Article title:

A prospective Drug Resistance surveillance to determine prevalence and burden Multi-Drug resistance among smear positive cases in Bhutan

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Introduction

As per the Global TB Report 2013, 8.6 million new TB cases were detected in 2012 and 1.3 million TB deaths (around 1.0 million among HIV-negative people and 0.3 million HIV-associated TB deaths). Most of these TB cases and deaths occur among men but the burden of disease among women is also high. The South East Asia Region (SEAR) WHO region accounts for 38% of morbidity and 39% mortality of the Global burden of Tuberculosis, with an estimated 4.5 million prevalence and 3.4 million incidence and 440000 deaths in 2013. Five among 11 member countries in the SEARO region are among the 22 high TB burden countries in the world with India alone accounting for 23% of the world's incidence and 21% of deaths for TB. Among all new TB cases detected in 2013 in the region, most cases occurred among young adults; age group 25-34 years and males are most affected compare to female with a male to female ratio of 1:5. (4)

In Bhutan, estimated TB prevalence and incidence rate of all forms of TB were 196 per 100,000 and 169 per 100000 population respectively in 2013. The notification rate of all forms of TB (new cases and relapses) and new bacteriologically confirmed cases were 143 per 100,000 and 56 per 100,000 population respectively. Treatment success for the cohort 2013 is 88% for new cases and 90% for all from of TB. The overall treatment success rate is maintained consistently above 90% since 2007.

The first TB drug resistance (DR) survey was conducted in 2010-2011 and since then TB drug resistance was conducted as surveillance because sample size require for survey as well as annual smear positive case reported amount to same number of smear positive cases require for representative of sample size for determining the drug resistance burden in the country. However, due to technical as well as logistic problem encountered in the past few previous years, lot of samples did not yield culture growth and complete drug susceptibility testing. As a result, samples with complete DST could not meet the minimum sample target to consider for analysis and interpretation. The objectives of the surveillance is to determine drug resistance and multi-drug resistance tuberculosis

(MDR-TB) prevalence and MDR-TB burden among new and previously treated smear positive cases reported in 2014.

Methods

Sampling strategy: We used 100% sampling method as per the WHO guideline for drug resistance surveillance because of less number of TB cases in the country. The surveillance was conducted from January to December 2014 in all hospitals and BHU-1 (total 35).

Case Enrolment– All sputum smear positive cases confirmed by smear microscopy at hospital and BHU-1 were enrolled for surveillance. Case investigation form was used to collect demographic and case history information to classify new smear positive or previously treated smear positive case to determine drug resistance and MDR-TB cases among new and previously treated cases. WHO standard definition was used to categories the case as follow.

Smear positive: Patients with at least two sputum smears positive for AFB under direct microscopy who has no history of past tuberculosis infection, OR, one sputum specimens positive for AFB and radiographic abnormalities consistent with PTB.

New smear cases: the presence of resistant strains of *M. tuberculosis* in a patient who, in response to direct questioning denies having had prior anti-TB treatment (for more than 1 month), OR, has no evidence of such history from adequate documentation if available with patients or hospitals.

Previously treated cases: the presence of resistant strains of *M. tuberculosis* in a patient who, in response to direct questioning admits having been treated for tuberculosis for 1 month or more OR, has evidence of such history from adequate documentation if available with patients or hospitals.

Laboratory Methods

Sputum collection and transportation: Two sputum samples (an early morning and spot samples) were collected in wide mouth container (Uricol 50 ml container) from each smear positive patient. To minimize chances of contamination, collection of independent samples were encouraged for surveillance in addition of routine samples collected for diagnostic purpose. Samples were refrigerated at 4^0C and transported to National Tuberculosis Reference Laboratory (NTRL) of the Public Health Laboratory (PHL) in the triple packaging container as per the international shipment standard. Sputum samples from each patient were accompanied by a patient information and sample shipment form. Lab technicians from hospitals were trained on sample collection, storage, packaging and transportation.

Sputum culture and identification: Samples were decontaminated using 1.5% NaOH NALC method and inoculated on two slopes of egg based Lowenstein-Jensen (L-J) and in the Mycobacterium Growth Indicator tubes liquid media as per the SOP's. The solid cultures were then incubated at a temperature of 37ºC and monitored weekly for growth up to 8 weeks and liquid culture in the MGIT 960 machine for six weeks.

