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Analysis of drug resistance and mutation profiles in *Mycobacterium tuberculosis* isolates in a surveillance site in Beijing, China

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Abstract

Objective: This study analyzed drug resistance and mutations profiles in *Mycobacterium tuberculosis* isolates in a surveillance site in Huairou District, Beijing, China.

Methods: The proportion method was used to assess drug resistance profiles for four first-line and seven second-line anti-tuberculosis (TB) drugs. Molecular line probe assays were used for the rapid detection of resistance to rifampicin (RIF) and isoniazid (INH).

Results: Among 235 strains of *M. tuberculosis*, 79 (33.6%) isolates were resistant to one or more drugs. The isolates included 18 monoresistant (7.7%), 19 polyresistant (8.1%), 28 RIF-resistant (11.9%), 24 multidrug-resistant (MDR) (10.2%), 7 pre-extensively drug-resistant (XDR, 3.0%), and 2 XDR strains (0.9%). A higher rate of MDR-TB was detected among previously treated patients than among patients with newly diagnosed TB (34.5% vs. 6.8%). The majority (62.5%) of RIF-resistant isolates exhibited a mutation at S531L in the DNA-dependent RNA polymerase gene. Meanwhile, 62.9% of INH-resistant isolates carried a mutation at S315T1 in the katG gene.

Conclusion: Our results confirmed the high rate of drug-resistant TB, especially MDR-TB, in Huairou District, Beijing, China. Therefore, detailed drug testing is crucial in the evaluation of MDR-TB treatment.

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Keywords
Mycobacterium tuberculosis, multidrug-resistant tuberculosis, phenotypic drug susceptibility testing, line probe assay, rifampicin, isoniazid, rpoB, katG

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Introduction

Tuberculosis (TB) remains a global public health threat.[1] Despite the development of newer drugs to combat this disease, drug resistance has emerged as a major concern.[2] Although the average time to treat patients with fully susceptible TB is 6 to 9 months, the treatment of drug-resistant TB may require 12 to 18 months or even longer, and the drugs are more expensive but less effective. In 2019, close to half a million people globally developed rifampicin (RIF)-resistant TB, of whom 78% had multidrug-resistant TB (MDR-TB). The three countries with the largest share of the global burden are India, China, and the Russian Federation. In 2019, 3.3% of new TB cases and 17.7% of previously treated cases globally involved MDR-TB/RIF-resistant TB. Conversely, in China, 7.1% of new cases and 23% of previously treated cases involved MDR-TB, exceeding the global averages.[3] As TB treatment has shifted from standardized therapies to more individualized approaches, information about drug resistance patterns in the population has gained increasing relevance and importance. Within the context of the high TB incidence in China, it is extremely important to pay attention to the drug resistance of TB bacteria to inform clinical decision-making concerning appropriate drug regimens.

The acquisition of drug resistance in *Mycobacterium tuberculosis* (MTB) is not attributable to horizontal transfer of resistance-determining genes or regions, but instead, drug resistance results from mutations in specific resistance-determining regions of the gene targets or their promoters or in the activating enzymes of anti-TB chemotherapeutic agents (caused by nucleotide substitution, insertion, or deletion).[4] Resistance to RIF is caused by mutations in the beta subunit of DNA-dependent RNA polymerase (rpoB) and is commonly found in the 81-bp hotspot region of the rpoB gene. Meanwhile, the mechanism of resistance to isoniazid (INH) is more complex. Many resistant organisms have mutations in the katG gene encoding catalase peroxidase, resulting in enzyme structure changes. These structural changes obviously reduce the transformation of INH to its bioactive form. Some INH-resistant organisms also have mutations in the inhA locus encoding a beta-ketoacyl-acyl carrier protein synthase.[5] Different mutations can result in functional differences, leading to different resistance levels or resistance to different drugs within or between drug classes.[6] In recent years, WHO endorsed molecular line probe assays (GenoType® MTBDRplus, Hain Lifescience, Nehren, Germany) for the rapid detection of resistance to RIF and INH. The method detects mutations in the rpoB gene that confer RIF resistance, mutations in the katG gene related to high-level INH resistance, and mutations in the regulatory region of the inhA gene related to low-level INH resistance.

