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Epidemiological and laboratory characteristics of multidrug-resistant tuberculosis patients in Bhutan, 2015-2019

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ABSTRACT

Background: Bhutan is no exception to the rising global threat of drug resistance tuberculosis (TB), particularly multidrug-resistant (MDR) TB. Although drug resistance surveillance has been carried out in Bhutan since 2010, limited analysis reports are available. Therefore, we looked at data from 2015–2019 to understand patient characteristics.

Method: To obtain data for MDR-TB from the past 5 years, we looked at manual registers and laboratory worksheets for all samples received at National TB Reference Laboratory. Epidemiological factors and laboratory variables were analyzed using descriptive statistics.

Result: Among 304 patients with MDR-TB, 85.20% (n=259) are new cases with no previous history of treatment. Those aged 16–25 years from both genders are affected more (46.05%, n=140) than other age groups. The majority (94.62%, n=264) of rifampicin resistance was found in the *MUT 3 rpoB* gene. For Isoniazid, 97.13% (n=271) resistance was seen in the *MUT1 band* of the *katG* gene.

Conclusion: A high number of MDR-TB cases among new patients and little variation in the resistance band pattern over 5 years could indicate uncontrolled ongoing transmission. Whole-genome sequencing for the samples is required to further understand the epidemiology of the resistance pattern.

INTRODUCTION

In the last 5 years, there has been a decline in tuberculosis (TB) cases worldwide. However, drug-resistant TB, particularly multidrug-resistant tuberculosis (MDR-TB) cases, has been on the rise as per the World Health Organisation's annual TB reports of the recent years. In 2018, 186,772 cases of MDR-TB were reported globally. Of the total cases, India (27%), China (14%) and the Russian Federation (9%) had the highest burden (World Health Organisation 2019). The Russian Federation reported 3.4% MDR-TB cases among new cases and >50% among previously treated cases, the highest reported figure globally. MDR-TB is a major global, regional and national concern (World Health Organisation, 2019).

A common risk factor for developing MDR-TB is previous treatment (Pradipta *et al.*, 2018). Other sociodemographic and clinical factors include older age, unemployment, non-completion or failure of TB treatment, severe adverse drug reaction, HIV co-infection, chronic obstructive pulmonary disease, and being infected with the Beijing *Mycobacterium Tuberculosis* strain (Fregona *et al.*, 2017; Ricks *et al.*, 2012).

Availability of reliable and timely drug sensitivity test reports is required for patient care, prevention of transmission and drug resistance development. Bacterial culture remains gold-standard test for diagnosis of TB. However, these tests require skill and facilities and take 8 or more weeks to complete. In recent years molecular tools such as line probe assay (LPA) and GeneXpert have been increasingly popular due to their ease of operation and turnaround time of a few hours. Samples such as sputum, cerebrospinal fluid, pleural fluid and urine can be tested, allowing diagnosis of both pulmonary and extrapulmonary TB (EP-TB). However, these tools are limited to detecting mutation in a particular-target gene and thus need further improvement (Nguyen *et al.*, 2019; Lange *et al.*, 2018).

Each year, in Bhutan, approximately 600 samples are screened for MDR-TB. According to the country's annual health bulletin, TB remains a major public health issue (https://www.moh.gov.bt/wp-content/uploads/ict-files/2017/06/health-bulletin-Website_Final.pdf). The first MDR-TB surveillance was conducted in 2010, with 12% of all TB cases reported as MDR-TB (Wangchuk *et al.*, 2013). Similarly, in the 2014 annual drug resistance surveillance report, 10.11% of new and 37.21% of previously treated cases had MDR-TB (Adhikar *et al.*, 2013).

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The introduction of liquid culture in 2013 and LPA in 2014 has allowed for more than 80% drug susceptibility testing (DST) completion for all smear-positive TB samples shipped to the National TB Reference Laboratory (NTRL).

Limited information on epidemiological and laboratory characteristics of MDR-TB is available from nationwide surveillance in Bhutan. Our study looks at those features for all MDR-TB patients diagnosed and recorded from 2015 to 2019. Our findings will aid in targeted, evidence-based intervention to control MDR-TB in Bhutan and improving the surveillance system. We also look at the diagnostic facilities available at NTRL to design data-guided protocols for scaling up screening of MDR-TB, extensively drug-resistant TB (XDR-TB) and contacts of patients.

