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Rapid Detection of *Mycobacterium tuberculosis* and Pyrazinamide Susceptibility Related to *pncA* Mutations in Sputum Specimens through an Integrated Gene-to-Protein Function Approach

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Testing the pyrazinamide (PZA) susceptibility of *Mycobacterium tuberculosis* isolates is challenging. In a previous paper, we described the development of a rapid colorimetric test for the PZA susceptibility of *M. tuberculosis* by a PCR-based *in vitro*-synthesized-pyrazinamidase (PZase) assay. Here, we present an integrated approach to detect *M. tuberculosis* and PZA susceptibility directly from sputum specimens. *M. tuberculosis* was detected first, using a novel long-fragment quantitative real-time PCR (LF-qPCR), which amplified a fragment containing the whole *pncA* gene. Then, the positive amplicons were sequenced to find mutations in the *pncA* gene. For new mutations not found in the Tuberculosis Drug Resistance Mutation Database (www.tbdreamdb.com), the *in vitro* PZase assay was used to test the PZA resistance. This approach could detect *M. tuberculosis* within 3 h with a detection limit of 7.8 copies/reaction and report the PZA susceptibility within 2 days. In an initial testing of 213 sputum specimens, the LF-qPCR found 53 positive samples with 92% sensitivity and 97% specificity compared to the culture test for *M. tuberculosis* detection. DNA sequencing of the LF-qPCR amplicons revealed that 49 samples were PZA susceptible and 1 was PZA resistant. In the remaining 3 samples, with new *pncA* mutations, the *in vitro* PZase assay found that 1 was PZA susceptible and 2 were PZA resistant. This integrated approach provides a rapid, efficient, and relatively low-cost solution for detecting *M. tuberculosis* and PZA susceptibility without culture.

The emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* seriously threatens the control of tuberculosis (TB). In 2011, the World Health Organization (WHO) estimated that there were 12 million cases of TB extant and that 1.4 million people died from TB worldwide (1, 2). Among the extant cases of TB, 630,000 cases were MDR-TB (3). Because the conventional *M. tuberculosis* diagnostic procedures used in hospitals, such as the Löwenstein-Jensen culture method (4), are time consuming, a number of new diagnostic approaches have been developed and have brought incremental improvements in *M. tuberculosis* detection and drug susceptibility testing (5–7). However, few of these approaches solved the problems facing pyrazinamide (PZA) susceptibility testing.

Pyrazinamide (PZA) is a first-line antituberculosis drug and plays a very important role in shortening the chemotherapy time for treating TB (8, 9). Considering the unique effect of PZA, it is important to identify PZA susceptibility, especially when dealing with MDR-TB cases. Due to the inhibitory effect of low pH on *in vitro* growth of *M. tuberculosis* isolates, many phenotypic methods (10, 11) have been developed to identify PZA susceptibility using high PZA breakpoint concentrations in culture medium with a pH of around 6.0. However, even using the method currently recommended by the Clinical and Laboratory Standards Institute, the Bactec 460TB assay system (12), false-resistant results and results that are inconsistent between different laboratories have been reported (13–16).

Since most PZA-resistant *M. tuberculosis* isolates (72 to 97%) carry mutations in the coding region of the *pncA* gene (GenBank accession number U59967) (17–19), genotypic methods to detect *pncA* mutations, especially direct sequencing of the *pncA* gene, are being considered more reliable and accurate methods to predict PZA susceptibility (20–24). A few PZA-resistant strains do not have *pncA* mutations, and new resistance mechanisms due to *rpsA* mutation have been proposed (25), but due to their rarity, *pncA* sequencing is still considered the most reliable method for detection of PZA susceptibility (20). An open-access database (Tuberculosis Drug Resistance Mutation Database [TB-DRMD; www.tbdreamdb.com]) has collected *pncA* mutations leading to PZA resistance found in previous studies (26). However, because PZA resistance mutations are highly diverse and found throughout the *pncA* gene, it is expected that clinical isolates with new *pncA* mutations will be encountered frequently. To link these new mutations to PZA phenotypic resistance usually requires going back to the traditional culture methods (27), which are time consuming. In our previous paper, we described the development of a rapid colorimetric test for PZA susceptibility (27) of clinical *M. tuberculosis* isolates using a PCR-based *in vitro*-synthesized-pyrazinamidase (PZase) assay. Because it judges PZA susceptibility by the activity of the synthesized PZase and not by individual mutations, it could be a good approach for linking the new mutations to PZA phenotypic resistance.