This study analyzed the drug resistance profiles and mutations of MTB isolates in a

surveillance site in Beijing, China (Huairou District) from 2009 to 2018. These profiles will provide basic information for developing an effective anti-TB regimen.

Patients and methods

Clinical isolates of MTB

The study was performed in accordance with the guidelines of the Helsinki Declaration and its later amendments or comparable ethical standards, and the protocol was approved by the Ethics Committee of Beijing Research Institute for Tuberculosis Control and Beijing Chest Hospital, Capital Medical University. Written informed consent was obtained from each patient before specimen collection. This study did not contain patient-identifiable data.

Study subjects were recruited from Huairou District in northern Beijing, China. Huairou District is located at 40°41′ N latitude 116°17′ E longitude with an area of 2123 km^2, and its population in 2019 was 384,000.

Patients with TB enrolled at Huairou Center for Disease Control and Prevention with at least one positive culture sample were included in the study. All patients were outpatients. Following global guidelines, the Huairou monitoring site collected at least two sputum samples from patients with TB and then inoculated all samples in acidified Lowenstein–Jensen (L-J) medium after routine pretreatment. Cultures with growing colonies were frozen and sent to Beijing Research Institute for Tuberculosis Control (Beijing, China) for further identification and drug susceptibility testing (DST). All culture-positive strains were tested for drug sensitivity to determine the optimal anti-TB regimen. In this study, 250 clinical isolates of MTB were consecutively collected from the sputum specimens of eligible patients from January 2009 to December 2018.

Definitions

Drug resistance patterns were classified according to the WHO definition of drug-resistant TB. Monoresistance was defined as resistance to a single first-line anti-TB drug. Polyresistance described resistance to multiple first-line anti-TB drug excluding both INH and RIF. Multidrug resistance was defined as resistance to both RIF and INH. Pre-extensively drug-resistant (pre-XDR)-TB described MDR-TB strains resistant to fluoroquinolones (FQ) or a second-line injectable drug, but not both. Extremely drug-resistant (XDR)-TB was defined strains that were additionally resistant to at least one FQ and at least one injectable agent.[7,8]

Laboratory identification and DST

Phenotypic DST was performed against first-line (RIF, INH, streptomycin [SM], ethambutol [EMB]) and second-line anti-TB drugs (levofloxacin [LFX], amikacin [AM], capreomycin [CPM], protionamide [PTO], p-aminosalicylic acid [PAS], kanamycin [KM], ofloxacin [OFX]) using the proportion method recommended by WHO on L-J medium. The critical concentrations of these anti-TB drugs were as follows: 40 μg/mL for RIF, 0.2 μg/mL for INH, 4.0 μg/mL for SM, 2.0 μg/mL for EMB, 2.0 μg/mL for LFX, 30 μg/mL for AM, 40 μg/mL for CPM, 40 μg/mL for PTO, 1 μg/mL for PAS, 30 μg/mL for KM, and 2.0 μg/mL for OFX. The critical growth proportion for drug resistance was 1% for all drugs. 2-Thiophenecarboxylic acid hydrazide (TCH) and p-nitrobenzoic acid (PNB) were used to identify mycobacteria simultaneously using the proportion method. PNB was added to the medium at a final concentration of 500 g/mL,

whereas TCH was used at a final concentration of 5 g/mL.

DNA extraction

Two loopfuls of mycobacterial colonies were suspended in 300 μL of sterile distilled water and heated in a water bath at 95°C for 20 minutes to isolate DNA from the bacteria. The solution was sonicated and centrifuged at $10{,}000 \times g$ for 5 minutes, after which the supernatant was collected and stored at −20°C for molecular related investigations.

Line probe assays (LPAs)

The molecular LPA is based on the reverse hybridization of amplified products with probes fixed on the membrane, including wild-type (WT) sequences and specific mutations in the regions of rpoB, katG, and the inhA promoter region.

During hybridization, if the isolated DNA does not bind to at least one WT probe or binds to any mutant probe, it was considered resistant. When all WT probes of a gene could be detected and there was no mutation detected in the inspected region, the sample was considered susceptible to the respective antibiotic.

Quality control (QC)

The MTB H37Rv reference strain was used for QC in the proportion method DST and molecular detection. This QC strain is sensitive to the first-line and second-line drugs tested in this study.

