Low prevalence of MDR-TB in Lao PDR: results from the first national anti-tuberculosis drug resistance survey

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Abstract

OBJECTIVE To present results of the first national anti-tuberculosis (TB) drug resistance survey conducted in Lao PDR between May 2016 and August 2017 to determine the prevalence of resistance to first-line anti-TB drugs among new and previously treated pulmonary TB cases in the country.

METHODS Patients with sputum smear-positive pulmonary TB were enrolled from 42 TB laboratories distributed in 40 clusters throughout the country. Survey sites were selected using probability-proportional-to-size sampling among all diagnostic centres in the country. In addition to smear microscopy, all patients underwent Xpert MTB/RIF testing and those found positive to Mycobacterium tuberculosis underwent sputum culture and drug susceptibility testing using the proportion method on solid Löwenstein–Jensen medium.

RESULTS Among 1006 eligible patients, 946 sputum smear-positive and Xpert MTB/RIF positive (Mycobacterium tuberculosis detected) patients were included in the survey, comprising 897 new and 49 previously treated TB cases. The prevalence of rifampicin-resistant TB was 1.2% (95% CI: 0.5–2.0%, n = 11/897) among new cases and 4.1% (95% CI: 0–9.6%, n = 2/49) among previously treated cases. Among the 946 TB cases confirmed by Xpert MTB/RIF, phenotypic drug sensitivity testing was available for 820 (776 new and 44 previously treated). The prevalence of multidrug-resistant TB (MDR-TB) was 0.5% (95% CI: 0–1.0%, n = 4/776) among new cases and 2.3% (95% CI: 0–6.7%, n = 1/44) among previously treated cases. No resistance to second-line injectable agents nor to fluoroquinolones was detected among MDR-TB patients.

CONCLUSIONS The first national anti-TB drug resistance survey in Lao PDR demonstrated an encouragingly low prevalence of MDR-TB. The results appear lower than previous WHO estimates, and in line with the routine surveillance based on Xpert MTB/RIF testing (conducted among 50% of presumptive TB patients in 2017). The country should continue to expand its Xpert MTB/RIF network and strive to achieve universal drug susceptibility testing.

KEYWORDS drug resistance survey, tuberculosis, multi drug-resistant TB, South East Asia

Introduction

Mycobacterium tuberculosis, the agent responsible for tuberculosis (TB) infection and disease, was declared a global emergency by WHO in 1993 [1]. Despite continuous efforts from governments and National TB programmes worldwide, TB remains a global threat and a major public health issue. In 2017, 10 million people fell ill with TB, and 1.6 million died from TB, with over 95% of TB deaths occurring in low- and middle-income countries [2]. Globally, TB incidence is falling at about 2% annually. This needs to accelerate to a 4–5% annual decline to reach the 2020 milestone of the End TB Strategy [3]. One challenge to this decline is the increasing resistance of Mycobacterium tuberculosis to TB drugs. Multidrug-resistant TB (MDR-TB), defined as TB resistant to at least rifampicin and isoniazid, the two most effective first-line drugs, and extensively drug-resistant TB
(XDR-TB), characterised by MDR strains resistant to second-line anti-TB injectable drugs and fluoroquinolones, are global threats. Therapeutic options for these patients are reduced, with less effective drugs, more frequent and severe side effects, and longer and more expensive treatment regimens [4].