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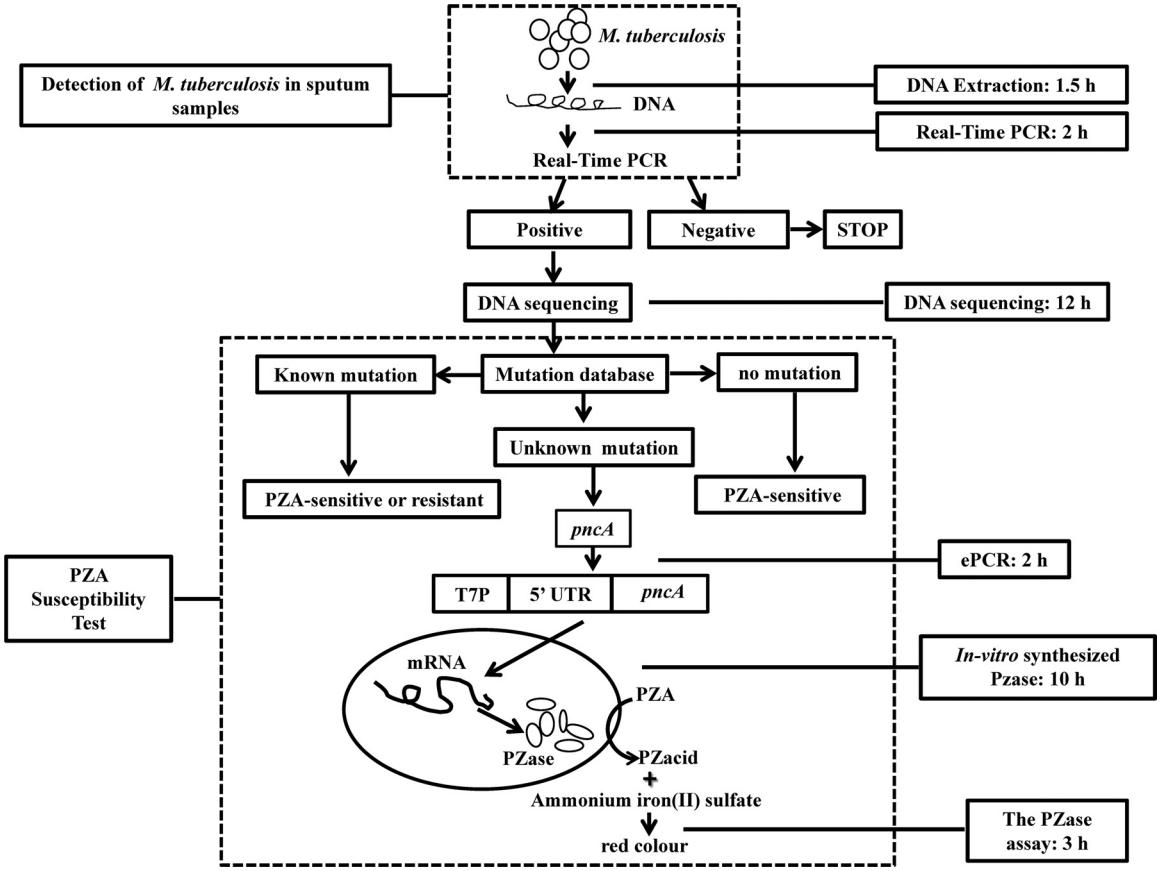

FIG 1 Schematic representation of the integrated GPF–MTB-PZase approach for rapid detection of *M. tuberculosis* and PZA susceptibility directly from sputum specimens.

In this study, we describe a new approach to combine a novel long-fragment quantitative real-time PCR (LF-qPCR) for *M. tuberculosis* detection with the sequencing of the DNA obtained and the *in vitro*-synthesized-PZase assay for rapid detection of PZA susceptibility directly from sputum specimens (called the gene-to-protein function–*M. tuberculosis* PZase [GPF–MTB-PZase] assay). The initial testing of 213 sputum specimens showed that this approach could detect *M. tuberculosis* within 3 h with a detection limit of 7.8 copies/reaction mixture and report the PZA susceptibility results within 2 days. This integrated approach provides a rapid and efficient novel solution for detecting *M. tuberculosis* and PZA susceptibility without culture.

MATERIALS AND METHODS

Bacterial strains and sputum specimen treatment. *M. tuberculosis* H37Ra (PZA sensitive) and *Mycobacterium bovis* BCG (PZA resistant) were maintained in our laboratory. Sputum specimens were collected from patients with suspected tuberculosis in Wuhan Tuberculosis Control Hospital (Wuhan, China) from December 2011 through February 2012. We enrolled consecutive adults with symptoms suggestive of pulmonary tuberculosis who were able to provide sputum specimens of at least 3.0 ml. Samples were obtained under informed consent with approval by the Ethical and Scientific Committee of Wuhan Tuberculosis Control Hospital and the Academic and Ethical Committee of Wuhan Institute of Virology, Chinese Academy of Sciences.