Statistical analysis

Categorized variables were analyzed using Fisher's exact test or Pearson's chi-squared test. $P < 0.05$ denoted statistical significance. All data were analyzed using SPSS 21.0 (IBM, Armonk, NY, USA).

Results

Resistance patterns

From 2009 to 2018, 250 mycobacterial isolates were submitted for drug resistance testing. Among these isolates, 15 were identified as nontuberculosis mycobacteria (NTM), giving an NTM isolation rate of 6%. These isolates were excluded from further analysis. Among the 235 strains of MTB, 156 (66.4%) strains were susceptible to all tested first- and second-line anti-TB drugs, and the remaining 79 (33.6%) isolates were resistant to at least one drug. The resistant strains included 28 (11.9%) RIF-resistant strains, and 24 of these strains were MDR-TB. The isolates included 18 monoresistant TB strains (7.7%), 19 polyresistant strains (8.1%), 7 pre-XDR strains (3.0%), and 2 XDR strains (0.9%).The rate of MDR-TB was higher in retreated cases (10/29 [34.5%]) than in new cases (14/206 [6.8%], $P < 0.05$, Table 1).

All 18 monoresistant isolates were from new cases. Of these, 11 were resistant to SM, and four (22.2%) were resistant to INH. Among the 19 polyresistant strains, 12 were resistant to two drugs, three were resistant to three drugs, and four were resistant to more than four drugs (Table 2).

Approximately 41.7% (10/24) of MDR-TB isolates were resistant only to first-line anti-TB drugs, whereas 20.8% of these isolates were resistant to RIF, INH, and SM. The drug resistance profiles of the seven pre-XDR and two XDR strains are presented in Table 3.

Drug-resistant TB grouped by sex and age

Of the TB isolates submitted for drug susceptibility testing in this study, 77.0% (181/235) were obtained from males. Among isolates obtained from males, 29.8% (54/181) were resistant to one or more anti-TB drugs, and 9.4% (17/181) were MDR-TB

Table 1. Distribution of different drug resistance types in new and previously treated cases.

	New cases (n = 206)		Previously treated cases (n = 29)		Total (n = 235)			
Drug resistance	n	%	n	%	n	%	χ^2	*P*
Any resistance	67	32.5	12	41.4	79	33.6	0.89	0.34
Monoresistance	18	8.7	0	0.0	18	7.7	2.74	0.10
Polyresistance	18	8.7	1	3.5	19	8.1	0.96	0.33
MDR-TB	14	6.8	10	34.5	24	10.2	21.25	<0.001
Pre-XDR–TB	3	1.5	4	13.8	7	3.0	13.39	<0.001
XDR-TB	1	0.5	1	3.5	2	0.9	2.65	0.1
Unclassified MDR-TB	10	4.85	5	17.2	15	6.4	6.53	0.01
Other	17	8.3	1	3.5	18	7.7	0.83	0.36

Pre-XDR TB is defined as MDR-TB with additional resistance to a fluoroquinolone or second-line injectable drug.
XDR TB is defined as MDR-TB with additional resistance to at least one fluoroquinolone and at least one injectable agent.
Unclassified MDR-TB represents MDR-TB isolates that cannot be classified as pre-XDR–TB or XDR-TB.
MDR-TB, multidrug-resistant tuberculosis; pre-XDR–TB, pre-extensively drug-resistant tuberculosis; XDR-TB, extensively drug-resistant tuberculosis.

Table 2. Drug resistance profiles of polyresistant strains.

Drug resistance	Number of isolates (%)
Resistance to two drugs	
INH + SM	8 (42.1)
INH + PTO	1 (5.3)
RIF + EMB	1 (5.3)
EMB + PTO	2 (10.6)
Resistance to three drugs	
RIF + SM + PTO	1 (5.3)
EMB + SM + PTO	1 (5.3)
SM + LFX + OFX	1 (5.3)
Resistance to four or more drugs	
INH + SM + EMB + PTO	1 (5.3)
INH + SM + LFX + OFX	1 (5.3)
INH + EMB + PTO + PAS	1 (5.3)
INH + EMB + PTO + LFX + OFX	1 (5.3)
Total	19 (100)

INH, isoniazid; SM, streptomycin; PTO, protionamide; RIF, rifampicin; EMB, ethambutol; LFX, levofloxacin; OFX, ofloxacin; PAS, p-aminosalicylic acid.

strains. Among isolates obtained from females, 46.3% (25/54) were resistant to at least one anti-TB drug, and 13.0% (7/54) were MDR-TB isolates. The rate of drug-resistant TB was higher in females than in males ($\chi^2 = 5.05$, $P < 0.05$).