A culture was reported as negative if no growth was observed after eight weeks for solid culture and six weeks for liquid culture. For the positive cultures; identification of Mycobacterium tuberculosis (MTB) was done based on phenotypic appearance of colonies on the solid media and by rapid identification test which detects MPT64 (a mycobacterial protein that is secreted by MTB cells during culture) for both solid and liquid positive culture.

Drug Susceptibility Testing (DST):

Only one MTB confirmed isolate for solid culture and MTB positive liquid culture for each patient was tested for primary resistance (rifampicin, isoniazid, ethambutol and streptomycin) using three different methods: LJ proportional method, MGIT 960 Liquid drug sensitivity testing method and line probe assay as per the SOP's.

Quality Control:

Solid culture and DST-Drug and drug free media were prepared based on requirement twice a month. Every new batch of LJ media prepared were tested for contamination and susceptibility with the standard H_{37}Rv strain. Similarly control strain; *M. tuberculosis* SM & RFP resistance, *M. tuberculosis* INH & EBM resistance were used to check quality of every new batch of drug LJ media prepared for DST. In case of any discrepancies results with control strains on drug free media; the whole batch of DST samples were considered as invalid and test was repeated. The DR and MDR isolates were also sent to the supra-national TB reference laboratory (STRL) in Bangkok-Thailand for reconfirmation.

MGIT 960 Liquid culture and Drug Sensitivity testing - The new lot of MGIT medium and enrichment was tested for quality control using H37RV and M. fortuitum strains and DSTusingH37RV, RF and SM resistant strain, INH and EMB resistant strain. Each set of DST hadH37RV strain along with the test samples as a control strain.

Line Probe Assay (LPA) - To validate the correct performance of the test and the proper functioning of kit constituents, each strip includes 5 control zones:a Conjugate Control zone [CC] to check the bindingof the conjugate on the strip and a correct chromogenic reaction.An Amplification Control [AC] to check for a successful amplification reaction. Three Locus Control zone (*rpoB, katG* and *inhA*) checking the optimal sensitivity of the reaction for each of the tested gene loci. Line probe assay had a negative strip run for every 11 samples hybridized after amplification of the bacterial DNA.

Data collection, Management and Analysis

A standard clinical form was used to obtain data on patients' demographic characteristics and history of TB treatment through a structured interview. The information was conducted by TB Unit In-charges of respective hospitals who were trained on collection of information as per the prescribed clinical form. Each smear positive case was assigned an identification number (site identification and case number) as recorded in the TB treatment registered maintained in every hospital. WHO software programme (4^{th} version) for Surveillance of Drug Resistance in Tuberculosis (SDRTB) was used for data compilation and management. Data analysis was done using Epi Info version 7.2 software that is freely available from CDC.

We have used total TB cases notified to NTCP in 2014 as denominator for estimation of MDR-TB burden. A total TB cases reported - 1082; all form of new TB cases – 1011 and previously treated cases - 71 respectively.

Results

Characteristics of patients: During 2014 DR surveillance from January – December 2014, a total of 535 patients enrolled from 33 hospitals and BHU-1 (**Figure 1**). From a total enrolled, 432 patients were new smear positive cases, 57 previously treated smear positive cases and 46 unknown smear positive cases (no adequate information to classify them). Of total 57 previously treated cases, 26.3% were treatment failure and 73.68% were relapsed cases respectively.

The median age of all TB cases is 26 years (interquartile [IQ] range21 - 40 years) **Figure 2** and 46.54% (n=249; 95% CI; 42.2-50.87) were female and 53.46% (n=286; 95% CI; 49.1-57.7) male respectively (**Figure 3**).