Within 4 h after sputum specimens were collected, the samples were processed to extract DNA for the GPF–MTB-PZase assay and subjected to microscopy with Ziehl-Neelsen staining and cultivation on solid Löwenstein-Jensen medium, respectively. The detailed extraction procedure is described in materials and methods in the supplemental material.

M. tuberculosis H37Ra and *M. bovis* BCG were collected from the Lowenstein-Jensen medium and then suspended in 0.5 ml of MilliQ water and incubated in a water bath at 100°C for 10 min. Cellular debris was pelleted at 13,000 rpm for 5 min, and the supernatants containing the genomic DNA were used as the templates for the LF-qPCR.

General procedure of the GPF–MTB-PZase assay. As shown in Fig. 1, the first step of the approach was detection of the *M. tuberculosis pncA* gene in the samples through a novel long-fragment quantitative real-time PCR (LF-qPCR). The major difference of this qPCR from other PCR methods was that a genomic fragment of *M. tuberculosis* containing the full-length *pncA* gene, including its putative promoter sequence, was amplified. The primers used for the LF-qPCR were RT-F and RT-R, shown in Table 1.

If a positive amplification was detected (meaning that *M. tuberculosis* was detected in the sputum), this amplicon was sequenced by Invitrogen, Inc. (Shanghai, China), using RT-F and RT-R as the sequencing primers. The resulting sequences were compared with the *pncA* sequence of *M. tuberculosis* H37Ra to identify mutations. When no mutation was found, the sample was considered PZA sensitive. If mutations were found, these mutations were searched in the TB Drug Resistance Mutation Database (TB-DRMD) (www.tbdreamdb.com) (26) to match any possible PZA resistance. If these mutations could not be found in the database, the second step, the *in vitro*-synthesized PZase assay, was initiated to test the PZA susceptibility.

For performing the *in vitro*-synthesized-PZase assay, a second *in vitro*

TABLE 1 Primers used for PCR amplification of *pncA* gene

Primer	Sequence
Standard-F	5′ CACCATCACCACTCGGGTGGTGGTTCCATCATCAA CGATCGGACGGATTTGTCGCTCAC 3′
Standard-R	5′ TGGTTAGTTAGTTATTACTACTTGTCAATGGTGAA TGCCCGATGAAGGTGTCGTAGAAG 3′
RT-F	5′ CGGACGGATTTGTCGCTCAC 3′
RT-R	5′ GCCCGATGAAGGTGTCGTAGAAG 3′
F-5	5′ TAATACGACTCACTATAGG**ATACTCCCCCACAACA GCTTACAATACTCCCCCACACAGCTTACAAATACT CCCCC**AGTCGCCCGAACGTAAGGAGGACGT 3′[a]
R-1	5′ ACCGCCGCCAACAGTTCATCCCGGT 3′

[a] The 5′ UTR sequence is shown in boldface. 5′ UTRs can enhance protein expression in the wheat germ system.

expression PCR (ePCR) was performed to introduce the expression elements, using the primers F-5 and R-1, shown in Table 1. After that, the ePCR amplicon was added into the cell-free wheat germ protein expression system (5 Prime, Inc., USA) to synthesize PZase. Finally, the PZase activity of the lysate was measured through the color developed due to the reaction of iron(II) with pyrazinoic acid, which was converted from PZA by the *in vitro*-synthesized PZase. A sample was considered resistant if the PZase synthesized from its *pncA* showed any reduction in activity (color absorption) compared with that from *M. tuberculosis* H37Ra; otherwise, it was considered susceptible. The test could be finished within 3 h for *M. tuberculosis* detection and within 48 h for PZA susceptibility testing.

The detailed conditions for performing LF-qPCR, ePCR, the *in vitro*-synthesizing of PZase, and the PZase activity assay are described in materials and methods in the supplemental material.

RESULTS

Primer design for LF-qPCR and ePCR. Because many mutations in the *pncA* gene, including in its putative promoter region, could lead to PZA resistance (28–31), the LF-qPCR product for sequencing should include the putative promoter fragment and the open reading frame (ORF). In order for the *in vitro*-synthesized PZase to include all the possible mutations within the ORF (561 bp), the primers used for ePCR should not overlap any bases within the ORF. Based on these considerations, the optimized primers were designed as shown in Table 1, and their positions relative to the *pncA* gene on the genome of *M. tuberculosis* are shown in Fig. 2. Based on this design, LF-qPCR amplified a 918-bp fragment, which included the whole putative promoter region and the ORF of the *pncA* gene.

