CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* ASSOCIATED WITH PULMONARY TUBERCULOSIS IN MIZORAM USING GENOMIC APPROACHES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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BY

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SUBMITTED

IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY OF MIZORAM UNIVERSITY, AIZAWL

CERTIFICATE

This is to certify that the thesis entitled "**Characterization of *Mycobacterium tuberculosis* associated with Pulmonary Tuberculosis in Mizoram using Genomic Approaches**" submitted to the Mizoram University in partial fulfillment for the degree of Doctor of Philosophy in Biotechnology is a record of research work carried out by **Christine Vanlalbiakdiki Sailo** under my personal supervision and guidance.

No part of this thesis has been reproduced elsewhere for any degree.

Dated: 13/06/2022

(N. Senthil Kumar)

Supervisor

DECLARATION

I, **Christine Vanlalbiakdiki Sailo**, hereby declare that the subject matter of this thesis entitled "**Characterization of *Mycobacterium tuberculosis* associated with Pulmonary Tuberculosis in Mizoram using Genomic Approaches**" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of **Doctor of Philosophy** in the Department of Biotechnology.

Date: 12/06/2022

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Place: Aizawl.

Date: **(CHRISTINE VANLALBIAKDIKI SAILO)**

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List of abbreviations used

ADA	:	Adenosine deaminase
AFB	:	Acid Fast Bacilli
AG	:	Arabinogalactan
AIDS	:	Acquired Immunodeficiency Syndrome
BAL	:	Bronchoalveolar Lavage
BCG	:	Bacille Calmette-Guerin
BMI	:	Basal Metabolic Index
CB-NAAT	:	Cartridge Based Nucleic Acid Amplification Test
CFU	:	Colony Forming Unit
CI	:	Confidence Interval
CT scan	:	Computed Tomography scan
Ct	:	Cycle threshold
DNA	:	Deoxyribonucleic acid
dNTP's	:	Deoxynucleotide triphosphates
DOTS	:	Directly Observed Treatment Short Course
DST	:	Drug Susceptibility Testing
DTC	:	District Tb Center
EDTA	:	Ethylenediamine tetraacetic acid
FNAC	:	Fine Needle Aspiration Cytology
GC	:	Growth Control
GOI	:	Government of India
H_2SO_4	:	Sulfuric Acid

HCW	:	Health Care Worker
HIV	:	Human Immunodeficiency Virus
ICMR	:	Indian Council of Medical Research
IEC	:	Institutional Ethics Committee
INH	:	Isoniazid
INR	:	Indian Rupee
IRL	:	Intermediate Reference Laboratory
KH_2PO_4	:	Potassium di-hydrogen phosphate
LJ	:	Lowenstein-Jensen
LPA	:	Line Probe Assay
LSP	:	Large Sequence Polymorphism
MA	:	Mycolic acid
MDR	:	Multidrug Resistant
MGIT	:	Mycobacterial Growth Indicator Tube
Mtb	:	*Mycobacterium tuberculosis*
Mtbc	:	*Mycobacterium tuberculosis complex*
Na_2HPO_4	:	Disodium Hydrogen Phosphate
NaLC	:	N-acetyl-l-cysteine
NaOH	:	Sodium Hydroxide
NTCP	:	National Tuberculosis Control Programme
NTI	:	National Tuberculosis Institute
NTP	:	National Tuberculosis Programme
OADC	:	Oleic acid, Albumin, Dextrose, Catalase
PANTA	:	Polymyxin, Amphotericin, Nalidixic acid, Trimethoprim, Azolicillin
PCR	:	Polymerase Chain Reaction

pH	:	Potential of Hydrogen
PMDT	:	Programmatic management of drug-resistant Tb
PPE	:	Personal Protective Equipment
Ptb	:	Pulmonary tuberculosis
PZA	:	Pyrazinamide
RD’s	:	Regions of Difference
RIF	:	Rifampicin
RNA	:	Ribonucleic acid
RNTCP	:	Revised National Tuberculosis Control Programme
RR	:	Rifampicin Resistant
RRDR	:	Rifampicin Resistance Determining Region
RT	:	Room Temperature
SIDA	:	Sewdish International Developmental Agency
SNP	:	Single Nucleotide Polymorphism
SPSS	:	Statistical Package for Social Sciences
STM	:	Streptomycin
Tb	:	Tuberculosis
TRC	:	Tuberculosis Chemotherapy Center
WHO	:	World Health Organization
XDR	:	Extensive Drug Resistant
ZN	:	Ziehl Neelsen

Chapter 1: Introduction and Review of Literature

1.1. Tuberculosis

Tuberculosis is an infectious, curable bacterial disease caused by *Mycobacterium tuberculosis* (Mtb). On a global scenario, it is among the top 10 causes of death and the principal cause of death by a single pathogen. The word tuberculosis (Tb) is derived from the word "tubercle"- a small lump or nodule (Wolinsky,1988). A probable reference of Tb existed in the Old Testament of the bible, Deuteronomy and Leviticus under the Hebrew word *Schachepheth* (Daniel et al., 1999). Similarly, across the centuries, Tb illness was known to the world by different names such as "Phtisis and consumption" in the 17th and 18th century due to the progressive wasting and consumptive condition (Figure 1); in Western Europe it was called "the robber of youth" due to the higher death rate among younger people and was also coined as "the white death" and "the great white plague" due to the extreme pallor of the affected (Barberis et al., 2017); in ancient Rome it was called "tabes" (World Tb Day, 2021). Scrofula (tuberculosis lymphadenitis) was called the "king's evil" in Europe where the people had the notion that affected persons could cure after a royal touch (Grzybowski and Allen, 1995). In 1960, consumption was also referred by a British author, John Bunyan as "The captain of all these men of death" (Bunyan, 2013); and "the graveyard cough" (Agarwal et al., 2017).

