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# Sputum PCR–Single-Strand Conformational Polymorphism Test for Same-Day Detection of Pyrazinamide Resistance in Tuberculosis Patients[▽]

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**Pyrazinamide is a first-line drug for treating tuberculosis, but pyrazinamide resistance testing is usually too slow to guide initial therapy, so some patients receive inappropriate therapy. We therefore aimed to optimize and evaluate a rapid molecular test for tuberculosis drug resistance to pyrazinamide. Tuberculosis PCR–single-strand conformational polymorphism (PCR-SSCP) was optimized to test for mutations causing pyrazinamide resistance directly from sputum samples and *Mycobacterium tuberculosis* isolates. The reliability of PCR-SSCP tests for sputum samples ($n = 65$) and *Mycobacterium tuberculosis* isolates ($n = 185$) from 147 patients was compared with four tests for pyrazinamide resistance: Bactec-460 automated culture, the Wayne biochemical test, DNA sequencing for *pncA* mutations, and traditional microbiological broth culture. PCR-SSCP provided interpretable results for 96% (46/48) of microscopy-positive sputum samples, 76% (13/17) of microscopy-negative sputum samples, and 100% of *Mycobacterium tuberculosis* isolates. There was 100% agreement between PCR-SSCP results from sputum samples and *Mycobacterium tuberculosis* isolates and 100% concordance between 50 blinded PCR-SSCP rereadings by three observers. PCR-SSCP agreement with the four other tests for pyrazinamide resistance varied from 89 to 97%. This was similar to how frequently the four other tests for pyrazinamide resistance agreed with each other: 90 to 94% for Bactec-460, 90 to 95% for Wayne, 92 to 95% for sequencing, and 91 to 95% for broth culture. PCR-SSCP took less than 24 hours and cost approximately $3 to $6, in contrast with the other assays, which took 3 to 14 weeks and cost $7 to $47. In conclusion, PCR-SSCP is a relatively reliable, rapid, and inexpensive test for pyrazinamide resistance that indicates which patients should receive pyrazinamide from the start of therapy, potentially preventing months of inappropriate treatment.**

Tuberculosis kills 1.7 million people annually (2), and antibiotic resistance increasingly complicates control (14). Rapid molecular tests for susceptibility to isoniazid and rifampin (rifampicin) (18) can guide treatment decisions within a day of laboratory diagnosis of tuberculosis disease (3). In contrast, pyrazinamide is another first-line antibiotic included in most tuberculosis treatment regimes, but the results of traditional culture-based pyrazinamide susceptibility testing are infrequently available because they require several weeks and generally involve tuberculosis culture in inhibitory acid media (25, 26). Consequently, little is known about the prevalence of pyrazinamide resistance, although one-third of retreated tuberculosis patients in Peru have pyrazinamide-resistant strains (23).

Pyrazinamide resistance is usually caused by a mutation in the *pncA* gene that disrupts pyrazinamidase activity, preventing conversion of pyrazinamide to its active form (17). The causative mutations may be detected in cultured isolates by sequencing or PCR–single-strand conformational polymorphism (PCR-SSCP) (3, 16). We modified the published protocols for PCR and SSCP (3, 16, 20) to allow pyrazinamide susceptibility testing directly on sputum, as well as enhanced testing of tuberculosis cultures, in order to guide treatment decisions more rapidly and reliably than current techniques.

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## MATERIALS AND METHODS

**Sputum samples.** Sputum samples were collected from individuals with a cough presenting for testing for suspected tuberculosis in peri-urban shantytowns and a human immunodeficiency virus (HIV) clinic and from patients in the first weeks of tuberculosis therapy. This research utilized excess anonymized sputum samples and *Mycobacterium tuberculosis* isolates that would otherwise have been discarded. Nonidentifiable clinical information that was recorded included whether the sample was collected before or during tuberculosis therapy. To increase the number of samples with pyrazinamide resistance, we selected some samples from patients with suspected multidrug-resistant tuberculosis. The 34 sputum samples that had negative microscopy, culture, and PCR test results were considered to be negative control samples. A total of 185 sputum samples from 147 tuberculosis patients were analyzed in this study, in addition to the negative control samples