METHOD

Sampling strategy

Any individual visiting hospital with signs and symptoms of TB, including cough for 2 weeks or more, unexplained weight loss, night sweats or coughing of blood, is sent to a laboratory to give 2 spot and 1 morning sputum sample. Similarly, someone suspected of EP-TB is sent to respective laboratories for sample collection. All smear-positive cases detected across more than 40 laboratories in Bhutan were included in this study. In addition, all presumptive TB patient samples received at NTRL for TB culture, including EP, sputum samples for TB screening for visa purposes, and smear-negative samples of individuals belonging to high-risk categories such as retreatment cases, close contacts of TB cases, patients with x-ray suggestive of TB or other symptoms of TB and immunocompromised individuals, were also included. EP samples were mainly sent from the country's capital, where the NTRL is located. The overall diagnostic algorithm is shown in Figure 1.

Laboratory methodology

Sputum collection and transportation: Two sputum samples were collected from each patient and transported to NTRL using triple packaging and maintaining a cold chain. Sputum samples from each patient were accompanied by a sample shipment form containing the patient's identification details.

Sputum culture and identification: Samples were decontaminated using an *N*-acetyl-L-cysteine-sodium citrate method and inoculated on 2 slopes of egg-based Lowenstein-Jensen and in liquid media using the Mycobacteria Growth indicator tubes (MGIT) in the BACTEC machine (BACTEC™MGIT™ 960 System, Becton Dickson and Company, MA, USA) for a maximum of 42 days. The solid cultures were then incubated at 37 °C and monitored weekly for growth for up to 8 weeks, and liquid culture in the MGIT 960 machine was incubated for up to 6 weeks and monitored daily for growth. For the positive cultures, identification of *Mycobacterium tuberculosis* complex (MTBC) was made based on the phenotypic appearance of colonies on the solid media and by rapid chromatographic identification test, which detects mycobacterial protein MPT64. Microscopy was performed on liquid positive cultures to check for cord formation.

Drug susceptibility testing (DST): Confirmed MTBC isolates for each patient were tested for the presence of first-line drug resistance using rapid molecular LPA. The LPA used is based on strip technology to diagnose TB and detect resistance to rifampicin and isoniazid through mutations in *rpoB* and both *inhA* and *katG* genes. The test was performed according to the manufacturer's protocol (Hain Life Science GmbH, Nehren, Germany). Parallel DST was conducted using the liquid DST method on the BACTEC machine mentioned above for rifampicin and isoniazid, and streptomycin and ethambutol (SIRE).

Data collection and analysis

All patient details were extracted from the web-based Tuberculosis Information and Surveillance System. For missing data, manual registers

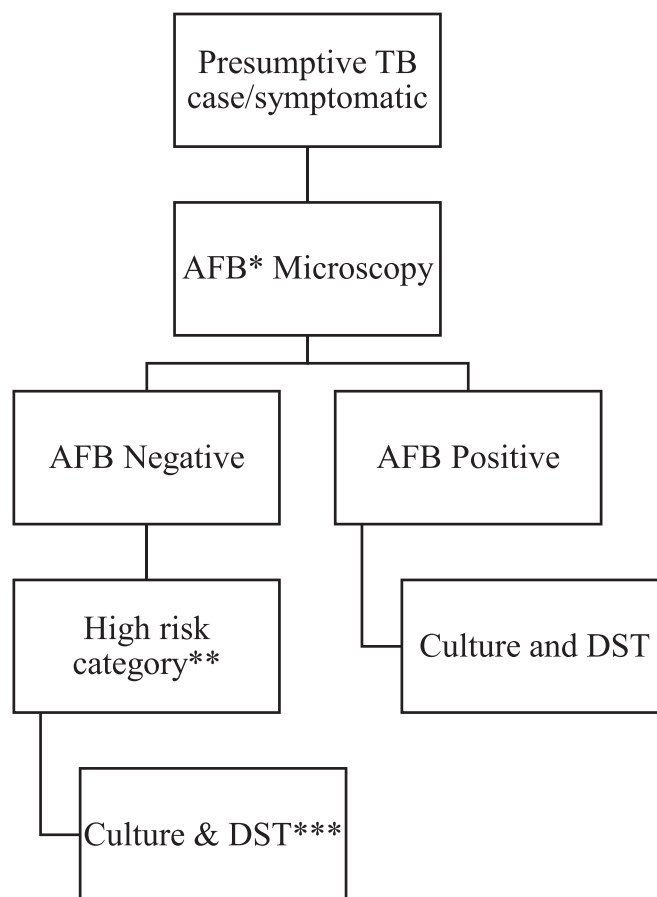


Figure 1. Diagnostic algorithm for MDR-TB patients in Bhutan

for pulmonary and EP-TB were also searched for records of MDR-TB for the previous 5 years (2015–19). Only cases which had confirmed MDR-TB results either by LPA or liquid DST were selected. Basic demographic details, including name, age and unique identification number, were cross-checked to avoid duplication.