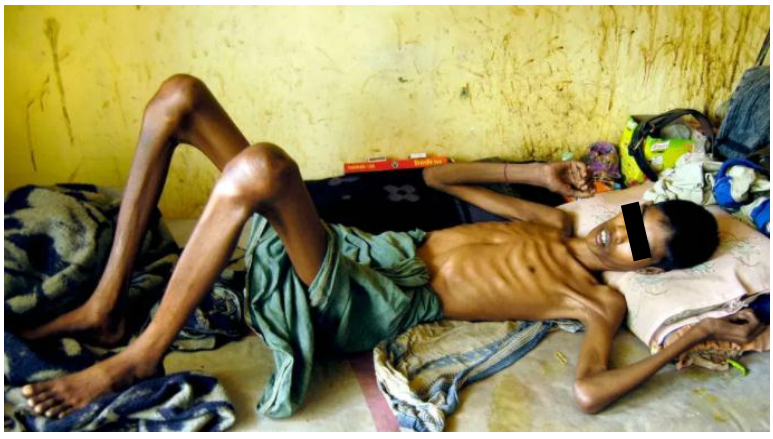


Figure 1. Tuberculosis patient consumed by the infection.

To describe the disease under a single heading, Johann Lukas Schonlein, a German physician coined the term "tuberculosis" in 1834 which was later used by Jean Antoine Villemin, Robert Koch and Herman Brehmer (Ferlinz, 1995). In 1868, Villemin, a French military doctor, first described the disease by inoculating the rabbits from man and cattle thus successfully transmitting the infection. He further demonstrated that scrofula (tuberculous cervical lymphadenitis) and pulmonary tuberculosis are separate manifestations of the same disease (Grange, 1984). Robert Koch, a German Physician and Microbiologist, isolated the organism and later announced the discovery of the tubercle bacilli in Berlin on 24 March 1882 (Schmidt, 1983).

Tracing back to its origin, tuberculosis is a remarkably ancient disease and is believed to survive over 70,000 years (MacDonald & Izzo, 2015). Their existence in Ancient Egypt arises from three sources namely, literature, art representation and remains of human. Egyptian mummies from 2400 BC disclose skeletal deformities that are characteristic of tuberculosis. Strong evidences suggest tuberculosis plaguing the Nile Valley and had been a significant cause of death in Ancient Egypt (Morse et al., 1964). The first documented written information describing Tb in India was as early as 3300 years ago and 2300 years ago in China (Morse, 1967; Brown, 1941). In 1912, the All-India Sanitary Conference which was held at Madras (now Chennai) passed a resolution through which the problem of Tb in India was first recognized (Mahadev and Kumar, 2003). The incorporation of Anti-tuberculosis funds such as King George V Thanks Giving in 1929 with King Emperor's fund led to the birth of the Tuberculosis Association in India in 1939 (Report of the Health Survey and Development Committee, 1959-1961).

Even though more than a century of Tb discovery by Robert Koch has passed, mankind is unable to eliminate this deadly disease till date.

1.2. Management of Tuberculosis during the past years

The attempt to control Tb has been a long battle. Literature survey shows that various attempts have been undertaken at different levels to control Tb as follows:

1906: Establishment of open-air sanatoriums in the Himalayas for the isolation and treatment of Tb in Tilonia near Ajmer (Sisodia et al., 2011).

1908: Another open-air sanatorium established in Almora (Sisodia et al., 2011).

1917: Establishment of Tb dispensary in Mumbai (Sisodia et al., 2011).

1948: Setting up of BCG Vaccine Laboratory, King Institute, Chennai, Tamil Nadu. In the same year, the first BCG vaccination programme was conducted in India (Central Tb Division. Tuberculosis Control India).

1951: Extension of mass BCG Vaccination was approved (Central TB Division. Tuberculosis Control India).

1962: BCG vaccination happens to be a part of the National Tuberculosis Control Programme (NTCP) (Central TB Division. Tuberculosis Control India).

1956: Establishment of Tuberculosis Chemotherapy Center (TRC), Chennai, renamed as National Institute for Research in Tuberculosis (Sisodia et al., 2011).

1961: National Tuberculosis Institute (NTI), Bangalore (Sisodia et al., 2011).

1962: National Tuberculosis Programme (NTP) was launched by the Govt. of India involving BCG vaccination and Tb treatment (https://www.nhp.gov.in/revised-national-tuberculosis-control-programme_pg)

1992: Joint review of NTP by GOI, WHO and SIDA (Swedish International Developmental Agency) where shortcomings of the programme were realised (Sisodia et al., 2011).

1993: WHO declared Tb a Global Health Emergency (World Health Organization, 1993).

1997: RNTCP (Revised National Tuberculosis Control Programme) started as a national programme to replace NTP. The internationally recommended DOTS (Directly Observed Treatment Short-Course) was officially launched. This indicates observation of patients during medication especially during the intensive phase (first two months of treatment) to guarantee the uptake of right drug combination for the appropriate duration. This programme strives to control Tb by interrupting the transmission chain using quick detection, identification and cure via. direct supervision of therapy thereby preventing the development of drug resistant forms in the community. The standard anti-Tb drugs used are isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin for a period of 6-8 months. (WHO. Communicable Diseases Cluster, 1999; Davies, 2003).

