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Comparative genomic analyses of multi-drug resistant *Mycobacterium tuberculosis* from Nepal and other geographical locations

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ABSTRACT

Nepal exhibits a tuberculosis (TB) incidence rate that is comparable to neighbouring high TB incidence countries. In addition, it records >500 cases of multi-drug resistant (MDR) TB each year. The objective of this study was to perform whole-genome bioinformatic analysis on MDR-TB isolates from Nepal ($n = 19$) to identify the specific mutations underlying their phenotypic resistance. In addition, we examined the dominant genotype among the Nepal MDR-TB isolates, the East-Asian Beijing sub-lineage, to determine its relatedness to a panel of 1274 genomes of international strains available from public databases. These analyses provided evidence that the XDR-TB isolates in our collection were not derived from importation of primary XDR-TB to Nepal but were more likely the result of acquisition of second-line drug resistance in Nepal. Resistance to fluoroquinolones was detected among a high proportion of the Nepal isolates. This has implications for the management of TB, including appropriate antimicrobial stewardship and susceptibility testing for fluoroquinolones and other second-line TB drugs, to minimise the development of XDR-TB among Nepal TB cases.

1. Introduction

In 2019 alone, it is estimated that there were 10 million new cases of tuberculosis (TB) worldwide including 465,000 incidences that were multi-drug resistant (MDR) or rifampicin resistant (RR) [1]. The majority of TB, approximately 97% of reported cases globally, occurs in low- to middle-income countries with only 2.9% and 2.6% of cases occurring in the World Health Organization (WHO) European and Americas Regions, respectively [2]. Nepal is a landlocked nation in South Asia whose nearest neighbours are designated by the World Health Organization as both high TB and high MDR-TB burden countries e.g. India, China, Pakistan, Bangladesh and Myanmar [3]. With a recorded population of 29 million in its 2020 census [4], Nepal experienced an estimated 69,000 cases of TB corresponding to an incidence rate per 100,000 of 238 which is comparable to the incidence rate reported for high TB burden countries such as India (193), Bangladesh (221), Pakistan (263) and Myanmar (322) (Table 1) [1].

TB is considered a major health problem by the Ministry of Health and Population in Nepal which has developed a National Strategy Plan for TB for 2016–2021 [5] with one of the key objectives being to reduce the incidence of TB in Nepal by 20% by 2021. Challenges to the plan include finding and treating an estimated 10,000 so-called "missing cases" of TB that are not registered and managed through the National Tuberculosis Control Program (NTP) [5]. Treatment of TB in Nepal is based on WHO recommended guidelines, but a significant challenge is the prevalence of drug resistance among TB in Nepal. 2.2% of new cases and 15% of previously-treated cases of TB in Nepal were MDR/RR in 2019 including 29 cases of laboratory-confirmed extensively-drug resistant tuberculosis (XDR-TB) [1]. Furthermore, the NTP Annual Report in 2015 highlighted that "one of the concerned aspects of drug-

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Table 1
TB cases in Nepal in comparison with its neighbouring countries.

Country	Population (million)	Total number of TB cases	TB incidence rate (per 100 000 population)	Number of MDR/RR-TB cases	Number of pre-XDR-TB or XDR-TB cases	Proportion of MDR that are pre-XDR or XDR
Nepal	29	69,000 (41,000-104,000)	238 (139-356)	593	84	14.2%
China	1,439	842,000 (717,000-978,000)	59 (50-68)	16,343	1,185	7.3%
India	1,380	2,590,000 (1,780,000-3,550,000)	193 (129-257)	49,679	8,982	18.1%
Bangladesh	165	360,000 (270,000-462,000)	221 (164-281)	1,113	67	6.0%
Pakistan	221	573,000 (409,000-764,000)	263 (185-346)	2,689	831	30.9%
Myanmar	54	167,000 (112,000-234,000)	322 (206-430)	2,368	178	7.5%
WHO SEA Region	2,021	4,270,000 (3,420,000-5,210,000)	211 (169-258)	64,970	9,672	14.9%
Global	7,768	9,870,000 (8,880,000-10,900,000)	127 (114-140)	157,842	25,630	16.2%

Fig. 1. Distribution of the Nepal *Mycobacterium tuberculosis* isolates ($n = 19$) collected in 2017. MDR-TB isolates were collected from different institutions across the Kathmandu District and Eastern regions in Nepal: Chandranigahapur, Rautahat; National Tuberculosis Control Center (NTCC), Bhaktapur; Nepal Anti Tuberculosis Association (NATA), Morang; Lalgadh, Dhanusha and National Medical College & Teaching Hospital (NMCTH), Birgunj.

resistance in Nepal is the high level of resistance to fluoroquinolones (26.4%), which leads to heavy burden of pre-XDR and XDR-TB among MDR-TB patients" [6]. It is not yet known whether this relates to the high mortality rates seen for MDR/RR-TB (15%) and pre-XDR-TB (20%) in Nepal [7].

In 2020, the South-East Asian region was responsible for the highest proportion of both MDR/RR-TB (41%, 64,970) and pre-XDR/XDR-TB (38%, 9672) cases globally [8]. Nepal contributed approximately 1% (593 cases) and 1% (84 cases) of the region's MDR/RR-TB and pre-XDR/XDR-TB cases, respectively [8]. Compared to its neighbouring countries, Nepal exhibited the third highest frequency of pre-XDR/XDR-TB among total MDR/RR-TB cases (14%) (Table 1) [8].

The purpose of this study was to determine the types of drug resistance mutations that are present in MDR-TB isolates from Nepal including mutations for resistance to second-line anti-TB drugs used for the treatment of MDR-TB. Furthermore, another objective of this work was to compare MDR-TB isolates collected from the Kathmandu District and Eastern regions in Nepal with a database of 1274 MDR-TB genomes from other jurisdictions to provide insights into the geographical origin of XDR-TB in Nepal. The implications of our findings for MDR-TB in Nepal are discussed.

2. Materials and methods

2.1. Study samples

The study encompasses 19 *M. tuberculosis* isolates that were collected from 19 separate TB-diagnosed patients between May 2017 and December 2017 in the Kathmandu central region and in the eastern regions of Nepal: Chandranigahapur, Rautahat ($n = 7$); National Tuberculosis Control Center (NTCC), Bhaktapur ($n = 5$); Nepal Anti Tuberculosis Association (NATA), Morang ($n = 5$); Lalgadh, Dhanusha ($n = 1$); and the National Medical College and Teaching Hospital (NMCTH), Birgunj ($n = 1$) (Fig. 1).

2.2. Collection of *M. tuberculosis* isolates

For the culturing of *M. tuberculosis*, clinical specimens were first decontaminated through treatment with a solution containing *N*-acetyl-L-cysteine and sodium hydroxide solution as previously described [9]. 200 μL of the concentrated sample was inoculated onto Löwenstein-Jensen (LJ) media (Becton Dickinson) which was then incubated at 37 °C with 5% CO_2 and monitored each week for growth. Phenotypic observation of colony morphology and pigmentation was performed before confirming the isolate as *M. tuberculosis* using the MPT 64 antigen test (SD Bioline). The patients, from whom MDR-TB *M. tuberculosis* was isolated in this study, were treated in accordance with the conventional treatment regimen in Nepal for MDR-TB (i.e. 8 months of combination treatment with kanamycin, cycloserine, ethoniamide, levofloxacin, pyrazinamide, followed by 12 months of combination treatment with cycloserine, ethionamide, levofloxacin, pyrazinamide) [7]. This chemotherapeutic regimen was in accordance with the previous treatment regimen for MDR-TB which was recommended by the World Health Organization [10]. The shorter treatment regimen (STR) for MDR-TB (4 to 6 months with kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, high dose isoniazid, followed by 5 months with moxifloxacin, clofazimine, pyrazinamide, ethambutol) [11], which is currently recommended by the WHO, was not selected as a treatment plan for patients from whom the MDR-TB isolates were examined in this study. For XDR-TB, the treatment regimen was 12 months with ethionamide, cycloserine, linezolid, bedaquiline (6 months), clofazimine, pyrazinamide, followed by 12 months with ethionamide, cycloserine, linezolid, clofazimine, pyrazinamide [7]. Ethics approval for this study was obtained from the Nepal Army Institute of Health Sciences Institutional Review Board (Reference #102).

