M. tuberculosis* strains were 100% and 84.1%, respectively. The mean turn around time of the CONRAS-liquid test was significantly shorter than that of the CONRAS-LJ test (mean, 9.4 and 14.5 days, respectively; *P*<0.001). In addition, no effect of growth-promoting substances on the growth of *M. tuberculosis* in a broth with acid pH was seen.

Conclusions: The CONRAS tests were rapid, cheap and easy to perform and interpret. Both tests should be evaluated on extended strain batteries in multicentre studies before they can be considered for use in susceptibility testing of *M. tuberculosis* to pyrazinamide in resource-limited settings.

Keywords: tuberculosis, susceptibility test, nitrate reductase assay, pyrazinamide

Introduction

Pyrazinamide is a first-line anti-tuberculosis (TB) drug administered in the first 2 months of standard TB treatment. Pyrazinamide is also important in the treatment of multidrug-resistant (MDR) TB (MDR-TB). The drug has a unique sterilizing effect by killing the semi-dormant *Mycobacterium tuberculosis* population in the acid environment that occurs during active inflammation. The introduction of pyrazinamide as an anti-TB drug has shortened the standard TB treatment from 9 to 6 months. Pyrazinamide is administered as a pro-drug and is converted into its active form, pyrazinoic acid (POA), by the bacterial enzyme pyrazinamidase. Pyrazinamidase is encoded by the *pncA* gene, and mutations in *pncA* are associated with pyrazinamide resistance.

It is important to perform antibiotic susceptibility testing (AST) to measure drug resistance not only before treatment, but also during the course of therapy to identify acquired resistance, especially in areas with a high incidence of MDR-TB. However, AST of pyrazinamide is difficult to perform. Despite its effective sterilizing effect *in vivo*, pyrazinamide is not active against *M. tuberculosis* in media with conventional pH (pH 6.8) since POA can only accumulate inside *M. tuberculosis* cells in an acid...
environment. Zhang et al. have shown that pyrazinamide-susceptible M. tuberculosis strains have a defect in the efflux pump mechanism for POA, whereas the naturally pyrazinamide-resistant Mycobacterium smegmatis has a more active POA efflux pump mechanism and can reduce the intracellular concentration of toxic POA. M. tuberculosis strains have reduced or no growth in media with low pH. The conventional proportional method, originally introduced to be performed on Löwenstein–Jensen (LJ) medium, has been used for AST of M. tuberculosis for several decades. For pyrazinamide susceptibility testing, the proportional method has traditionally been performed on Middlebrook 7H10 agar at pH 5.5 with a pyrazinamide concentration of 25–50 mg/L. However, ~10% of M. tuberculosis isolates fail to grow sufficiently in media with pH 5.5.

The inoculum size affects the pH in the media and is important to control in AST of pyrazinamide. It has been shown that an inoculum size of ≥10^7 cells/mL increases the pH in the medium from 5.6 to 6.6, whereas an inoculum size of 10^6 cells/mL gives a minor increase (~0.3 units) in pH.8

Another alkalizing factor is fetal bovine serum (FBS), which at a concentration of 10% increases the pH in the medium from 5.5 to 6.5. A pyrazinamide concentration of 60 mg/L at pH 6.5 is inactive against M. tuberculosis, resulting in false-resistant test results. Thus, it is crucial to find the correct balance between pH in the medium, inoculum size and drug concentration in order to perform reliable susceptibility testing of pyrazinamide.

The BACTEC 460TB system (Becton Dickinson Biosciences, Sparks, MD, USA) is one of the commercial systems that offers AST of pyrazinamide using a critical concentration of 100 mg/L pyrazinamide in 7H12 broth at pH 6.0. However, problems with false-resistant or inconsistent test results for pyrazinamide in the BACTEC 460TB system have been reported. Moreover, Miller et al. have shown that 3.5% of 428 M. tuberculosis strains tested had inadequate growth in the 7H12 broth used for pyrazinamide susceptibility testing in the BACTEC 460TB system. The BACTEC MGIT 960 (Becton Dickinson Biosciences), MB/BacT (Organon Teknika, Turnhout, Belgium) and BacT/Alert 3D (bioMérieux, Inc., Durham, NC, USA) systems also offer susceptibility testing of pyrazinamide. However, AST by automatic broth systems requires costly equipment and reagents, and may not be affordable in many countries. A cheaper alternative for susceptibility testing of pyrazinamide was suggested by Heijts and Sanchez in 2000. They recommended testing for pyrazinamide susceptibility by using Middlebrook 7H10 agar supplemented with 10% FBS as the growth-promoting additive instead of the more often used 10% oleic acid/albumin/dextrose/catalase (OADC), pH 6.2 (acidified by phosphate buffer), and a pyrazinamide concentration of 900–1200 mg/L. Alternative methods for assessing pyrazinamide susceptibility that are not dependent on growth of the M. tuberculosis strains in acid medium are sequencing of pncA, the gene associated with pyrazinamide resistance, and Wayne's assay, where the pyrazinamidase activity is evaluated. Most pyrazinamide-resistant M. tuberculosis strains have lost their pyrazinamidase activity, thereby giving a negative test result in Wayne's assay.

