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Direct Detection of Pyrazinamide Resistance in *Mycobacterium tuberculosis* by Use of *pncA* PCR Sequencing

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ABSTRACT An in-house-developed *pncA* sequencing assay for analysis of pyrazinamide (PZA) resistance was evaluated using 162 archived *Mycobacterium tuberculosis* complex (MTBC) isolates with phenotypic PZA susceptibility profiles that were well defined by analysis of Bactec MGIT 960 PZA kit and PZase activity data. Preliminary results showed 100% concordance between *pncA* sequencing and phenotypic PZA drug susceptibility test (DST) results among archived isolates. Also, 637 respiratory specimens were prospectively collected, and 158 were reported as MTBC positive by the Abbott Realtime MTB assay (96.3% sensitivity [95% confidence interval {CI}: 92.2% to 98.7%]; 100% specificity [95% CI: 99.2% to 100.0%]). Genotypic and phenotypic PZA resistance profiles of these 158 MTBC-positive specimens were analyzed by *pncA* sequencing and Bactec MGIT 960 PZA kit, respectively. For analysis of PZA resistance, *pncA* sequencing detected *pncA* mutations in 5/5 (100%) phenotypic PZA-resistant respiratory specimens within 4 working days. No *pncA* mutations were detected among PZA-susceptible specimens. Combining archived isolates with prospective specimens, 27 were identified as phenotypic PZA resistant with *pncA* mutation. Among these 27 samples, 6/27 (22.2%) phenotypic PZA-resistant strains carried novel *pncA* mutations without *rpsA* and *panD* mutations. These included 5 with mutations (a deletion [Del] at 383T [Del383T], Del 380 to 390, insertion of A [A Ins] at position 127, A Ins at position 407, and G Ins at position 508) in *pncA* structural genes and 1 with a mutation (T-12C) at the *pncA* promoter region. All six of these strains had no or reduced PZase activities, indicating that the novel mutations might confer PZA resistance. Additionally, 25/27 phenotypic PZA-resistant strains were confirmed multidrug-resistant tuberculosis (MDR-TB) strains. As PZA is commonly used in MDR-TB treatment regimens, direct *pncA* sequencing will rapidly detect PZA resistance and facilitate judicious use of PZA in treating PZA-susceptible MDR-TB.

KEYWORDS drug-resistant TB, molecular diagnosis, *Mycobacterium tuberculosis*, pyrazinamide

Tuberculosis (TB) has been a global public health problem for decades. In 2017, estimates of 10.0 million new TB cases and 1.7 million TB-related deaths were reported worldwide (1). The situation was worsened by the presence of multidrug-resistant TB (MDR-TB), which is defined by bacillary resistance to both isoniazid (INH) and rifampin (RIF). Rapid diagnosis of drug resistance is essential for timely initiation of optimal treatment to quickly contain infection at the source.

Pyrazinamide (PZA) is a prodrug that is activated by mycobacterial pyrazinamidase (PZase) encoded by *pncA*. The resultant activated product (pyrazinoic acid) can provide a sterilizing effect to *Mycobacterium tuberculosis* complex (MTBC) infection (2). PZA

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plays a critical role in the treatment of both drug-susceptible TB and MDR-TB. Its unique sterilizing property of killing persister bacilli has been proven to shorten the treatment period of drug-susceptible TB from 12 months to 6 months (3, 4). PZA is also recommended by World Health Organization (WHO) for MDR-TB treatment regimens (5). Inclusion of PZA in PZA-susceptible MDR-TB may improve MDR-TB treatment outcomes (6, 7). Potential synergistic activities among PZA, important second-line drugs, and novel drugs (such as bedaquiline, delamanid, and pretomanid) highlight its importance in both current and future MDR-TB treatment (5, 8).

Currently, growth-based drug susceptibility tests (DST) using an MGIT 960 PZA kit are widely regarded as representing the gold standard for determining MTBC drug susceptibility to PZA. However, there are limitations to the current PZA resistance tests. The long turnaround time of mycobacterial culture means phenotypic drug susceptibility tests may take up to months to complete, which leads to diagnostic delays. The tests could take even longer for drug-resistant MTBC strains with an even lower replication rate (9). Moreover, PZA phenotypic DSTs are rarely performed in routine clinical settings as the test is time-consuming and technically demanding. The low pH medium and the stringent inoculum size of the assays often lead to inconsistent and nonreproducible results which deter clinicians from relying on these tests for guidance. PZA resistance has been reported to be predominantly associated with *pncA* mutations, leading to reduced or lost PZase activity and failure to activate PZA (10, 11). Nevertheless, mutations in *pncA* are diverse in nature and scattered throughout the whole *pncA* gene without any hot spot regions (12). This makes probe-based real-time PCR assay approaches challenging**.** Other molecular approaches such as *pncA* sequencing and use of the commercial Genoscholar PZA-TB II assay (NIPRO Corporation, Japan) have been evaluated for analyzing *pncA* mutations in previous studies (13–15). However, evaluation studies of these platforms included only purified isolates or specimens subjected to genolysis with confirmed smear-positive and MTBC culture results that were positive for acid-fast bacilli (AFB) (13–15). In response to the current threat of MDR-TB pandemic, a rapid and highly accurate PZA resistance diagnostic tool is, therefore, urgently needed (16).