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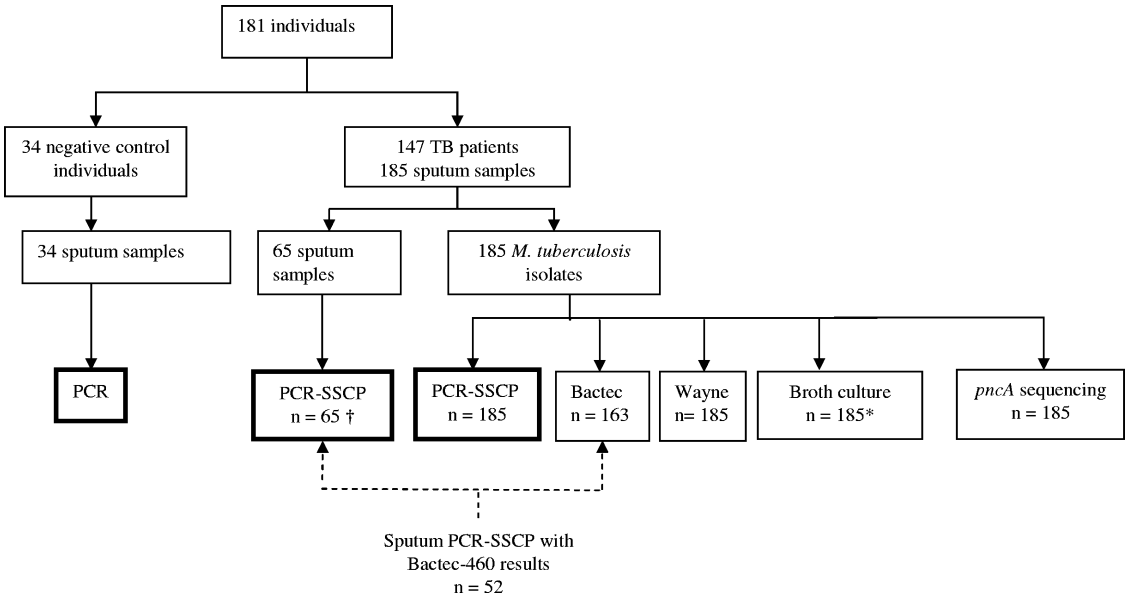


FIG. 1. Standards for reporting of diagnostic accuracy (STARD) flow diagram of procedures for sputum samples and *M. tuberculosis* isolates. *, the broth culture test was done in duplicate, and 147 of the 185 samples yielded concordant results (see text); †, sputum sample was tested with SSCP for 65 of the 185 samples (see text); TB, tuberculosis.

(see Fig. 1). Single reference isolates of *Mycobacterium smegmatis*, *Mycobacterium avium*, and *Mycobacterium kansasii* were also tested by PCR-SSCP.

**Procedures.** All tests were done by technicians who were blinded to clinical details and to the results of other assays. Fifty PCR-SSCP results were blindly read independently by three technicians to assess concordance.

**Microbiology.** Sputum samples were decontaminated and tested for the presence of mycobacteria by auramine microscopy (8, 10) and the microscopic observation drug susceptibility assay (8, 11). Species were confirmed by IS*6110* PCR (10), and sequencing of the *pncA* gene confirmed that none of the clinical samples were *Mycobacterium bovis* (which has a specific C169G mutation). *M. bovis* was used as a positive control for pyrazinamide resistance in all PCR-SSCP assays. The gold standard test for pyrazinamide susceptibility was considered to be the Bactec-460 commercial assay (Becton Dickinson, Sparks, MD) that was performed for 163/185 (88%) of isolates according to the manufacturer's instructions, with a critical concentration of 100 μg/ml pyrazinamide (15). All 185 isolates were also tested by traditional microbiological broth subculture, with visualization of mycobacterial growth in acidified (pH 6.0) Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase containing pyrazinamide concentrations doubling from 6.25 to 800 μg/ml (17). Because of variability in microbiological pyrazinamide susceptibility testing (26), duplicate *Mycobacterium tuberculosis* isolates were derived separately from the same sample and subjected to this test. Only concordant duplicate results were included in the broth culture data analysis; discordant duplicates were considered to have indeterminate results with this test. However, isolates with discordant duplicate broth culture results were included in all other data analyses. For broth culture, a critical concentration of ≥200 μg/ml was used because this was the optimal cutoff determined by receiver-operator curve analysis compared with the Bactec-460 assay (which utilized 100 μg/ml [data not shown]).

**DNA.** DNA from sputum samples was extracted by boiling 0.5 ml of the 2 ml decontaminated sample in Chelex (10). Isolates from positive cultures were extracted using the proteinase K phenol-chloroform method (22).