Of all patients with drug-resistant TB, only one was younger than 20 years. The proportion of isolates resistant to anti-TB drugs ranged from 10.1% (61–70 years old) to 21.5% (51–60 years old). No MDR-TB isolates were obtained from patients younger than 20 years or those aged 61 to 70 years. The proportions of MDR-TB ranged from 4.2% (51–60 years of age) to 37.5% (31–40 years of age). Most patients with MDR-TB were aged 31 to 60 years old (Figure 1).

Detection of mutations associated with drug resistance using LPAs

Of the 28 RIF-resistant strains diagnosed via DST, 24 were correctly identified using LPAs, giving a coincidence rate of 85.7%. The majority (15/24 [62.5%]) of RIF-resistant isolates carried the S531L mutation in the rpoB gene diagnosed by loss of the WT8 band and the presence of the MUT3 band. One RIF-resistant isolate carried a mutation at D516V in the rpoB gene,

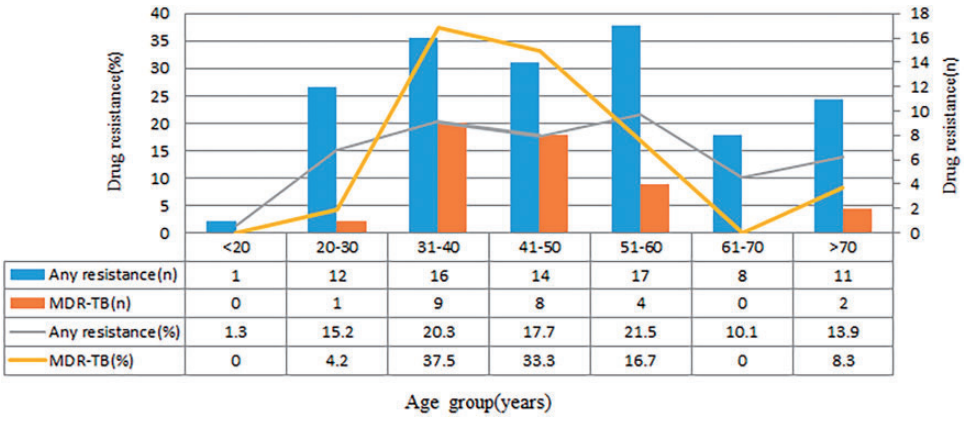
Table 3. Drug resistance profiles of MDR-TB.

	New cases (n = 14)		Previously treated cases (n = 10)		Total cases (n = 24)	
Drug resistance	n	%	n	%	n	%
MDR-TB resistant to first-line anti-TB drugs only						
INH + RIF	1	7.1	1	10.0	2	8.3
INH + RIF + SM	5	35.7	0	0.0	5	20.8
INH + RIF + SM + EMB	1	7.1	2	20.0	3	12.5
Subtotal	7	50.0	3	30.0	10	41.7
Pre-XDR–TB						
INH + RIF + SM + LFX + OFX	2	14.3	0	0	2	8.3
INH + RIF + SM + EMB + OFX	0	0.0	1	10.0	1	4.2
INH + RIF + SM + LFX + PTO + OFX	1	7.1	1	10.0	2	8.3
INH + RIF + EMB + LFX + PTO + OFX	0	0.0	1	10.0	1	4.2
INH + RIF + SM + EMB + AK + CPM + PTO + KM	0	0.0	1	10.0	1	4.2
Subtotal	3	21.4	4	40.0	7	29.2
XDR-TB						
INH + RIF + SM + EMB + AK + CPM + KM + OFX	1	7.1	0	0.0	1	4.2
INH + RIF + SM + EMB + LFX + AK + CPM + PTO + PAS + KM + OFX	0	0.0	1	10.0	1	4.2
Subtotal	1	7.1	1	10.0	2	8.3
Other drug resistance patterns						
INH + RIF + PTO	0	0.0	1	10.0	1	4.2
INH + RIF + SM + PTO	2	14.3	1	10.0	3	12.5
INH + RIF + SM + EMB + PTO	1	7.1	0	0.0	1	4.2
Subtotal	3	21.4	2	20.0	5	20.8
Total	14	100	10	100	24	100