Optimization of the LF-qPCR assay. The specificities of the primers RT-F and RT-R for the detection of *M. tuberculosis* were first evaluated by a BLAST search of the NCBI database (www.ncbi.nlm.nih.gov). No matches to the primers were found except in *M. tuberculosis* and *M. bovis* BCG genomes. The specificity of the LF-qPCR was further checked using DNA genomes extracted from a few strains of bacteria, including *Escherichia coli* J53. No positive amplification was detected.

During the initial LF-qPCRs, we observed primer dimers in no-template control (NTC) tubes. From the melting curves (see Fig. S1 in the supplemental material), we could see that the melting temperature (T_m) of the *pncA* gene amplicon was significantly higher than that of the primer dimers. By acquiring the fluorescence signal at 85°C, just below the T_m of the *pncA* amplicon and above the T_m of the primer dimers, the nonspecific fluorescence of SYBR green dye due to the primer dimers was eliminated, i.e., the cycle threshold (C_T) values of NTC tubes were undetermined.

After optimization, the detection limit of the LF-qPCR assay was determined based on a 10× dilution series of the standard *pncA* template. The standard curve had a linear coefficient (R^2) of 0.998 with a slope of −3.5232 (Fig. 3). The LF-qPCR assay demonstrated a large dynamic range of at least 7 log with a detection limit of approximately 7.8 copies/reaction.

Specificity and sensitivity of LF-qPCR for detection of *M. tuberculosis* in sputum. To assess the sensitivity and specificity of the LF-qPCR method for detection of *M. tuberculosis*, 213 sputum specimens were tested by the LF-qPCR and by the smear and culture methods routinely used in the Tuberculosis Control Center (Wuhan, China) for clinical diagnosis. The LF-qPCR yielded 53 positive specimens. Among them, 5 specimens were found culture negative. Using the culture results as the gold standard for comparison, the overall sensitivity and specificity of the LF-qPCR were 92% and 97%, respectively, as shown in Table 2. Among patients with culture-positive tuberculosis, the sensitivity of the LF-qPCR was 100% for smear- and culture-positive cases and 89% for smear-negative and culture-positive cases.

DNA sequencing results. All 53 positive LF-qPCR products were sequenced. The sequencing results showed that 49 of the isolates had wild-type *pncA* sequences and were therefore PZA susceptible. In the remaining 4 isolates, different mutations were found, as shown in Table 3. Among them, sample 8879 had an upstream mutation at nucleotide −11, resulting in an A→G change (29), which was considered PZA resistant according to the

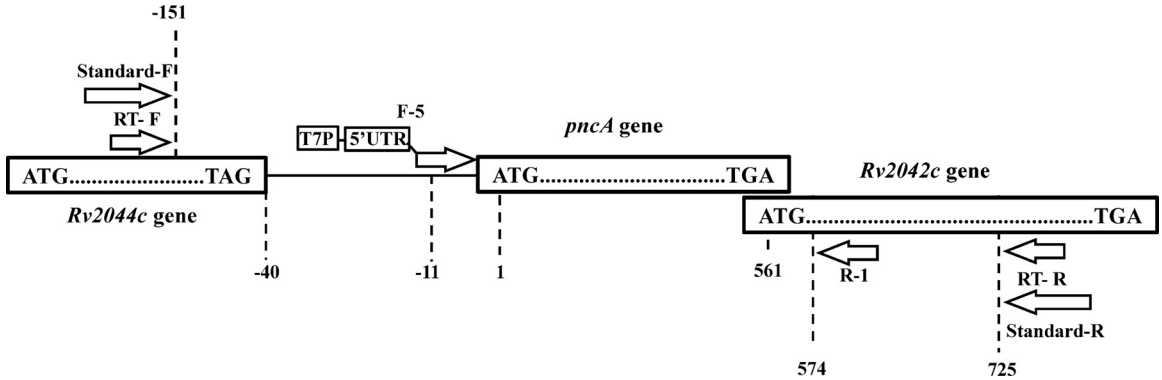

FIG 2 Schematic representation of *pncA* gene and primer positions. Primers Standard-F and Standard-R were used to generate standard templates. Primers RT-F and RT-R were used for the LF-qPCR. ePCR was performed using F5 (with a 5′ UTR sequence) and R-1 as the primers.