**PCR.** PCR was performed in 25-μl reaction mixtures (3, 16, 20). Sputum samples were preamplified using P1/6 primers (16), and a second nested PCR was then performed using inner primers (3). Isolates were amplified using the second nested PCR alone (3). This produced an amplicon of 561 bp. To control for possible PCR contamination in each PCR batch, two negative controls were added using buffer aliquoted after DNA extraction, in the final phase of the PCR preparation. To further verify the PCR, one positive control was added—a smear-negative, culture-positive (i.e., paucibacillary) sample that was decontaminated and processed in parallel with the samples under testing. PCR results were analyzed only if the negative controls had a negative PCR result and the positive control was PCR positive.

**Precautions to prevent nested PCR contamination.** Precautions to prevent nested PCR contamination included the following: preparing buffers and PCR mix and performing DNA extraction in a separate room with dedicated materials and pipettes; handling the first inner PCR products in a separate laminar flow hood from the final outer PCR products; and handling all PCR products in a dedicated electrophoresis room in which protective clothing was used to prevent cross-contamination. These precautions have proved to be essential in our last decade of mycobacterial nested PCR work to prevent carryover contamination from causing false-positive molecular amplification results.

**SSCP.** The 561-bp PCR product was denatured at 98°C for 10 min in an equal volume of formamide buffer (16) and cooled on ice for 10 min, and then 40 μl was electrophoresed. For electrophoresis, a 0.8× mutation detection enhancement gel was used (16 by 18 cm; Cambrex, Rockland, ME) (20) with 3 W constant power in 0.6× TBE (Tris, boric acid, and EDTA) for 20 h at 12°C and then stained with ethidium bromide. DNA size markers were not used because the SSCP technique differentiates DNA amplification products on the basis of secondary conformational structure rather than molecular weight. Consequently, control samples with known pyrazinamide resistance and sensitivity were used for comparison with the clinical samples being tested. Our modifications to the PCR-SSCP technique consisted of adapting the combinations of primers used with the electrophoresis conditions to improve the discriminatory resolution of the PCR-SSCP products. These gels were interpreted by comparing the patterns of bands with pyrazinamide resistance and susceptible control samples that were also run in all gels (see Fig. 2). Pyrazinamide susceptibility has a characteristic pattern of bands in the PCR-SSCP gel, and any differences from this pattern were interpreted as indicating pyrazinamide resistance.

**Wayne biochemistry.** Pyrazinamidase activity was assayed by the Wayne technique on sputum culture isolates (8, 16). Reaction colors from pink to red were recorded and considered positive enzymatic reactions (9, 21).

**DNA sequencing.** The *pncA* gene was sequenced after PCR, using P1 and P6 primers (16) with an ABI 3100 sequencer in one direction.

**Statistical analysis.** Statistical analysis was performed with Stata version 9.0. The gold standard for pyrazinamide resistance was considered to be the Bactec-460 result (6, 16, 25). Comparisons of sensitivity/specificity between the different tests were performed with the proportion test for the binomial distribution.

## RESULTS

Figure 1 shows the tests performed on the 99 sputum samples and 185 *M. tuberculosis* isolates used in this research. Table 1 shows the characteristics of the *M. tuberculosis* isolates,

TABLE 1. Molecular, biochemical, and microbiological characteristics of the *Mycobacterium tuberculosis* isolates[e]