MDR-TB, multidrug-resistant tuberculosis; INH, isoniazid; RIF, rifampicin; SM, streptomycin; EMB, ethambutol; pre-XDR–TB, pre-extensively drug-resistant tuberculosis; LFX, levofloxacin; OFX, ofloxacin; PTO, protionamide; AK, amikacin; CPM, capreomycin; KM, kanamycin; XDR-TB, extensively drug-resistant tuberculosis.

whereas another isolate carried a mutation at H516D. Six isolates without either WT or mutant bands were considered uncertain or unknown mutations, and their probes were not included in the nitrocellulose strips of LPAs. One isolate exhibited a heterogeneous resistance mutation pattern in which all wild-type bands were observed and one mutant band (MUT1) was also present.

Among the 41 INH-resistant strains diagnosed by DST, 85.4% (35/41) featured mutations in the katG gene or inhA promoter region. Of the 35 INH-resistant strains harboring gene mutations, 62.9% (22/35) carried the S315T1 mutation in the katG gene, indicating high-level resistance, whereas 5.7% (2/35) of the strains carried the C15T mutation in the inhA gene, indicating low-level resistance. Two isolates featured mutations in both the katG and inhA genes. In seven (20%) katG mutants, both WT and mutant bands were absent, and thus, other mutations may not have been detected. A heterogeneous resistance mutation pattern was found in two isolates with all WT probes present along with the presence of one mutant band (Table 4).



	<20	20-30	31-40	41-50	51-60	61-70	>70
Any resistance(n)	1	12	16	14	17	8	11
MDR-TB(n)	0	1	9	8	4	0	2
Any resistance(%)	1.3	15.2	20.3	17.7	21.5	10.1	13.9
MDR-TB(%)	0	4.2	37.5	33.3	16.7	0	8.3

Figure 1. Number and proportion of tuberculosis isolates with reported drug resistance by age group and resistance pattern.
MDR-TB, multidrug-resistant tuberculosis.

Discussion

In this study, we investigated the drug resistance distribution and mutation patterns of MTB strains isolated from patients with TB in Huairou District, a surveillance site in Beijing. Our study revealed that 33.6% (79/235) of the isolates were resistant to at least one anti-TB drug, and 11.9% (28/235) of the isolates were resistant to RIF. RIF is a key anti-TB drug that is used for the initial treatment and retreatment of patients with TB. However, more than 90% of TB strains that are resistant to RIF are also resistant to INH,[9] which has decreased the rate of successful treatment. In our study, 85.7% (24/28) of RIF-resistant strains were MDR-TB. Therefore, the continuous monitoring of RIF resistance patterns is important and meaningful.

Compared with patients with newly diagnosed TB in this study (6.8%) in this study, the prevalence of MDR-TB in the retreatment cases was relatively high (34.5%), in line with earlier studies.[10,11] The higher rate of MDR-TB in patients with previously diagnosed TB might be attributable to several factors, including improper chemotherapy regimens, insufficient or irregular drug supply, patient defaulting, and a lack of supervision for treatment regimens.

Women were more likely to exhibit drug resistance than men, as reported previously.[12,13] Most patients with MDR-TB in our study were between 31 and 60 years old, demonstrating that preventative and control measures for MDR-TB should also target young and middle-aged patients. The higher frequency of MDR-TB in the middle-aged group suggests the possibility of MDR-TB transmission in the community because of the high mobility of youth.[14] Therefore, we should focus on detecting infection and interrupting the transmission routes of patients. Patients younger than 20 years had the lowest rate of drug resistance, possibly because of full-time supervision by a guardian and good treatment compliance.

We identified seven pre-XDR–TB and two XDR-TB strains. FQ and second-line injectable drugs are extremely effective against MDR-TB. If resistance to FQ or second-line injectable drugs exists, then the treatment of MDR-TB will be more complicated. The identification of patients

Table 4. Pattern of gene mutations detected by line probe assays.