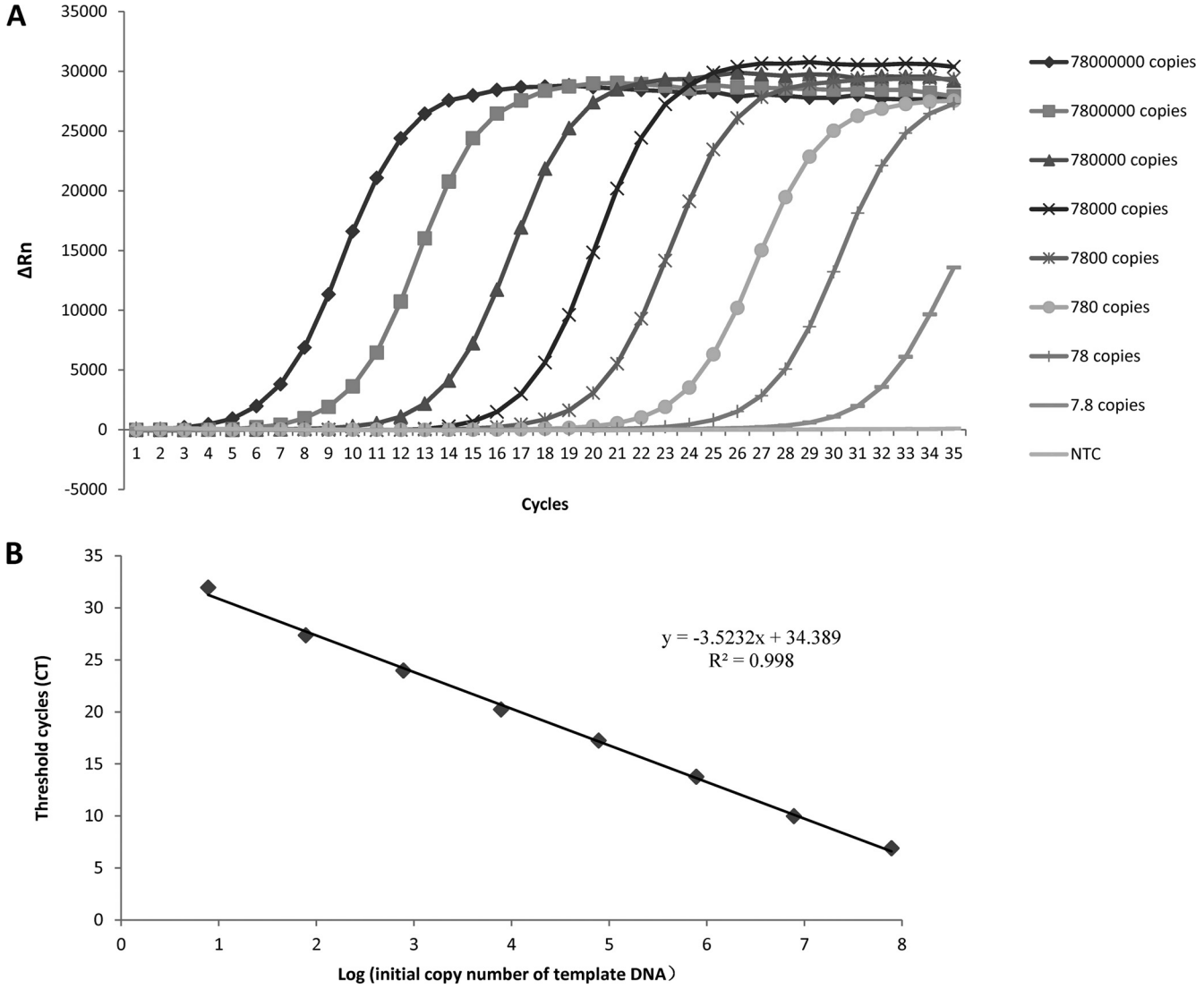

FIG 3 Typical amplification curves (A) and the standard curve (B) of SYBR green-based LF-qPCR for the detection of the *pncA* gene. NTC, no-template control; ΔRn, baseline-corrected normalized reporter.

search of the TB-DRMD database. However, the mutations in the other 3 samples could not be found in the TB-DRMD. Therefore, these 3 mutations were considered new and their links to PZA susceptibility were tested by the *in vitro*-synthesized-PZase assay.

ePCR for adding the *in vitro* expression elements. In order for the wheat germ system to express protein directly from PCR products, a T7 promoter sequence is needed to attach to the forward primers. There are also reports that some 5′ untranslated region (5′ UTR) sequences can enhance protein expression in the