In this study, we aimed to evaluate an algorithm for *M. tuberculosis* detection using the Abbott Realti*m*e MTB assay and subsequent in-house-developed genotypic PZA resistance detection targeting the PZA resistance-related *pncA* gene. The in-house-developed *pncA* PCR-sequencing assay allows detection of PZA resistance MTBC directly from respiratory specimens. With a sequencing size corresponding to 828 base pairs (NC_000962.3 positions 2288495 to 2289322), our sequencing assay covered not only the entire 561 bp of the *pncA* gene but also the putative promoter region that regulates *pncA* expression (10).

To the best of our knowledge, this publication represents the first prospective evaluative study of an in-house-developed *pncA* sequencing platform for direct detection of PZA resistance in MTBC by the use of both AFB smear-positive and AFB smear-negative respiratory specimens. The accuracy of the sequencing platform results were also evaluated using archived MTBC clinical isolates collected over a 14-year period. Detection of genotypic PZA resistance by *pncA* sequencing was compared with detection by the use of phenotypic DSTs with a Bactec MGIT 960 PZA kit as well as the PZase activity in the subsequent mycobacterial cultures.

MATERIALS AND METHODS

Archived MTBC clinical isolate collection. Archived clinical isolates collected between April 2003 and January 2017 from Queen Mary Hospital and Grantham Hospital were available for this study and were stored at −80°C prior to usage. Resuscitation of archived MTBC clinical isolates was performed using Lowenstein-Jensen (LJ) medium and Middlebrook 7H9 supplemented with oleic acid dextrose catalase (OADC). The cultures were incubated at 37°C and were supplemented with 5% CO_2.

Respiratory specimen collection. Respiratory specimens were available from newly diagnosed patients suffering from lower respiratory infection between May and December 2017 in five general hospitals (inpatients) and in 13 chest clinics of the Department of Health (outpatients) in Hong Kong. After being subjected to direct AFB smear microscopy using fluorescence auramine O and confirmed by Ziehl-Neelsen staining, all respiratory specimens were decontaminated by the conventional *N*-acetyl-L-

cysteine-NaOH method as described previously (17). Processed sediments were then inoculated onto a Bactec MGIT 960 mycobacterial detection system (Becton, Dickinson, Baltimore, MD, USA) and LJ medium (bioMérieux, Marcyl'Etoile, France) at 37°C.

Specimen DNA extraction and Abbott Realtime MTB assay. For each processed specimen, 500-μl samples were taken for TB detection using the Abbott Realtime MTB assay. Processed specimens were inactivated and subjected to DNA extraction using an Abbott m2000*sp* instrument as previously described (18, 19). The extracts were then transferred onto an Abbott m2000*rt* real-time PCR thermocycler for real-time PCR. Upon completion, either "MTBC detected" or "MTB not detected" and a manufacturer defined cutoff cycle number (Cn) of 40.0 were reported. For samples reported as "MTBC Positive" by the Abbott Realtime MTB assay, specimen DNA was extracted from another 500-μl volume of processed sediments using a Roche Amplicor respiratory specimen preparation kit (Roche Diagnostics, Berlin, Germany) for in-house *pncA* sequencing platform analysis.

Genotypic identification of culture isolates. Mycobacterial DNA was extracted from all culture isolates using a Roche Amplicor respiratory specimen preparation kit as described previously (20). The presence of MTBC was confirmed by an in-house PCR assay, and species identification of nontuberculosis mycobacterium (NTM) was confirmed using 16S rRNA sequencing as described previously (20, 21).

Phenotypic drug susceptibility testing (DST). PZA susceptibility was defined by phenotypic DST, which was conducted using a Bactec MGIT 960 PZA kit and a Bactec MGIT 960 SIRE kit (Becton, Dickinson, Baltimore, MD, USA). The critical concentrations used were 100 mg/liter for PZA, 0.1 mg/liter for INH, and 1.0 mg/liter for RIF.

Pyrazinamidase (PZase) activity assay. The PZase activity assay was performed using a modified Wayne procedure as described previously (22). The PZase activity assay was interpreted independently by 2 well-trained observers. *M. tuberculosis* H37Rv and a clinical isolate of *M. bovis* collected from the diagnostic microbiology laboratory of Queen Mary Hospital in Hong Kong were used as positive and negative controls, respectively.