2.3. Drug susceptibility testing

Confirmed isolates of *M. tuberculosis* were tested for susceptibility to anti-tubercular drugs using the gold standard phenotypic LJ method. For phenotypic drug susceptibility testing of the *M. tuberculosis* isolates, the LJ proportion method was used. *M. tuberculosis* isolates obtained from previously-treated patients presenting with TB and from treatment non-responders, as well as isolates displaying resistance to rifampicin were tested genetically for drug resistance using line probe assays (LPA) at the National Tuberculosis Control Center as noted previously [5]. GenoType@MTBDRplus (Version 2) was used for first-line drugs isoniazid and rifampicin, and GenoType@MTBDRsl (Version 1) was used for second-line drugs that included fluroquinolone and injectable anti-TB drugs, as per the manufacturer's instructions (Hain Lifescience, Nehren, Germany). In January 2021, the WHO revised its definition of XDR-TB. The WHO now defines XDR-TB as "MDR/RR-TB that is resistant to a fluoroquinolone and either bedaquiline or linezolid (or both)" [12]. In addition, it officially defines pre-XDR-TB as TB that fulfils the definition of MDR/RR-TB and is also resistant to any fluoroquinolone [12]. In this study, the Nepal and international genomic data are derived from XDR-TB isolates that were collected and classified prior to the 2021 definition of XDR-TB. Hence, for the purpose of the genomic comparison, the pre-2021 XDR-TB designation is used here.

2.4. Genomic DNA purification

DNA libraries were prepared with genomic DNA extracted as previously described [13]. Briefly, bacterial pellets were collected from liquid culture by centrifugation (8000 ×*g* for 8 min) and resuspended in phosphate-buffered saline containing lysozyme. The DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) and the High Pure PCR Template Preparation Kit (Roche, Germany) were used to purify and elute contaminant-free, high-molecular weight DNA. Genomic DNA was quantified by fluorometry using the Qubit™ dsDNA HS Assay kit (Invitrogen, Australia).

2.5. Next-generation sequencing

Purified genomic DNA was tagged and amplified using a Nextera® XT DNA Library Preparation Kit and Nextera® XT Index Kit as per the manufacturer's instructions. The libraries were cleaned using Agencourt AMPure XP beads. A normalization step was used to obtain a pooled DNA library concentration of 15 pM. The pooled library was loaded into an Illumina MiSeq v2 (2 × 150-bp paired-end reads) cartridge for sequencing. A quality check of the raw FASTQ sequencing reads was performed using FASTQC [14] to determine the total number of reads and sequencing quality scores. The sequence reads were then passed through Trimmomatic [15] to filter poor quality reads (using phred quality score threshold of 30) as well as to remove the Nextera XT index library adapters. The output reads were mapped to the *M. tuberculosis* H37Rv reference genome (GenBank accession number NC_000962.3) using the Bowtie2 plugin in Geneious Prime (https://www.geneious.com) [16]. All isolates were sequenced with a coverage of between 93.5% and 98.9% with respect to the chromosome of the H37Rv reference genome. Investigation into accessory genomes of the isolates was not included as the acquisition of resistance in *M. tuberculosis* to the drugs examined in this study is not driven by horizontal gene transfer of resistance plasmids or other mobile genetic elements, but through chromosomal mutations [17–19]. Paired-end sequence reads were also assembled de novo using the SPAdes plugin in Geneious Prime and then exported as contigs files in FASTA format.

2.6. In silico genetic lineage, spoligotype, and drug resistance mutation determination

The *M. tuberculosis* raw genomic sequence data (fastq.gz files) were uploaded to the TGS-TB database (https://gph.niid.go.jp/tgs-tb/) to determine the genetic lineage, in silico spoligotype, and genetically-encoded drug resistance mutations. TB-Profiler database (https://tbdr.lshtm.ac.uk/) was also used to verify the lineages and spoligotype profiles of the isolates. The drug resistance profile of each isolate was confirmed using the PhyResSE database (http://phyresse.org/). The WHO Catalogue of mutations in the *M. tuberculosis* genome complex and their association with drug resistance was used as a standard reference for the interpretation of mutations conferring resistance to the genetic markers of resistant variants identified [20].

2.7. Phylogenetic relatedness determination among Nepal TB isolates

Phylogenetic comparisons were performed between the Nepal isolates to provide a correlation with spoligotyping and also determine any clonal clusters among the TB isolates within the same sublineages. For phylogenetic analysis, maximum likelihood trees were obtained using PhyML [21] in Geneious Prime (https://www.geneious.com). The H37Rv reference-mapped sequences were aligned using the MUSCLE plugin with default settings. To overcome any incongruence among the different regions of the genomes under investigation during the phylogenetic analyses, the alignment files were analysed using ModelTeller (http://modelteller.tau.ac) to select for the most suitable substitution model for phylogeny reconstruction. The substitution models available included General Time Reversible (GTR) [22], Hasegawa-Kishono-Yano (HKY85) [23], Jukes-Cantor (JC69) [24], Kimura (K80) [25], Felsenstein 81 (F81) [26], and Tamura-Nei (TN93) [27]. To test the reliability of the tree branches, bootstrap analyses with 1000 replicates were performed.

Pairwise analysis of single nucleotide polymorphisms (SNP) was conducted whereby each Nepal MDR-/XDR-TB genome was nominated as the reference genome against which the raw FASTQ sequences of the other isolates were assembled, and a core SNP alignment was generated using Snippy (https://github.com/tseemann/snippy). The presence of a SNP was defined using a minimum nucleotide variant frequency of 95% and a minimum read depth of 20. Gubbins (https://github.com/sange

Table 2
43-spacer-based spoligotyping of the MDR and XDR *M. tuberculosis* isolates from Nepal ($n = 19$).