TABLE 2 Comparison of LF-qPCR with smear and culture methods for detection of *M. tuberculosis* in sputum specimens

Parameter	Result by smear and/or culture	No. positive by LF-qPCR/ total no. (%)
Sensitivity	Culture positive	48/52 (92)
	Smear positive and culture positive	17/17 (100)
	Smear negative and culture positive	31/35 (89)
Specificity	Culture negative (no tuberculosis)	156/161 (97)

TABLE 3 PZA susceptibility results for *M. tuberculosis* clinical samples tested by *pncA* sequencing and the GPF-MTB-PZase assay[a]

Sample	*pncA* sequencing result and mutation	PZase mutation	PZA susceptibility determined by:	
			Sequencing	*In vitro*-synthesized-PZase assay
218	C(89) → A	Ala → Asp	NA	Resistant
271	A(521) → G	Glu → Gly	NA	Susceptible
8333	C(151) → G	His → Asp	NA	Resistant
8879	A(−11) → G	NA	Resistant	NA

[a] NA, not available. The mutations revealed by sequencing in the first 3 samples could not be found in the TB-DRMD, and therefore their links to PZA susceptibility cannot be identified. The mutation in sample 8879 was a PZA-resistant mutation included in the TB-DRMD database, and it was not necessary to perform the PZase assay for this sample.

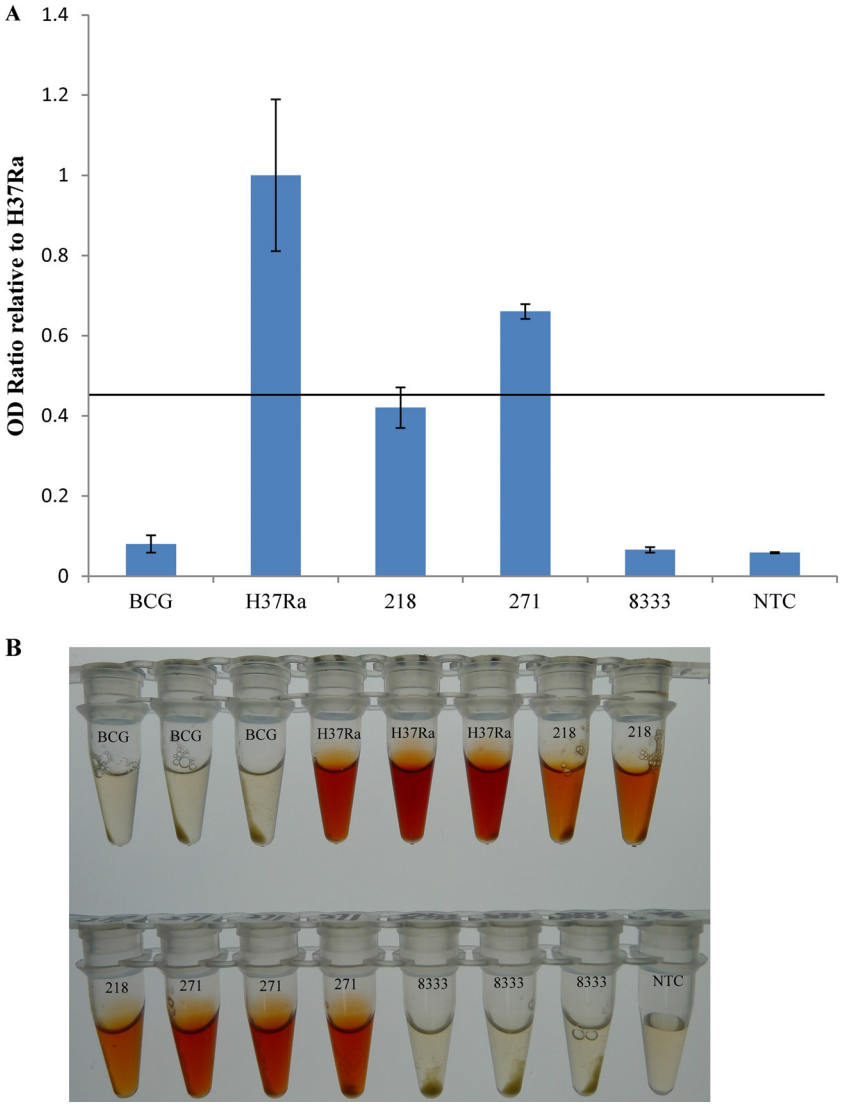

FIG 4 *In vitro*-synthesized-PZase activities of clinical samples. (A) OD ratios of samples relative to *M. tuberculosis* H37Ra. (B) Photograph showing the color difference between samples. Clinical samples are labeled with their sample numbers. NTC, no-template control.

wheat germ system (27, 32). Therefore, ePCRs were performed using F5 (with a 5′ UTR sequence) and R-1 as the primers and the LF-qPCR amplicons of the 3 samples with new mutations, i.e., samples 218, 271, and 8333, as the DNA templates. The ePCR could effectively amplify the *pncA* genes of *M. tuberculosis* H37Ra, *M. bovis* BCG, and the 3 samples (see Fig. S2 in the supplemental material).