Several growth-promoting supplements have been suggested to enhance the growth of M. tuberculosis in media with conventional (pH 6.8) or reduced pH. The BACTEC 460TB system supplements the broth used in the pyrazinamide susceptibility test with reconstitution fluid (RF), which contains the growth promotion substance polyoxyethylene stearate (POES). Other studies have demonstrated a growth-promoting effect on M. tuberculosis by adding epidermal growth factor (EGF) or fastidious organism supplement (FOS) to media with conventional pH. Bermudez et al. demonstrated an increased growth of Mycobacterium avium and M. tuberculosis in Middlebrook 7H9 broth supplemented with EGF. FOS is a growth-promoting supplement of BSA and haemin used in cultures of sterile body fluids. Szpinda demonstrated an increased M. tuberculosis culture sensitivity (by 20%) and a decrease of 5.4 days in the detection time by adding FOS to Middlebrook 7H9 broth.

New modifications for AST have been introduced. The nitrate reductase assay (NRA) on LJ medium has been demonstrated to be a good alternative to the more expensive automatic AST systems for rifampicin and isoniazid. The colorimetric nitrate reductase-based antibiotic susceptibility (CONRAS) test has also been applied successfully in broth, and the test demonstrated a good performance for rifampicin and isoniazid, and a moderate performance for streptomycin and ethambutol. Resistance to pyrazinamide by M. tuberculosis as a marker of pyrazinamide resistance assessed by the NRA has recently been described. However, testing of M. tuberculosis susceptibility to pyrazinamide in the NRA has not been described previously. Thus, the aim of this study was to evaluate the effect of different growth-promoting supplements on M. tuberculosis in broth with acid pH, and to evaluate the CONRAS test in liquid broth (the CONRAS-liquid test) and on solid medium (the CONRAS-LJ test) with pH 6.0 for susceptibility testing of M. tuberculosis to pyrazinamide.

Materials and methods

Strains

A total of 77 nitrate-positive M. tuberculosis strains, including M. tuberculosis H37Rv (ATCC 27294), were included in the study. Sixty-six strains were collected during the national drug resistance survey conducted by the Medical Research Council of South Africa in 2001–2002. The strains were obtained from the Haukeland University Hospital, Bergen, Norway. Thirty-three (42.9%) of the 77 M. tuberculosis strains were resistant to pyrazinamide by the BACTEC 460TB system. Two strains were excluded from the evaluation of the CONRAS-liquid test due to non-mycobacterial contamination (Gram-positive cocci). The identity of strains was confirmed by AccuProbe® Culture Identification Tests (Gen-Probe Inc., San Diego, CA, USA) and spoligotyping. The nitrate-positive reference strain M. tuberculosis H37Rv (ATCC 27294) and a nitrate-negative clinical strain of the Mycobacterium avium-intracellulare complex were used as controls in the CONRAS tests. The strains were coded and tested blindly. The purity of each mycobacterial strain was checked on blood agar plates [Colombia agar base (Oxoid, Basingstoke, UK) with 5% sheep blood], which were incubated at 37°C for 48 h.

Growth-promoting substances

Ten 3-week-old M. tuberculosis strains were suspended in 7H9 broth (as defined below) and adjusted to a turbidity equal to that of a no. 0.5 McFarland standard before diluting 1:5 in 7H9 broth. Each bacterial suspension (110 µL) was inoculated into the following tubes: six tubes containing 1 mL of 7H9 broth, pH 6.8; six tubes containing acid 7H9 broth (as defined under the section on CONRAS-liquid); six tubes containing acid 7H9 broth with EGF (Sigma-Aldrich Norway AS, Norway); final...
CONRAS-liquid test, pilot study

The CONRAS-liquid test was performed as previously described24,25 with modifications. In an initial pilot study including 19 M. tuberculosis strains, the CONRAS-liquid test was used as a pyrazinamidine concentration of 100 mg/L. The M. tuberculosis strains were coded and tested blindly. Lyophilized pyrazinamide (Becton Dickinson Biosciences) was reconstituted with RF provided in the pyrazinamide kit, as recommended by the manufacturer. Three-week-old M. tuberculosis cultures grown on LJ medium were suspended in sterile tubes with glass beads containing 4 mL of Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with 10% OADC, 0.5% glycerol, 0.05% Tween 80 and 850 mg/L NaNO3 (7H9 broth) and acidified by 1 M KH2PO4 (pH 4.2) to pH 6.0 (acid 7H9 broth). The bacilli suspensions were adjusted to turbidities equal to that of a no. 0.5 McFarland standard. From each bacterial suspension 110 μL was added to four sterile polystyrene tubes (Sarstedt, Numbrecht, Germany) containing 1 mL of acid 7H9 broth (growth control tubes) and two tubes containing 1 mL of acid 7H9 broth and pyrazinamide. A 110 μL aliquot of a 1/100 dilution of the bacterial suspension was added to one tube containing 1 mL of acid 7H9 broth (1/100 growth control).