PCR amplification and DNA sequencing for *pncA*. All archived clinical isolates and "MTBC detected" specimens were subjected to *pncA* PCR amplification and sequencing targeting a 828-bp region covering the 561-bp *pncA* gene and the promoter region. The *pncA* PCR was performed using primers pncA_1F (5′-CGGCGTCATGGACCCTATATC-3′) and pncA_1R (5′-GCCCGATGAAGGTGTCGTAG-3′). The PCR mixture consisted of 1× PCR buffer (Qiagen, Hilden, Germany), 1× Q solution (Qiagen, Hilden, Germany), a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP) (Life Technologies, CA, USA), 1.5mM $MgCl_2$, a 0.4 μM concentration of each primer (Integrated DNA Technologies, IA, USA), and 5 U HotStar *Taq* Plus DNA polymerase (Qiagen, Hilden, Germany). The PCR was carried out under the following conditions: initial denaturation at 96°C for 8 min, followed by 40 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a final elongation step at 72°C for 10 min. The PCR products were subjected to Sanger sequencing using an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Consensus sequences of *pncA* were assembled and edited using the Staden Package (version 2.0.0) and compared with data from the reference *M. tuberculosis* H37Rv strain by the use of the BioEdit sequence alignment editor (version 7.0.5). In cases of novel *pncA* mutations detected in PZA-resistant MTB, *rpsA* and *panD* sequencing were performed as described previously to exclude PZA resistance corresponding to *rpsA* and/or *panD* mutations (10, 23).

Cross reactivity of *pncA* PCR amplification. To determine the specificity of *pncA* PCR, DNA from 10 NTM clinical strains was subjected to *pncA* PCR amplification. These strains were selected in order to represent the most common pathogenic NTM species identified in Hong Kong (24). Mycobacterial DNAs from *M. tuberculosis* H37Rv, *M. bovis*, and *M. africanum* were used as positive controls.

Statistical analysis. The diagnostic performance of Abbott Realtime MTB assay was evaluated with reference to mycobacterial culture results. The sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) were calculated based on bacteriology information. The diagnostic performance of *pncA* sequencing was evaluated using the PZA susceptibility test as the reference standard. Any nonsynonymous mutations detected by *pncA* sequencing were regarded as representing genotypic resistance, and the percentage of concordance was calculated based on "MTBC detected" specimens with positive MTBC cultures and interpretable *pncA* sequencing results.

RESULTS

Drug susceptibility patterns and PZase activity. Using MGIT SIRE and MGIT PZA kits, phenotypic resistance to INH, RIF, and PZA for the 326 MTBC culture isolates, including 162 from archived MTBC strains and 164 isolates from respiratory specimens, was determined. Among these isolates, 266/326 were pan-susceptible strains, 19/326 were INH monoresistant, 4/326 were RIF monoresistant, and 37/326 were MDR-TB. PZA resistance was identified in 2/266 (0.8%) pan-susceptible strains and 25/37 (67.6%) MDR-TB strains (Table 1; see also Table 2). Using a modification of the Wayne method, the levels of PZase activity of the 326 MTBC culture isolates were determined. Among the 299 PZA-susceptible strains, 100% (299/299) showed positive PZase activity. For the 27 PZA-resistant strains, 26/27 (96.3%) strains showed negative PZase activity and 1/27 (3.7%) strain exhibited weak PZase activity (Table 1; see also Table 2).

Analytical performance of *pncA* sequencing on archived isolates. A total of 162 archived isolates were collected in this study to evaluate the analytical performance of

TABLE 1 Pyrazinamide susceptibility and *pncA* mutation profile of 162 archived purified *M. tuberculosis* isolates

MGIT PZA (100 mg/liter) susceptibility test result (no. of isolates)	No. of strains	PZase activity	Sanger PCR sequencing result for *pncA*		
			SNP location(s)	Nucleotide change[a]	Amino acid change(s)
Susceptible ($n = 140$)[b]	140	Positive	WT[d]	WT	WT
Resistant ($n = 22$)[c]	1	Negative	38	tTc→tCc	F13S
	1	Negative	40	Tgc→Cgc	C14R
	3	Negative	196	Tcg→Ccg	S66P
	1	Negative	202	Tgg→Agg	W68R
	2[e]	Negative	269	aTc→aGc	I90S
	1	Negative	374	gTc→gAc	V125D
	6	Negative	485	gGt→gAt	G162D
	1	Negative	545	tTg→tCg	L182S
	2	Negative	127	A Ins at 127	Frameshift at codon 43
	1	Negative	407	A Ins at 407	Frameshift at codon 136
	1	Negative	508	G Ins at 508	Frameshift at codon 170
	1	Negative	383	Deletion 383 T	Deletion of codon 128
	1	Negative	380–390	Deletion, 380 to 390	Deletion of codons 127–130

[a]Uppercase sequence characters represent the nucleotide change.
[b]Among 140 PZA-susceptible isolates, 6 were MDR-TB, 10 were INH monoresistant, 2 were RIF monoresistant, and 122 were pan-susceptible strains as determined by use of the MGIT 960 SIRE kit.
[c]Among 22 PZA-resistant isolates, 20 were MDR-TB by MGIT 960 SIRE kit.
[d]WT, wild type.
[e]Two PZA-resistant isolates from the same patient were PZA monoresistant.