Spoligotyping Pattern (Spacers 1-43)																																							Isolate	Lineage	Sub-Lineage	Collection Date	Location
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43					
•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	H37Rv (NC_000962.3)	4 (Euro-American)	LAM		
																																•	•	•	•	•	•	•	•	•	•	•	NP1701X	2 (East-Asian)	Beijing	Sep-17	NATA, Morang
																																		•	•	•	•	•	•	•	•	•	•	•	NP1702X	2 (East-Asian)	Beijing	May-17	NATA, Morang
																																		•	•	•	•	•	•	•	•	•	•	•	NP1711	2 (East-Asian)	Beijing	May-17	NTCC, Bhaktapur
									•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	NP1712	3 (East-African Indian)	CAS	May-17	NATA, Morang
			•	•	•				•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	NP1713	3 (East-African Indian)	CAS	Jun-17	Chandranigapur, Rautahat
			•	•	•				•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	NP1714	2 (East-Asian)	Beijing	Jul-17	Chandranigapur, Rautahat
																																		•	•	•	•	•	•	•	•	•	•	•	NP1715	2 (East-Asian)	Beijing	Jul-17	Chandranigapur, Rautahat
																																		•	•	•	•	•	•	•	•	•	•	•	NP1716	2 (East-Asian)	Beijing	Jul-17	Chandranigapur, Rautahat
																																												NP1718	3 (East-African Indian)	CAS	Jul-17	Chandranigapur, Rautahat
			•						•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	NP1719	2 (East-Asian)	Beijing	Aug-17	NATA, Morang
																																		•	•	•	•	•	•	•	•	•	•	•	NP1720	2 (East-Asian)	Beijing	Aug-17	Chandranigapur, Rautahat
																																		•	•	•	•	•	•	•	•	•	•	•	NP1721	2 (East-Asian)	Beijing	Aug-17	NTCC, Bhaktapur
			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	NP1722	4 (Euro-American)	LAM	Aug-17	Lalgadh, Dhanusha
																																		•	•	•	•	•	•	•	•	•	•	•	NP1723	2 (East-Asian)	Beijing	Sep-17	Chandranigapur, Rautahat
																																		•	•	•	•	•	•	•	•	•	•	•	NP1724	2 (East-Asian)	Beijing	Sep-17	NMCTH, Birgunj
			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	NP1725	4 (Euro-American)	LAM	Sep-17	NTCC, Bhaktapur
																																		•	•	•	•	•	•	•	•	•	•	•	NP1726	2 (East-Asian)	Beijing	Nov-17	NTCC, Bhaktapur
																																		•	•	•	•	•	•	•	•	•	•	•	NP1727	2 (East-Asian)	Beijing	Nov-17	NATA, Morang
																																		•	•	•	•	•	•	•	•	•	•	•	NP1728	2 (East-Asian)	Beijing	Dec-17	NTCC, Bhaktapur

Each spoligotype pattern is a combination of 43 spacers denoted by white and black spacers which were determined in silico using the TGS-TB, PhyResSE and TB-Profiler online databases. A dotted box represents the presence of a specific spacer at that location while an empty box represents the absence of a spacer. The reference genome *M. tuberculosis* H37Rv version 3 (NC_000962.3) was used. Abbreviations: LAM, Latin American-Mediterranean; CAS, Central Asian Strain; NTCC, National Tuberculosis Control Center; NATA, National Anti-Tuberculosis Association.

r-pathogens/Gubbins) was then used to process the resulting SNP alignment to predict regions of homologous recombination within each isolate cluster. This resulted in the generation of three separate SNP scores for the phylogenetic comparison of isolates: total number of SNPS; number of SNPs inside homologous recombinant regions; and number of SNPs outside homologous recombinant regions.

2.8. Comparison of Beijing sub-lineage of Nepal MDR-/XDR-TB isolates with international isolates

The European Nucleotide Archive (ENA) online database (https://www.ebi.ac.uk/ena/) was used to download publicly available DNA sequences of *M. tuberculosis* belonging to the Beijing sub-lineage of the East Asian Lineage 2. Additionally, the genome sequences were filtered for MDR-TB and XDR-TB genomes that were submitted between 2010 and 2019, inclusive. The quality of the genome sequences was further filtered for a minimum genome coverage of 93% with respect to the reference H37Rv genome (GenBank accession number NC_000962.3). In total, 1274 genome sequences were available for this study (Supplementary Table 1 and Supplementary Table 2).

Phylogenetic relationships were determined between the Lineage 2 isolates from Nepal and those which were downloaded from the ENA database. The raw read sequences were mapped to the reference genome of the Beijing sub-lineage of the East Asian Lineage 2 of *M. tuberculosis*, CCDC5180 strain (GenBank accession number NC_017522.1) using Snippy (https://github.com/tseemann/snippy) and the genomes were aligned using Gubbins [28,29]. The default settings in Snippy were used for SNP calling with the following criteria: i) minimum nucleotide variant frequency of 95%; and ii) minimum number of reads covering the variant position of at least 20. Maximum likelihood trees rooted to the reference genome using PhyML were generated from the multiple core genome alignments in Geneious Prime (https://www.geneious.com). Default parameters were used with the best substitution model as determined by ModelTeller (http://modelteller.tau.ac) with 1000 bootstrap replicates.

Downloaded genomes of the international *M. tuberculosis* strains were filtered for XDR-TB isolates for comparison with the Lineage 2 isolates from Nepal. After constructing a phylogenetic tree of these isolates, a core genome multi-locus sequence typing (cgMLST) analysis was conducted using the SeqSphere+ version 6 software (Ridom GmbH, Munster, Germany) of the international *M. tuberculosis* that clustered closely with the isolates from Nepal. The default settings used included removing the shorter of the two genes overlapping by more than four bases and the removal of genes with an internal stop codon in more than 80% of all query genomes from the scheme. The mapped reads of each isolate were queried against a pre-defined cgMLST scheme consisting of 2891 target genes [28]. The number of allelic differences was calculated to determine the minimum spanning tree between these isolates for the detection of clusters within each lineage type. An allelic distance threshold of $\leq$12 was used as an indicator of members of a possible cluster [30,31].

3. Results

3.1. Spoligotypes and genetic lineages of Nepal MDR-/XDR-TB isolates

In silico spoligotyping of the *M. tuberculosis* MDR-/XDR-TB isolates ($n = 19$) from Nepal collected in 2017 showed that 73.7% ($n = 14$) of them belonged to the Beijing sub-lineage of the East Asian Lineage 2 (Table 2). Other lineages detected include the Central Asian Strain (CAS) sub-lineage of the East-African Indian Lineage 3 ($n = 3$), and the Latin American-Mediterranean (LAM) sub-lineage of the Euro-American Lineage 4 ($n = 2$).

Table 3
Mutations in genes associated resistance to with first line and second line anti-tuberculosis drugs among the Nepal MDR- and XDR-TB patient isolates ($n = 19$).