| Characteristic | Amino acid change | No. of samples | No. (%) of resistant samples according to: | | | |
|---|---|---|---|---|---|---|
| | | | Wayne test | PZA broth culture[b] | Bactec-460 | PCR-SSCP |
| Mutations known to be associated with resistance | | | | | | |
| A35C | D12A | 1 | 0 (0) | — | 1 (100) | 0 (0) |
| T40G | C14G | 1 | 0 (0) | — | 1 (100) | 1 (100) |
| IN GG at 188 | FSHIFT | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| C211T | H71Y | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| A226C | T76P | 1 | 1 (100) | — | 1 (100) | 1 (100) |
| C364T | Q122STOP | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| A410C | H137P | 1 | 1 (100) | — | 1 (100) | 1 (100) |
| T11C | L4S | 2 | 2 (100) | 2 (100) | 2 (100) | 0 (0) |
| A29G | Q10R | 2 | 2 (100) | 2 (100) | 2 (100) | 0 (0) |
| C123A | Y41STOP | 2 | 2 (100) | 2 (100) | 2 (100) | 2 (100) |
| T254C | L85P | 2 | 2 (100) | 1/1 (100)[b] | 2 (100) | 2 (100) |
| C161T | P54L | 3 | 3 (100) | 1/2 (50)[b] | 3 (100) | 3 (100) |
| A403C | T135P | 6 | 6 (100) | 5/5 (100)[b] | 6 (100) | 6 (100) |
| C309G | Y103STOP | 9 | 9 (100) | 9 (100) | 8/8 (100) | 9 (100) |
| A152G | H51R | 13 | 12 (92) | 9/9 (100)[b] | 13 (100) | 13 (100) |
| A407G[a] | D136G | 1 | 0 (0) | 0 (0) | 0 (0) | 1 (100) |
| Subtotal | | 47 | 47 | 36 | 46 | 47 |
| Novel mutations | | | | | | |
| G71A | G24D | 1 | 0 (0) | 0 (0) | 1 (100) | 1 (100) |
| T100G | Y34D | 1 | 0 (0) | 1 (100) | 1 (100) | 1 (100) |
| C185T | P62L | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| G232T | G78C | 1 | 0 (0) | 1 (100) | 1 (100) | 1 (100) |
| G356T | W119L | 1 | 1 (100) | — | 1 (100) | 1 (100) |
| T347C | L116P | 1 | 1 (100) | 0 (0) | 1 (100) | 1 (100) |
| IN 18 bp at 258 | IN 6 aa | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| IN 106 bp at 279 | IN 35 aa | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| DE 10 bp at 293 | DE 3 aa | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| IN GA at 377 | FSHIFT | 1 | 1 (100) | 1 (100) | 1 (100) | 0 (0) |
| IN T at 521 | FSHIFT | 1 | 1 (100) | — | 1 (100) | 1 (100) |
| DE CA at 285 | FSHIFT | 2 | 2 (100) | 2 (100) | 2 (100) | 2 (100) |
| DE 81 bp at 359 | DE 27 aa | 2 | 2 (100) | 1/1 (100)[b] | 2 (100) | 2 (100) |
| A36G | D12G | 2 | 1 (50) | 2 (100) | 2 (100) | 0 (0) |
| T280C | F94L | 2 | 1 (50) | — | 2 (100) | 1 (50) |
| G314A | G105D | 2 | 2 (100) | 2 (100) | 2 (100) | 2 (100) |
| A143C[d] | K48T | 4 | 1 (25) | 0/2 (0)[b] | 3/4 (75) | 4 (100) |
| DE T at 490 | FSHIFT | 5 | 5 (100) | 5 (100) | 5 (100) | 5 (100) |
| G145A | D49N | 27 | 26 (96) | 20/20 (100)[b] | 27/27 (100) | 27 (100) |
| Subtotal | | 57 | 57 | 43 | 57 | 57 |
| No mutation | None | 81 | 1 (1) | 3/68 (4)[b] | 7/60 (5)[c] | 0 (0) |
| Subtotal | | 81 | 81 | 68 | 60 | 81 |
| Total | | 185 | 185 | 147 | 163 | 185 |

[a] A previously reported mutation causing pyrazinamide resistance in Lowenstein-Jensen testing (12), but in the current research, as shown in the table above, duplicate isolates of this strain were both pyrazinamide susceptible in Bactec-460 testing and additional duplicate isolates were also both pyrazinamide susceptible in pyrazinamide broth culture testing (all MICs, <100).

[b] — and indicated values, the 38 isolates with discordant results in duplicate pyrazinamide (PZA) susceptibility tests in broth culture. These 38 discordant results are excluded from the broth culture column but are included in all other analyses (see Materials and Methods).

[c] Two of 37 isolates with no mutation had a PZA-resistant Bactec-460 result.

[d] One of four isolates with an A143C mutation had a PZA-susceptible Bactec-460 result.

[e] The 185 isolates had the following pattern of antimicrobial resistance: isoniazid, 107/161 (67%); rifampin, 101/160 (63%); multidrug-resistant tuberculosis, 93/161 (58%); streptomycin, 65/160 (41%); and ethambutol, 32/161 (20%). FSHIFT, nucleotide insertions or deletions resulting in frameshift; IN, insertion; DE, deletion; aa, amino acid.