***In vitro* synthesis of PZase and PZase assay.** After the *in vitro* synthesis reactions were completed, the activity of the *in vitro*-synthesized PZase was measured in terms of the optical density (OD) ratio relative to the OD of *M. tuberculosis* H37Ra and the corresponding color difference (Fig. 4). The cutoff value to judge PZA susceptibility was determined as we described previously (27). Among the 3 samples, sample 271 was determined to be PZA susceptible because it exhibited PZase activity higher than the cutoff value. Samples 218 and 8333 exhibited PZase activities lower than the cutoff value and, therefore, were determined to be PZA resistant. The difference in color between the PZA-susceptible samples and the PZA-resistant samples was so significant that it could be easily discriminated even by the naked eye (Fig. 4).

DISCUSSION

Because of the many problems associated with the phenotypic methods for testing PZA susceptibility of *M. tuberculosis*, such as their lengthiness, high cost and low accuracy, DNA sequencing of the *pncA* gene is being used more and more. Since PZA resistance mutations are highly diverse and found throughout the *pncA* gene, including its putative promoter region, amplification of the full-length *pncA* gene and establishing a database to associate the genetic mutations with PZA phenotypic resistance are needed for testing PZA susceptibility by DNA sequencing. In the current methods, the full-length *pncA* gene is normally amplified from clinical isolates and seldom directly from sputum specimens. A possible reason may be because sputa contain many substances to inhibit PCR. It is clear that if the full-length *pncA* gene of *M. tuberculosis* can be amplified directly from sputum specimens,

eliminating the time needed to isolate the *M. tuberculosis* bacilli, or better, if the PCR primers are specific enough, detection of *M. tuberculosis* can be achieved simultaneously with obtaining the *pncA* sequence. While establishing a database to associate the genetic mutations with PZA phenotypic resistance is simple, it may be not easy, or even possible, for the database to include all the possible genetic mutations of the *pncA* gene in clinical *M. tuberculosis* isolates. Due to the wide spread of *M. tuberculosis* and the highly diverse *pncA* mutations associated with PZA resistance, it is expected that new mutations of *pncA* gene are sure to be found in some clinical samples. When this happens, certain phenotypic methods are still needed to link the new mutations with the PZA phenotypic susceptibility. Based on these considerations, we designed the integrated GPF–MTB-PZase approach to combine *M. tuberculosis* detection with PZA susceptibility analysis.

From the results in this study, we can see that it is possible to specifically amplify the full-length *pncA* gene directly from sputum specimens. Although the amplicon length is as long as 918 bp, the amplification efficiency was close to that of conventional PCR under the optimized conditions. The long length of the amplicon also provides an additional advantage by allowing the amplicon to be discriminated from the primer dimers by measuring the SYBR green fluorescence at a temperature slightly lower than the melting temperature of the amplicon. With 92% sensitivity and 97% specificity compared to the gold standard culture method, the LF-qPCR demonstrated its potential to be used for *M. tuberculosis* detection. Compared with other genetic methods for *M. tuberculosis* detection, SYBR green dye is less expensive than the probes, such as TaqMan probes and molecular beacons, used by many qPCR methods.

In addition to its use for *M. tuberculosis* detection, the long amplicon contains the full length of the *pncA* gene, including its putative promoter region, which facilitates the direct use of the amplicon as the template for DNA sequencing to find mutations, therefore avoiding lengthy culture methods. At the same time, the DNA sequencing result can be used to double check whether the correct fragment was amplified by the LF-qPCR, thus avoiding false-positive results and further improving the detection specificity of the LF-qPCR.