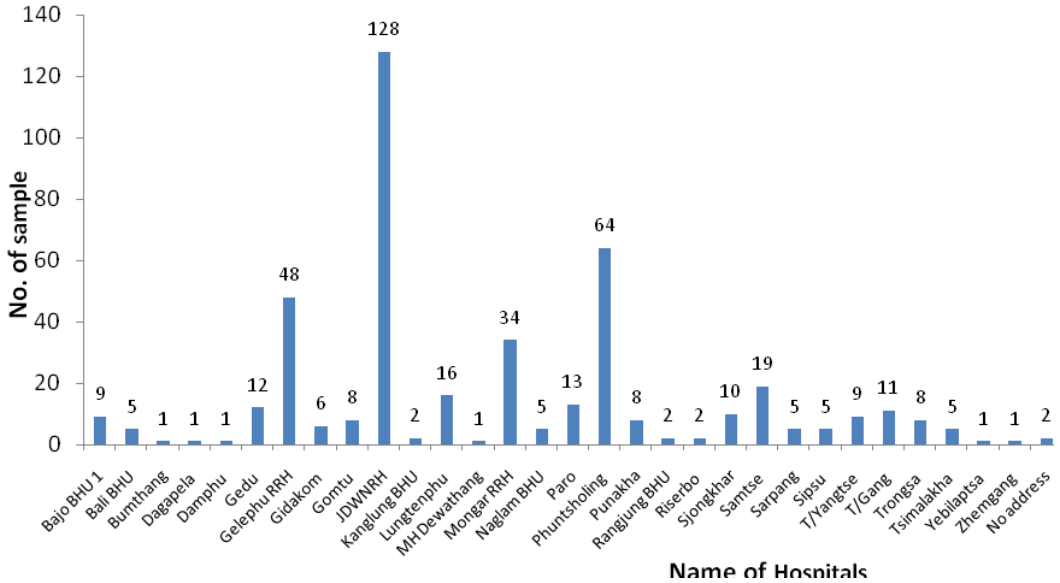


Figure 1: Number of smear positive samples received from hospitals and BHU-1

Culture and DST results: Of 535 enrolled patients, 86.25% (n=461; 95% CI;82.9 – 89) yielded growth, 13.08% (n=70; 95% CI; 10.8 – 16.8) yielded no growth and 0.19%(n=1; 95% CI; 0.01 –1.2) contaminated (**Table 1**). From a total 461culture growth samples, 96.09% (n= 443) completed DS; that comprises new cases - 82.62% (n=366), previously treated cases - 9.71% (n=43 and unknown cases - 7.67% (n=34). However, 4.5% (n=21) could not processed for DST because of inadequate colonies (scanty growth).

Number of samples processed for DST by three different methods available at NTRL are 281 samples (63.43%) by Line Probe Assay, 146 samples (32.96%) by Liquid culture, and 16 samples (n=3.7%) by

solid culture. In this surveillance, DST for streptomycin and ethambutol is not conducted as most DST was performed by LPA.

Table 1: Summary of culture results

Categories	Number of sample received (%) (n=535)	Culture results (%)			DST (%)	
		Growth	No Growth	Contamination	Completed	Not done
New Smear positive/negative	432(80.74%)	384(89.89%)	45(10.42%)	0	366(95.56)	17(4%)
Previously treated	57 (10.65%)	44(77.19%)	12(21.05%)	1(1.75%)	43(97.72%)	1(2%)
Unknown	46 (8.59%)	33(71.74%)	13(28.26%)	0	34	
	Overall Rate	461(86.17%)	70(13.08%)	1(0.19%)	443(96.41%)	

Drug resistance prevalence:

Total 366 new cases completed DST from 432 new cases enrolled for the surveillance. The prevalence of drug resistance to any of the primary drugs was 25% (n = 95; 95% CI;21.6-30.82). Any resistance to rifampicin was 10.11% (n=37; 95% CI;7.3-13.7) and isoniazid 21.58% (n=79; 95% CI;17.5-26.2).

Among 57 previously treated enrolled cases, 43 cases completed DST. About 55% (n=24, 95% CI; 39.8-70.92) showed resistance to at least one primary drug. Any resistance to rifampicin was 44.19% (n=19, 95% CI;29.08-60.12) and isoniazid 48.84% (n=21, 95% CI;33.31-64.54).

Among unknown cases (cases having incomplete information to classify), resistance to rifampicin was 26.47% (n=9; 95% CI; 12.88-44.36) and isoniazid 29.4% (n=10; 95% CI; 15.10-47.48).