81 (44%) of which had no mutation in the pyrazinamidase gene, 57 (31%) of which had novel mutations, and 47 (25%) of which had mutations previously known to be associated with pyrazinamide resistance. No silent mutations in the *pncA* gene were found. The Bactec-460 results ($n$ = 163) demonstrated that when any mutation occurred in the *pncA* gene, the strains were nearly always resistant; only 2/103 (1.9%) strains with mutations (K48T and D136G) were sensitive according to

TABLE 2. Sensitivity and specificity of pyrazinamide susceptibility tests[a]

| Susceptibility method | No. of isolates | % (No. of isolates) | |
|---|---|---|---|
| | | Sensitivity | Specificity |
| Bactec-460 as gold standard | | | |
| Sputum PCR-SSCP[b] | 52 | 85.7 (24/28) | 95.8 (23/24) |
| Isolate PCR-SSCP | 163 | 85.2 (92/108)* | 96.4 (53/55) |
| Wayne | 163 | 84.3 (91/108)* | 100 (55/55) |
| Broth culture | 125 | 89.0 (73/82) | 95.4 (41/43) |
| DNA sequencing | 163 | 93.5 (101/108) | 96.4 (53/55) |
| DNA sequencing for *pncA* mutations as gold standard | | | |
| Sputum PCR-SSCP[c] | 59 | 92.6 (25/27) | 100.0 (32/32) |
| Isolate PCR-SSCP | 185 | 91.4 (95/104) | 100.0 (81/81) |
| Wayne | 185 | 87.5 (91/104) | 98.8 (80/81) |
| Broth culture | 147 | 92.4 (73/79) | 95.6 (65/68) |

[a] Considering Bactec-460 to be the gold standard test for pyrazinamide susceptibility, sensitivity and specificity of the PCR-SSCP test were not significantly different from those of the Wayne test and broth culture. The sensitivity, but not the specificity, of sequencing was significantly higher than those of the isolate PCR-SSCP test and the Wayne test (*). Considering the presence of *pncA* mutations to be the gold standard test for pyrazinamide susceptibility, there were no significant differences in sensitivity and specificity between these four tests.

[b] In subgroup analysis, the sensitivity of the sputum PCR-SSCP test for smear-positive samples compared with that of the Bactec-460 assay was 88% (21/24) and that for smear-negative samples was 75% (3/4).

[c] In subgroup analysis, the sensitivity of the sputum PCR-SSCP test for smear-positive samples compared with that of DNA sequencing was 91% (21/23) and that for smear-negative samples was 100% (4/4).

Bactec-460 results. Sixty strains had no mutation, and 7 (12%) of these were resistant, according to Bactec-460 results. There was high concordance between Bactec-460 and *pncA* gene sequencing (94%) (Table 2).

**PCR-SSCP interpretation.** PCR-SSCP provided clearly interpretable results for 46/48 (96%) smear-positive sputum samples; 13/17 (76%) smear-negative, culture-positive sputum samples; and all 185 *M. tuberculosis* isolates (Table 2 and Fig. 2). Thus, 9.2% of sputum PCR-SSCP and 2.4% of all PCR-SSCP tests had uninterpretable results because no PCR amplification products were visualized, and this was more likely for sputum samples than for isolates ($P < 0.0001$) and more likely for relatively paucibacillary smear-negative samples than for smear-positive sputum samples ($P < 0.02$). There was 100% concordance between the 50 blinded gel rereadings by three observers.

**PCR-SSCP accuracy.** PCR-SSCP diagnosis of pyrazinamide resistance had 85% sensitivity and 96% specificity compared with Bactec-460 tests for the 163 *M. tuberculosis* isolates that had both results (Table 2). Fifty-two of these isolates had previously been tested directly as sputum samples that had 86% sensitivity and 96% specificity compared with Bactec-460 (Table 2). The sensitivity and specificity of the PCR-SSCP to predict *pncA* mutations (Table 2) were 93% and 100%, respectively, for sputum samples and 91% and 100%, respectively, for cultured isolates.