2000: Poor outcomes in patients infected with Mtb resistant to isoniazid and rifampicin (multi-drug resistant Tb) upon standard regimen treatment (DOTS) were

realized. Thus, WHO and partners launched DOTS-Plus for the appropriate management of multi-drug resistant Tb, this includes second line agents that are more expensive, more difficult to administer, and often poorly tolerated (Mitchison & Nunn, 1986; Espinal et al., 2000; WHO. Treatment of Tuberculosis,1997).

2007: Programmatic management of drug resistant tuberculosis (PMDT) was launched under revised national Tb control programme (RNTCP), whose role is defined as "all associated functions related to providing services based in the Tb strategy in order to achieve the targets set for drug-resistant Tb in the Global Plan to Stop Tb 2011–2015" (Companion Handbook- WHO, 2014).

World Health Organisation's (WHO) End Tb Strategy has set the target for Tb elimination globally at 2035. However, India has set its own target at 2025 (Kumar, 2017; Uplekar et al., 2015).

1.3. Evolution in the Drugs used for the treatment of Tuberculosis

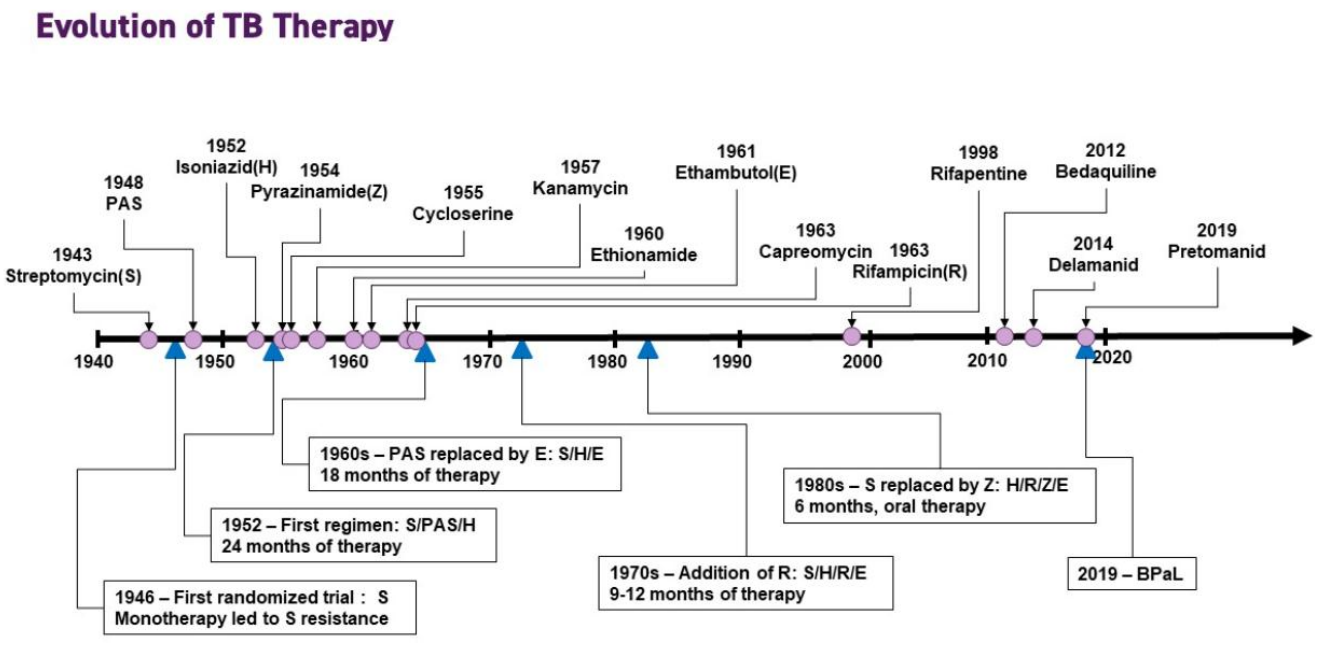


Figure 2. Timeline in the evolution of drugs used to treat tuberculosis

(Source: Tballiance.org)

In the earlier days, Tb was treated using a single drug (monotherapy) which resulted in drug monoresistance. Various antibiotics have been discovered in rapid succession over the past many years (Figure 2) and have proven clinical efficacy towards treatment of tuberculosis. Due to the existence of monoresistance, standard initial drug treatment regimens have been designed and introduced, which encompasses the simultaneous utilization of four drugs (combination therapy), for achieving better outcome. Despite the functional combination therapy, highly resistant Mtb strains have emerged in many settings. Studies analysing the emergence of drug resistant forms of Tb reported that multi-drug resistance is a 'man-made' phenomena that could possibly arise from various factors such as – poorly administered therapy, non-compliance of the patient, poor quality or counterfeit drugs, improper absorption of the drugs through the gastro intestinal tract or due to the presence of the bacteria in protected compartments of the body like the lung cavities where antibiotics cannot penetrate properly, or where their activity is limited by pH or some other functional constraints (Gillespie 2002; Mitchison 1998; Warner 2006). To sum up, tuberculosis is nearly always curable, provided the patients are administered the right drugs with a right dosage for the right duration at the right time without any interruption.