The tubes were incubated at 37°C with gentle shaking. The NRA was performed by adding sequentially the three nitrate test reagents into the test tube (25 μL of concentrated hydrochloric acid, 50 μL of 0.2% sulphanilamide and 50 μL of 0.1% N-1-naphthyl-ethylenediamine dihydrochloride). The resulting colour changes were read visually after 5 min and interpreted as described previously.24 The antibiotic-containing tubes were tested if the colour intensity in the growth control tube was equal to or greater than that of the 3+ standard tube. The colour intensity of the 1/100 growth control tube of a strain was compared with the intensity of the colour standard tubes. A strain was classified as resistant if the colour intensity of the antibiotic-containing tube was two or more colour intensity gradations above that of the 1/100 growth control tube.

In the pilot study the CONRAS-liquid test was performed on days 4, 7, 10, 14 and 21. No strains turned out to be positive (colour intensity equal to or greater than that of the 3+ standard tube) on day 4. Thus, for the main study, the CONRAS-liquid test was performed on days 7, 10, 14 and 21.

CONRAS-liquid test, main study

The CONRAS-liquid test was evaluated on 75 M. tuberculosis strains with an initial pyrazinamidine concentration of 100 mg/L. The M. tuberculosis strains were coded and tested blindly. If a strain was resistant to 100 mg/L pyrazinamide, the test was repeated with higher pyrazinamidine concentrations (300 and 900 mg/L). Growth-negative strains in acid 7H9 broth were retested in the same broth. After the code was broken, AST results that were discrepant between the CONRAS-liquid test and the BACTEC 460TB system were retested by the CONRAS-liquid test.

CONRAS-LJ test, pilot study

The CONRAS-LJ test was performed as previously described22 with modifications: standard LJ medium containing 1000 mg/L KNO3 with and without pyrazinamide incorporated was used. Each LJ slant consisted of 4.5 mL of medium. The pH in the medium was adjusted to pH 6.0 by addition of 1 M KH2PO4 (pH 4.2). In an initial pilot study, 19 M. tuberculosis strains were coded and tested blindly in the CONRAS-LJ test at pyrazinamide critical concentrations of 100 and 900 mg/L.

One loopful of bacteria from 3-week-old M. tuberculosis cultures on LJ medium (conventional pH) were suspended in 3 mL of PBS in sterile tubes with glass beads. The suspensions were adjusted to a turbidity equal to that of a no. 1 McFarland standard. For each isolate, 200 μL of the bacterial suspension was inoculated onto three LJ slants (pH 6.0, growth control tubes), 200 μL of the bacterial suspension was inoculated onto two LJ slants (pH 6.0) with pyrazinamide and 200 μL of a 1/10 dilution of the bacterial suspension was inoculated onto one LJ slant (pH 6.0; 1/10 growth control).

The tubes were incubated at 37°C and examined on days 7, 14 and 21. When mycobacterial growth was observed (by the help of a magnifying glass) on the surface of the medium, 500 μL of a mixture of the NRA reagents was added to one of the growth control tubes. If a colour intensity equal to or greater than that of the 3+ standard tube was obtained, the corresponding antibiotic-containing tubes and the 1/10 growth control tube were also tested by the NRA. An isolate was considered resistant to pyrazinamide if the colour intensity observed in the antibiotic-containing tube was greater than that of the 1/10 growth control tube.22

CONRAS-LJ test, main study

Standard LJ medium, pH 6.0, containing 1000 mg/L KNO3 with and without pyrazinamide incorporated was used. The 77 M. tuberculosis strains evaluated were coded and tested blindly and initially tested at a pyrazinamide concentration of 900 mg/L. Strains resistant to 900 mg/L pyrazinamide were further tested at a pyrazinamide concentration of 1200 mg/L.

Radiometric BACTEC 460TB

AST of the South African strains was performed as described previously.28 Susceptibilities of M. tuberculosis strains from Haukeland University Hospital to pyrazinamide were determined by standard procedures for pyrazinamide in a BACTEC 460TB instrument (Becton Dickinson Biosciences). The pyrazinamide critical concentration used was 100 mg/L.

Sequencing of the pncA gene

The pncA gene of the M. tuberculosis strains from South Africa was sequenced as described previously.28 The pncA gene of strains obtained from the Haukeland University Hospital was amplified from each isolate using primers P1 and P6.28 The expected size of the PCR product was 720 bp, which included the full length of the pncA gene (561 bp) as well as 104 bp of the upstream sequence and 55 bp of the downstream sequence.