Table 3 shows the percentage of agreement between the results of the sputum PCR-SSCP, isolate PCR-SSCP, Bactec-460, broth culture, Wayne, and DNA sequencing assays. Agreement between the results of the PCR-SSCP tests and other assays varied from 89% to 97%. This was similar to how frequently the results of the four other tests for pyrazinamide resistance agreed with each other: 90 to 94% for Bactec-460; 90 to 95% for Wayne; 92 to 95% for sequencing; and 91 to 95% for broth culture. Agreement between the gold standard Bactec-460 assay and other tests varied from 89% (for isolate PCR-SSCP) to 94% (for DNA sequencing). There was 100% concordance between PCR-SSCP results from direct sputum testing and

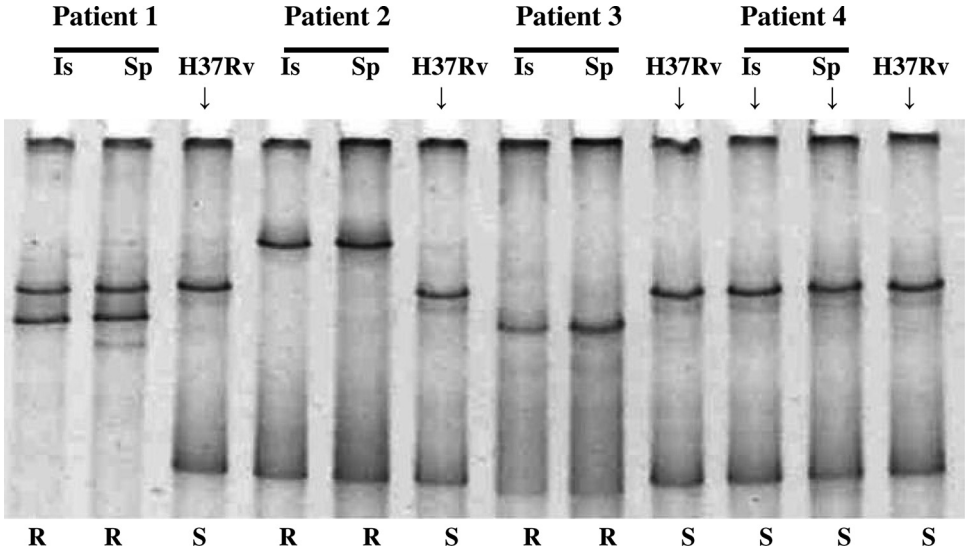


FIG. 2. PCR-SSCP of DNA of *Mycobacterium tuberculosis* isolates and their corresponding sputum samples. The PCR-SSCP gel photograph shows results for direct sputum testing (Sp) and cultured *M. tuberculosis* isolates (Is) from four patients who had concordant pyrazinamide resistance (R) or susceptibility (S) in all tests, as indicated below the photograph. Results for H37Rv *M. tuberculosis* that is pyrazinamide susceptible are also shown. Pyrazinamide susceptibility has a characteristic pattern (marked with an arrow); any difference from this pattern indicates pyrazinamide resistance. Note that the *pncA* mutation was H51R for patient 1; L85P for patient 2; G105D for patient 3; and no mutation was detected for patient 4. For details of these mutations see the text and Table 1.

TABLE 3. Agreement between pyrazinamide susceptibility tests[a]

| Test | % Agreement of results for: | | | | |
|---|---|---|---|---|---|
| | Wayne test | Broth culture | Bactec-460 | Sputum PCR-SSCP | Isolate PCR-SSCP |
| DNA sequencing | 92.4 | 93.9 | 94.5 | 96.6 | 95.1 |
| Wayne test | | 95.2 | 89.6 | 91.5 | 89.7 |
| Broth culture | | | 91.2 | 93.2 | 89.1 |
| Bactec-460 | | | | 90.4 | 89.0 |
| Sputum PCR-SSCP | | | | | 100 |

[a] The percentage of agreement is shown between all the assays used in this research.

subsequent PCR-SSCP testing of the isolates cultured from those sputum samples.

**Other test accuracy.** Compared with Bactec-460, sensitivity and specificity for broth culture were 89% and 95%, and those for Wayne biochemistry were 84% and 100%, respectively (Table 2). If weak Wayne results were considered resistant, then the sensitivity of the Wayne test tended to increase (from 84% to 91%; $P = 0.2$). The sensitivity of *pncA* sequencing was significantly higher than that of PCR-SSCP and Wayne biochemical testing (both $P < 0.05$). There were no significant differences between the sensitivities of other tests. Specificities did not differ significantly between any of the five tests (all $P > 0.05$).