1.4.Various categories of existing and novel drugs for Tuberculosis

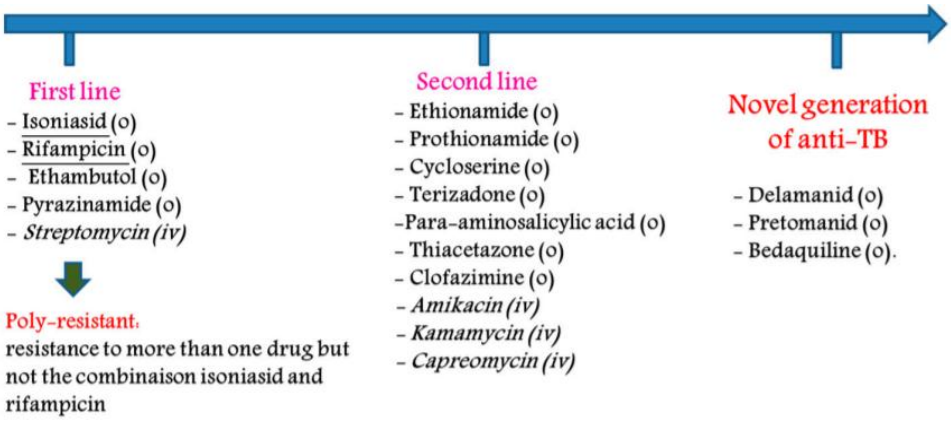


Figure 3. Drugs to tackle tuberculosis (o: oral; iv: intravenous administration)

(Source: Mignani et al., 2018)

As per the Global Tuberculosis Programme, World Health Organization, the types of Drug-resistant Tb are as follows:

- **Mono-resistance Tb:** resistance to only one first-line anti-TB drug.
- **Poly-resistance Tb:** resistance to more than one first-line anti-TB drug, other than both isoniazid and rifampicin.
- **Multidrug resistance (MDR) Tb:** resistance to two of the most potent anti-TB drugs, isoniazid and rifampicin.
- **Extensive drug resistance (XDR):** Along with multidrug resistance, resistance to any fluoroquinolones, and at least one of the three second-line injectable drugs (capreomycin, kanamycin and amikacin).
- **Rifampicin resistance (RR):** Detected using phenotypic or genotypic methods, with or without resistance to other anti-TB drugs. It includes any resistance to rifampicin, in the form of mono-resistance, poly-resistance, MDR or XDR.

1.5. Mechanism of action and mode of drug resistance of first line ATT drugs

Isoniazid (INH): INH is non-toxic to the bacteria, however is a prodrug activated by the mycobacterial enzyme *kat*G (Zhang et al., 1992). It targets the mycolic acid synthesis and thereby inhibits cell wall production (Zhang, 2005). However, INH activity is deemed to be limited to actively growing tubercle bacilli and not against bacilli under anaerobic environment (Nath & Ryoo, 2013). The genetic background related to INH resistance is rather complex in which several genes have been associated such as *kat*G, *inh*A, *ahp*C and *kas*A. Among these, the two most common mutations are associated with *kat*G and *inh*A, where 50-90% INH-resistant strains mutate at codon 315 (Ser→Thr) of the *kat*G gene and 20-35% mutates at *inh*A (Zhang et al., 1992; Ando et al., 2010).

Rifampicin (RIF): It is bactericidal and the most effective anti-Tb drug currently available. It has the ability to act against both actively growing and slowly metabolizing bacilli (Mitchison, 1979). It inhibits DNA-dependent RNA polymerase leading to quelling of RNA synthesis resulting in cell death. This occurs as a result of the binding of the drug in the polymerase subunit, facilitating direct blocking of RNA elongation. Previous studies have frequently stated the presence of upto 95% of the Rifampicin Resistance (RR) to be associated with the *rpo*B gene mutations in the 81-bp hotspot region (Rifampicin Resistance Determining Region, RRDR, codons 507-533) (Kaur et al.,2016; Reddy et al.,2017). The SNPs involved are single amino

acid substitutions (93%), in-frame deletions (4%), and insertions (3%) (Telenti et al., 1993; Valim et al., 2000; Blanchard, 1996).

Ethambutol (EMB): It is a bacteriostatic agent that hinders with the synthesis of cell wall arabinogalactan (AG) and lipoarabinomannan and is active only for growing bacilli (Takayama & Kilburn, 1989; Jackson et al., 2013). It also synergizes with other drugs including rifamycins, aminoglycosides and quinolones by increasing the permeability of mycobacterial cell wall and is mainly active during the intensive phase (first 2 months of treatment) (Onyebujoh et al., 2005). Mutations in the *emb*CAB locus are associated with ethambutol resistance where, *emb*A and *emb*B genes are associated with arabinogalactan synthesis while *emb*C is involved in lipoarabinomannan synthesis (Mikusová et al., 1995; Jankute et al., 2012). Mutations at codons 306, 406 and 497 are the most commonly detected within the *emb*B gene (Wang et al., 2016).

Pyrazinamide (PZA): The pyrazinamidase (PZase) enzyme converts this pro-drug to pyrazinoic acid (active form) (Konno et al., 1967). Unlike other Tb drugs, PZA kills non-replicating persisters (dormant/sermi-dormant bacilli) making it an ideal drug for treating drug-susceptible as well as drug-resistant Tb (Zhang et al., 2013). A key feature of pyrazinamide is its ability to exhibit its activity in acidic environments such as macrophages (Mitchison, 1985). PZA is only active against *M. tuberculosis* at acid pH (e.g. 5.5) (McDermott & Tompsett, 1954). It exerts its maximum effect during the early intensive phase (2 months of therapy) (Starke, 2012). Few genes have been implicated in the resistance towards PZA drug. These are *pnc*A gene encoding the enzyme pyrazinamidase, RpsA (ribosomal protein S1) and a recently identified *pan*D gene (Zhang et al., 2014).

