BNP immunoassays. We found better agreement between the results obtained with the IRMA and ADVIA methods (Fig. 1D; see also Fig. 1D in the online Data Supplement). We also observed a significant difference between the results obtained with these two methods (\( P = 0.0032 \)); the mean (SD) difference was 2.1 (140.8) ng/L.

All immunoassay methods could differentiate between healthy individuals and patients with different degrees of heart failure as well as between patients with mild (NYHA classes I and II) and severe (NYHA classes III and IV) heart failure (Table 1B).

We tested the diagnostic accuracy of immunoassay methods for BNP and NT-proBNP by ROC curve analysis. All immunoassay methods clearly differentiated between the group of healthy individuals and the two groups of cardiac patients with mild (NYHA classes I and II) or severe (NYHA classes III and IV) heart failure [areas under the curves (AUC), 0.865–0.999]. The electrochemiluminescence immunoassay for NT-proBNP (AUC = 0.954; 95% confidence interval, 0.920–0.978) showed the best power, compared with the other immunoassays (AUC values ranging from 0.865 for the ADVIA to 0.902 for the IRMA; \( P < 0.01 \)), for separating healthy individuals from patients with mild symptoms of heart failure. The MEIA method showed different diagnostic characteristics compared with other the BNP immunoassays (McNemar \( \chi^2 \) test, \( P = 0.0036 \) vs ADVIA, 0.0083 vs IRMA, and 0.0153 vs POCT TRIAGE) in this group of patients, whereas there were no significant differences in performance among the other immunoassays. All immunoassay methods performed well (AUC values, 0.982–0.999) in differentiating between healthy individuals and patients with severe heart failure.

The main goal of this study was to evaluate the analytical performance of several BNP and NT-proBNP immunoassays in samples subjected to the same preanalytical conditions. This protocol was chosen to better focus on performance differences among the tested methods to reduce as much as possible the other confounding causes of variability typical of multicenter studies or metaanalysis of published data. However, the clinical results of this study cannot be extrapolated to other clinical settings. Comparison of our results with those of other recent studies (7–14) suggests that diagnostic accuracy can strongly depend on patient selection and on the cardiac natriuretic peptide assayed (BNP, N-terminal pro-A-type natriuretic peptide, or NT-proBNP), as well as on the analytical performance and diagnostic accuracy of the immunoassay chosen.

References


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New PCR-Based Assay for Detection of Common Mutations Associated with Rifampin and Isoniazid Resistance in Mycobacterium tuberculosis, Soetlana Dubiley,1,* Angelina Mayorova,2 Anna Ignatova,3 Eugene Kirillov,2 Valentina Stepanishina,2 Alexander Kolesnikov,1 and Igor Shemyakin2 1Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS, Moscow, Russia; 2State Research Center for Applied Microbiology, Obolensk, Moscow Region, Russia; *address correspondence to this author at: Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS, 16/10 Miklukho-Maklaya, Moscow, 1179997 Russia; fax 7-095-3357103, e-mail lana@ibch.ru

Tuberculosis imposes a major burden of death and disease on the human population. Each year, tens of millions of people become infected with Mycobacterium tuberculosis, several millions develop clinical disease, and more than 2 million die of tuberculosis (1, 2). The successful treatment of tuberculosis depends heavily on timely diagnostics and selection of an adequate treatment strategy. Use of genotyping tools based on molecular techniques decreases the time necessary for the detection of drug resistance in M. tuberculosis from several weeks to a few days or even less, and a patient’s treatment regimen can
be adjusted more rapidly to account for any detected drug resistance. Several mutations conferring resistance to the first-line drugs used for antituberculosis treatment have been described [for a review, see Ref (3)], making detection of these mutations by molecular biology techniques feasible. Rifampin and isoniazid belong to the first-line drugs used for treatment of tuberculosis (4). Resistance to rifampin, one of the most efficient antitubercular drugs, is conferred mainly by mutations in the rpoB gene. Ninety-six percent of rifampin-resistant cases are attributable to point mutations, insertions, or deletions located in an 81-nucleotide segment of the rpoB gene (5). Although resistance to isoniazid can be conferred by mutations in several different genes, mutations in katG represent the most frequent cause of the resistance (3, 6). Here we describe the allele-specific depletory PCR (ASD-PCR) assay, which was developed for the detection of mutations associated with M. tuberculosis resistance to first-line drugs, specifically isoniazid and rifampin.