**Controls.** None of the negative controls yielded positive PCR results. All 34 control samples that were microscopy, culture, and IS*6110* PCR negative were also PCR-SSCP negative. Reference isolates of *M. smegmatis* and *M. avium* did not yield PCR products, and a reference isolate of *M. kansasii* yielded a pattern that was different from those of pyrazinamide-susceptible strains of *M. tuberculosis*, consistent with the intrinsic phenotypic resistance of *M. kansasii* to pyrazinamide.

**Pretreatment samples.** The 219 samples (185 positive and 34 negative controls) were collected from 181 individuals. Sensitivities and specificities between PCR-SSCP and the other tests did not differ significantly between 58% of the samples collected before and 42% of the samples collected after 2 weeks of tuberculosis therapy (all $P > 0.05$; data not shown).

**Repeat testing.** PCR-SSCP results for 10 sputum samples (three smear-positive and seven smear-negative, culture-positive samples) were retested because the first PCR yielded no detectable product. Repeat testing was done in duplicate with both 20% and 200% of the original DNA concentration in order to dilute inhibitors and increase detection sensitivity, respectively, and both of these strategies yielded positive results in four of these 10 cases.

**Speed.** The PCR-SSCP sputum test provided results usually on the same day and always within 24 h of starting this analysis. In contrast, 2 to 7 weeks of initial culture to obtain the isolate was done prior to the microbiological, Wayne biochemical, or DNA sequencing tests. After subculture, the microbiological assay then took an additional 9 days, the Wayne biochemistry an additional 7 days, and DNA sequencing an additional 2 days—i.e., 24 to 51 days total.

**Costs.** The approximate local per-sample costs for each test are stated as consumables + labor cost ($5/hour) = total in United States dollars, as follows: $1.36 + $3.00 = $3.36 for the sputum PCR-SSCP test; $1.50 + $2.00 = $3.50 for sputum culture (8, 11), followed by $2.60 + $4.00 = $6.60 for broth culture; $0.27 + $3.00 = $3.27 for the Wayne assay; $40.00 + $3.00 = $43.00 for Bactec-460; and $14.10 + $3.00 = $17.10 for DNA sequencing.

## DISCUSSION

Pyrazinamide is one of the four first-line drugs for tuberculosis treatment and plays a key role, sterilizing organs in animal studies and in humans, reducing relapse rates from 22% to 8% (1). However, pyrazinamide resistance is common in some countries (23), and resistance testing is usually too slow to influence initial therapy and is infrequently available in the resource-poor settings where drug-resistant tuberculosis occurs most. Consequently, patients with pyrazinamide-resistant tuberculosis are usually treated for months inappropriately with this hepatotoxic drug and consequently fail to benefit from substitution therapy with another drug to prevent relapse. In this research, we demonstrate a same-day test for pyrazinamide resistance that is sufficiently rapid to allow adequate therapy to be administered for pyrazinamide-resistant strains, potentially preventing relapse. This may allow appropriate therapy to be administered earlier to patients with multidrug-resistant tuberculosis and retreatment cases. Whether laboratory diagnosis of *M. tuberculosis* was by direct sputum testing or by culture, PCR-SSCP determined pyrazinamide susceptibility with sensitivity and specificity that were similar to those of the much slower microbiological and biochemical tests.

Studies of small numbers of selected strains have identified *pncA* genotype-phenotype discrepancies in pyrazinamide susceptibility (3, 4, 7, 16, 24). However, our study suggests that the PCR-SSCP genotypic test has sufficient specificity to be used as a rapid screening tool for pyrazinamide resistance directly from sputum samples at high risk for pyrazinamide resistance. Furthermore, we found that earlier PCR-SSCP protocols distinguished poorly between susceptible and resistant strains (3, 16) and our modifications made the PCR-SSCP test results simple to read, with bands that are clearly separated. These modifications appeared to be crucial to reliability and to allow direct sputum testing.

Culture methods for determining pyrazinamide resistance are notorious for providing inconsistent results (5). This is exemplified by the data presented here, in which there was imperfect agreement between pyrazinamide susceptibility tests, both between different tests and between duplicate replicates of the same test on the same sample. This difficulty and unreliability of pyrazinamide resistance testing cause most tuberculosis patients globally to receive empirical pyrazinamide therapy without the benefit of susceptibility testing. Consequently, the capacity of the PCR-SSCP test for same-day determination of pyrazinamide susceptibility directly on sputum specimens at low cost makes this an important test for optimizing tuberculosis therapy.