Isolate	DST method	First Line Drugs							Second Line Drugs						Drug Resistance
		INH		RIP		EMB		PZA[c]	STR		FQ		AMK[c], CPM, KM		
NP1701X	PSD	(R)	ahpC_promoter (-88G>A) [b](0.80) fabG1_promoter (-8T>C) [b](0.99)	(R)	rpoB (Thr400Ala, Acc/Gcc) [b](1.00) rpoB (Ser450Leu, tCg/tTg) [b](1.00)	(R)	embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Ala134Val, gCc/gTc) [b](1.00)	(R)	rpsL (Lys43Arg, aAg/aGg) [b](1.00)	(R)	gyrA (Ser91Pro, Tcg/Ccg) [b](0.99)	(R)	rrs (1401A>G) [b](0.99)	XDR
NP1702X	LPA	(R)	ahpC_promoter (-88G>A) [b](0.80) fabG1_promoter (-8T>C) [b](0.99)	(R)	rpoB (Thr400Ala, Acc/Gcc) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Ala134Val, gCc/gTc) [b](1.00)		rpsL (Lys43Arg, aAg/aGg) [b](1.00)		gyrA (Ser91Pro, Tcg/Ccg) [b](0.99) gyrA (Asp94Gly, gAc/gGc) [a](0.27)		rrs (1401A>G) [b](0.99)	XDR
NP1711			katG (Asp419His, Gac/Cac) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Asp354Ala, gAc/gCc) [a](0.82)			rpsL (Lys43Arg, aAg/aGg) [b](1.00)					MDR
NP1712			katG (Ser315Thr, aGc/aCc) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Val180Phe, Gtc/Ttc) [b](1.00)		rpsL (Lys43Arg, aAg/aGg) [b](1.00)		gyrA (Asp94Gly, gAc/gGc) [b](0.99)			MDR
NP1713			katG (Ser315Thr, aGc/aCc) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)					gyrA (Asp94Gly, gAc/gGc) [b](0.99)			MDR
NP1714	PSD	(R)	fabG1_promoter (-15C>T) [b](1.00) katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)	(R)	embA_promoter (-11C>A) embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Leu27Pro, cTg/cCg) [b](1.00)	(R)	rpsL (Lys43Arg, aAg/aGg) [b](1.00)	(R)	gyrA (Asp94Gly, gAc/gGc) [b](0.99)	(S)		MDR
NP1715	PSD	(R)	fabG1_promoter (-15C>T) [b](1.00) katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)	(R)	embB (Gln497Arg, cAg/cGg) [b](0.97)	pncA (Gly132Ala, gGt/gCt) [b](1.00)	(R)	rpsL (Lys43Arg, aAg/aGg) [b](1.00)	(R)	gyrA (Asp94Gly, gAc/gGc) [b](0.99)	(S)		MDR
NP1716	PSD	(R)	katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)	(R)	embB (Met306Val, Atg/Gtg) [b](0.97)		(R)	rpsL (Lys43Arg, aAg/aGg) [b](1.00)	(R)	gyrA(Asp94Asn, gac/aac) [b](0.99)	(S)		MDR
NP1718			katG (Ser315Thr, aGc/aCc) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Met175Val, Atg/Gtg) [b](1.00)		rpsL (Arg86Pro, cGa/cCa) [b](1.00)		gyrA (Ser91Pro, Tcg/Ccg) [b](0.99)			MDR
NP1719	PSD	(R)	katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)	(R)	embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Leu27Pro, cTg/cCg) b(1.00)	(R)	rpsL (Lys43Arg, aAg/aGg) [a](0.97)	(R)	gyrA (Asp94Gly, gAc/gGc) [b](0.99)	(S)		MDR
NP1720	LPA	(R)	katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)					rpsL (Lys43Arg, aAg/aGg) [b](1.00)		gyrA (Asp94Asn, Gac/Aac) [a](0.89)			MDR
NP1721	PSD	(R)	katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)	(R)	embB (Met306Val, Atg/Gtg) [b](0.97)		(R)	rpsL (Lys43Arg, aAg/aGg) [b](1.00)		gyrA (Asp94Tyr, Gac/Tac) [b](0.039)			MDR
NP1722			katG (Ser315Thr, aGc/aCc) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Gln497Arg, cAg/cGg) [b](0.97)							rrs (514A>C) [b](0.98)	MDR
NP1723	LPA	(R)	katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Gly108Arg, Gga/Cga) [b](1.00)		rpsL (Lys43Arg, aAg/aGg) [b](1.00)					MDR
NP1724	LPA	(R)	katG (Ser315Thr, aGc/aCc) [b](1.00)	(R)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)			rpsL (Lys43Arg, aAg/aGg) [b](1.00)		gyrA (Ala90Val, gCg/gTg) [b](0.99)			MDR
NP1725			katG (Ser315Thr, aGc/aCc) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)	pncA (Thr142Ala, Acg/Gcg) [b](1.00)							MDR
NP1726			katG (Ser315Thr, aGc/aCc) [b](1.00)		rpoB (Ser450Leu, tCg/tTg) [b](1.00)		embB (Met306Val, Atg/Gtg) [b](0.97)			rpsL (Lys43Arg, aAg/aGg) [b](1.00)		gyrA (Asp94Gly, gAc/gGc) [b](0.99)			MDR

(*continued on next page*)

Table 3 (*continued*)

Isolate	DST method	First Line Drugs				Second Line Drugs			Drug Resistance
		INH	RIP	EMB	PZA[c]	STR	FQ	AMK[c], CPM, KM	
NP1727		katG (Ser315Thr, aGc/aCc) [b](1.00)	rpoB (Ser450Leu, tCg/tTg) [b](1.00)	embB (Met306Val, Atg/Gtg) [b](0.97)		rpsL (Lys43Arg, aAg/aGg) [b](1.00)	gyrA (Asp94Tyr, Gac/Tac) [b](0.99)		MDR
NP1728	PSD	(R) fabG1_promoter (-15C>T) [b](1.00) katG (Ser315Thr, aGc/aCc) [b](1.00)	(R) rpoB (Asp435Val, gAc/gTc) [b](1.00)	(R) embB (Met306Val, Atg/Gtg) [b](0.97)		(R) rpsL (Lys88Arg, aAg/aGg) [b](1.00)		gyrA (Asp94Gly, gAc/gGc) [b](0.99)	MDR

[a] Genetic mutations in resistance genes were identified in silico as high confidence using the *PhyResSE, TGS-TB* and *TB-Profiler* databases unless indicated in the brackets.

[b] Gene mutation specificity for resistance to anti-tubercular drugs was based on the WHO's "Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance" [20].

[c] PZA and AMK were not included in the phenotypic DST. Phenotypic DST for each drug, where available, is shown as resistant (R) or susceptible (S). SNPs are presented with the original and mutated amino acid and 3 nucleotides with the codon position. Insertions are presented with the nucleotide position where the polymorphism occurred. Abbreviations: DST, drug susceptibility test; PSD, phenotypic solid DST; LPA, Line Probe Assay; INH, isoniazid; RIP, rifampicin; EMB, ethambutol; PZA, pyrazinamide; STR, streptomycin; FQ, fluoroquinolones; AMK, amikacin; CPM, capreomycin; KM, kanamycin; MDR, Multi-drug resistance to first line antibiotics (INH & RIP); XDR, Extensively-drug resistance to INH and RIP plus FQ and one of the second line injectable antibiotics (AMK, CPM or KM).

3.2. Determination of genetic mutations associated with resistance to first-line TB drugs

Genetic resistance in *M. tuberculosis* to first-line TB drugs is characterised by acquired genetic chromosomal mutations [32]. For each of the 19 MDR-/XDR-TB isolates, software platforms PhyResSE and TGS-TB were used detect to anti-tuberculosis drug resistance mutations in genes located on the chromosome of *M. tuberculosis* [18,32] (Table 3). Laboratory-based results were available for 11 of the 19 Nepal *M. tuberculosis* isolates. The test methods were phenotypic solid drug sensitivity testing (PSD, $n = 7$) and line probe assay (LPA, $n = 4$) (Table 3). Of the Nepal MDR-/XDR-TB isolates for which drug-susceptibility data were available, there was 100% concordance with the WGS-based detection of high-confidence mutations in the *katG* and *fabG1* genes for isoniazid resistance and in the *rpoB* gene for rifampicin resistance.

The XDR-TB isolates, NP1701X and NP1702X, exhibited high-confidence resistance mutations for all four first-line antibiotics (Table 3). For both XDR-TB isolates, isoniazid resistance was associated with the *fabG1-inhA* promoter (−8T>C) [33] and *ahpC* promoter (−88G>A) [34] gene variants. Mutation of the *rpoB* gene (Thr400Ala, Acc/Gcc) underlay rifampicin resistance in both NP1701X and NP1702X, while a second *rpoB* (Ser450Leu, tCg/tTg) variant was also detected in isolate NP1701X [35]. The variants conferring resistance to pyrazinamide and ethambutol were in the *pncA* gene (Ala134Val, gCc/gTc,) [36,37], and *embB* gene (Met306Val, Atg/Gtg) [38,39], respectively.