Lao PDR is a landlocked country in South East Asia. Even though not classified as a High Burden Country (HBC), it is surrounded by countries that are all listed in at least one of the three HBC lists used by WHO for the period 2016–2020 [5]. The National TB Control Programme (NTCP) was established in 1995 with the support of WHO and Damien Foundation, Belgium. In the same year the directly observed treatment short-course was introduced in the country and reached full coverage in 2005, with high treatment success rates since then (88% among new and relapse cases, 85% among previously treated cases in the 2015 cohort). In 2010 capacity for TB culture was established at the National TB reference laboratory (NTRL) with the support from the Korean Institute of Tuberculosis (KIT). In 2010–2011 the first National TB prevalence survey was successfully conducted [6]. In 2010 the first MDR-TB case was identified using culture and Line Probe Assay test at Centre of Infectiology Lao Christophe Mérieux [7] and the NTCP started the programmatic management of drug-resistant TB in Vientiane in 2011 using a 24-month second-line drug regimen based on WHO guidelines for the programmatic management of MDR-TB [8], while the true burden of MDR-TB was unknown. In 2014 NTRL started using the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) for rapid molecular diagnosis of rifampicin resistance, while developing capacity for phenotypic drug susceptibility testing on solid media for all first-line drugs as well as fluoroquinolones and second-line injectable TB drugs. The shorter 9-month MDR-TB regimen [9] was introduced as an operational research project in 2013 and recommended by the regional Green Light Committee as standard treatment for MDR-TB patients sensitive to fluoroquinolones and second-line injectable TB drugs in 2016 [8].

In 2016 and 2017, a total of 4981 and 5730 TB cases of all forms were reported by NTCP, respectively (NTCP source). Concurrently the Lao PDR National TB Control Programme (NTCP) and National TB Reference Laboratory (NTRL) conducted the first national anti-TB drug resistance survey to determine the prevalence of resistance to first-line anti-TB drugs (isoniazid, rifampicin, streptomycin, ethambutol) among TB patients and selected second-line drugs among MDR-TB patients: kanamycin, capreomycin and ofloxacin among new and previously treated TB cases.

Method

Survey design

A national cluster sample survey-study of smear-positive pulmonary TB patients was undertaken from May 2016 to August 2017. The sample size was based on WHO’s estimation of the prevalence of rifampicin resistance among new pulmonary TB cases (4.5% in year 2014) [10], using an internationally recommended approach for cluster sample surveys [11].

Sampling strategy

Lao PDR is divided into 17 provinces and one central municipality comprising the capital city. Each province is further divided into districts. Each district has its own hospital with a TB diagnostic centre. Overall in the country there are 164 TB diagnostic centres including five central hospitals. A total of 40 clusters were selected, each with a target sample size of 26 consecutive new sputum smear-positive cases. The 40 clusters were selected using a probability-proportional-to-size sampling, from all 164 diagnostic centres in the country, after first excluding diagnostic centres with fewer than 10 notified cases in 2014 (representing 8% of the total new smear positive pulmonary cases diagnosed in the country). Some large diagnostic centres constituted more than one cluster. For centres with a small number of notified cases, additional supplementary centres were selected to contribute to the target sample size, provided they were similar in terms of location, catchment area, and type of health facility. Both selected and supplementary centres enrolled consecutive cases in parallel until the required 26 new cases were reached. In total, 42 diagnostic centres from district, provincial, and central level constituted the 40 clusters (Table 1; Figure 1).

Intake of patients

Patient enrolment was completed within 14 months and was preceded by a one-month pilot survey in several diagnostics centres, in order to test data collection tools and to sample transport and laboratory processes. Due to the high TB burden, some centres were able to reach the target sample size for new smear positive TB patients within 3 months, while others required the full 14 months due to the low number of TB cases.

Inclusion and exclusion criteria

New cases and newly registered previously treated cases with a finding of at least 10 bacilli in 100 high powered
field on sputum smear microscopy (corresponding to at least a 1+ result using the International Union Against Tuberculosis and Lung Disease recommended grading of sputum smear microscopy results) were included in the survey. Patients with sputum smear-negative or scanty pulmonary TB, patients with extra-pulmonary TB and patients already on treatment were excluded from enrolment.

Statistical analysis

Data were analysed using Stata version 14 from Stata Corp.

The number of patients for which Mycobacterium tuberculosis was detected using the Xpert MTB/RIF assay represented the denominator for the calculation of the prevalence of rifampicin resistance. The denominator for the calculation of the prevalence of MDR-TB and resistance to isoniazid, ethambutol and streptomycin was the number of patients whose culture was positive and therefore phenotypic drug susceptibility testing could be performed for first-line and selected second-line drugs.