Direct PCR-SSCP testing of sputum reduces not only the delay but also the biohazard associated with manipulating *M. tuberculosis* isolates that contain mycobacterial concentrations several orders of magnitude higher than sputum. The material costs of the PCR-SSCP test compared favorably with those of the other assays. The PCR-SSCP test required molecular bi-

ology equipment and expertise available in most microbiology laboratories in industrialized nations but present in only a minority of laboratories in the developing world. The equipment costs required for the PCR-SSCP test were similar to those required for the enzyme-linked immunosorbent assay technique widely used for HIV-testing, but much higher than those for traditional microbiological drug susceptibility testing. The technical skills required and staff labor costs for PCR-SSCP were comparable to those required for enzyme-linked immunosorbent assays, but we found traditional microbiological drug susceptibility testing to be considerably more technically demanding than the PCR-SSCP test.

Genetic tests for drug susceptibility are problematic for isoniazid because resistance is associated with multiple different genes (13), whereas genetic tests are relatively straightforward for rifampin resistance that is invariably caused by mutations in a single gene (*rpoB*) (19). As confirmed by our study, it is known that *pncA* mutations are strongly associated with pyrazinamide resistance, suggesting that the pyrazinamide resistance mechanism appears to be driven mainly by this single gene. This fundamental principle, together with the fact that *pncA* silent mutations are rare, supports the use of the PCR-SSCP test to predict pyrazinamide resistance. However, the multiplicity of mutations associated with pyrazinamide resistance in our study implies that genetic probe-based testing for pyrazinamide resistance is likely to be complex.

We found that only 1.9% of *pncA* mutations were not associated with pyrazinamide resistance but 12% of isolates with no *pncA* mutations were pyrazinamide resistant, limiting the sensitivity of the PCR-SSCP test for detecting pyrazinamide resistance and suggesting that an alternate mechanism is responsible for these cases of pyrazinamide resistance. Smaller studies have reported poorer concordance between molecular and microbiological tests for pyrazinamide susceptibility (25). The reasons for the discrepancy with our study may be that strains from other regions have different resistance mechanisms or that the variety of microbiological pyrazinamide susceptibility tests used may have had different accuracies (5). Therefore, it will be important to reevaluate the PCR-SSCP test prior to clinical use in other regions.

The main limitation of this study is the lack of patient clinical details because anonymized samples were used. However, this does not alter the conclusion of the study that the PCR-SSCP test rapidly determined pyrazinamide susceptibility within a day of laboratory diagnosis of tuberculosis. Also, the high cost of the Bactec-460 assay prevented us from using this commercial assay for every sample. Indeed, the challenge of funding pyrazinamide susceptibility testing with the only Bactec-460 machine in this country highlights the advantage of the much less expensive PCR-SSCP test that we evaluated. Although the PCR-SSCP test had encouraging results for three samples of *Mycobacterium* species other than *M. tuberculosis*, further assessment is required.

In response to the findings presented here, our ongoing research is assessing the reliability of SSCP for a larger number of sputum specimens. We used different DNA extraction techniques for sputum samples and tuberculosis isolates because these were the routine standard operating procedures in our laboratory for these specimens. Specifically, the proteinase K phenol-chloroform method had previously been our standard technique for DNA extraction and was used in the current research for extracting DNA from isolates. We had recently found that the much simpler Chelex technique had a similar DNA extraction yield for sputum samples and that the DNA product had acceptable stability, and we therefore used this technique for sputum samples. We now aim to assess the relative DNA yields and stabilities from both techniques when used for processing both sputum samples and tuberculosis isolates in order to determine the optimal DNA extraction protocol.

In contrast to currently available tests, the tuberculosis sputum PCR-SSCP test is rapid and thus clinically relevant, with the potential to guide the decision of whether pyrazinamide should be included at the initiation of tuberculosis therapy. This is important because the global use of 6-month "short-course" oral chemotherapy for tuberculosis depends upon the action of pyrazinamide. Therefore, if the PCR-SSCP test indicates pyrazinamide resistance at the time of diagnosis, then therapy should be modified to substitute alternative medications to prevent relapse and to avoid unnecessary administration of this potentially hepatotoxic drug. Thus, this PCR-SSCP technique represents a significant improvement upon the weeks of delay required by current tests. The PCR-SSCP test may therefore allow earlier initiation of optimal therapy, with the potential to reduce morbidity, mortality, and tuberculosis transmission.

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We have no conflicts of interest in relation to this work.

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