The overall prevalence of any resistance among new, previously treated and unknown cases was 25.51% (n =113; 95%; CI, 21.57–29.80) **Table 2**.

Table 2: Anti-Tuberculosis drug resistance pattern among categories (new cases, previously treated cases and unknown cases)

Pattern of Resistance	New cases		Previously Treated		Unknown		All cases	
	Number (%)	95% CI	Number (%)	95% CI	Number (%)	95% CI	Number (%)	95% CI
Total patients	N=366		N=43		N=34		N=443	
Susceptible to all	287(78.42)	73.77-82.45	19(44.19)	(29.08-60.12)	24(70.59)	(52.5-84.90)	330(74.49)	(70.12-78.43)
Any Resistance	79(21.58)	17.55-26.23	24(55.81)	39.88-70.92	10(29.41)	15.10-47.48	113(25.51)	21.57-29.88
Any Resistance to:								
RMP	37(10.11)	7.31-13.78	19(44.19)	29.08-60.12	9(26.47)	12.88-44.36	65(14.67)	11.58-18.39
INH	79(21)	17.55-26.23	21(48.84)	33.31-64.54	10(29.41)	15.10-47.48	110(24.83)	20.93-29.18
INH+RMP Resistant (MDR)	37(**10.11**)	7.31-13.78	16(**37.21**)	22.98-53.27	9(**26.47**)	12.88-44.36	62(**14.00**)	10.97-17.66
Mono Resistance to:								
RMP	0	0	3(6.98)	1.46-19.06	0	0	3(0.68)	0.18-2.14
INH	42(11.48)	8.48-15.30	5(11.63)	3.89-25.08	1(2.94)	0.07-15.33	48(10.84)	8.17-14.20

Figure 2: Non-multi-drug resistance cases by age group among categories (new cases, previously treated cases and unknown cases).

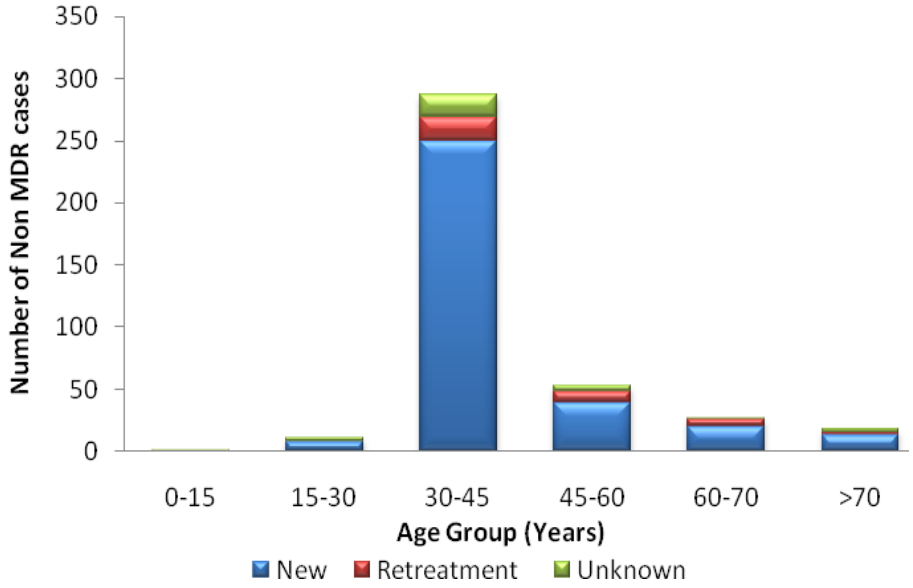
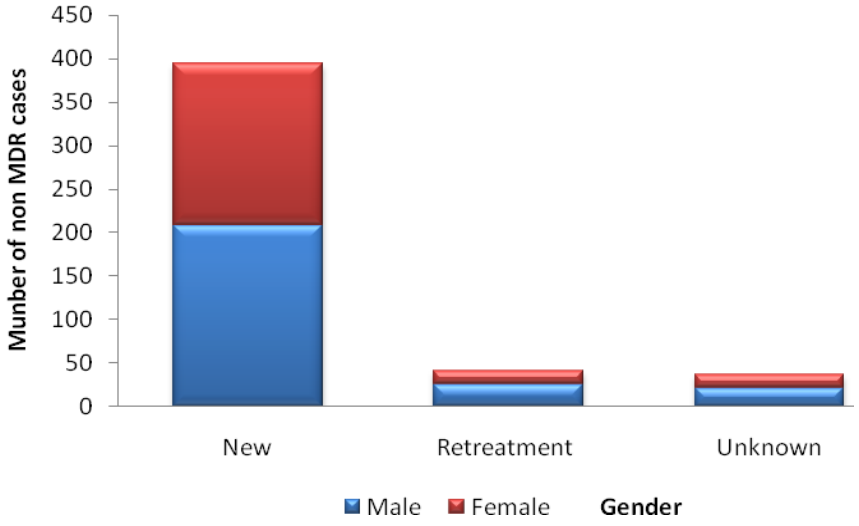