Wayne’s assay

The pyrazinamidase activity of the M. tuberculosis strains from South Africa28 and Haukeland University Hospital were evaluated according to Wayne’s procedure, as described previously.16 The pyrazinamidase activity assay was considered positive (and, thus, the strain interpreted as pyrazinamide susceptible) if a pink band was seen in the upper part of the test medium.
Pyrazinamide susceptibility testing of M. tuberculosis

Statistical analyses

The performance of the CONRAS-liquid test and the CONRAS-LJ test in comparison with the BACTEC 460TB system was evaluated in terms of sensitivity, specificity, and positive and negative likelihood ratios. A positive likelihood ratio >10 or a negative likelihood ratio <0.1 was considered to indicate excellent test performance, whereas ratios >5 or <0.2 were taken to indicate adequate performance.29,30 The two-tailed paired t-test was used to compare the mean turn around times for the CONRAS-liquid and CONRAS-LJ tests, and the independent two-tailed t-test was used to compare the times needed for CONRAS test completion for susceptible and resistant strains. A P value <0.05 was considered as significant. The agreement between the CONRAS test results and the BACTEC 460TB test results was estimated by 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.31

Results

Growth-promoting substances

Ten M. tuberculosis isolates were tested in conventional 7H9 broth (pH 6.8) and in acid 7H9 broth (pH 6.0) with and without growth-promoting substances (EGF, FOS and RF). Figure 1 demonstrates growth of the strains in the different media from day 0 to day 14. Cultivation in 7H9 broth (pH 6.0), with and without growth-promoting substances, resulted in a similar number of nitrate-positive isolates to those in conventional 7H9 broth (pH 6.8) from day 3 to day 7 (Figure 1). Thus, further pyrazinamide susceptibility testing in the CONRAS-liquid test was performed in 7H9 broth (pH 6.0) without the addition of any growth-promoting substances.

CONRAS-liquid test

Seventy-five M. tuberculosis isolates, including H37Rv (ATCC 27294), were tested by the CONRAS-liquid test for susceptibility to 100 mg/L pyrazinamide in 7H9 broth (pH 6.0). Five (6.7%) of the 75 strains showed inadequate growth and failed to generate a positive test result in the acid 7H9 broth, and were retested. On retesting, all strains generated a positive test result. Strains resistant to 100 mg/L pyrazinamide were tested at higher pyrazinamide concentrations (300 and 900 mg/L). The test performance of the CONRAS-liquid test is shown in Tables 1 and 2. An overview of discrepant test results between the CONRAS-liquid test using 100 mg/L pyrazinamide, the BACTEC 460TB system, pncA sequencing and Wayne’s assay is provided in Table 3.

CONRAS-LJ test

In a pilot study with 19 M. tuberculosis strains (7 pyrazinamide susceptible and 12 pyrazinamide resistant by the BACTEC 460TB system) the critical concentration of pyrazinamide in the CONRAS-LJ test was evaluated. The strains were tested for susceptibility to 100 mg/L pyrazinamide and resistant strains were tested at a higher pyrazinamide concentration (900 mg/L). The pilot showed that the CONRAS-LJ test performed better using a pyrazinamide critical concentration of 900 mg/L compared with a pyrazinamide critical concentration of 100 mg/L (two and four discordant test results, respectively, when the BACTEC 460TB system was used as the reference test).

In the main study, a total of 77 M. tuberculosis strains, including the 19 strains from the pilot, were evaluated in the CONRAS-LJ test using 900 mg/L pyrazinamide. The test performance of the CONRAS-LJ test is shown in Tables 4 and 5. An overview of discrepant test results between the CONRAS-LJ test using 1200 mg/L pyrazinamide, the BACTEC 460TB system, pncA sequencing and Wayne’s assay is provided in Table 6.

Turn around time

The average time required to obtain a susceptibility test result by the CONRAS-liquid test was 9.4 days, compared with 14.5 days for the CONRAS-LJ test. The mean turn around time of the CONRAS-liquid test was significantly shorter than that of the CONRAS-LJ test (P<0.001). Susceptibility test results by the CONRAS-liquid test were available in 7 days for 53% of the strains, in 10 days for 85% of the strains, in 14 days for 95% of the strains and in 21 days for 100% of the strains. The corresponding numbers for the CONRAS-LJ test were as follows: 7 days for 21%, 14 days for 71% and 21 days for 100%. AST results for pyrazinamide-susceptible strains were not significantly likely to be available before those for strains with pyrazinamide resistance in the CONRAS-liquid test (mean, 9.3 and 9.4 days, respectively; P=0.88) or in the CONRAS-LJ test (mean, 14.7 and 14.5 days, respectively; P=0.85).

Discussion

TB is still a major problem worldwide, with ~9 million people developing active disease and 2 million TB deaths annually.32 AST of M. tuberculosis strains helps to optimize TB treatment on an individual basis, which is especially important in areas with known resistance against anti-TB drugs. Unfortunately, areas with drug resistance are areas with limited resources in their healthcare system. Thus, rapid, reliable and low cost antibiotic susceptibility tests are urgently needed. We have developed nitrate reductase-based susceptibility tests to enable susceptibility testing of pyrazinamide, an important first-line anti-TB drug. Since it has been shown that M. tuberculosis strains have reduced growth in acid environments, three
potential growth-promoting substances, RF, FOS and EGF, were evaluated in acid 7H9 broth.

Miller et al. have shown that POES, which is an ingredient in the RF used to promote growth of M. tuberculosis in the susceptibility testing of pyrazinamide in the BACTEC 460TB system, can inhibit M. tuberculosis growth. However, the inhibitory effect of POES was not observed in the present study. The RF supplement did not, on the other hand, result in more rapid positive test results in the CONRAS-liquid test. Animal sera (fetal equine serum found in FOS, or FBS) and EGF are supplements that have been suggested to enhance the growth of M. tuberculosis cells in growth media. However, addition of these supplements did not result in more rapid positive test results in the CONRAS-liquid test using media with pH 6.0. Thus, RF, FOS and EGF were not included in susceptibility testing of the clinical M. tuberculosis isolates to pyrazinamide.