Drug resistance patterns					
RIF resistance pattern (rpoB gene, n = 24)				Mutations detected	No. (%) of strains
WT probes		Mutant probes			
WT		MUT1		Unknown	1 (4.2%)
ΔWT3		—		Unknown	1 (4.2%)
ΔWT3, ΔWT4		MUT1		D516V	1 (4.2%)
ΔWT7		—		H526R, H526P, H526Q, H526N, H526L, H526S, H526C	3 (12.5%)
ΔWT7		MUT2B		H526D	1 (4.2%)
ΔWT8		—		S531P, S531Q, S531W, L533P	2 (8.3%)
ΔWT8		MUT3		S531L	15 (62.5%)
INH resistance pattern (n = 35)					
katG		inhA			
WT probes	Mutant probes	WT probes	Mutant probes	Mutations detected	No. (%) of strains
WT	MUT1	WT	—	Unknown	1 (2.9%)
ΔWT	—	WT	—	Unknown	7 (20%)
ΔWT	MUT1	WT	—	S315T1	22 (62.9%)
WT	—	WT	MUT1	Unknown	1 (2.9%)
WT	—	ΔWT1	MUT1	C15T	2 (5.7%)
ΔWT	MUT1	ΔWT2	MUT3B	S315T1, T-8A	2 (5.7%)

RIF, rifampicin; rpoB, DNA-dependent RNA polymerase; WT, wild-type pattern with all respective bands visible; ΔWT, lack of hybridization to the wild-type probe; MUT, mutation; INH, isoniazid.

with pre-XDR–TB will help clinicians to monitor these patients closely and prevent the progression to XDR-TB, which is more difficult to treat.

Because of the slow growth of MTB bacilli, delays in the detection of resistant strains can occur when conventional phenotypic assays are used. Molecular tools can be used to diagnose drug-resistant TB in a timely manner.[15] Many studies demonstrated that LPAs have promising sensitivity and specificity.[16,17] In our study, the S531L mutation in the rpoB gene was the most prevalent (62.5%). This result is consistent with the results of a study in Beijing.[18] The most common mutation leading to INH resistance was the S315T substitution in katG, which is related to high-level INH resistance, as reported previously.[19] Understanding the potential prevalence of katG and inhA mutations, together with the local incidence of TB and drug-resistant TB, can better inform clinicians and prompt them to prescribe more effective drugs for TB treatment. High-dose INH may be a viable treatment option for individuals with isolates carrying only inhA mutations, whereas ethionamide may be a suitable alternative when only a katG mutation is present.[20]

We identified three isolates with heterogeneous resistance mutation patterns in which all WT bands were detected together with one mutation band. TB involving

infection by a heterogeneous MTB population may be associated with reinfection events or an evolutionary genetic variation of a single infection.[21] Heterogeneous resistance cannot be detected using routine laboratory methods such as culture and other molecular tests.

The major advantages of LPAs are its more rapid detection speed and shorter time to diagnosis.[22] Patients are able to receive timely and appropriate treatment, thereby preventing the transmission of drug-resistant strains. Despite the increased costs of laboratory testing, the use of the LPAs decreases the total health care costs for Chinese patients with MDR-TB.[23] More importantly, patients are not exposed to ineffective empirical therapy, which prevents disease progression and improves clinical outcomes for patients with MDR-TB. However, extremely few patients with MDR-TB were resistant to only RIF and INH resistance in our study. By focusing only on RIF and/or INH resistance, other important and relevant resistance patterns can be overlooked. In addition, LPAs failed to detect all mutations in or outside the common gene. Molecular detection techniques must be improved to characterize other mutations. Therefore, LPAs can only be used as a complementary method for the rapid detection of MDR-TB strains in high-burden countries opposed to a substitute for traditional drug sensitive methods.

In conclusion, we determined the drug resistance and mutations profiles in Huairou District, which provided basic data for TB prevention and treatment in this district as well as Beijing. There was a high proportion of drug-resistant TB n Huairou District. The use of standardized regimens for patients with previously treated TB could amplify and spread resistance through selective pressure, increasing the risk of MDR-TB and XDR-TB. Therefore, effective individualized treatment regimens should be designed for patients according to the results of traditional or molecular drug sensitivity testing.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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