Figure 3: Non-multi-drug resistance cases by gender (new cases, previously treated cases and unknown cases).



Multi-Drug resistance prevalence and burden

The overall prevalence of MDR-TB among new, previously treated and unknown cases were 13.99% (n= 62; 95% CI 10.97-17.66). MDR-TB among new cases is 10% (n=37; CI;7.31-13.78), previously treated cases 37.21% (n=16, 95% CI; 22.98.4-53.27) and unknown cases 26.47% (n=9; 95% CI; 12.88-44.36) respectively.

The median age among the MDR-TB was 27 years (interquartile [IQ] range21-38 years) Figure 4. The gender ratio was similar to general TB cases (**Figure 5**).

By occupation, most affected was house-wife (27.7%), students (25.9%) and farmers (18.5%) respectively (**Figure 6**).

Based on MDR-TB prevalence of 2010-2011, the estimated number of MDR-TB among new case smear positive is 50 and previously treated 25 respectively. From current surveillance MDR-TB prevalence finding, the estimated number of MDR-TB among new smear positive is 101 and previously treated 27 respectively (**Table 3**). The estimated number of MDR-TB among new cases compared to 2010-2011 DR survey has been increase by 2-fold whereas estimated of MDR-TB among previously treated remain same.

Figure 4: Multi-drug resistance cases by age group among categories (new cases, previously treated cases and unknown cases).

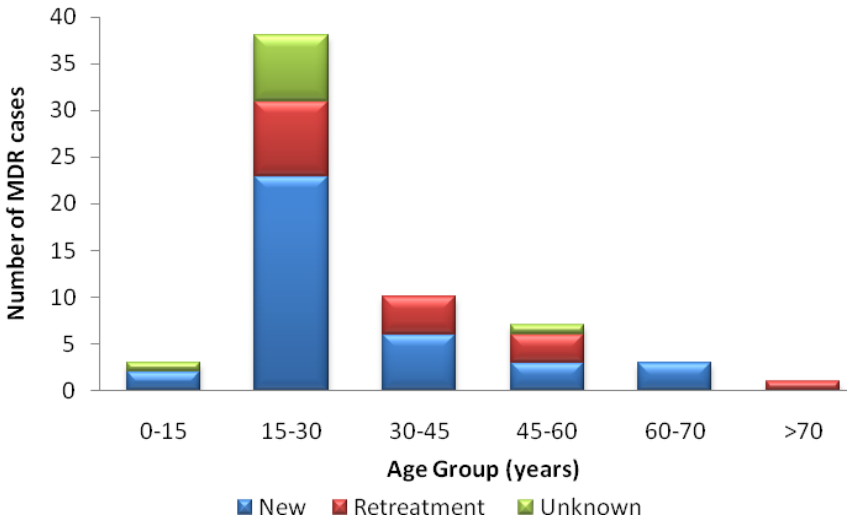


Figure 5: Multi-drug resistance cases by gender among categories (new cases, previously treated cases and unknown cases).