For the calculation of rifampicin resistance and MDR-TB prevalence, a logistic regression with robust standard errors was used to account for the clustered survey design.

Multiple imputation was performed to account for missing data, and results were compared to crude, unadjusted values.
Survey procedures

When a presumptive TB patient presented at the diagnostic centre, a spot sputum was collected. The patient was asked to come back the day after with a second morning sputum as per national guidelines. An additional third spot morning sputum sample was collected for this survey when the patient returned with the morning sample.

The laboratory technician performed direct smear microscopy on all samples collected. If at least 10 bacilli were seen in 100 high powered field (grading 1+) on at least one smear, the patient was eligible and one sample was tested by the Xpert MTB/RIF assay at one of the eleven provincial laboratories equipped with the Genexpert instrument. An HIV rapid screening test was performed subsequently for each TB patient identified as per the national technical guidelines.

Since 2004 all diagnostic centres in Lao PDR participate in an external quality assessment (EQA) programme (based on the APHL consensus document on EQA microscopy, 2000) conducted on a quarterly basis, which includes:

- On-site evaluation of local TB microscopy services with regular visits by the provincial TB coordinator, district TB manager, as well as an annual visit by the National TB Reference Laboratory (NTRL).
- Blinded re-checking of district laboratory samples of all slides performed quarterly by the province laboratory (first level control), while the NTRL rechecks discrepant results between the district laboratory and the province assessor (second level control).

EQA results in 2016 and 2017 showed satisfactory score countrywide (Figure 2) and the same quality.
control was performed for the microscopy results in the frame of the survey.

Eligible patients were interviewed using a specifically designed questionnaire including a consent form, and two samples were sent to the NTRL.

Upon reception at the NTRL, samples were processed for culture examination. Patients were classified based on the outcomes of the microbiology results (Figure 3).

**Laboratory examination**

In all diagnostic centres, samples were stained with carbol fuchsin by Ziehl–Neelsen method then followed by high-powered fields (1000×) microscopic examination [12]. Cetylpyridinium Chloride (CPC) 0.25% + NaCl 2% was added in a 1:1 ratio as soon as direct smear microscopy result was available to maintain the viability of the bacilli and reduce risks of contaminant growth in smear positive samples.

At the NTRL, specimens were transferred into 50-ml sterile Falcon tubes. Sterile distilled water was added to the tube to the 50-ml mark. Tubes were centrifuged at 3000 g for 15 min at room temperature. The supernatant was discarded and the pellet resuspended in 2 ml of phosphate buffer [13]. Two slopes of Löwenstein–Jensen (LJ) media were inoculated per specimen processed and incubated at 37 °C horizontally for 3 days, caps partially loose. After 3 days, if no contamination was observed, the tubes were straightened up with caps fully tightened and incubated for up to 8 weeks prior to being discarded as negative if no growth was observed. All visible colonies were confirmed for AFB by microscopy, susceptibility testing to para-nitrobenzoic acid (PNB) (500 µg/ml) and rapid identification using SD Bioline rapid test. Strains confirmed as being *Mycobacterium tuberculosis* were subcultured to obtain pure colonies for phenotypic DST.

A loop of colonies was scraped from the subculture, transferred into a tube containing glass beads and vortexed to separate the colonies. The concentration of bacilli was adjusted to a Mac Farland No 1 standard. A 10-fold dilution was performed until the level of a $10^{-5}$ dilution was reached. One hundred microlitre of the $10^{-3}$ dilution was inoculated onto 2 LJ drug-free slopes and onto one slope of each of 7 the drug-containing LJ media. One hundred microliter of the $10^{-5}$ dilution was inoculated onto 2 LJ drug-free slopes. Resistance was interpreted according to the proportion method principle based on the number of colonies observed [14, 15]. Xpert MTB/RIF testing was performed following the manufacturer’s instructions [16].