Among the MDR-TB isolates, isoniazid resistance was caused by either of the following two variants of the *katG* gene, (Ser315Thr, aGc/aCc) ($n = 16$) or (Asp419His, Gac/Cac) ($n = 1$) [40,41]. Rifampicin and ethambutol resistance were conferred by mutations in the *rpoB* gene ($n = 17$) [35]. High confidence resistance mutations were also detected in the *pncA* ($n = 7$) and *embB* ($n = 16$) loci of the MDR-TB isolates [20,36,38,39] (Table 3).

3.3. Identification of further mutations associated with resistance to second-line TB drugs

Second-line drug susceptibility data were available for isolate NP1701X and showed resistance to fluoroquinolones and second-line injectables (amikacin, capreomycin, and kanamycin) in agreement with the detection of high-confidence resistance mutations in the *gyrA* (Ser91Pro, Tcg/Ccg) [42] and *rrs* (1401A>G) loci [43], respectively (Table 3). Only first-line drug susceptibility (LPA) was performed for isolate NP1702X confirming resistance to isoniazid and rifampicin, however, the WGS analysis permitted detection of high-confidence resistance mutations to fluoroquinolones and second-line injectables (Table 3).

The XDR-TB isolates, NP1701X and NP1702X, also possessed genetically-encoded resistance to streptomycin in the *rpsL* gene (Lys43Arg, aAg/aGg) [43]. The MDR-TB isolates exhibited genetically-encoded resistance to streptomycin plus fluoroquinolones ($n = 12$), streptomycin only ($n = 2$), fluoroquinolones only ($n = 1$), or second-line injectables only ($n = 1$) (Table 3).

3.4. SNP-based analysis of Nepal MDR-TB isolates

A maximum-likelihood SNP-based phylogenetic tree was constructed for the *M. tuberculosis* isolates and the *M. tuberculosis* H37Rv (NC_000962.3) genome was used as the reference. The best-fit nucleotide substitution model selected for the phylogenetic reconstruction of these isolates was the Felsenstein model (F81) [26] (Fig. 2). Their SNP-based phylogeny was concordant with their lineages identified by spoligotyping where isolates of the same lineage were shown to cluster together (Fig. 2). Among the isolates belonging to the Beijing sub-lineage of East-Asian lineage 2, the XDR-TB isolates, NP1701X and

NP1702X, clustered most closely with pre-XDR-TB Nepal isolate NP1727 than with other MDR-TB isolates.

3.5. Comparative genomics of sequenced Nepal and international isolates of XDR-TB

A global assessment of the genomic diversity of XDR-TB strains of *M. tuberculosis* Beijing sub-lineage of East Asian Lineage 2 collected from Nepal in this study and from other international locations. A search through the European Nucleotide Archive (ENA) online database (https://www.ebi.ac.uk/ena/) for MDR- and XDR-TB isolates reported between 2010 and 2019 resulted in 1274 genomes that were publicly available, which consisted of 1032 MDR and 242 XDR *M. tuberculosis* isolates. Details of these isolates are provided in Supplementary Tables 1 and 2.

Phylogenetic comparisons were performed between the *M. tuberculosis* isolates from Nepal in this study and international isolates of XDR-TB (Fig. 3). The best-fit nucleotide substitution model selected for the alignment of this group of genomes was the Hasegawa-Kishono-Yano (HKY85) model [23]. Among the international XDR-TB isolates ($n = 242$), five Nepal isolates which included the two XDR-TB isolates, NP1701X and NP1702X, shared a common node with isolates ($n = 17$) from South Africa collected from 2010 to 2017 (Fig. 3). Despite sharing a common node, a clear separation between the isolates from both countries was evident. From the cgMLST analysis based on 2891 core genome target genes, the two XDR-TB isolates from Nepal were shown to be clonally related to one another with no allelic differences between them (Fig. 4). They were also found to be more closely related to other pre-XDR isolates from Nepal, such as NP1727 with 14 allelic differences detected. Using a previously described SNP threshold of ≤12 SNPs for identifying clonally related or non-unique isolates [30,31], SNP pairwise comparisons of their core genomes detected a difference of 22 SNPs between the XDR-TB isolates and theirnearest Nepal isolate in the cgMLST analysis, NP1727 (Fig. 4). As shown using cgMLST, the closest phylogenetic relatives among the sequenced international XDR-TB isolates were separated from the Nepal MDR-/XDR-TB genomes by at least 72 alleles or 132 SNPs. The separation distance between the international and Nepal XDR-TB isolates was even greater (Fig. 4). Phylogenetic comparisons were also made with sequenced international isolates of MDR-TB from 2010 to 2019. ModelTeller identified best-fit substitution models in the reconstruction of each of the phylogenetic trees. The Nepal XDR-TB isolates, NP1701X and NP1702X, positioned most closely with MDR-TB isolates from Nepal than with MDR-TB isolates from other countries in the years analysed (Supplementary Fig. 1).

4. Discussion

A previous study found that among 153 cases of TB (49 new and 104 previously treated) in Nepal in 2013, the highest rate of resistance recorded was towards streptomycin [44]. In our study, mutations encoding resistance to streptomycin were present in 16/19 (84.2%) of the MDR-TB isolates from Nepal. Streptomycin is used in the treatment of Category II TB cases in Nepal, i.e. retreatment cases including failures, relapses and defaults [45], and is also a Group B second-line injectable that is used in the treatment of MDR-TB internationally [46,47]. Among the isolates, resistance mutations were also prevalent for ethambutol (94.7%, 18/19) and pyrazinamide (63.2%, 12/19) which are key first-line drugs in the treatment of drug-susceptible TB [48] and in addition, are used as Group D1 second-line drugs for treating MDR-TB [46,47]. Of concern also is that a high level of resistance mutations to