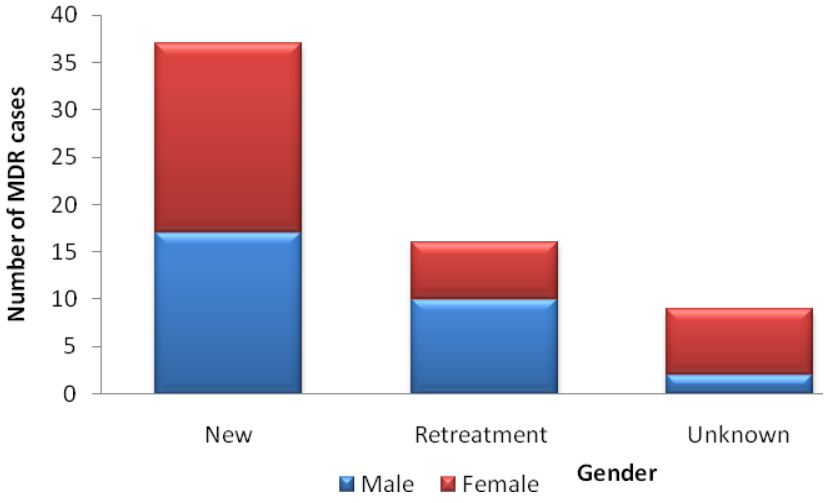


Figure 6: Multi-drug resistance cases by occupation

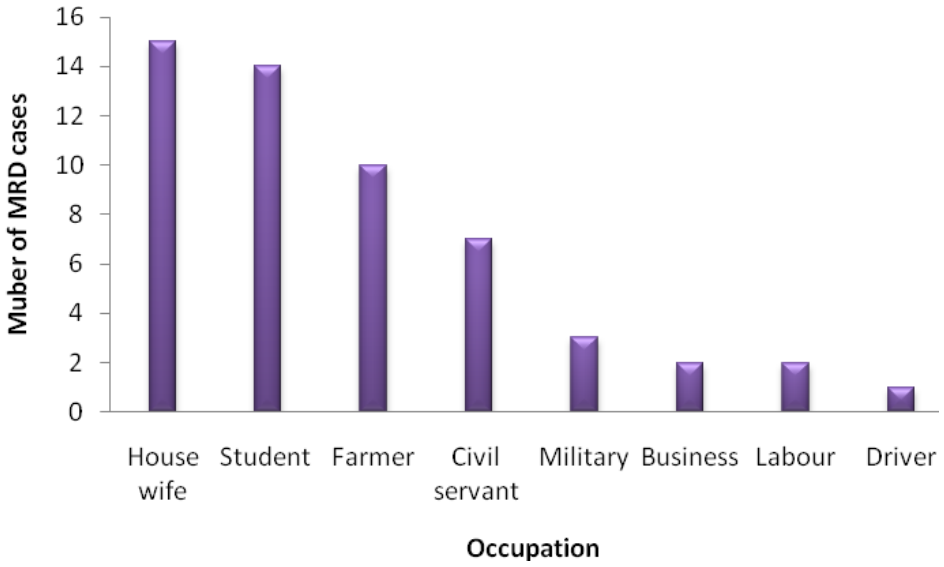


Table 3: MDR-TB burden estimation among new and previously treated cases

MDR-TB	Prevalence as per 2010-2011 DR survey	Estimated MDR-TB burden based on 2014 TB cases	Prevalence as per 2014 DR surveillance	Estimated MDR-TB burden based on 2014 TB cases
New case	5%	50 cases	10.1%	101 cases
Previously treated cases	35%	25 cases	37.2%	26 cases
Total		**75 cases**		**127 cases**

Discussion

This is the second national anti-TB drug resistance surveillance conducted in Bhutan by National Tuberculosis Reference Laboratory under the Public Health Laboratory. The first national anti-TB drug resistance was conducted as survey in 2010-2011. In this DR surveillance, we observed the overall culture growth rate 86.25% which is 8% improved from 78.2% during previous survey. This improvement in bacilli recovery by culture is attributed to the introduction of liquid culture using MGIT 960. The liquid culture has higher sensitivity than solid culture (7) and acceptable contamination rate in liquid culture is usually higher (15-20%) compared to solid culture (5-10%). In this surveillance, we observed low contamination rate and that is because contaminated samples were repeatedly re-cultured after confirming the samples using concentrated AFB microscopy by Zheil Nelson method. The

low culture growth and high contamination rate is usually associated with delay in sample shipment in our context. The current shipment mechanism takes longer time to reach NTRL (average 7-10 days from hospital where PHL has courier service; contract agreement with Bhutan Post and 20-45 days where there is no courier service) and invariably impact bacilli recovery and contamination during the culture. The sample shipment remain as the main challenge for DR surveillance and it will continue to remain as challenge until there is a viable sample shipment solution.