pncA sequencing. Of the 162 archived isolates, 22/162 (13.6%) were phenotypically PZA resistant and 140/162 (86.4%) were phenotypically PZA susceptible as determined by the use of the MGIT PZA kit. All isolates were positive for *pncA* PCR, with *pncA* mutations detected in all 22 phenotypic PZA-resistant isolates but not in 140 PZA-susceptible isolates. *pncA* sequencing detected known PZA resistance-related missense mutations in 16 PZA-resistant strains (F13S [$n = 1$], C14R [$n = 1$], S66P [$n = 3$], W68R [$n = 1$], I90S [$n = 2$], V125D [$n = 1$], G162D [$n = 6$], and L182S [$n = 1$]) (Table 1). Novel mutations were identified in the other 6 PZA-resistant strains, which included two deletion (Del) patterns (Del 383 T [$n = 1$] and Del 380 to 390 [$n = 1$]) and three insertion (Ins) patterns (insertion of A [A Ins] at 127[$n = 2$], A Ins at 407[$n = 1$], and G Ins at 508 [$n = 1$]). All

TABLE 2 Rapid diagnosis of pyrazinamide resistance using *pncA* sequencing on 164 "MTBC culture-positive" direct specimens[a]

AFB smear	No. of specimens ($n = 164$)	Abbott realtime MTB result	Sanger sequencing for *pncA* on respiratory specimens[b]				*M. tuberculosis* culture isolates	
			pncA PCR result	SNP location	Nucleotide change	Amino acid change(s)	MGIT PZA [100 mg/liter][c]	PZase activity
Positive ($n = 28$)	1	+	+	−12	T→C	Promoter region	R	Weakly positive
	1	+	+	188	gAc→gGc	D63G	R	Negative
	1	+	+	226	Act→Cct	T76P	R	Negative
	1	+	+	422	cAg→cCg	Q141P	R	Negative
	24[d]	+	+	WT	WT	WT	S	Positive
Negative ($n = 136$)	1	+	+	175	Tcc→Ccc	S59P	R	Negative
	111[e]	+	+	WT	WT	WT	S	Positive
	18[f]	+	−	NA	NA	NA	S	Positive
	6[g]	−	N.D.	N.D.	N.D.	N.D.	S	Positive

[a]Abbreviations: NA, not applicable; N.D., not done.
[b]All results from *pncA* PCR Sanger sequencing performed on direct specimens were concordant with the results seen with subsequent culture isolates.
[c]In this study, all five phenotypically PZA-resistant specimens were confirmed to represent MDR-TB by the use of an MGIT 960 SIRE kit.
[d]Among 24 AFB smear-positive specimens phenotypically susceptible to PZA, 4 were MDR-TB, 4 were INH monoresistant, and the remaining 16 were pan-susceptible to all first-line antibiotics.
[e]Among the 111 AFB smear-negative specimens phenotypically susceptible to PZA, 2 were MDR-TB, 2 were RIF monoresistant, 5 were INH monoresistant, and the remaining 102 were pan-susceptible to all first-line antibiotics.
[f]Eighteen AFB smear-negative MTB-positive pan-susceptible specimens showed negative *pncA* PCR results. No *pncA* mutations were detected in subsequent culture isolates.
[g]Six AFB smear-negative pan-susceptible specimens were reported to be "MTB-Negative" by the Abbott Realtime MTB assay. Therefore, no *pncA* sequencing was performed on these six direct specimens. No *pncA* mutations were detected in subsequent culture isolates.