This study evaluates two CONRAS tests for assessing susceptibilities of M. tuberculosis isolates to pyrazinamide. The CONRAS-liquid test was evaluated using three pyrazinamide concentrations; 100, 300 and 900 mg/L. The CONRAS-LJ test was evaluated using two pyrazinamide concentrations; 900 and 1200 mg/L. In the CONRAS-liquid test the specificity increased from 83.7% to 100% and from 84.4% to 100% when the pyrazinamide concentration increased, using the BACTEC 460TB system and pncA sequencing, respectively, as reference methods. However, the sensitivity decreased from 87.5% to 62.5% and

### Table 1. Performance of the CONRAS-liquid test compared with BACTEC 460TB as the reference test for 75 M. tuberculosis strains

<table>
<thead>
<tr>
<th>CONRAS-liquid</th>
<th>susceptible</th>
<th>resistant</th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/L PZA</td>
<td>36</td>
<td>4</td>
<td>87.5 (70.1 – 95.9)</td>
<td>83.7 (68.7 – 92.7)</td>
<td>5.38 (2.70 – 10.7)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>28</td>
<td></td>
<td></td>
<td>0.15 (0.06 – 0.38)</td>
</tr>
<tr>
<td>300 mg/L PZA</td>
<td>42</td>
<td>12</td>
<td>62.5 (43.7 – 78.3)</td>
<td>97.7 (86.2 – 99.9)</td>
<td>26.9 (3.80 – 190)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20</td>
<td></td>
<td></td>
<td>0.38 (0.25 – 0.60)</td>
</tr>
<tr>
<td>900 mg/L PZA</td>
<td>43</td>
<td>12</td>
<td>62.5 (43.7 – 78.3)</td>
<td>100 (89.8 – 100)</td>
<td>infinite</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td></td>
<td></td>
<td>0.38 (0.24 – 0.59)</td>
</tr>
</tbody>
</table>

CONRAS-liquid, colorimetric nitrate reductase-based antibiotic susceptibility test in Middlebrook 7H9 broth, pH 6.0; PZA, pyrazinamide; CI, confidence interval.

### Table 2. Performance of the CONRAS-liquid test compared with pncA sequencing as the reference test for 75 M. tuberculosis strains

<table>
<thead>
<tr>
<th>CONRAS-liquid</th>
<th>wild-type</th>
<th>mutation</th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/L PZA</td>
<td>38</td>
<td>2</td>
<td>93.3 (76.5 – 98.8)</td>
<td>84.4 (69.9 – 93.0)</td>
<td>6.00 (3.02 – 11.9)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>28</td>
<td></td>
<td></td>
<td>0.08 (0.02 – 0.38)</td>
</tr>
<tr>
<td>300 mg/L PZA</td>
<td>44</td>
<td>10</td>
<td>66.7 (47.1 – 82.1)</td>
<td>97.8 (86.8 – 99.9)</td>
<td>30.0 (4.25 – 212)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20</td>
<td></td>
<td></td>
<td>0.34 (0.21 – 0.57)</td>
</tr>
<tr>
<td>900 mg/L PZA</td>
<td>45</td>
<td>10</td>
<td>66.7 (47.1 – 82.1)</td>
<td>100 (90.2 – 100)</td>
<td>infinite</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td></td>
<td></td>
<td>0.33 (0.20 – 0.55)</td>
</tr>
</tbody>
</table>

CONRAS-liquid, colorimetric nitrate reductase-based antibiotic susceptibility test in Middlebrook 7H9 broth, pH 6.0; PZA, pyrazinamide; CI, confidence interval.
from 93.3\% to 66.7\%, respectively. The increase in false-
susceptible test results with increasing drug concentrations
indicates that the pyrazinamide concentrations of 300 and
900 mg/L were somewhat high. Likewise, in the CONRAS-LJ
test, the specificity of the CONRAS-LJ test increased (from 70.5\%
and from 64.6\% to 77.1\%) with increasing pyrazinamide
concentrations. The sensitivity remained stable (100\%). The
reduced number of false-resistant test results with an increased
pyrazinamide concentration (1200 mg/L) suggests that a higher
pyrazinamide concentration was more suitable. However, the
CONRAS-LJ test using 1200 mg/L pyrazinamide produced seven
false-resistant and no false-susceptible test results when the
BACTEC 460TB system was used as the reference test, suggesting
that a somewhat higher pyrazinamide critical concentration may
be required.

The CONRAS-liquid test with a pyrazinamide concentration of
100 mg/L demonstrated a good performance (kappa = 0.70)
and the CONRAS-LJ test with a pyrazinamide concentration of
1200 mg/L performed excellently (kappa = 0.82) when the
BACTEC 460TB system was used as the reference test. Both
nitrate reductase-based tests performed well when
pncA
sequencing was used as the reference test (kappa values:
0.76 for the CONRAS-liquid test and 0.72 for the CONRAS-LJ
test).