The World Health Organization (WHO) estimates of the MDR-TB prevalence in Bhutan is 2.2% (0–5.6) among new TB cases and 15% (0–40) among previously treated cases respectively [1]. However, DR survey conducted in 2010-2011 observed MDR-TB prevalence 5% among new TB cases and 35% among retreatment cases, which is 2-fold higher than the estimate. The current DR surveillance also shows high MDR-TB prevalence among new cases 10.1% and previously treated cases 37.21% respectively. Compared to 2010-2011 MDR-TB baseline for new cases, MDR-TB prevalence from this surveillance shows increased by 2-fold which is 5-fold higher than WHO estimate. However, among previously treated cases, MDR-TB case has been increased by only 2% from 2010-2011 baseline but it is still 2-fold higher than WHO estimate. We also observed combined MDR-TB cases increased from 12% (2010-2011) to 14% (2014). The current surveillance and the survey conducted in 2010-2011 suggest that MDR-TB is the major public health concern in the country.

We also found the current MDR-TB prevalence is comparatively high both among new and previously treated cases compared to the MDR-TB prevalence reported in the SEARO region. The SEARO population-weighted mean of MDR-TB reported by six countries (few Indian states, Indonesia, Myanmar, Nepal, Sri Lanka and Thailand) is 2.8% (95% CLs, 1.9–3.6) among new cases, 18.8% (95% CLs, 13.3–24.3) among previously treated cases and 6.3% (95% CLs, 4.2–8.4) among combined cases [9]. The highest MDR-TB reported among the SEARO member states is 4.0% in Myanmar [11] however, 9.9% MDR-TB was also reported in North India [12]. The MDR-TB prevalence of Bhutan is also very high; both among new and previously treated cases compared to the global weighted mean of MDR-TB which is 2.9% (95% CLs, 2.2–3.6) among new cases, 15.3% (95% CLs, 9.6–21.0) among previously treated cases and 5.3% (95% CLs, 3.9–6.7) among all TB cases respectively.

The median age among the MDR-TB is 27 years (interquartile [IQ] range 21 -38 years) with 53.23% female and 46.77% of male. The MDR-TB cases age pattern is similar to global finding but we found 61.29% cases are among age group 15-30 years, which is comparatively younger age group than global finding. By occupation, house-wife, students and farmer in combined contribute around 72% suggesting these occupational groups are at risk for MDR-TB. While congregation could be the possible risk factor among students for acquiring MDR, we believe the life style could be the factor associated among house-wifes because all cases are young and urban dweller. The source for farmer could be possibly from students who acquired MDR-TB infection in the schools.

Based on prevalence of MDR-TB among new and previously treated cases of 2010-2011 DR survey and 2014 surveillance, and taking denominator of TB cases of 2014 cohort, we estimated overall MDR-TB burden 75 (new case – 50 and previously treated 25) and 127 (new case – 101 and previously treated 26) cases respectively. Based on classification used by WHO, Bhutan MDR-TB burden could be rated as moderate-high burden [9, 10]. Comparatively the MDR-TB has increased 2-fold over 3 years, which is quite alarming. If the same increasing trend continues, MDR-TB will be public health disaster. It is difficult to comprehend 2-folds increase of MDR-TB prevalence among new cases when compared to 2010-2011 baseline. However, we believe that the introduction of LPA would have contributed to increase in MDR-TB detection complemented by liquid culture and DST but more importantly we still believe that the increase of MDR-TB cases could have been attributed by is failure in strict implementation of DOTS and infection control in the country.