Furthermore, when the *pncA* gene from a clinical sample is found to have new mutations not available in the current PZA resistance mutation database, the LF-qPCR amplicon is used as the template for ePCR to introduce the expression elements to the ORF of the *pncA* gene. Because the ePCR primers and the LF-qPCR primers form a nested PCR, it is easy to get enough ePCR products with high purity, which is required by the wheat germ system to synthesize PZase. This advantage also renders it possible to perform the *in vitro*-synthesized-PZase assay directly from sputum without the lengthy isolation step and the associated problem of biosafety during the isolation, whereas in our previously published method (27), clinical isolates were usually needed because only one round of PCR was used. It is well known that isolating *M. tuberculosis* from sputum needs a long time, ranging from a few weeks to months. Therefore, the current approach could reduce the overall testing time (from sputum to results) to less than 2 days, which is a significant improvement for PZA susceptibility testing of *M. tuberculosis*. Therefore, the LF-qPCR provides many advantages and plays a key role in our approach.

The results of testing the 213 sputum specimens revealed 24.4% to be culture positive and 24.9% positive by the LF-qPCR. The higher positive percentage of LF-qPCR is understandable because it is more sensitive than culture, as studied using many other PCR detection methods. After checking the clinical data, it was found that the 5 LF-qPCR-positive and culture-negative specimens were from patients who were being treated for suspected tuberculosis. Since treated patients can still harbor mycobacteria long after culture for mycobacteria has become negative (33), the LF-qPCR results might not be false positive for these 5 specimens. After sequencing, it was found that the *pncA* gene from most of the clinical samples was wild type (including the 5 LF-qPCR-positive and culture-negative samples) and only about 7.5% of the samples (4 samples) contained mutations. Because we did not discriminate the primary patients from the re-treated patients, this low frequency of *pncA* mutation seems consistent with the other epidemiology data for TB in China (34). However, among these 4 samples, 3 samples contained *pncA* mutations not reported before. The high chance of finding new mutations in clinical samples necessitates having a fast phenotypic method to link the new mutations with PZA phenotypic resistance.

As we demonstrated previously (27), the *in vitro*-synthesized-PZase assay is fast and can be used to detect all kinds of resistance due to *pncA* mutations. However, because the *in vitro* assay consists of many steps and requires the reagents to be stored at under −80°C, it may not be convenient for all laboratories. The integration of the *in vitro*-synthesized-PZase assay with the DNA sequencing provides a more effective solution because only newly found mutations need testing by the PZase assay. As shown above, using the 213 sputum specimens in this study as an example, only 3 samples needed to be analyzed by the PZase assay to determine the PZA susceptibility. This low percentage (1.4%) may imply that the average cost of the GPF–MTB-PZase per patient will be low, which is very important for developing countries with heavy burdens of TB, which commonly suffer from limited resources.

Something else to note is that the PZase assay found different PZase activities for the 3 samples (Fig. 4). The mutation A521G (with alanine replaced by glycine at position 521; sample 271) did not change the PZase activity much; therefore, this sample was PZA sensitive. Another mutation, C89A (sample 218), slightly reduced the PZase activity. Although it was considered PZA resistant, this sample may be PZA sensitive under higher PZA concentrations. The last mutation, C151G (sample 8333), deactivated the PZase significantly, which indicated that this sample was highly resistant to PZA. These results demonstrated again that PZA resistance mutations in the *pncA* gene are not only highly diverse but also may confer different levels of resistance to PZA, as we found previously (27).

The last but not the least thing to note is that, although the current GPF–MTB-PZase method can work well for testing PZA resistance due to *pncA* mutations, it will not be able to test PZA resistance unrelated to *pncA* mutations. This limitation might miss a significant number of resistant strains. In a recent report (35), only a 45.7% correlation was found between PZA resistance and the presence of a mutation in the *pncA* gene for strains isolated in Rio de Janeiro, Brazil. Therefore, the application of the GPF–MTB-PZase assay would need to be done with care in regions with a high ratio of PZA resistance without *pncA* mutations. But as mentioned in the introduction, since many other studies have found a strong relationship between *pncA* mutations and PZA resistance, we believe that the GPF–MTB-PZase method would be useful in practice for fast screening for *M. tuberculosis* and earlier

identification of PZA resistance related to *pncA* mutations, especially since the current phenotypic methods for determining PZA susceptibility are very slow.

In conclusion, the novel GPF–MTB-PZase method integrates the long-fragment qPCR for *M. tuberculosis* detection and the sequencing of the DNA obtained in tandem with the *in vitro*-synthesized-PZase assay for a PZA susceptibility assay. This integrated approach provides many advantages not available by other methods for *M. tuberculosis* detection and PZA susceptibility assay. Because of its rapidity, simplicity, and relatively low cost, the GPF–MTB-PZase assay may be quite useful for TB screening and fast testing of PZA susceptibility related to *pncA* mutations.

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