### Table 3. Discrepant test results between the CONRAS-liquid test using 100 mg/L pyrazinamide, the BACTEC 460TB system, pncA sequencing and Wayne's assay

<table>
<thead>
<tr>
<th>Strain identity</th>
<th>CONRAS-liquid 100(^a) (retest result)</th>
<th>BACTEC 460TB</th>
<th>pncA sequencing</th>
<th>Wayne's assay</th>
<th>CONRAS-liquid 300(^b)</th>
<th>CONRAS-liquid 900(^c)</th>
<th>CONRAS-LJ 1200(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>R (R)</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>23</td>
<td>R (S)</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>30</td>
<td>R (S)</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>40</td>
<td>NG (S and S)</td>
<td>R</td>
<td>T &gt; C pos 452, L151S</td>
<td>–</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>41</td>
<td>R (S)</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>46</td>
<td>R (R)</td>
<td>S</td>
<td>sm</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>51</td>
<td>R (R)</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>67</td>
<td>S (S)</td>
<td>R</td>
<td>A &gt; G pos 35, D12G</td>
<td>–</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>73</td>
<td>R (S)</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>76</td>
<td>S (S)</td>
<td>R</td>
<td>sm</td>
<td>–</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>77</td>
<td>R</td>
<td>R</td>
<td>wt</td>
<td>–</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>78</td>
<td>S (S)</td>
<td>R</td>
<td>wt</td>
<td>–</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>79</td>
<td>R</td>
<td>R</td>
<td>wt</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

CONRAS-liquid, colorimetric nitrate reductase-based antibiotic susceptibility test in Middlebrook 7H9 broth, pH 6.0; CONRAS-LJ, colorimetric nitrate reductase-based antibiotic susceptibility test on Löwenstein–Jensen medium, pH 6.0; S, susceptible; R, resistant; NG, no growth; wt, wild-type; sm, silent mutation.

\(^a\)100 mg/L pyrazinamide.
\(^b\)300 mg/L pyrazinamide.
\(^c\)900 mg/L pyrazinamide.
\(^d\)1200 mg/L pyrazinamide.

### Table 4. Performance of the CONRAS-LJ test compared with BACTEC 460TB as the reference test for 77 M. tuberculosis strains

<table>
<thead>
<tr>
<th>No. of isolates with the following BACTEC 460TB results</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONRAS-LJ susceptible resistant</td>
<td></td>
</tr>
<tr>
<td>900 mg/L PZA susceptible</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>33</td>
</tr>
<tr>
<td>1200 mg/L PZA susceptible</td>
<td></td>
</tr>
<tr>
<td>susceptible</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
</tr>
</tbody>
</table>

CONRAS-LJ, colorimetric nitrate reductase-based antibiotic susceptibility test on Löwenstein–Jensen medium, pH 6.0; PZA, pyrazinamide; CI, confidence interval.

from 93.3\% to 66.7\%, respectively. The increase in false-
susceptible test results with increasing drug concentrations
indicates that the pyrazinamide concentrations of 300 and
900 mg/L were somewhat high. Likewise, in the CONRAS-LJ test,
the specificity of the CONRAS-LJ test increased (from 70.5\% to
84.1\% and from 64.6\% to 77.1\%) with increasing pyrazinamide
concentrations. The sensitivity remained stable (100\%). The
reduced number of false-resistant test results with an increased
pyrazinamide concentration (1200 mg/L) suggests that a higher
pyrazinamide concentration was more suitable. However, the
CONRAS-LJ test using 1200 mg/L pyrazinamide produced seven
false-resistant and no false-susceptible test results when the
BACTEC 460TB system was used as the reference test, suggesting
that a somewhat higher pyrazinamide critical concentration may
be required.

The CONRAS-liquid test with a pyrazinamide concentration of
100 mg/L demonstrated a good performance (kappa = 0.70)
and the CONRAS-LJ test with a pyrazinamide concentration of
1200 mg/L performed excellently (kappa = 0.82) when the
BACTEC 460TB system was used as the reference test. Both
nitrate reductase-based tests performed well when pncA
sequencing was used as the reference test (kappa values: 0.76 for the CONRAS-liquid test and 0.72 for the CONRAS-LJ test).
It has previously been shown that BACTEC 460TB using a single pyrazinamide concentration of 100 mg/L may produce false-resistant or inconsistent test results, and suggestions that the recommended gold standard for pyrazinamide susceptibility testing would give more correct pyrazinamide susceptibility test results if the critical concentration of 100 mg/L was increased have been made. Thus, the high numbers of false-susceptible test results in the CONRAS-liquid test with pyrazinamide concentrations of 300 and 900 mg/L compared with the BACTEC 460TB system may have been true-susceptible test results. However, 10 of the 12 false-susceptible test results in the CONRAS-liquid test when pyrazinamide concentrations of 300 or 900 mg/L were used had non-synonymous mutations in the \textit{pncA} gene and all 12 strains had lost their pyrazinamidase activity as determined by Wayne's test (data not shown).