**Data entry and analysis**

Data were entered in Filemaker Pro (v12, Filemaker Inc., Santa Clara, USA). All information on the request forms and the questionnaires was transcribed into the database and cross-checked at the end of the survey.

**Ethical approval**

This study was approved by the National Ethics Committee and the Department of Communicable Diseases Control of the Ministry of Health of Lao PDR. The protocol was reviewed and validated by WHO and KIT. The data of the survey are owned by NTCP of Lao PDR. The decision on dissemination and/or publication of the data was taken with the NTCP Manager.

**Results**

**Microbiology culture results and identification**

A total of 1006 patients were eligible for enrolment (with direct microscopy positive ≥1+) of whom 948 (94.2%) were new cases and 58 (5.8%) were previously treated cases (Figure 2).
Among 1006 eligible patients, *Mycobacterium tuberculosis* was confirmed in 946 patients by the survey diagnostic algorithm. Of those, 897 (94.8%) were new cases and 49 (5.2%) were previously treated cases. The ratio of new: retreatment among total notified cases in Lao PDR in 2017 (95:5) was similar to the one observed in the survey participants.

Among eligible patients, *Mycobacterium tuberculosis* was not confirmed in 60 patients who were then excluded from the survey: 22 patients had a negative Xpert MTB/RIF test result (17 new cases and 5 previously treated cases); and for 38 patients (34 new cases and 4 previously treated cases) an Xpert MTB/RIF test result was not available.

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**Figure 3** Algorithm of the study. \(^{(1)}\)1 0–99 acid fast bacilli in 100 fields using the International Union Against Tuberculosis and Lung Disease recommended grading of sputum smear microscopy results. \(^{(2)}\) NTRL, national tuberculosis reference laboratory. \(^{(3)}\) Xpert MTB/RIF+: *Mycobacterium tuberculosis* detected by Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA). \(^{(4)}\) Xpert MTB/RIF−: *Mycobacterium tuberculosis* not detected by Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA). \(^{(5)}\) DST, drug susceptibility testing. \(^{(6)}\) I, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; KM, kanamycin; CPM, capreomycin; OFX, ofloxacin. \(^{(7)}\) SRL, supranational reference laboratory (Korean Institute of Tuberculosis). [Colour figure can be viewed at wileyonlinelibrary.com]
Of the 946 patients for whom *Mycobacterium tuberculosis* was confirmed, 820 had positive culture: (776 (94.6%) new cases and 44 (5.4%) previously treated cases), 103 had negative culture (100 (97.1%) new cases and 3 (2.9%) previously treated cases), and for 23 patients culture was contaminated (21 (91.3%) new cases and 2 (8.7%) previously treated cases). These 126 patients without culture information were included in the analysis of rifampicin resistance prevalence since they all had Xpert MTB/RIF testing results (Figure 4).

The characteristics of the patients enrolled in the survey were compared to the characteristics of those eligible for enrolment and no significant differences were found. The mean age of patients was 49 years. Characteristics of patients enrolled in the survey are presented in Table 2.

The distribution of the missing data on isoniazid resistance (for the 126 patients who had no culture results available due to either contamination, negative cultures or insufficient growth for drug susceptibility testing) was analysed among different groups based on the patients’ treatment history, gender, age, and distribution by cluster. Missing data were proportionally equally distributed among the different groups for each of the chosen variable.