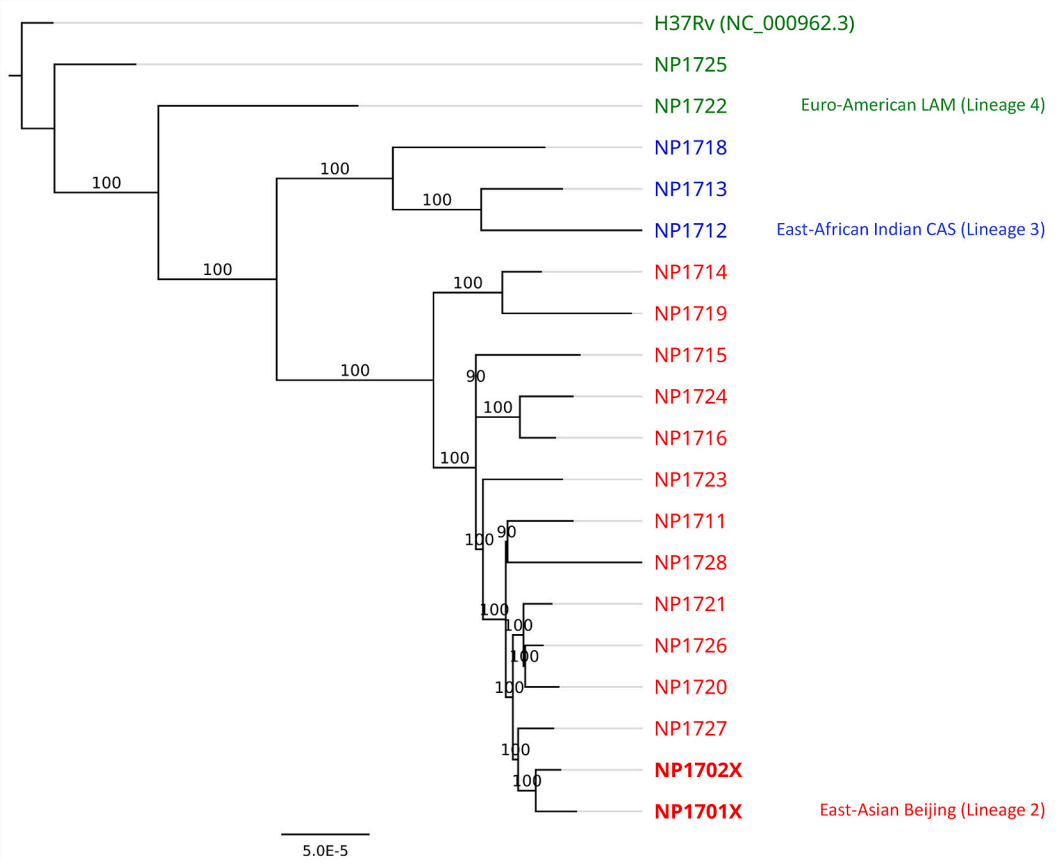

Fig. 2. Maximum-likelihood phylogenomic tree based on the alignment of whole genome sequencesof the 19 *Mycobacterium tuberculosis* isolates collected in Nepal in 2017. The Nepal TB genomes were mapped to reference genome *M. tuberculosis* H37Rv version 3 (NC_000962.3). The PhyML tree rooted to the reference genome was constructed using the Felsenstein (F81) substitution model. Branch labels indicate support values for 1000 bootstrap replicates.

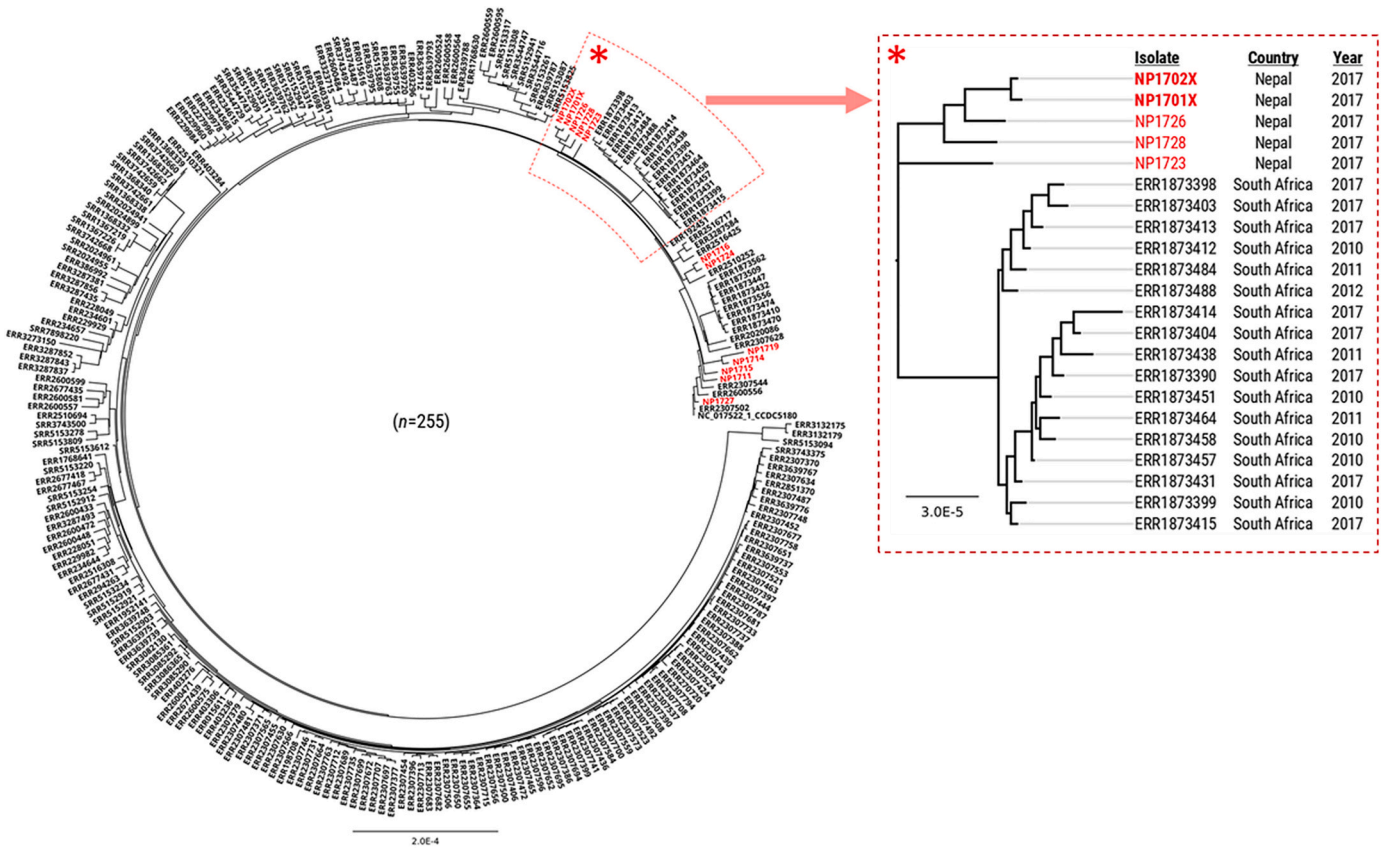

Fig. 3. Phylogenetic comparison of XDR-TB strains of *Mycobacterium tuberculosis* Beijing sub-lineage of East Asian Lineage 2 collected in Nepal in this study and in other international studies. XDR-TB isolates (n = 242) reported between 2010 and 2019 were downloaded via the European Nucleotide Archive (ENA) database (https://www.ebi.ac.uk/ena/) (Supplementary Table 1 and Supplementary Table 2). The Nepal Beijing sub-lineage isolates (in red) (n = 12) included both MDR-TB and XDR-TB (bold) isolates. A SNP-based maximum likelihood (PhyML) phylogenetic tree was generated from the *M. tuberculosis* isolates that were rooted and mapped to reference genome of the Beijing sub-lineage of the East Asian Lineage 2 of *M. tuberculosis*, CCDC5180 (Gen Bank accession number NC_017522.3). The best-fit substitution model selected was the Hasegawa-Kishono-Yano (HKY85) model with 1000 bootstrap replicates. The clade of international isolates from South Africa (n = 17) that clustered most closely with 5 isolates from Nepal (including the 2 XDR-TB isolates) is outlined with a red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the Group A class of second-line TB drugs, fluoroquinolones, was identified in the Nepal MDR-TB isolates (78.9%, 15/19). Such isolates are considered pre-XDR-TB in that an additional single base pair mutation can be sufficient to shift them to being fully XDR.

A paper in the *Lancet* referred to a decrease in the success rate for the treatment of MDR-TB in Nepal to 67% but the reasons behind this have not been established [49]. Our work shows that MDR-TB isolates from Nepal carry resistance mutations to several of the second-line drugs that are used in the treatment of MDR-TB in particular, fluoroquinolones, streptomycin, ethambutol and pyrazinamide (Table 3). In the absence of routine second-line drug-susceptibility testing of TB isolates in Nepal, it is possible that a significant proportion of MDR-TB patients may receive treatment regimens for which the infecting TB isolates already harbour resistance against a number of components of therapeutic drug combinations.