Data were collected for epidemiological variables such as age, gender, location (district), infection site and treatment history. Frequencies and percentages were calculated for the epidemiological variables. Laboratory variables included detection of mutation in rifampicin and isoniazid. Results for rifampicin and isoniazid were compared between molecular and liquid DST reports to check for percentage agreement. The band pattern for rifampicin and isoniazid were analyzed to check for variations in mutation bands. Statistical analysis was done using RStudio (version 1.2.5033).

Ethics approval

The study has been approved by the Research Ethics Board of Health (REBH) under the Ministry of Health in Bhutan (Ref. No. REBH/Approval/2021/081).

RESULT

Sociodemographic characteristics

A total of 304 patients were diagnosed with MDR-TB between 2015 and 2019 at NTRL. There were 273 (89.80%) pulmonary and 31 (10.20%) EP samples. EP samples received included samples collected using fine-needle aspiration cytology technique (n=8), pleural fluid (n=6), pus (n=10), tissues (n=4), cerebrospinal fluid (n=1) and

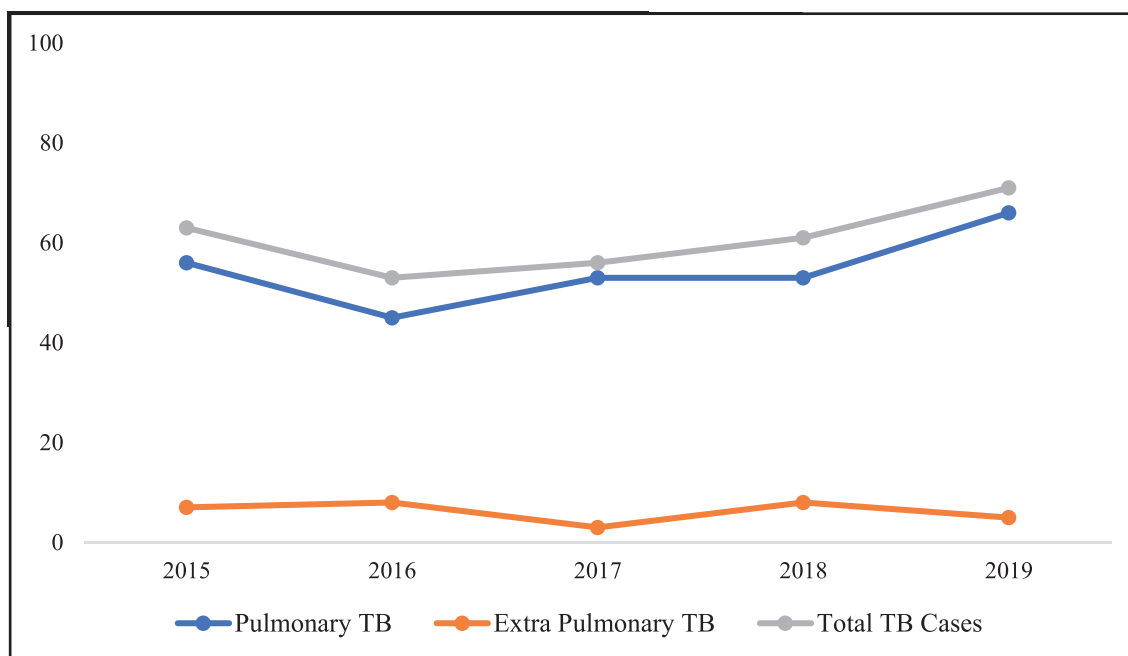


Figure 2. Number of MDR-TB cases detected among pulmonary (PTB) and extra-pulmonary (EP TB) samples from 2015-2019

Table 1
Demographic characteristics of MDR-TB patients from 2015-2019

Variables	Number	Percentage	
Age (Years)	<16	19	6.25
	16-25	140	46.05
	26-35	75	24.67
	36-45	28	9.21
	46-55	20	6.58
	56-65	14	4.61
	>65 years	8	2.63
Gender	Female	180	59.21
	Male	124	40.79
Site	Pulmonary	273	89.8
	Extra Pulmonary	31	10.2
Type	New	259	85.2
	Previously treated	24	7.89
	Unknown	21	6.91

ascitic fluid (n=2). The average age of patients was 29.72 years (SD 13.83) and ranged from 28 days to 90 years. Among the total cases, 259 (85.20%) were new cases, 24 (7.89%) had a previous history of treatment, and 21 (6.91%) had unknown treatment history. The sociodemographic characteristics are outlined in Table 1.