The accuracy and reproducibility of classic allele-specific PCR assays depend greatly on the careful selection of amplification conditions, primer design, and template concentration. Elongation through the mismatched duplex leads to accumulation of mutant template fully complementary to the initially mismatched primer, yielding the false-positive amplicon. Depending on the nature of the mismatch composition, the elongation efficiency of the mismatched duplex in a single round of PCR can vary from 0.0001% to 1% of the elongation efficiency of the perfectly matched duplex (7). We found that inclusion of an additional “auxiliary” pair of primers amplifying an arbitrarily chosen conserved region of the M. tuberculosis genome concurrently with the allele-specific PCR reaction efficiently suppresses unwanted amplification from the 3'-terminally mismatched allelic primer. The auxiliary primer pair always forms perfect duplexes with complementary regions of DNA, thereby yielding efficient amplification of M. tuberculosis DNA fragments unrelated to the target sequence. If both allele-specific and auxiliary primer pairs match perfectly to the DNA, efficient accumulation of both amplicons takes place in the same tube. In cases in which the allele-specific primer forms 3'-terminal mismatches, auxiliary amplicon accumulates with much greater efficiency (Fig. 1A).

Many of the techniques designed for the identification of bacilli of the M. tuberculosis complex rely on detection of the IS6110 element (8). A species-specific genetic marker of M. tuberculosis, mtp40, has been described (9). Use of this genetic marker as an auxiliary amplicon in the ASD-PCR assay permits identification of the organism as either M. tuberculosis (using mtp40 as marker) or another M. tuberculosis complex bacterium (using IS6110 as the marker), while simultaneously identifying mutations conferring drug resistance. Although there are several indications that the sequence of mtp40 can be disrupted by IS6110 insertion (10) and that mycobacteria outside of the M. tuberculosis complex can harbor IS6110 (11), such exceptions can be considered as rare. A 622-bp fragment of mtp40 and a 1085-bp part of the IS6110 element were elaborated as auxiliary amplicons.

The PCR mixture contained 0.1 μM allele-specific forward primer, 0.1 μM reverse primer, genomic DNA of M. tuberculosis, 50 μM each of the deoxynucleotide triphosphates (dNTPs), 2 U of Taq polymerase (Promega), 2 mM MgSO4, 25 mM KCl, and 20 mL/L dimethyl sulfoxide in 10 mM Tris-HCl, pH 8.85. For ASD-PCR, 0.2 μM auxiliary primers (mpFor and mpRev and/or ISFor and ISRev) was added to the reaction mixture. The sequences of all primers used in this study are listed in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue2/. The amount of genomic DNA varied from 10 pg to 100 ng per 50 μL in different experiments. Genomic DNA of M. tuberculosis was purified from cultured cells as described by van Soolingen et al. (12). Amplification was carried out in a DNA Engine PTC-200 thermal cycler (MJ Research, Inc.) under the following conditions: initial denaturation at 96 °C for 5 min, followed by 30–50 cycles with denaturation at 95 °C for 40 s, annealing at 62 °C for 20 s, and elongation at 72 °C for 60 s. After completion of cycling, 15-μL aliquots of the PCR mixture were analyzed by electrophoresis in 1% agarose gels.

Production of allelic and auxiliary amplicons in the same PCR reaction leads to competition between the amplicons for polymerase and dNTPs. Introduction of additional amplicons causes depletion of major reaction components before the amount of the amplicon extended from the 3'-terminally mismatched primer reaches a significant concentration. We speculated that lowering the concentrations of the input dNTPs renders the ASD-PCR assay specificity virtually independent from the amount of the input DNA template. The optimum range of dNTP concentrations, in which efficient and specific PCR still occurs while accumulation of illegitimate product is suppressed, was determined empirically to be 30–50 μM (data not shown). At higher dNTP concentrations, accumulation of the misprimed amplicon is possible during late cycles of PCR. The results (Fig. 1B) showed that under optimized conditions, accrual of the auxiliary amplicon efficiently suppressed accumulation of the false-positive PCR product. Under the same conditions, conventional allele-specific PCR assays showed dramatically reduced reliability, whereas the ASD-PCR assay with either of the auxiliary amplicons completely retained the capacity to differentiate between alleles. Thus, with an ASD-PCR assay, results can be obtained at the stage when all reactions reach their plateaus. Although the time in which the amplicon amount reaches plateau depends on the concentration of the input template, this concentration can be ignored in the analysis of amplification data at the late cycles of the PCR.

To determine whether the power of the ASD-PCR assay to differentiate between alleles depended on the amount of the input genomic DNA, we set up a series of ASD-PCR reactions with the amount of input DNA varying from 10 pg to 100 ng. The auxiliary amplicon used was the 622-bp
fragment of the mtp40. As shown in Fig. 1 of the online Data Supplement, the ASD-PCR assay remained specific in the range of template concentrations spanning at least five orders of magnitude. Under the described conditions, the method permitted efficient mutation detection with as little as 10 pg of input genomic DNA.