Streptomycin (STM): Streptomycin was the first drug used in the treatment of Tb. It is an aminoglycoside antibiotic originally isolated from the bacteria *Streptomyces griseus* (Daniel, 2005). It is bactericidal and acts by interfering with ribosomal protein synthesis (Ball et al., 1975). It kills only the actively growing tubercle bacilli and inactive against intracellular forms (Mitchison, 1985). Unlike pyrazinamide, it is active in neutral or alkaline conditions (Onyebujoh et al., 2005). Mutations for streptomycin resistance were found in 16S rRNA (*rrs*) and ribosomal protein S12 (*rps*L) genes (Springer et al., 2001; Nair et al., 1993). The most common mutations

reported are clustered around nucleotides 530 and 915 (Finken, 1993), and at 514 for *rrs* gene, accounting for about 15.8% of the resistance and at codon 43 and 88 for *rps*L, accounting for about 63.3% (Wang et al., 2019).

1.6. Taxonomy and Classification of *Mycobacterium tuberculosis*

Kingdom **:** Bacteria
Phylum **:** Actinobacteria
Order **:** Actinomycetales
Suborder **:** Corynebacterineae
Family **:** Mycobacteriaceae
Genus **:** Mycobacterium
Species **:** *Mycobacterium tuberculosis*

1.7. Morphology, cell characteristics and Pathogenesis of Mtb infection

Mtb are irregular rods (0.3–0.5 μm diameter) of variable length (Wayne & Kubica, 1986). It is an obligate or facultative intracellular bacterium (Vandal et al., 2009). It has a genome size of 4.4 million base pairs encoding 4,000 genes (Cole et al., 1998). They are acid-fast bacterium detectable by Ziehl–Neelsen stain and are not Gram-positive or Gram-negative. (Koch & Mizrahi, 2018). Unlike other bacteria, the cell wall of Mtb has a thick peptidoglycan (PG) layer similar to that of gram-positive cell and an outer thick waxy layer called mycolic acid (MA) that mimics gram negative cell. It also has a polysaccharide layer called arabinogalactan (AG) which serves to connect peptidoglycan with the outer mycolic acid layer (Maitra et al., 2019) (Figure 4). The MA is an important virulence factor and also helps to evade the host innate immunity (Forrellad et al., 2013). The distinctive cell wall structure allows low permeability for drugs and has many efflux pumps (Jarlier & Nikaido, 1994; Brennan & Nikaido, 1995).

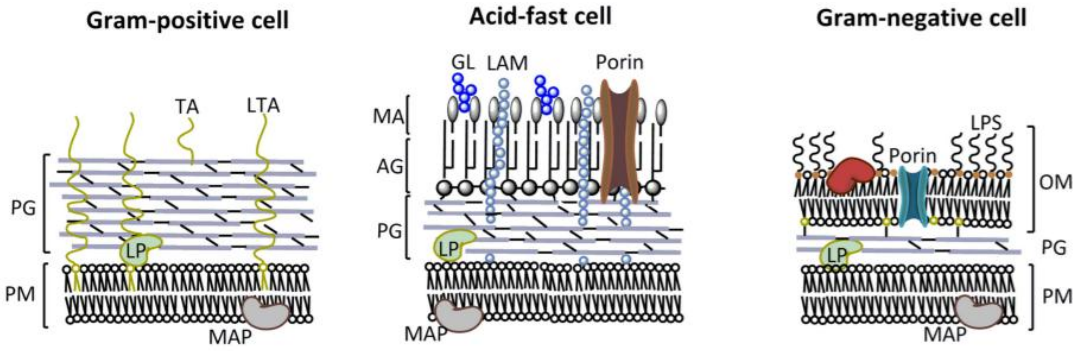


Figure 4. Comparison between acid-fast and non-acid-fast cell wall

(Source: Maitra et al., 2019)

Transmission

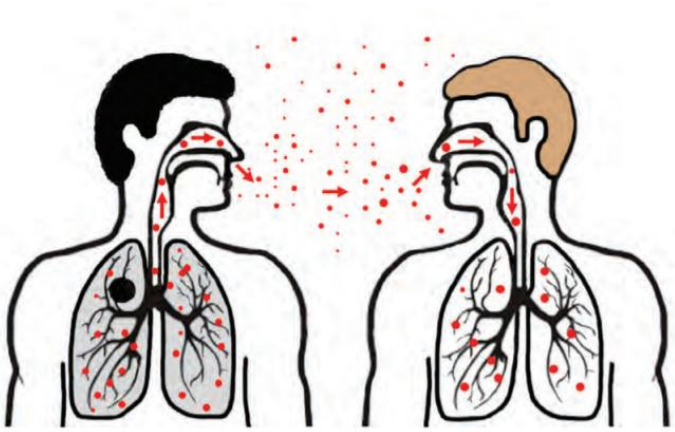


Figure 5. Transmission of pulmonary tuberculosis from one person to another

The act of coughing by an individual with active pulmonary Tb leads to the formation of mycobacteria-laden droplet nuclei, which can persist in the air for several hours. This act of coughing or sneezing may also expel the bacteria. Tb is transmitted when the infectious droplet nuclei that contains the viable bacilli (aerosol) are inhaled; it then goes down into the respiratory tract and are ingested by alveolar macrophages where the bacilli are either destroyed or inhibited (Figure 5). However, few may replicate intracellularly and get released when macrophages die (https://www.cdc.gov/Tb/education/corecurr/pdf/chapter2 .pdf).

Usually, phagocytosis begins with the binding of pathogens to the receptor protein on surface of the phagocytic cells. After a series of interaction, the phagosome fuses

with the cell's lysosomes, forming a phagolysosome that results in acidification and finally destruction of the pathogens (Casem, 2016).