The highest number of MDR-TB cases detected was in 2019 (n=71; 23.36% of total samples), and the lowest was in 2016 (n=53; 17.43%). The distribution of MDR-TB cases in pulmonary and EP-TB patients from 2015 to 2019 is shown in Figure 2. Of the total samples received, 113 (37.17%) were from Jigme Dorji Wangchuck National Referral Hospital in Thimphu. The distribution of samples according to shipment location is shown in Figure 3.

Laboratory characteristics

LPA and liquid DST

MDR-TB samples were diagnosed using LPA or liquid DST, or both. Of the total 304 cases, 205 (67.43 %) patients were diagnosed as MDR-TB by liquid DST and 279 (91.78 %) using LPA. Of the 192 samples with test results for both LPA and liquid DST, 180 (93.30%) were MDR-TB

positive with both tests, while 4 were by LPA only and 8 were by liquid DST only. The percentage agreement for MDR-TB diagnosis between the two methods was 93.75%. Details of this comparison are shown in Table 2.

Mutation patterns

The mutation patterns were compared among new MDR-TB cases, previously treated cases and those with unknown treatment history. The most common band patterns in all case types and across years were missing Wild Type 8 (WT8) gene and presence of Mutation 3 (MUT 3) for *rhoB* gene, and missing wild type (WT) and presence of MUT1 for *KatG* gene. Resistance to *inhA* gene was seen in 5 new cases, 1 previously treated case and 1 unknown history case. The different pattern combinations according to treatment history are shown in Table 3. The band pattern did not show many variations across years of diagnosis. Table 4 shows the variation in the mutation band from 2015 to 2016.

DISCUSSION

The average age of MDR-TB patients in Bhutan in 2015–19 was 29 years (range: 28 days to 90 years) which is slightly lower than that found in India (32.15 ± 13.19) (Venkatesh et al., 2018). A study in Taiwan in 2007–14 found an average age of 53.4 ± 18 years, much higher than for Bhutanese MDR-TB patients (Chuang et al., 2016). The difference between the male and female population for MDR-TB varies across countries. For example, in India, the majority (2/3) of MDR-TB cases were found among men, whereas in Ukraine, women had 2.5 times greater odds of MDR-TB than men (Venkatesh et al., 2018; Pavlenko et al., 2018). In Bhutan, the ratio between men to women was 1.4:1. Further studies are required to understand the underlying factors influencing the age and gender distribution of MDR-TB in Bhutan. For a small landlocked nation of approximately 700,000 people, the impact of MDR-TB, including hospitalization for up to 6 months, is a concern.

Of total MDR-TB detected, 89.80% was from pulmonary samples. The EP samples were received only from Thimphu district since timely shipment of samples from other districts is a challenge (Adhikari et al., 2013). Although the National TB program in Bhutan reports a high percentage of EP-TB in the population, there is likely a major gap in laboratory diagnosis (Jamtsho et al., 2013). There is an urgent need to

Table 2
Comparison of Line Probe Assay test results with liquid drug sensitivity test (DST) (n=192)

		Liquid DST			
		MDR-TB	Isoniazid Mono Resistant	Rifampicin Mono Resistant	Sensitive to Rifampicin & Isoniazid
Line Probe Assay	MDR-TB	180(93.75)	1(0.52)	0	3(1.56)
	Isoniazid Mono Resistant	3(1.56)	0	0	0
	Rifampicin Mono Resistant	2(1.04)	0	0	0
	Sensitive	3(1.56)	0	0	0

Table 3
Frequency of drug resistance pattern according to treatment history for MDR-TB