In this surveillance, we observed rifampicin resistance with other primary drugs 14.7% (44% among previously treated cases and 10% among new cases). While there is no rifampicin mono-resistance among new cases, we found 6.9% rifampicin mono-resistance among previously treated. Rifampicin mono-resistance for both new and previously treated case was not observed in previous survey. Similarly, we observed isoniazid resistance with other primary drugs 24.8% (48.8% among previously treated cases and 21% among new cases). Isoniazid mono-resistance among new cases is 11.6%, compared to 0.5%; which is increased of 10% from previous survey. Isoniazid mono-resistance observed among previously treated cases is 11.4% but isoniazid mono-resistance was not observed in previous survey. The development of rifampicin mono-resistance could have been attributed by introduction of 6-month TB regimen with 4 months of rifampicin for the continuation from 2010. This requires strictly monitoring of directly observed therapy and adherence and ensured treatment completion. If DOTS fails this could potential lead to increase in DR or MDR-TB in the country [14]. The increased mono-resistance and MDR cases observed in the country would have been attributed to stepwise selection of mutants due to drug resistance conferring genes [10]. It is generally accepted that the development of drug resistance (DR) and MDR-TB is caused by inadequate treatment, *i.e.* regimens with an inadequate number of drugs to which the bacilli are susceptible, an inadequate dose or dosing frequency, an inadequate quality of the drugs, or an inadequate adherence to the regimen. In most studies, the use of an inappropriate TB treatment regimen and the patient inadequate adherence to treatment is found as risk factors for acquiring MDR-TB. Therefore, appropriate treatment regime and full adherence to treatment is essential to ensure that the patient is cured (13). Unlike other countries we believe that the main risk factor for development of DR or MDR-TB in Bhutan is adherence/compliance to treatment because inadequate quality and quantity of drug are addressed by following the standard treatment regime as per the national guideline developed by NTCP and drugs are centrally procured and distributed to all health facilities. Directly Observed Therapy (DOT) was introduced in Bhutan in 1997 to improve adherence to treatment, however DOT implementation and

practice in the health facilities and among the TB patients was never assessed and monitored. In addition, risk factor study conducted among MDR-TB in 2011-2012 also found that 81% of MDR-TB patient who are interviewed for MDT-TB risk factor study did not practice DOT at home during the TB treatment [14]. The high rate of MDR-TB (14%) and resistance to any primary drugs (55.81 %) among previously treated patient further raise concerns about the quality of DOT and adherence to treatment.

Our main findings of this surveillance suggest ongoing transmission of drug resistant strains in the general population. This further implies weakness in infection control measures, which was also highlighted as one of the main risk factor among MDR-TB cases in Bhutan where 70% of MDR-TB cases never used face-mask to prevent transmission [15]. Therefore, priority should also be accorded to TB infection control training for health care workers in the TB diagnostic and treatment centres especially those hospitals, which offer comprehensive TB/HIV care including DR or MDR-TB patients.

Conclusion and Recommendations concern

The drug resistance surveillance of 2014 suggests that the MDR-TB is the public health concern and MDR-TB has increased 2-fold from 2010-2011 Drug Resistance survey. We therefore recommend strengthening and implementing appropriate interventions critical to control and reduce MDR-TB cases in the country.

1. NTCP to focus on improving the quality of directly observed therapy and develop interventions to support patient adherence in order to prevent development of acquired drug resistance.
2. NTCP to improve infection control in TB diagnostic and treatment centers. Efforts towards TB infection control including ensuring adequate ventilation for inpatient wards and outpatient waiting areas, provision of protective wear for patients and most importantly effective treatment among drug susceptible cases should be ensure to minimize emergence of new MDR-TB cases.
3. NTRL should strengthen the specimen referral system to improve quality of DR surveillance.
4. NTRL should improve turnaround time in detecting MDR-TB using appropriate technology available.
5. Conduct operational study to establish risk factors for development of MDT-TB and risk factor among occupation (house-wifes, students and farmer) MDR-TB cases.
6. Conduct studies to establish whether MDR-TB cases are due to reactivation of latent disease or transmission of new infections and whether there exists predominance of a particular MTB strain among drug resistant patients as described elsewhere [16].

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