To counteract the problem of underlying resistance to second-line drugs in Nepal MDR-TB cases, and the risk of progression to XDR-TB, further drug susceptibility testing (DST) of *M. tuberculosis* isolates from MDR-TB cases in Nepal is required. This enhanced-level of DST would enable the selection only of drugs for which MDR-TB isolates from patients are susceptible and would assist in avoiding treatment regimens with lower success rates. It is also important that the previously-reported delay in the diagnosis of TB in Nepal [50] is minimised. This is required in order to prevent onward transmission of MDR-TB strains of *M. tuberculosis* and reduce the organism's potential to acquire the small number of additional mutations needed to become XDR. In addition, concerns have been expressed regarding widespread inappropriate usage of antibiotics in Nepal in both the human health sector and in animal agriculture [51,52]. It may therefore be necessary for the use of second-line anti-tubercular drugs, such as fluoroquinolones, in non-TB settings in Nepal to be examined in terms of antimicrobial stewardship for the purposes of reducing background levels of resistance.

The majority of the Nepal *M. tuberculosis* MDR-/XDR-TB isolates (n = 19) examined here belonged to the Beijing sub-lineage of the East Asian Lineage 2 (73.7%, n = 14) with the remaining isolates belonging to the CAS sub-lineage of the East-African Indian Lineage 3 (n = *3*), and the Latin American-Mediterranean (LAM) sub-lineage of the Euro-American Lineage 4 (n = *2*) (Table 2). Earlier studies have also reported a dominance of East Asian Lineage 2 (including the Beijing sub-lineage) among MDR-TB cases in Nepal [53–55]. Among the isolates from Nepal sequenced in this study, there were two isolates that were XDR-TB. We were interested in whether these isolates may have developed extensive-drug resistance in Nepal, or whether they were most likely circulating already as XDR-TB isolates in other countries. We therefore performed an analysis to determine the relatedness of the Nepal XDR-TB isolates to MDR-TB isolates collected in Nepal and to 242 XDR-TB isolates collected in other countries. To minimise bias during phylogenetic inferencing, best-fit substitution modelling was included in the pipeline.

The *M. tuberculosis* samples sequenced in this study were collected from multiple locations in Nepal (Fig. 1). Despite the multiple locations sampled in Nepal, the XDR-TB Nepal genomes collected in Morang in the Easternmost Province No. 1 of Nepal still clustered most closely with

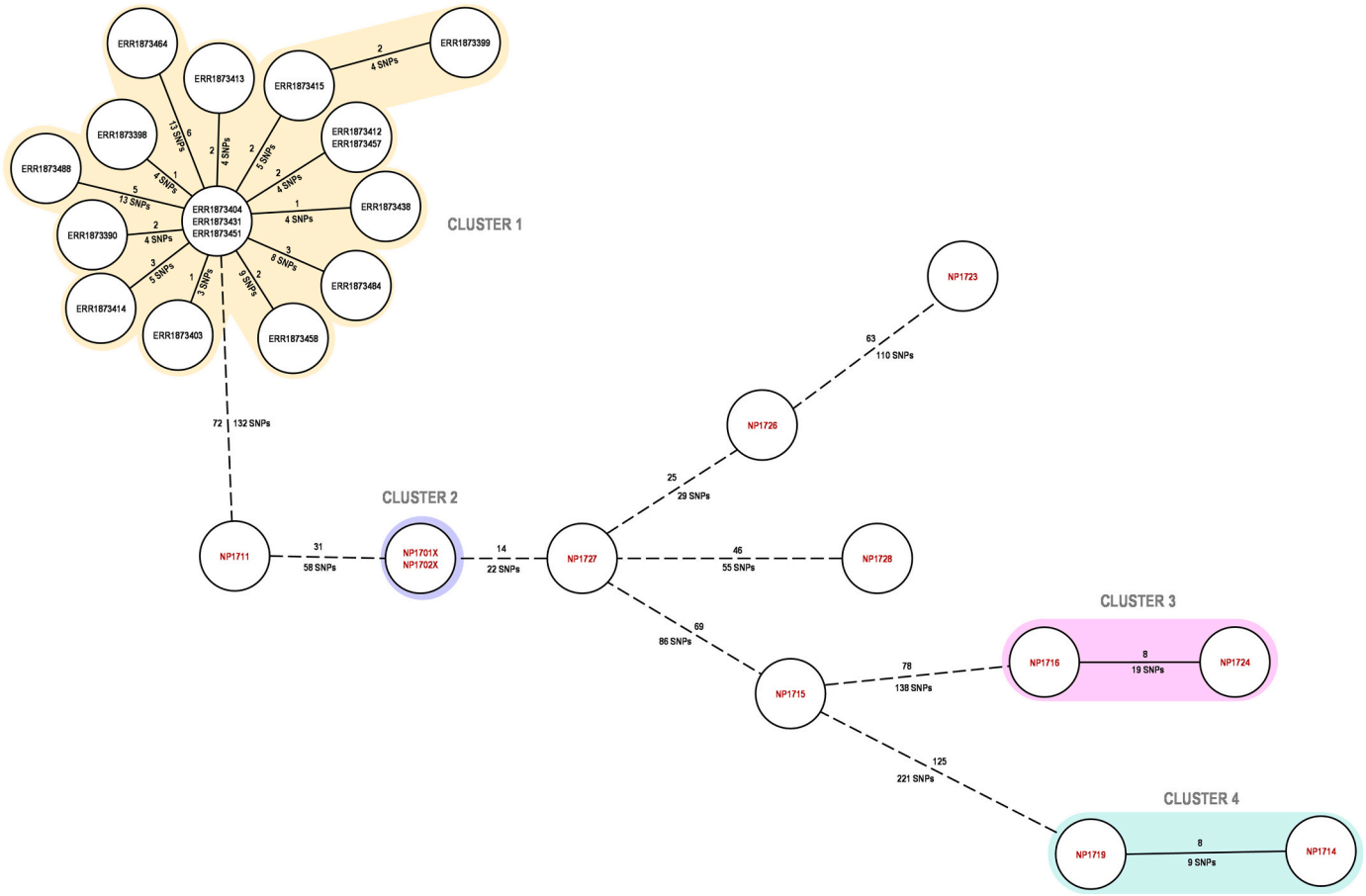

Fig. 4. Core genome Multi-Locus Sequence Type (cgMLST) comparions of *Mycobacterium tuberculosis* Beijing sub-lineage of East-Asian lineage 2 isolates from Nepal with XDR-TB international isolates. A minimum-spanning tree of 28 isolates was constructed using Ridom SeqSphere+ software. Isolate clusters were defined when neighbouring isolates exhibited 12 or less allelic differences between them (coloured bands). Isolates with zero allelic differences between them are grouped in the same circle. The number of allelic differences between isolates and SNPs detected within the core genome are shown on the connecting lines (>12 alleles are shown as detached lines). The Cluster 2 Nepal XDR-TB isolates, NP1701X and NP1702X, are most closely related to pre-XDR-TB and MDR-TB isolates from Nepal than the international XDR-TB isolates from South Africa (downloaded via the European Nucleotide Archive (ENA) database (https://www.ebi.ac.uk/ena/)) (Supplementary Table 2).

MDR-TB isolates from other locations in Nepal i.e. Rautahat in Province No. 2 (NP1723), and Bhaktapur in the Bagmati Province of Nepal (NP1726 and NP1728), than with XDR-TB isolates from other locations internationally (Fig. 3). Furthermore, the recombination-filtered SNP distances between the XDR-TB isolates collected in this study and the Nepal MDR-TB isolates studied here, including isolates from Morang, are too high (ranging from 37 to 124 SNPs) to indicate recent or household transmission based on the established SNP threshold for *M. tuberculosis* of ≤5 SNPs [30,31].