Band	Gene region or Mutation	New Cases (235)	Previously treated (24)	Unknown (20)	Frequency (Total) n=279
rpoB					
WT1	506-509	0	0	0	0
WT2	510-513	0	0	0	0
WT3	513-517	1(0.43)	1(4.17)	1(5.00)	3(1.08)
WT4	516-519	2(0.85)	0	1(5.00)	3(1.08)
WT5	518-522	1(0.43)	0	0	1(0.36)
WT6	521-525	1(0.43)	0	1(5.00)	2(0.72)
WT7	526-529	9(3.83)	2(8.33)	0	11(3.94)
WT8	530-533	223(94.89)	20(83.33)	20(100)	263(94.27)
MUT 1	D516V	0	0	1(5.00)	1(0.36)
MUT 2	H526Y	4(1.70)	1(4.17)	0	5(1.79)
MUT 2B	H526D	6(2.55)	0	0	6(2.15)
MUT 3	S531L	223(94.89)	22(91.67)	19(95.00)	264(94.62)
katG					
WT	315	229(97.45)	22(91.67)	20(100)	271(97.13)
MUT 1	S315T1	230(97.87)	21(87.50)	20(100)	271(97.13)
MUT 2	S315T2	0	0	0	0
inhA					
WT1	-15/-16	5(2.13)	1(4.17)	1(5.00)	7(2.51)
WT2	-8	3(1.28)	1(4.17)	1(5.00)	5(1.79)
MUT 1	C15T	5(2.13)	1(4.17)	0	6(2.15)
MUT 2	A16G	0	0	0	0
MUT 3A	T8C	0	1(4.17)	0	1(0.36)
MUT 3B	T8A	0	0	0	0

Table 4
Frequency of drug resistance pattern from 2015-2019 in Pulmonary MDR-TB

Band	Gene region or Mutation	2015(52)	2016(50)	2017(56)	2018(53)	2019(68)	Total(279)
rpoB							
WT1	506-509	0	0	0	0	0	0
WT2	510-513	0	0	0	0	0	0
WT3	513-517	0	2(4.00)	0	0	1(1.47)	3(1.08)
WT4	516-519	1(1.92)	1(2.00)	0	0	1(1.47)	3(1.08)
WT5	518-522	1(1.92)	0	0	0	0	1(0.36)
WT6	521-525	0	1(2.00)	0	1(1.89)	0	2(0.72)
WT7	526-529	4(7.69)	3(6.00)	0	2(3.77)	2(2.94)	11(3.94)
WT8	530-533	46(88.46)	46(92.00)	55(98.21)	51(96.23)	65(95.59)	263(94.27)
MUT 1	D516V	0	1(2.00)	0	0	0	1(0.36)
MUT 2A	H526Y	3(5.77)	2(4.00)	0	0	0	5(1.79)
MUT 2B	H526D	2(3.85)	2(4.00)	0	0	2(2.94)	6(2.15)
MUT 3	S531L	46(88.46)	44(88.00)	56(100.00)	53(100.00)	65(95.59)	264(94.62)
katG							
WT	315	49(94.23)	48(96.00)	55(98.21)	52(98.11)	67(98.53)	271(97.13)
MUT 1	S315T1	49(94.23)	48(96.00)	56(100.00)	52(98.11)	66(97.06)	271(97.13)
MUT 2	S315T2	0	0	0	0	0	0
inhA							
WT1	-15/-16	2(3.85)	3(6.00)	0	1(1.89)	1(1.47)	7(2.51)
WT2	-8	0	3(6.00)	0	1(1.89)	1(1.47)	5(1.79)
MUT 1	C15T	2(3.85)	2(4.00)	0	1(1.89)	1(1.47)	6(2.15)
MUT 2	A16G	0	0	0	0	0	0
MUT 3A	T8C	0	0	0	0	1(1.47)	1(0.36)
MUT 3B	T8A	0	0	0	0	0	0