We used the ASD-PCR assay for identification of seven point mutations responsible for most of the drug-resistant tuberculosis cases encountered in reference clinics in Russia (13, 14). Six mutations in the rpoB gene (D516V, H526D, H526Y, H526R, H526L, and S531L) and one mutation in the katG gene (S315T) were interrogated. Using the ASD-PCR assay, we achieved highly accurate detection of the katG S315T mutation, which accounts for >90% of isoniazid resistance in Russia (15). Detection of six mutations in the rpoB gene, which together are responsible for ~90% of rifampin resistance (14, 15), was equally efficient. We used the ASD-PCR assay to analyze 39 clinical isolates of M. tuberculosis whose drug resistance was characterized by microbiological methods [as described Viljanen et al. (16)] and DNA sequencing (for primer details, see Table 1 in the online Data Supplement). Typical results of the ASD-PCR assay are presented in Fig. 1C. The ASD-PCR results were in perfect agreement with the sequencing and microbiological data.

ASD-PCR can be used for detection of drug-resistant isolates in a background of wild-type bacilli. We investigated the detection limit of the ASD-PCR assay for identifying the mutant M. tuberculosis present as the minor subpopulation against a background of wild-type bacilli. Wild-type (H37Rv) and mutant DNAs were mixed at different ratios (0–100%) and subjected to ASD-PCR. Reactions were carried out for 50 cycles with 10 ng of DNA mixture. Our results suggest that for all seven mutations analyzed, the ASD-PCR method permits detection of at least 5% mutant sequences in the DNA sample (see Fig. 1D). This sensitivity approaches that of amplification refractory mutation system (17) and phenotypic methods.

In conclusion, development of fast, economical, and technically affordable molecular techniques, such as ASD-PCR, for detection of drug-resistant tuberculosis would facilitate the rapid response required to limit the extent and severity of multiple drug-resistant tuberculosis (MDR-TB) transmission and infection, decrease the incidence of primary MDR-TB, and improve the care of patients with MDR-TB in a cost-effective manner. The
ASD-PCR assay described here can be easily adapted for the identification of other drug-resistant mutations in *M. tuberculosis*. It can also serve as a simple and efficient tool for general single-nucleotide polymorphism analysis.

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**Estimate of Biological Variation of Laboratory Analytes**

**Based on the Third National Health and Nutrition Examination Survey**

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Laboratory analytes for individuals are subject to several sources of variation, including biological variation, pre-analytical variation (specimen collection), analytical variation (bias and imprecision), and postanalytical variation (reporting of results). Biological variation consists of within-person (WP) and between-person (BP) variation. These components of biological variation are used to set analytical quality specifications for bias and imprecision, evaluate serial changes in individual analytes, and assess the clinical utility of population-based reference intervals.

Desirable quality specifications for imprecision (I), bias (B), and total error have been related to the WP CV (CV_w) and the BP CV (CV_v) of laboratory analytes (1–3). Imprecision should be ideally less than one half of the CV_W, and bias should be <0.25[(CV_v)^2 + (CV_g)^2]^{1/2}. The quality specification for total error is to be less than kl + B, where k = 1.65 at α = 0.05. The total CV (CV_v) can be estimated assuming that the CVs of all sources are measured at the same analyte mean and that pre- and postanalytical sources of variation are negligible. The CV_v = [(CV_g)^2 + (CV_w)^2]^{1/2}, where the analytical CV (CV_a) equals the laboratory method imprecision (CV_i) if there is no bias present.

Estimates of CV_w and CV_v for laboratory analytes were derived from the Third National Health and Nutrition Examination Survey (NHANES III) conducted from 1988 to 1994 (4, 5). NHANES III was a cross-sectional survey that collected data on the civilian noninstitutionalized US population through questionnaires and medical examinations, including laboratory analytes. NHANES III used a stratified, multistage probability design to collect a nationally representative sample. The laboratory methods including imprecision (CV_i) for NHANES III have been described (6).

The BP and WP means, SDs, and CVs for 42 general biochemical, nutritional, immunologic, environmental, and hematologic analytes are listed in Table 1. The BP and WP variations were estimated on 24,978 and 2426 sample persons, respectively. The WP sample, ~10% of the sample persons, was recruited for a second analyte measurement. The WP sample was not selected randomly, but with the goal for obtaining approximately equal proportions of males and females with one half between 20 and 39 years of age and one half over 40 years of age. When possible, the second examinations were scheduled at the same time of day as the first examinations. Compared with the BP sample, the WP sample was older (mean age, 42.9 vs 30.8 years), had more non-Hispanic whites (42.2%