Among the XDR-TB isolates reported between 2010 and 2019, the Beijing sub-lineage of the East Asian Lineage 2 of *M. tuberculosis* is not limited to the Asian continent but is also present in Europe, Africa and South America (Fig. 5). The SNP-based phylogeny showed that of the XDR-TB genomes sequenced globally, the most closely-related international isolates of XDR-TB, to the Nepal XDR-TB isolates, were collected in South Africa. However, cgMLST and pairwise SNP analyses found that there are at least 72 allelic and 132 SNP differences, respectively, between the South African isolates and the closest related Nepal MDR-/XDR-TB isolates in our study (Fig. 4). In contrast, the Nepal XDR-TB isolates exhibited lower numbers of allelic and SNP differences to MDR-TB and pre-XDR-TB isolates collected in Nepal (Fig. 4). This provides evidence that acquisition of further antibiotic resistance by MDR-TB to develop into XDR-TB is more likely to have occurred in the local isolates examined in this study, rather than from direct import from other countries as primary XDR-TB isolates. Combined with concerns over the level of second-line drug resistance in Nepal TB isolates, this finding highlights the need for further work to reduce the prevalence of pre-XDR-TB in Nepal which has previously been associated with a relatively high mortality rate of 20% [7].

There are a number of limitations of the study that should be acknowledged. Whole genome sequencing with short reads has a limited ability to fully resolve sequence containing repetitive regions or large structural variations [56]. For *M. tuberculosis*, the GC-rich content of the genes encoding the proline-glutamate (PE) and proline-proline-glutamate (PPE) motif family of proteins can introduce errors including gaps into sequence assemblies [57]. Also, the main *M. tuberculosis* reference strain used in this study H37Rv [58] may introduce some potential bias during assembly due to the presence of genomic regions in TB isolates that are not found in the H37Rv genome due to genomic divergence. However, a previous study by Lee and Behr using 7 different reference genomes of *M. tuberculosis* reported a negligible effect of the reference genome on phylogenetic and epidemiological studies of *M. tuberculosis* [59]. Also, nucleotide substitution modelling was incorporated into the analysis pipeline to determine the best fit model for inferring phylogeny reconstruction in this study [60]. Incongruency between the models was not evident with respect to the placement of the XDR-TB isolates from Nepal which were found to cluster more closely with MDR-TB isolates from Nepal collected in this study that with non-Nepal international isolates of XDR-TB (Fig. 2, Fig. 3 and Supplementary Fig. 1).

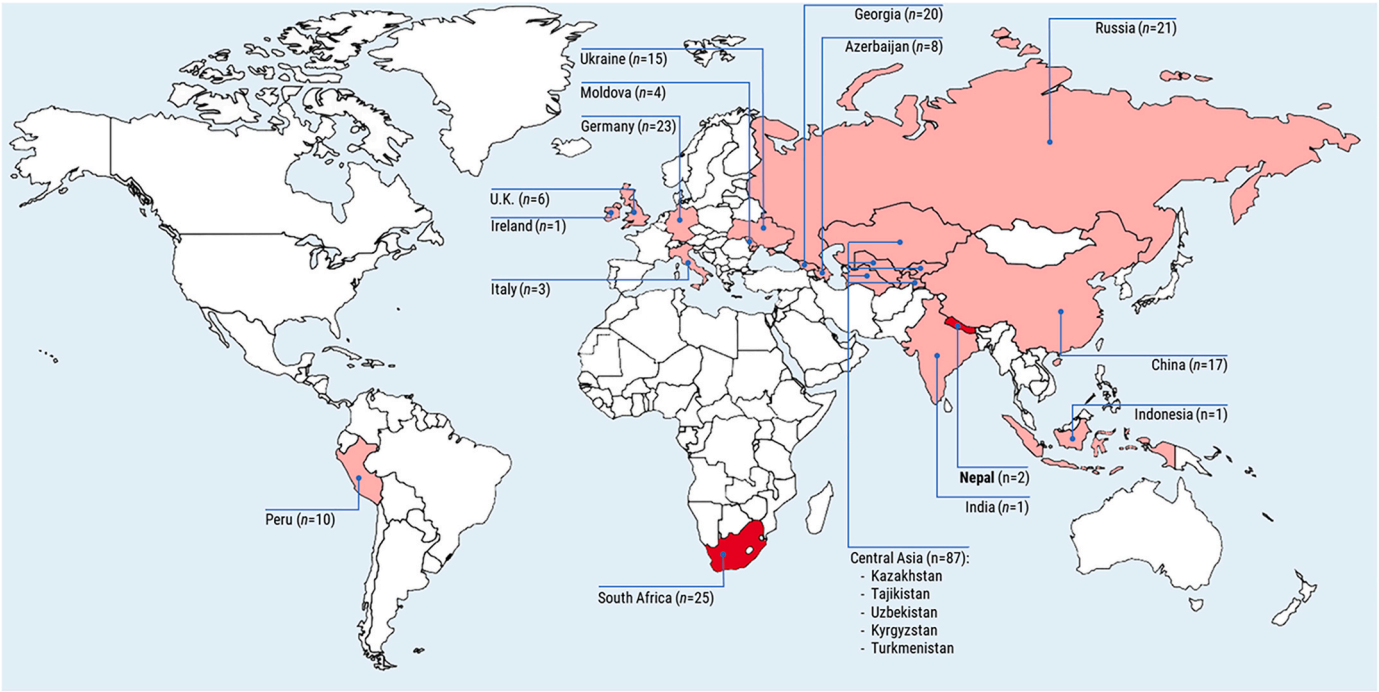

Fig. 5. Global distribution of the XDR Beijing sub lineage of the East Asian lineage 2 *Mycobacterium tuberculosis* isolates ($n = 242$) reported between 2010 and 2019. There was no information about the origin of one ($n = 1$) isolate. In this study, comparative genomics showed that of the XDR-TB genomes sequenced globally, the most closely-related international isolates of XDR-TB, to the Nepal XDR-TB isolates ($n = 2$), were collected in South Africa (dark red). International isolates were downloaded via the European Nucleotide Archive (ENA) database (https://www.ebi.ac.uk/ena/) (Supplementary Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In summary, MDR-TB remains a major public health problem in Nepal. In this study, the use of whole genome sequencing has allowed for the detection of genetically-encoded resistance to fluoroquinolones among a high proportion of the MDR-TB isolates examined. Furthermore, we identified evidence that isolates from two cases of XDR-TB in Nepal were more likely to have evolved from locally transmitted MDR-TB rather than being introduced as primary XDR-TB from other countries. The newly-introduced definition of XDR-TB by the WHO, together with the high background resistance to fluoroquinolones in MDR-TB cases in Nepal, emphasise the need for expanded susceptibility testing for second-line TB drugs including Group A drugs bedaquiline and linezolid. Such information will be integral to the surveillance and control of XDR-TB in Nepal.

Author contribution

Conceptualization, KWCL, SSG, RFO; Data curation, KWCL, SSG, MP, US, SK, RFO; Funding acquisition, RFO; Investigation, KWCL, SSG, RFO; Methodology, KWCL, SSG, MP, YIS, US, SK, RFO; Project administration, RFO; Resources, KWCL, MP, YIS, SKR, GRG, KA, RC, GG, RFO; Supervision, RFO; Validation, KWCL, RFO; Roles/Writing - original draft, KWCL, RFO; Writing - review & editing, KWCL, RKC, RFO.

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Data archiving

Sequence read files for the MDR-TB isolates sequenced in this work have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) BioProject ID PRJEB48270.

Declaration of Competing Interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110278.

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