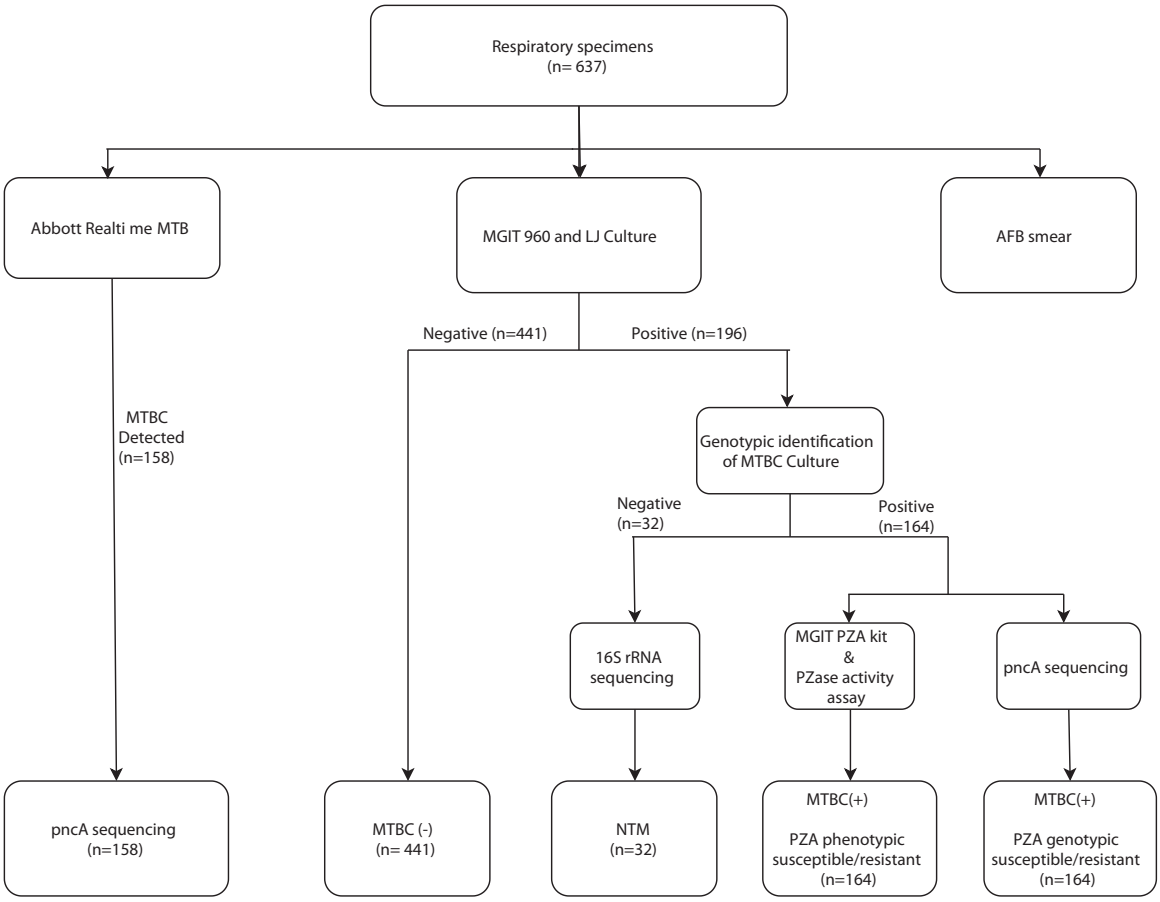

FIG 1 Workflow of evaluating in-house *pncA* sequencing for the detection of *pncA* mutation and PZA resistance in respiratory specimens. Abbreviations: MTBC, *M. tuberculosis* complex; NTM, nontuberculosis mycobacterium.

PZA-resistant archived isolates showed negative PZase activity in the modified Wayne test. Overall, PZA resistance results determined by *pncA* sequencing among archived isolates were 100% concordant with the MGIT PZA kit results (Table 1).

Mycobacterial culture and AFB smear on respiratory specimens. The overall workflow for respiratory specimens processing is illustrated in Fig. 1. A total of 637 respiratory specimens were prospectively collected from 427 newly diagnosed patients suspected with lower chest infection. Among the 637 specimens, 164/637 (24.8%) specimens from 109 patients were culture positive for MTBC and 32/637 (5.0%) specimens from 19 patients were culture positive for NTM (*M. avium* [$n = 6$], *M. abscessus* [$n = 3$], *M. colombiense* [$n = 1$], *M. chelonae* [$n = 4$], *M. fortuitum* [$n = 2$], *M. gordonae* [$n = 2$], *M. intracellulare* [$n = 6$], *M. kansasii* [$n = 4$], *M. scrofulaceum* [$n = 1$] *M. parascrofulaceum* [$n = 1$], *M. neoaurum* [$n = 1$], and *M. xenopi* [$n = 1$]). The remaining 441 specimens were negative for mycobacterial culture. Among the 164 bacteriologically confirmed specimens, 28/164 (17.1%) were AFB smear positive. The rates of culture positivity in MGIT 960 and LJ medium were 89.6% (147/164) and 79.9% (131/164), with mean times to detection of 20.7 days ($\pm$ 0.8 days) and 47.9 days ($\pm$ 2.6 days), respectively.

Of the 164 MTBC culture-positive specimens, the Abbott Realti*m*e MTB assay reported 158/164 (96.3%) as "MTBC detected." "MTBC not detected" results were reported for 473/473 (100%) MTBC culture-negative specimens. No indeterminate result due to the presence of a PCR inhibitor was obtained. The Abbott Realti*m*e MTB assay exhibited no cross-reactivity for 32 NTM specimens, and these specimens were reported as "MTBC not detected." Among the 52/637 (8.2%) AFB smear-positive and the 585/637 (91.8%) AFB smear-negative specimens, the Abbott Realti*m*e MTB assay demonstrated sensi-

tivities of 100% and 95.6% with specificities of 100% and 100%, respectively. Overall sensitivity, specificity, positive predictive values, and negative predictive values and corresponding 95% confidence intervals (CI) determined by the Abbott Realtime MTB assay were 96.3% (95% CI: 92.2% to 98.7%), 100% (95% CI: 99.2% to 100.0%), 100% (95% CI: 100%), and 98.8% (95% CI: 97.2% to 99.4%), respectively. All 158 specimens reported as "MTBC positive" and the corresponding isolates were analyzed by in-house-developed *pncA* sequencing.