On the other hand, various studies have reported that Mtb shows its superiority to other bacteria by its ability to inhibit the fusion of phagosomes with lysosomes thereby suppressing the acidification and enhancing their survival, replication and persistence of the bacilli in a low acid environment (pH~36.2) within the macrophage (Sturgill-Koszycki et al., 1994; Armstrong & Hart, 1971). Upon survival, these bacilli spreads by either lymphatic channels or through haematogenous route and settle to more distant anatomical sites, thus causing infection (Figure 6). This entire process triggers the immune system for a systemic response (Smith, 2003).

Certain factors like bacillary load and the cough frequency of the index case influences the chance of Tb transmission. Determinants of infectiousness is attributed to high bacillary load indicated by (a). Sputum smear-positivity (b). Presence of cavitation in the lungs on chest radiograph (c). The vicinity and exposure duration (Acuña-Villaorduña et al., 2018). The infectious dose of tuberculosis had been reported to be only a few bacilli lodging in the distal alveoli of the lungs. However, an active form of the disease is clinically evident only when the bacterial population reaches 10^8-10^{10} organisms (Shimao 1987; Gillespie 2002). Initiation of the right drug with the right dosage can effectively reduce the transmission (Dharmadhikari et al. 2014).

Approximately 5 - 10% of the infected individuals develop the disease within 2 - 5 years after infection (Anderson et al., 2018), while for the rest, the innate immunity reacts by either fully eliminating the infection or leading to a state of persistency called latent-Tb infection without clinical evidence of active disease thus, becoming a potential reservoir for active tuberculosis upon waning of immunity (Simmons et al., 2018; WHO. Latent tuberculosis infection, 2018). The risk of infection among Tb household contacts is about 30 % (Singh et al., 2005).

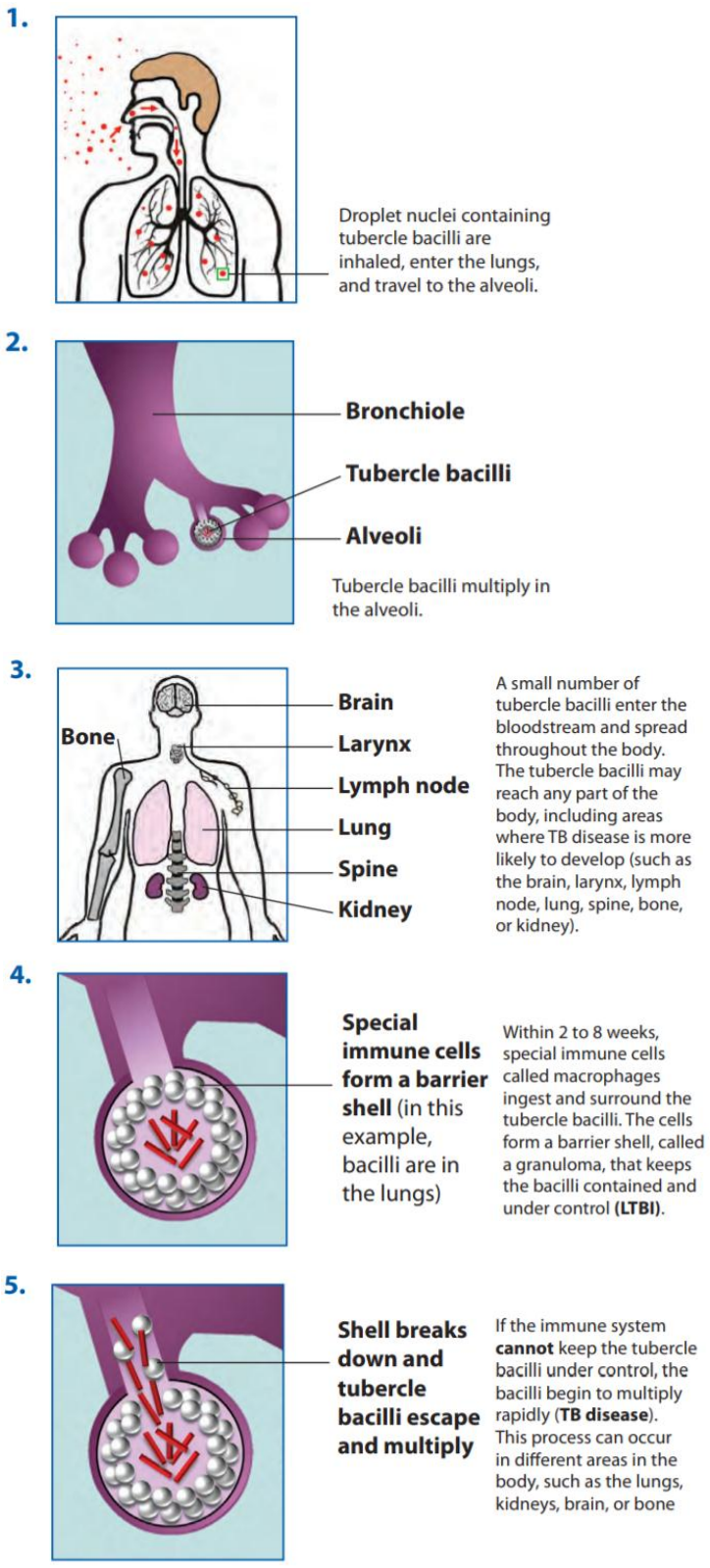


Figure 6. Pathogenesis of tuberculosis

(Source: Transmission and Pathogenesis of tuberculosis. CDC. https://www.cdc.gov/Tb/education/corecurr/pdf/chapter2.pdf)