Several factors can affect the susceptibility testing of \textit{M. tuberculosis} to pyrazinamide and may cause disagreement between the reference and the test candidate. Zhang et al. have previously demonstrated that the presence of 0.5% BSA may increase the MIC of pyrazinamide, which in turn may result in false-resistant pyrazinamide test results. Also an increase in medium pH by large bacterial inocula or a short incubation time due to accelerated growth rate can cause false-resistant results compared with the reference test. In this study, five of seven false-resistant test results in the CONRAS-LJ test (1200 mg/L pyrazinamide) and six of seven false-resistant test results in the CONRAS-liquid test (100 mg/L pyrazinamide) using BACTEC 460TB as the reference were positive on day 7 (the first day of reading), which may support the hypothesis that an accelerated growth rate can result in false-resistant test results. The four false-resistant test results that were interpreted as true-susceptible test results when retesting in the CONRAS-liquid test, using the BACTEC 460TB system as the reference, may be explained by: large bacterial

### Table 5. Performance of the CONRAS-LJ test compared with \textit{pncA} sequencing as the reference test for 77 \textit{M. tuberculosis} strains

<table>
<thead>
<tr>
<th></th>
<th>CONRAS-LJ</th>
<th>BACTEC 460TB</th>
<th>pncA sequencing</th>
<th>Wayne's assay</th>
<th>CONRAS-liquid 100°</th>
<th>CONRAS-liquid 300°</th>
<th>CONRAS-liquid 900°</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{pncA} sequencing results</td>
<td>wild-type</td>
<td>mutation</td>
<td>Sensitivity % (95% CI)</td>
<td>Specificity % (95% CI)</td>
<td>Likelihood ratio positive (95% CI)</td>
<td>Likelihood ratio negative (95% CI)</td>
<td></td>
</tr>
<tr>
<td>900 mg/L PZA susceptible</td>
<td>31</td>
<td>0</td>
<td>100 (85.4 – 100)</td>
<td>64.6 (49.4 – 77.4)</td>
<td>2.82 (1.98 – 4.51)</td>
<td>0.00 (0.00 – 0.16)</td>
<td></td>
</tr>
<tr>
<td>resistant</td>
<td>17</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1200 mg/L PZA susceptible</td>
<td>37</td>
<td>0</td>
<td>100 (85.4 – 100)</td>
<td>77.1 (62.3 – 87.5)</td>
<td>4.36 (2.68 – 8.31)</td>
<td>0.00 (0.00 – 0.14)</td>
<td></td>
</tr>
<tr>
<td>resistant</td>
<td>11</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CONRAS-LJ, colorimetric nitrate reductase-based antibiotic susceptibility test on Löwenstein–Jensen medium, pH 6.0; PZA, pyrazinamide; CI, confidence interval.

### Table 6. Discrepant test results between the CONRAS-LJ test using 1200 mg/L pyrazinamide, the BACTEC 460TB system, \textit{pncA} sequencing and Wayne's assay

<table>
<thead>
<tr>
<th>Strain identity</th>
<th>CONRAS-LJ</th>
<th>BACTEC 460TB</th>
<th>pncA sequencing</th>
<th>Wayne's assay</th>
<th>CONRAS-liquid 100°</th>
<th>CONRAS-liquid 300°</th>
<th>CONRAS-liquid 900°</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>R</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>23</td>
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<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>34</td>
<td>R</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>35</td>
<td>R</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>37</td>
<td>R</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>46</td>
<td>R</td>
<td>S</td>
<td>sm</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>51</td>
<td>R</td>
<td>S</td>
<td>wt</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>76</td>
<td>R</td>
<td>R</td>
<td>wt</td>
<td>–</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>77</td>
<td>R</td>
<td>R</td>
<td>wt</td>
<td>–</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>78</td>
<td>R</td>
<td>R</td>
<td>wt</td>
<td>–</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>79</td>
<td>R</td>
<td>R</td>
<td>wt</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

CONRAS-LJ, colorimetric nitrate reductase-based antibiotic susceptibility test on Löwenstein–Jensen medium, pH 6.0; CONRAS-liquid, colorimetric nitrate reductase-based antibiotic susceptibility test in Middlebrook 7H9 broth, pH 6.0; S, susceptible; R, resistant; wt, wild-type; sm, silent mutation.

a100 mg/L pyrazinamide.
b300 mg/L pyrazinamide.
c900 mg/L pyrazinamide.
d1200 mg/L pyrazinamide.
inocula, which could increase the pH in the medium resulting in inactivation of pyrazinamide; the MIC for the strain tested being close to the breakpoint between pyrazinamide susceptibility and resistance; or accidentally not adding pyrazinamide to the test.