Diagnostic performance of in-house-developed *pncA* sequencing on respiratory specimens. The *pncA* gene was successfully sequenced in 140 (88.6%) specimens, including all ($n = 28$) of the AFB-smear-positive specimens and 112/130 (86.2%) of the AFB smear-negative specimens (Table 2). *pncA* mutations (including T-12C [$n = 1$], S59P [$n = 1$], D63G [$n = 1$], T76P[$n = 1$], and Q141P[$n = 1$]) were detected in five respiratory specimens, all of which were subsequently confirmed to be phenotypically resistant to PZA by using MGIT 960 PZA phenotypic DST. None of the 135 PZA-susceptible specimens harbored *pncA* mutations (Table 2). Therefore, our *pncA* sequencing assay was 100% concordant with the MGIT 960 PZA phenotypic DST in determination of PZA resistance in *pncA* PCR-positive respiratory specimens. Follow-up *pncA* sequencing performed on subsequent isolates showed identical results with their corresponding specimens.

Regarding the 6 false-negative specimens reported by the Abbott Realtime MTB assay and the 18 *pncA* PCR-negative specimens, the subsequent isolates gave no mutation by *pncA* sequencing and were phenotypically susceptible to PZA (Table 2). In addition, *pncA* PCR exhibited no cross-reactivity with 10 NTM strains, including *M. avium*, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. gordonae*, *M. fortuitum*, *M. kansasii*, *M. colombiense*, *M. xenopi*, and *M. scrofulaceum*.

DISCUSSION

The demand for rapid detection of drug-resistant TB from direct specimens is ever-increasing. Review of the current literature showed that the levels of *pncA* mutation concordance in PZA-resistant strains ranged from 72% to 99%, suggesting that *pncA* is a good indicator for detecting PZA resistance (25). The diagnostic performance of the use of the *pncA* sequence to predict PZA resistance has been previously evaluated (10, 15, 26, 27), but those retrospective evaluations were conducted on clinical isolates or AFB smear-positive specimens only. In our current study, apart from using archived MTBC clinical isolates collected over a 14-year period to demonstrate test accuracy, we also extended our findings by prospectively evaluating our in-house-developed *pncA* sequencing for genotypic identification of PZA resistance in both AFB smear-positive and AFB smear-negative respiratory specimens. To the best of our knowledge, this was the first prospective study to have evaluated the diagnostic performance of *pncA* sequencing in clinical specimens collected from TB intermediate-burden regions.

In this study, the Abbott Realtime MTB assay was used to primarily detect the presence of MTBC in respiratory specimens. Concordant with our previous studies (18, 19), the Abbott Realtime MTB assay demonstrated high sensitivity (95.6%) and specificity (100%). The results demonstrated that the Abbott Realtime MTB assay is a suitable platform for rapid screening of MTBC-positive specimens. Specimens reported as "MTBC detected" by the Abbott Realtime MTB assay were subsequently selected for in-house-developed *pncA* sequencing.

The in-house-developed *pncA* sequencing method uses a PCR-based platform that screens the entire *pncA* sequence and upstream promoter for any potential PZA resistance-related mutations. As PZA resistance-related mutations are scattered along the entire *pncA* gene without any hot spot regions, the sequencing-based approach of our assay can provide exact mutation profiles in the targeted DNA sequence. Compared to other commercially available PZA line-probe assays such as the Genoscholar PZA-TB II assay, which uses 48 wild-type probes for *pncA* mutation detection (14), our in-house-developed assay can identify the exact mutation patterns and thus prevent false

reports of PZA resistance that are due to the presence of silent mutations. This is critical for early diagnosis of drug resistance and pivotal for optimal TB infection control and patient care. In this study, in-house-developed *pncA* sequencing demonstrated 100% sensitivity among AFB smear-positive specimens. In addition, the current evaluation was also extended to include AFB smear-negative specimens and demonstrated an acceptable sensitivity level of 86.2%. With overall specificity of 100% and no cross-reactivity to NTM pathogens, our result suggested that the in-house-developed *pncA* sequencing system could be a highly accurate diagnostic tool for PZA resistance detection.