1.8. Risk factors of Tuberculosis infection

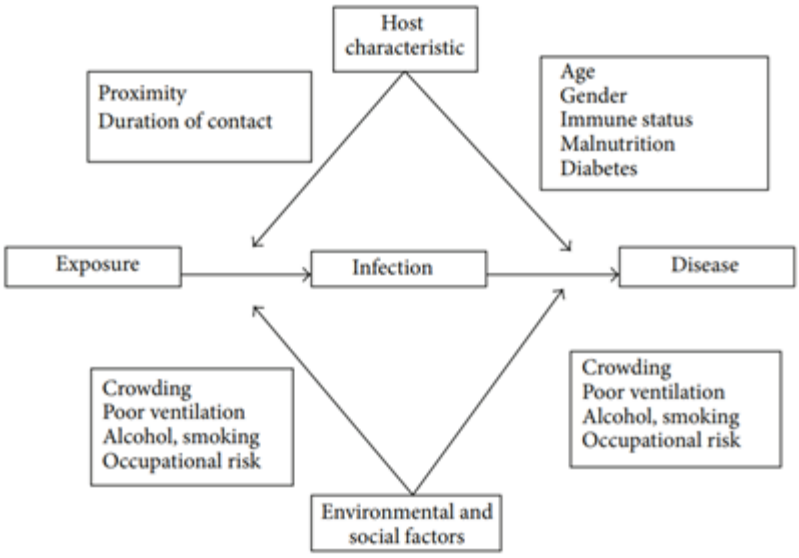


Figure 7. Risk factors for tuberculosis infection and disease *(Narasimhan et al., 2013)*

Tuberculosis is a multifactorial disease. Well established risk factors of tuberculosis can be summarised as: (a) bacillary load and proximity to an infectious case (b) immunosuppressive state, diabetes, malnourishment, health-care workers, extremes of age, socioeconomic and behavioural factors, tobacco, alcohol and demographic (ethnic) factors (Narasimhan et al., 2013) (Figure 7).

Bacillary Load – Smear positive cases are more infectious (Opie, 1926; Shaw & Wynn-Williams, 1954), wherein a new smear positive case can lead to two new cases, one of which is likely to be infectious. In addition, a patient with an untreated sputum positivity has the ability to infect approximately 10 individuals/year (Maher, 2009; Nadia & Enarson, 2003).

Proximity to infectious case of Tb - Studies have shown that close contacts of infectious Tb cases (household contact, health care workers etc.) are at a high risk of being infected and are likely to develop the active disease (Narain et al., 1966; Joshi et al., 2006; Devadatta et al., 1970; Andrews et al., 1960; Kamat et al., 1966).

Immunosuppressive state – HIV Co-infection is the most frequent risk factor that directly contributes to the active Tb disease (Corbett et al., 2003), where cell mediated immunity plays a main role. HIV infection weakens this cell mediated immunity resulting in increased risk of contracting the infection as well as causing dissemination of the infection leading to extrapulmonary Tb. Either way, HIV co-infection worsens the severity of Tb diseases while additionally, the replication of the HIV virus accelerates in Tb infected individuals (Collins et al., 2002; Sharma et al., 2005).

Diabetes – Diabetes directly impairs both innate and adaptive immunity and hence have a chance of threefold increased risk of developing Tb (Jeon et al., 2008). Diabetic mice have shown high bacillary load when infected with the bacilli (Martens et al., 2007).

Tobacco smoking – Studies have reported the presence of a two-fold increased risk of Tb infection among smokers (Lin et al., 2009) and active smoking increases the risk of extrapulmonary TB (Maurya et al., 2002; Lin et al., 2007) as well as its recurrence and severity (Yen et al., 2014; Chuang et al., 2015). Previous studies have reported that alveolar macrophages of smokers are impaired and secretes lower levels of cytokines which are necessary for early responses to pathogens (McCrea et al., 1994). The biological explanations attributable for the susceptibility to pulmonary Tb due to smoking tobacco are reduced phagocytic ability of alveolar macrophages (Sopori et al., 2002), negative influence on mucociliary clearance (Houtmeyers et al., 1999) as well as nicotine induced lymphopenia (Arcavi & Benowitz, 2004).

Alcohol consumption – Meta-analysis have shown that consumption of alcohol is associated with higher risk of Tb (Imtiaz et al., 2017) due to alteration in the signalling molecules responsible for production of cytokines (Szabo, 1997).

Socioeconomic and behavioural factors – Tb affects the poorest of the poor where people with lower socio-economic status are subjected to several risk factors including malnutrition, crowding, limited safe cooking practices (Gupta et al., 2004; Feleke et al., 2019). An advanced state of the Tb disease as well as extra-pulmonary Tb was found among unemployed individuals and further, the proportion of smokers and alcohol abusers were also higher among the unemployed (Przybylski et al., 2014).

Young age – Children are at a high risk of developing Tb infection where young children in the age group of 0-4 are highly susceptible to the infection due to vulnerability of their immune system (Marais et al., 2007). Studies have shown that children below 2 years of age contract Tb infection from household source case while >2 years contract from the community. The highest risk for Tb related deaths occurred during infancy and gradually declines with increasing age (Ellman et al., 1954; Marais, 2009).

Healthcare Workers (HCW) – Not all hospitals or Tb isolation wards will have a desired space and a proper cross ventilation system. Hence, HCW are at higher-than-average risk for contracting Tb infection due to the frequent direct contact with patients as well as sharing room space and air with infectious patients (Baussano et al., 2011).

Single marital status – Various studies from African countries (Lienhardt et al., 2005; Gustafson et al., 2004) China (Wang et al., 2005) and Poland (Przybylski et al., 2014) have reported single marital status as an independent factor for contracting Tb. The plausible mechanism of this association could be that being single suggests absence of family support and susceptibility to Tb could increase at times due to psycho-social stress (Cain et al., 2008).