Sequencing of the \textit{pncA} gene and testing for pyrazinamidase activity are alternative ways to assess pyrazinamide susceptibility. Previous studies have shown that 72\%–98\% of the strains resistant to pyrazinamide have mutations in the \textit{pncA} gene.\textsuperscript{5,6} It has been shown that high-level (900 mg/L) pyrazinamide-resistant strains in the BACTEC 460TB system have mutations in the \textit{pncA} gene, while low-level resistant strains (MIC, 200–300 mg/L) have a wild-type \textit{pncA} gene,\textsuperscript{12,35} resulting in false-susceptible test results if \textit{pncA} sequencing was used as the only antibiotic susceptibility test. Moreover, questions have been raised about the critical concentration of pyrazinamide used in the BACTEC 460TB system being too low, and strains with MIC values slightly above the breakpoint concentration of 100 mg/L pyrazinamide used in the reference test and absent \textit{pncA} mutations may, in effect, be pyrazinamide susceptible. Thus, 4 of the 33 pyrazinamide-resistant \textit{M. tuberculosis} strains as assessed by the BACTEC 460TB system in this study were wild-type (3 strains) or had a silent mutation (1 strain) in the \textit{pncA} gene, and 2 of these strains were susceptible in the CONRAS-liquid test, at all pyrazinamide concentrations tested (100, 300 and 900 mg/mL). Others suggest that mutations in unknown mycobacterial targets of pyrazinamide account for the remaining resistant \textit{M. tuberculosis} strains with a wild-type \textit{pncA} gene, or the activation of an efflux pumping mechanism transporting the drug out of the cells.\textsuperscript{12}

The need for reduced pH in the media is an argument for performing susceptibility testing of \textit{M. tuberculosis} to pyrazinamide by methods not dependent on growth, i.e. identification of mutations in pyrazinamide-associated genes by molecular methods. This is in agreement with the WHO expert group that recommends the use of molecular line probe assays for identification of MDR \textit{M. tuberculosis} strains in areas with a high incidence of patients infected with MDR-TB. Previous studies have shown that MDR \textit{M. tuberculosis} strains have a high frequency of pyrazinamide resistance,\textsuperscript{8,32} resulting in an ethambutol monotherapy for the patient. A rapid and reliable line probe assay for pyrazinamide susceptibility testing would provide valuable information in guiding the individual treatment regimen in areas with high MDR-TB incidence. Sekiguchi et al.\textsuperscript{34} have recently developed a line probe assay for identification of mutations in the \textit{pncA} gene. By testing 225 \textit{M. tuberculosis} clinical isolates using the BACTEC 460TB system and Wayne’s assay as reference tests, they found a sensitivity and specificity of 100\%. However, we have previously shown in a study on 130 \textit{M. tuberculosis} isolates from South Africa (71 MDR and 59 fully susceptible to rifampicin, isoniazid, ethambutol and streptomycin) that 43 isolates were resistant to pyrazinamide by BACTEC 460TB. Nine percent of the pyrazinamide-resistant isolates did not have a mutation in the \textit{pncA} gene and for many isolates Wayne’s test was difficult to interpret. Moreover, costly reagents and instruments and the requirement for skilled laboratory personnel limit their use in countries with limited resources.

Both CONRAS tests evaluated were easy to interpret; the test is quantitative since the colony numbers can be recorded visually; and it may be less prone to contamination, although in our study neither of the two CONRAS tests was prone to contamination. All strains tested by the CONRAS-LJ test yielded an interpretable result on the first evaluation, whereas five strains had to be retested in the CONRAS-liquid test before a test result could be obtained. Uneven distribution of bacilli in liquid suspensions or unviable bacilli may explain why some strains were negative when tested for drug susceptibility. Salfinger and Heifets\textsuperscript{33} have previously shown that resistant strains in particular have reduced or no growth in medium with pH 5.5. Three of the five strains in this study that had to be retested in the CONRAS-liquid test were resistant by the BACTEC 460TB system. The advantages of the CONRAS-liquid test were: a significantly shorter turn around time than the CONRAS-LJ test; and a reduced amount of medium and antibiotic needed since the CONRAS-liquid test was performed using a smaller volume and with a lower pyrazinamide concentration than the CONRAS-LJ test, resulting in reduced costs (total volume in the CONRAS-liquid test, 1.11 mL and 100 mg/L pyrazinamide; total volume in the CONRAS-LJ test, 4.5 mL and 1200 mg/L pyrazinamide).

In summary, the two CONRAS tests for susceptibility testing of \textit{M. tuberculosis} to pyrazinamide were rapid, cheap and easy to perform and interpret. We recommend performing the CONRAS-liquid test in Middlebrook 7H9 broth supplemented with 10\% OADC, pH 6.0 and with a pyrazinamide concentration of 100 mg/L. Further studies have to be performed on a larger panel of strains with varying susceptibilities to pyrazinamide before an optimal critical concentration of pyrazinamide in the CONRAS-LJ test can be recommended. Both tests have to be evaluated on extended strain batteries in multicentre studies before the NRAs can be recommended for the use of pyrazinamide susceptibility testing of \textit{M. tuberculosis} strains in clinical settings.

Acknowledgements

We thank Ms Matsie Mphahlele, Dr Karin Weyer and Dr Bernard Fourie, MRC, South Africa and Haukeland University Hospital, Norway for the \textit{M. tuberculosis} isolates used in this study, and Geir Egil Eid for statistical advice.

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Transparency declarations

None to declare.

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\textsuperscript{1} Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. \textit{Tuberce 1985; 66}: 219–25.