In the current study, all phenotypically PZA-resistant MTBC cultures were found to carry *pncA* mutations as identified by in-house-developed *pncA* sequencing. The results were further supported by a modified Wayne's test with reduced levels or an absence of PZase activity. Meanwhile, no *pncA* polymorphism was displayed in any of the PZA-susceptible strains from either respiratory specimens and archive isolates. The result suggested that silent mutations and natural polymorphisms in *pncA* are not common among PZA-susceptible strains. On the other hand, PZA resistance is mainly associated with *pncA* mutations in our locality, despite the association of *rpsA* and *panD* mutations with PZA-resistant MTBC strains (10, 11). Similarly to previous studies, our cohort of *pncA* mutations included missense mutations, deletions, and insertions dispersed along the *pncA* gene and its promoter regions without any hot spot regions (19, 25). This indicates that *pncA* sequencing is most likely to be the most suitable diagnostic tool for genotypic prediction of PZA resistance due to its ability to accurately identify mutations throughout the whole *pncA* gene and its promoter regions.

Overall, the operation of *pncA* sequencing was deemed to be simpler and less technically demanding than that of the MGIT 960 PZA kit. Our combined turnaround time of 4 working days includes specimen digestion and decontamination (0.5 day), MTBC detection by the Abbott Realtime MTB assay (0.5 day), *pncA* sequencing (2 days), and data analysis and reporting (1 day). This is much shorter than the average of 35.2 days ($\pm$ 3.8 days) for phenotypic DST. As analyses using the 3130 Genetic Analyzer as described in this study can be selectively performed on a maximum of 45 specimens per batch, *pncA* sequencing can be used for small-to-large-scale PZA resistance detection. The frequency of *pncA* sequencing can be adjusted according to the workload of the clinical laboratory and can provide a more efficient choice for PZA resistance detection.

In our sample cohort, six phenotypically PZA-resistant strains were found to carry a novel *pncA* mutation(s). These novel *pncA* mutations included five in *pncA* structural genes and one at the *pncA* promoter region. As there are no additional mutations in *rpsA* and *panD* mutations in these six isolates (data not shown), these novel *pncA* mutations were postulated to be correlated with PZA resistance. Interestingly, one phenotypic PZA-resistant specimen (HKU14621) harboring a novel *pncA* promoter mutation at T-12C was found to have weak PZase activity. PZase analysis of this particular strain was performed in triplicate, and consistent weak PZase activities were detected. In a previous study conducted by Marttila et al. (28), instead of a T-12C mutation, a promoter mutation at the same nucleotide (position T-12G) was identified in TB clinical isolates with phenotypic PZA resistance. In this study, we demonstrated that a nucleotide transition from thymine to cytosine at position 12 upstream of the *pncA* gene could also result in PZA resistance. As the T-12C mutation was located in the promoter region of *pncA*, the mutation is very likely to affect the expression level of *pncA*, causing the weakened PZase activity but phenotypic PZA-resistant profile in HKU14621. In-depth investigation is still required in order to delineate the molecular characteristics associated with the abnormal PZase activity and phenotypic PZA profile of this TB strain.

The in-house-developed *pncA* sequencing approach has its limitations. It is notable that *pncA* mutations might not necessarily correlate with PZA resistance. According to a comprehensive study conducted by Whitfield et al. (29), *pncA* polymorphisms at codon positions 35 and 130 could also exist among PZA-susceptible strains. Although