**Table 2** Characteristics of the 946 eligible patients for whom *Mycobacterium tuberculosis* was confirmed

|                      | New cases | Previous treated cases | Total cases |
|----------------------|-----------|------------------------|-------------|
|                      | #         | %                      | #           |
| Total                | 897       | 95                     | 49          |
| Gender               |           |                        |             |
| Male                 | 612       | 68                     | 49          |
| Female               | 285       | 32                     | 14          |
| Age group, years     |           |                        |             |
| 0–24                 | 78        | 9                      | 3           |
| 25–34                | 115       | 13                     | 4           |
| 35–44                | 142       | 16                     | 10          |
| 45–54                | 187       | 21                     | 9           |
| 55–64                | 194       | 22                     | 9           |
| ≥65                  | 181       | 20                     | 14          |

Figure 4 Microbiology flowchart. [Colour figure can be viewed at wileyonlinelibrary.com]
**Drug resistance patterns**

Table 3 shows the prevalence of resistance to first-line anti-TB drugs. The prevalence of rifampicin resistance was 1.2% (95% CI: 0.5–2.0%) and 4.1% (95% CI: 0–9.6%) of new cases and previously treated cases, respectively. The prevalence of MDR-TB was 0.5% (95% CI: 0–1.0%) among new cases and 2.3% (95% CI: 0–6.7%) among previously treated cases.

As there was little difference between imputed and crude values, multiple imputation was not needed for the calculation of prevalence of resistance. No resistance to second-line drugs was detected among patients with resistance to rifampicin. The results did not reveal any hot-spot for resistance with a homogenous distribution of the resistant cases throughout the country.

**Discussion**

This survey provides the first nationally representative data of the prevalence and pattern of resistance to TB drugs in Lao PDR.

In 2017, the estimated prevalence rates of MDR/RR-TB in the WHO South East Asia Region were 3.4% (95% CI: 2.5–4.4%) among newly detected cases and 19% (95% CI: 9.6–31%) among previously treated cases [2], and the region accounts for 30% of the global estimated MDR/RR-TB cases among notified pulmonary TB cases worldwide [17]. Our study shows a relatively low rate of MDR/RR-TB among new cases compared to the findings from similar studies in Cambodia (1.8% (95% CI: 0.77–2.8%)) [18], Thailand (2.2% (95% CI: 1.5–2.9%)) [19], Vietnam (4.1% (95% CI: 2.6–5.5%)) [20], Myanmar (5.1% (95% CI: 3.2–7%)) [21] and China (7.1% (95% CI: 5.6–8.7%)) [22]. In 2017, the WHO estimated that 18% of rifampicin-resistant TB are sensitive to isoniazid [2] whereas the proportion is much higher in our study, although numbers are very small (63%, n = 7/11). Kurbatova et al. [23] found that strains resistant to rifampicin but sensitive to isoniazid were more likely to be susceptible to all other anti-TB drugs than MDR-TB isolates. This could partially explain the high levels of treatment success observed in rifampicin-resistant TB patients on treatment (84% in the 2015 cohort), as the 9-month short regimen, which includes isoniazid, is the standard treatment for rifampicin resistance TB in Lao PDR. Therefore, patients with rifampicin-resistant TB and isoniazid-susceptible strains may benefit from more therapeutic options and experience better treatment outcome than MDR-TB patients.

The global averages of isoniazid resistance without concurrent rifampicin resistance were 7.1% (95% CI: 6.2–8.0%) in new TB cases and 7.9% (95% CI: 5.9–10%) in previously treated TB cases [2]. The risk we observed in our study was also similar between new cases with 4.3% (95% CI: 2.9–5.9%) and previously treated cases with 4.5% (95% CI: 0.6–15.5%), which points to continued primary transmission of this form of TB. For these patients the recommended regimen is rifampicin, ethambutol, pyrazinamide and levofloxacin for 6 months [24]. However, in Lao PDR there is no screening of this form of TB for presumptive TB patients, as the choice for the treatment regimen is based on the rifampicin resistance status. Denkinger et al. [25] demonstrated that detection of INH-resistance has minimal impact on transmission of TB, MDR-TB, and INH-monoresistant TB. Nonetheless, this should be monitored as these TB patients may experience poorer treatment outcomes.