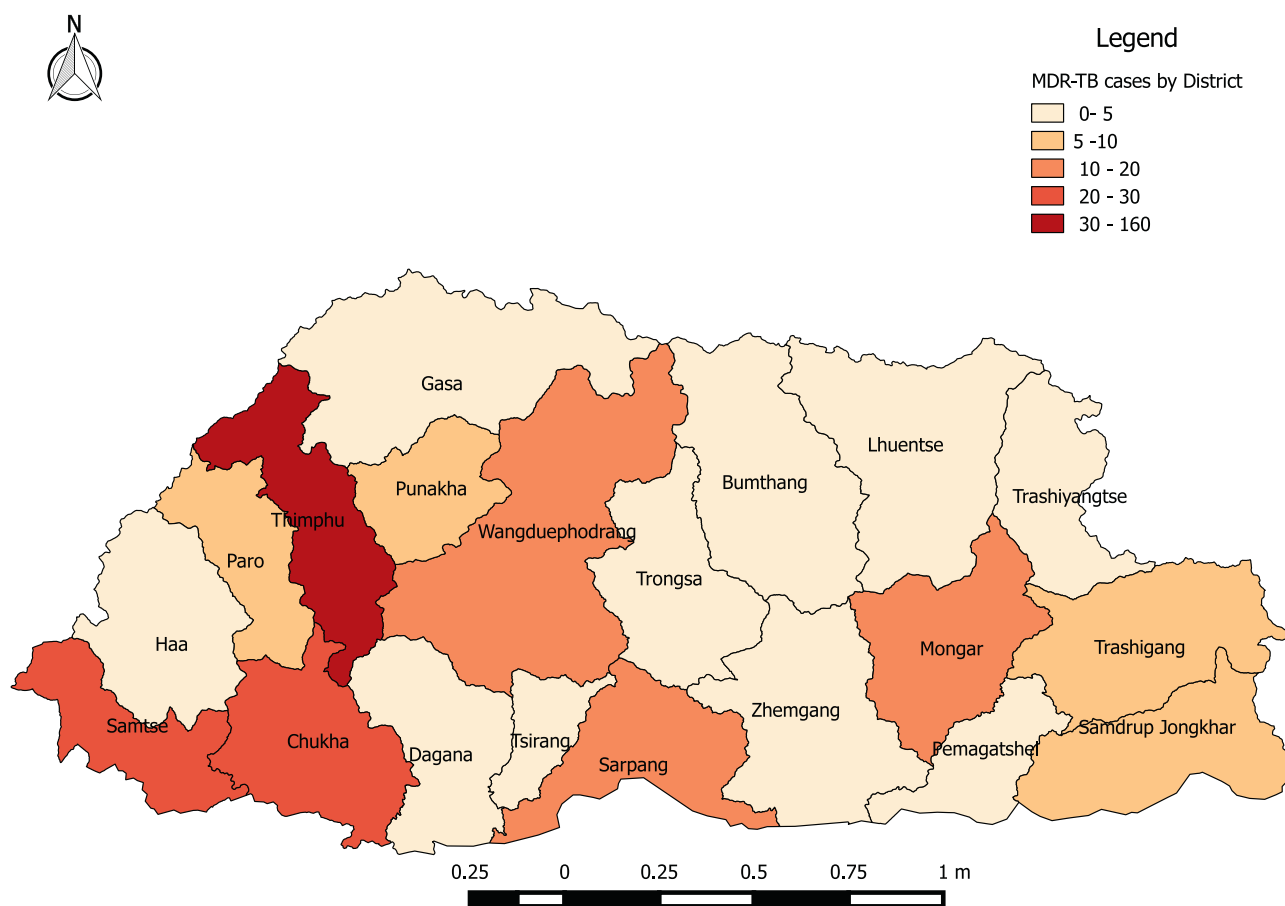


Figure 3. Distribution of MDR-TB cases in Bhutan by districts

advocate for lab diagnosis at regional and district hospitals to better estimate the EP-TB burden in the country. In resource-poor counties, almost 71% of EP-TB detected is in smear-negative samples from fluorescent microscopy (Metaferia et al., 2018). Moreover, a growing body of GeneXpert implementation literature suggests that MTB/IF provides rapid diagnosis in 50%–80% of EP-TB cases (Lawn and Zumla, 2012). Therefore, using GeneXpert assays at peripheral hospitals as a diagnostic tool for EP-TB would enhance case finding.

We found that 259 (85.20%) samples were new (primary) MDR-TB cases. Although the risk of MDR-TB is higher among retreatment cases, it is concerning to see that we have such large numbers of primary MDR-TB. Studies on the molecular epidemiology of cases are needed to establish the causes of such high prevalence (Chisompola et al., 2020). There are also very few studies on the risk factors for acquired and transmitted drug resistance. In Lima, Peru, a study found that patients with higher socioeconomic status had a 3-fold increased risk for transmitted resistance than those with lower status (Odone et al., 2016).