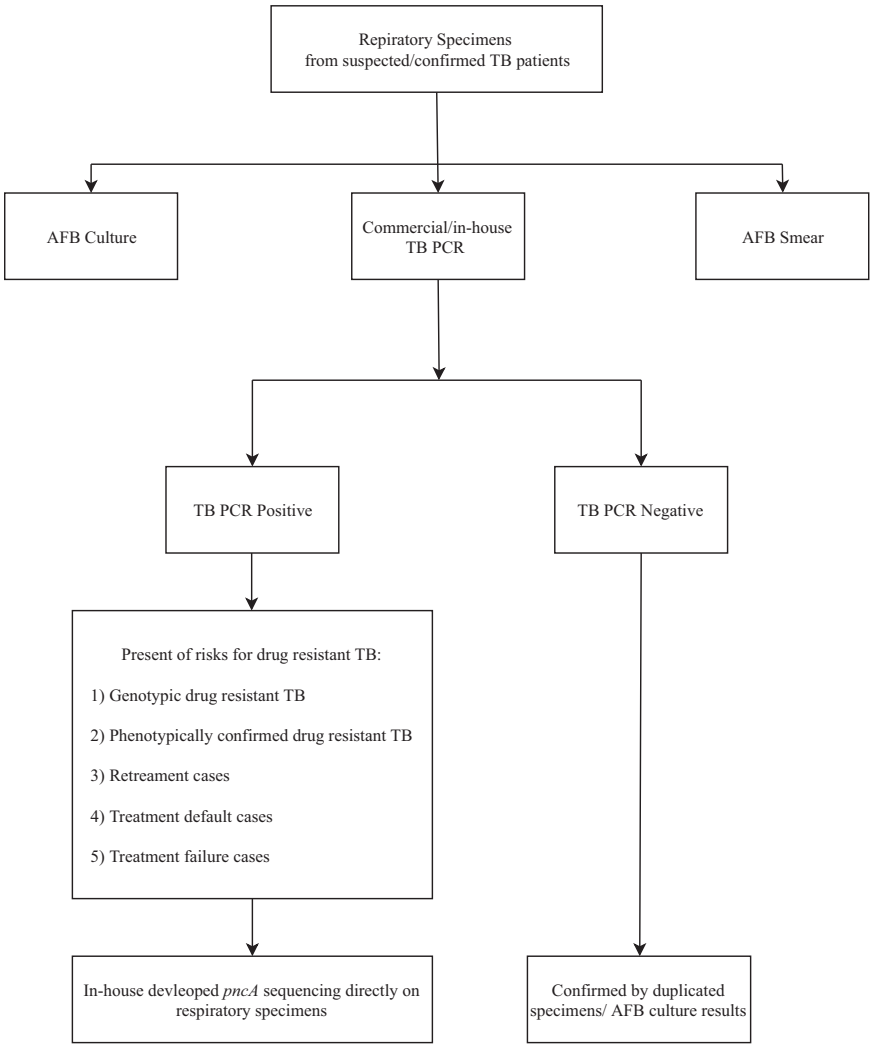

FIG 2 Recommended algorithm for the implementation of genotypic detection of PZA resistance by in-house-developed *pncA* sequencing. Abbreviation: MTBC, *M. tuberculosis* complex.

we demonstrated 100% concordance between *pncA* mutations and phenotypic PZA resistance in clinical strains in our current study, it is still important to consider the potential occurrence of *pncA* mutations in phenotypic PZA-susceptible cases. In addition, our study did not explore all of the possible *pncA* mutants globally even though we have reflected a realistic representation of the clinical setting in our locality. With the effective implementation of directly observed short-course treatment, we could identify only a relatively small number of phenotypic PZA-resistant clinical specimens. The use of a low number of strains/specimens in this study could potentially result in missing other low-frequency PZA resistance mechanisms such as mutations in *rpsA* and *panD*. Thus, a multicenter study with a larger cohort is clearly warranted. Nevertheless, among the 37 MDR-TB isolates included in the current cohort, 25/37 (65.6%) were resistant to PZA. This is similar to the PZA resistance prevalence of 31% to 89% among MDR-TB reported in other studies (9, 30). Considering the high cost of PCR sequencing and the high PZA resistance rate among MDR-TB in not only our locality but also other regions (31), we have suggested an algorithm for selectively applying direct *pncA* PCR sequencing in respiratory specimens or culture isolates. The algorithm is particularly crucial for early chemotherapeutic guidance for treatment of patients at risk of drug-resistant TB (Fig. 2), including those with genotypic drug-resistant TB or MDR-TB detected by commercial molecular assays (32, 33) or phenotypical DST methods,

retreatment cases (34), treatment defaulters (35), and treatment failure cases (36). Referring to the recommended algorithm (Fig. 2), a total of 164/637 specimens could be identified as "MTBC detected" by the Abbott Realti*m*e MTB assay. Among these 164 specimens, 22 strains, including 11 MDR-TB, 2 mono-RIF-resistant TB, and 9 mono-INH-resistant TB strains, would have qualified for *pncA* sequencing, providing a more cost-efficient routine application. Alongside rapid diagnosis of bacillary resistance to rifampin, isoniazid, fluoroquinolones, and second-line injectable drugs, direct *pncA* sequencing can further inform clinicians to formulate the most optimal regimen against drug-resistant TB.

Among the 164 MTBC culture-positive specimens, six AFB smear-negative specimens were reported as "MTBC not detected" by the Abbott Realti*m*e MTB assay, which may be accounted for by the paucibacillary nature of these specimens. Follow-up *pncA* sequencing performed on subsequent isolates revealed no *pncA* mutation, and the specimens were shown to be phenotypically susceptible to PZA, with positive PZase activity. On the other hand, 18 AFB smear-negative but "MTBC detected" specimens failed to amplify during *pncA* PCR step, which might also be attributable to low bacterial load. It is noted that Abbott Realti*m*e MTB assay detects MTBC using multiple copies of IS*6110*, in contrast to *pncA* sequencing, which detect resistance in only the single-copy *pncA* gene. The difference in copy numbers could account for the difference in sensitivities between the Abbott Realti*m*e MTB assay and *pncA* PCR.

In conclusion, results from the use of in-house-developed *pncA* sequencing as a molecular-method diagnostic algorithm exhibited high sensitivity and specificity, and the technique was able to provide an accurate and reliable PZA resistance profile among respiratory specimens within four working days. With the flexible and user-friendly nature of its workflow, *pncA* sequencing could potentially serve as a rapid and high-throughput diagnostic tool for clinical routine service situated in TB intermediate-burden regions. As PZA plays a critical role in MDR-TB treatment regimen, direct *pncA* sequencing will rapidly detect PZA resistance and facilitate judicious use of PZA in the treatment of PZA-susceptible MDR-TB.

Ethics approval. This study has been approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (reference UW 12-309).

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