In our study, the prevalence of resistance to streptomycin among new cases was 4.5%, whereas it was 6.2% in Thailand [26], 8.1% in Cambodia [27], 8.7% in Myanmar [28] and 30% in Vietnam [20]. This rate could
be explained by the fact that streptomycin is freely accessible on the market and people use it to treat other diseases. However, former re-treatment regimens with first-line TB drugs and added streptomycin is no longer recommended by WHO and not in use anymore in the country.

Overall, resistance to TB drugs rate in Lao PDR seems lower than in neighbouring countries, which can be due to several reasons. Historically, Lao PDR TB programme closely supervised the intensive phase of the category 1 TB treatment (2RHZE/6HE) while the continuation phase did not contain Rifampicin (switching to category 1 regimen 2RHZE/4RH occurred in 2010). Another explanation could be the fact that TB drugs are well-controlled and not used to treat other diseases in Lao PDR, which limits the emergence of resistance. In addition, the use of 4 FDC combined with DOT helped prevent the acquisition of resistance during treatment. Social determinants such as poverty could also explain to some extend the low transmission of drug resistance. Most Lao people almost never travel, due to a sedentary community-based lifestyle, and because of limited financial resources. Thus, there is little population exchange between regions in Lao PDR or with neighbouring countries. The National TB Strategic Plan 2017–2020 of the National TB Control Programme has planned the scaling up of more sensitive TB molecular diagnosis tools that also give rapid information on the rifampicin resistance status for at least 90% of the presumptive TB patients by 2020. Accuracy of detection with microscopy is estimated to be 10 000 organisms/ml [29], implying an estimated 50% of cases undiagnosed [30]. The accuracy of detection with Xpert MTB/RIF is estimated to be 131 organisms/ml [31, 32]. Therefore, the introduction of the Xpert MTB/RIF technology enables earlier diagnosis and treatment of more TB patients with a high treatment success rate nationwide.

This study has a number of limitations. Firstly, the survey was not designed to estimate the resistance rate among previously treated patients. The number of previously treated cases enrolled was small, resulting in a less precise estimate of the prevalence of resistance in this group. Secondly, the study was designed as a cluster sample survey with probability-proportional-to-size sampling, meaning that not all TB patients in the country were recruited. However, the survey was designed to be representative of the entire TB patient population.

Thirdly, the objective of the survey was to estimate the prevalence of drug resistance among new and previously treated smear positive pulmonary TB cases only. However, microscopy has low sensitivity and the performance of laboratory technicians may vary between centres. Therefore, it could be of interest to conduct subsequently another drug resistance survey based on the principle of a National TB prevalence survey in which we would enrol patients using other tools such as X-ray device. We would then be able to compare the resistance rate among positive and negative smear results patients. Fourthly, access to the healthcare system in Lao PDR is challenging; 70% of the country is covered by forest and mountains. This may decrease the representation of people living in remote areas, as our survey only captured patients who were able to access health care. The Lao Front for National Construction recently listed 49 ethnicities consisting of more than 160 ethnic groups [33]. Some groups rely on traditional medicine and would be missed by health services. The WHO TB country profile for Lao PDR reported 50% treatment coverage among the estimated incident new and relapse TB cases of all forms in 2017.

**Conclusion**

Lao PDR’s first national TB drug resistance survey in 2016–2017 allowed to determine the magnitude of drug-resistant TB in the country. Levels of resistance appear lower than previous WHO estimates, and in line with the routine surveillance based on Xpert MTB/RIF testing (conducted among 50% of presumptive TB patients in 2017). None of the patients with rifampicin resistance in the survey sample had any resistance to fluoroquinolones or second-line injectable TB drugs (i.e. no pre-XDR-TB or XDR-TB). Lao PDR should continue to expand its Xpert MTB/RIF network and strive to achieve universal drug susceptibility testing to maintain a surveillance baseline and determine trends overtime. The National TB Control Programme should continue reinforcing efforts to improve adherence to medication of patients on 1st line regimens as a means to limit emergence of resistance (in 2016 treatment cohort of new and relapse cases about 7% of cases were lost to follow up or without data).

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