Several MDR-TB cases were from the national referral hospital (Thimphu), which could be due to the high mobility of the Bhutanese population and the better health care facilities available at the national referral hospital. The issue of MDR-TB patient overload in some districts compared with others could be reduced by additional diagnostic and treatment facilities at peripheral hospitals. From 2017 to 2019, there has been a steady recorded increase in MDR-TB which may be due to the introduction of GeneXpert assay in district hospitals in 2016. MDR-TB and XDR-TB have also risen worldwide in the same period (World Health Organisation, 2019). However, according to Bhutan's National TB guidelines, MDR-TB screening is still mainly for smear-positive TB cases, so the cases counted may be lower than the actual number present in the community as smear-negative. Screening all contacts of MDR-TB cases

using GeneXpert would lead to enhanced case finding (Creswell et al., 2014).

The overall agreement between LPA and liquid DST was high at 93.75% (n=192). However, for a small Bhutanese population, missing even a single case of MDR-TB by relying only on genotypic or phenotypic diagnosis could incur a considerable clinical and public health cost. In total, 4 samples tested as MDR-TB by LPA were missed by the DST liquid test and 8 by LPA, which may be due to different phenotypic and genotypic characteristics. Also, since LPA targets only *rpoB*, *katG* and *inhA* gene, a variation in detection might have occurred. Due to a lack of data for GeneXpert reports, a comparison between GeneXpert, LPA and liquid DST could not be carried out in this study.

The common mutation pattern did not differ between primary and acquired MDR-TB. More than 95% of rifampicin resistance is attributed to mutation in the *rpoB* gene occurring within an 81-base pair core region (codon 507 to 533). Between 31% to 97% of isoniazid resistance has been attributed to *katG* mutations (at codon 315) (Kalokhe et al., 2013). Similarly, mutation in *rpoB* gene *katG* gene were the most common mutations in the current study samples. Similar findings were previously reported from Bhutan in suspected TB samples processed at NTRL from 2014 to 2016 (Pelden et al., 2021). Whole-genome sequencing and a complete epidemiological picture would be required to understand these variations. For isoniazid, *KatG* mutation is reported as high-level resistance compared with *inhA* (Soolingen et al., 2000); we saw less *inhA* resistance in previously treated MDR-TB cases, which indicates a poorer prognosis in those patients. The band patterns from 2015 to 2019 did not show many variations, which could indicate ongoing transmission of the same strains (Ektefaie et al., 2021). Further investigation using contact tracing and the results from whole-genome sequencing will be helpful to estimate and monitor the transmission of MDR-TB in Bhutan.

The spread of MDR-TB could be due to ongoing transmission, and not only to the development of drug resistance over time; however, this hypothesis needs support from genome sequence analysis. Improved contact tracing methods need to be put in place, and due to the mobility of the Bhutanese population, contact and collaboration are needed for case follow up.

Strengths and limitations

The history of new and previously treated cases requires further verification as many samples are sent under an unknown category. In addition, variables such as occupation, comorbidities and socioeconomic status had several missing data and hence could not be analyzed. Interpretation of data for the mutation pattern was limited due to genome sequence data not being available. However, this study included samples from a nationwide survey, and therefore we were able to get representative data for the Bhutanese population despite the limitations. In addition, the samples analyzed were tested only at the NTRL, the country's only laboratory with phenotypic DST facilities and LPA, eliminating potential errors from variations in test conditions or staff.

CONCLUSION

The high number of MDR-TB cases among new patients and little variation in the resistance band pattern over 5 years requires further investigation. Collaboration between NTRL, the National TB Control Program and various regional and district hospitals needs to be strengthened, including in-depth discussion on technical and diagnostic aspects to be updated. Whole-genome sequence and analytical studies need to be carried out to investigate sources for MDR-TB cases, especially in new and EP samples. Active screening of MDR-TB patients for second-line drug sensitivity screening requires monitoring and increased vigilance. The diagnostic protocol needs frequent evaluation to address the growing burden of MDR-TB cases so that enhanced case detection is maintained in the community. In addition, the economic and social impact of high MDR-TB cases among the economically productive age group needs to be studied.

Conflict of Interest

No conflict of interest to declare.

Contributions

Lila Adhikari: Study concept, manuscript writing, data analysis and interpretation

Sonam Wangchuk: Critical revision of manuscript

Pavitra Bhujel, Pema Lhaden, Sherab Zangmo, Ugyen Dorji and Karchung Tshering: Assistance in data collection, analysis and manuscript editing

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