BMI – Lower BMI levels have been attributed as an independent factor for active Tb (Lu et al., 2021). Individuals with low-BMI may predispose to Tb re-activation in the lungs which could be explained by the presence of a congenital bullae in the apex of the lungs in 15% of the population which can enlarge in low BMI males (Casha & Scarci, 2017).

BCG vaccination – The BCG vaccine existed for more than 80 years and is still the most commonly used licensed vaccine worldwide. Various study trials have shown that it had a protective efficiency of 60-80% in children with acute forms of Tb, especially meningitis (Rodrigues et al., 2011; Trunz et al., 2006). However, geographical variations in their efficacy against pulmonary diseases exists (Colditz et al., 1994; Lienhardt et al., 2005; Zodpey et al., 2007). In a trial conducted in UK, the efficacy of BCG vaccination in adolescents during the first 5 years after the uptake of vaccine was around 84%, declining to 68% after 5-10 years since vaccination and to 63% after 10-15 years since vaccination (Hart & Sutherland, 1977). A study from

Ireland reported higher incidence of Tb among BCG unvaccinated population as compared to vaccinated population (Sweeney et al., 2019).

Cross-ventilation – Proper ventilation system has the potential to dilute the airborne pathogens and potentially reduce the risk of transmission of infections to individuals residing in a common space (Lygizos et al., 2013).

Condition of housing – An earlier study have observed the influence of damp soil and damp houses in the predisposition of tuberculosis (Kober et al., 1915). A South African study had also reported that households which are damp are a significant risk factor contributing to childhood tuberculosis (Jafta et al., 2019).

Water source – A review on tuberculosis transmissions by waste waters had raised concerns as well as addressed the possibility of tuberculosis transmission where sewages from tuberculosis sanatorium contained the bacteria that is capable of producing the disease when infected in guinea pigs (Greenberg et al., 1957). Few reports have focused on water supply sources indirectly accounting to TB infection (Steentoft et al., 2006). However, though the modes of transmission of tuberculosis have been well elucidated, a possible chance of indirect-infection through consumption of contaminated water have been found (Sailo et al., 2022).

1.9. Genes responsible for drug resistance in tuberculosis and lineages of Mtbc

Tuberculosis in humans and animals is caused by *Mycobacterium tuberculosis complex* (Mtbc) comprising of homogenous mycobacteria species (Pesciaroli et al., 2014). Resistance to first line anti-tuberculosis drugs like Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), Ethambutol (EMB), Streptomycin (STM) has been attributed to specific mutations in specific genes (Tekwu et al., 2014). For instance, several mutations with varying degree of association have been reported for *rpo*B (RIF resistance) (Yue et al., 2003). With the advent of GeneXpert, where primary screening involves PCR-based detection of Mtb DNA as well as a targeted SNP based PCR in the *rpo*B gene of Mtb, the rationale behind using *rpo*B SNP in the primary detection stage is that *rpo*B serves as a surrogate marker for Rifampicin since >90 % of Rifampicin resistant isolates also exhibited resistance

towards Isoniazid (Boehme et al., 2011). Thus, a positive result would mean confirmation of both tuberculosis as well as multi-drug resistant infection.

The Mtbc phylogeny strictly includes human pathogens *M. tuberculosis* sensu stricto (Mtb) and *M. africanum (*Brites & Gagneux, 2012*)* that are classified into different lineages according to their phylogeographic structure, which is hypothesized to reflect adaptation to the human populations in which they cause disease. Animal-adapted Mtbc strains includes *M. bovis* (Clifton-Hadley et al., 1995), *M. microti* (Smith et al., 2009), *M. pinnipedii (*Kiers et al., 2008*)*, *M. caprae* (Schoepf et al., 2012*)*, *M. orygis* (Gey et al., 2012), *M. mungi* (Alexander et al., 2010), *M. suricattae*. Zoonitic transmission of Mtbc to humans mainly involves *M. bovis.* The association of lineages with specific host populations led to the nomenclature of Mtbc lineages mirroring geographic regions in which they predominate (Gagneux et al., 2006). *Mycobacterium tuberculosis* strains harbour genomic insertions or deletions [Large Sequence Polymorphism (LSPs)], otherwise known as Regions of Difference (RDs). RDs are polymorphisms where the mutations occur only once in the evolutionary history of a species. They are unique, irreversible and do not show homoplasy (Hirsh et al., 2004) as a result of which RDs are contemplated as markers used to identify Mtb to its respective lineages (Tsolaki et al., 2004). Mtbc phylogenies constructed using whole genome sequence data were in agreement with those generated using LSPs (Comas et al., 2010). Human-adapted Mtbc strains are grouped into seven lineages which are classified as “ancient” (lineages 1,5,6 & 7) or “modern” (lineages 2,3 & 4) according to the presence or absence of the tuberculosis-specific deletion (TbD1) region (Figure 8). While modern lineages (lineages 2 & 4) have spread globally, most ancient lineages (lineage 5, 6 & 7) remain geographically restricted to specific regions (de Jong et al., 2010). Other lineages have recently been identified: Lineage 8 (African Great Lakes region), Lineage 9 (East/West Africa). (Nebenzahl-Guimaraes et al., 2016; Ngabonziza et al.,2020; Coscolla et al., 2021). Previous studies have also shown that different lineages of Mtb varied with regard to the site of disease (Click et al., 2012), clinical presentation and duration of disease (Thwaites et al., 2008), it’s progression (Thwaites et al., 2008) and risk for relapse (Burman et al., 2009).

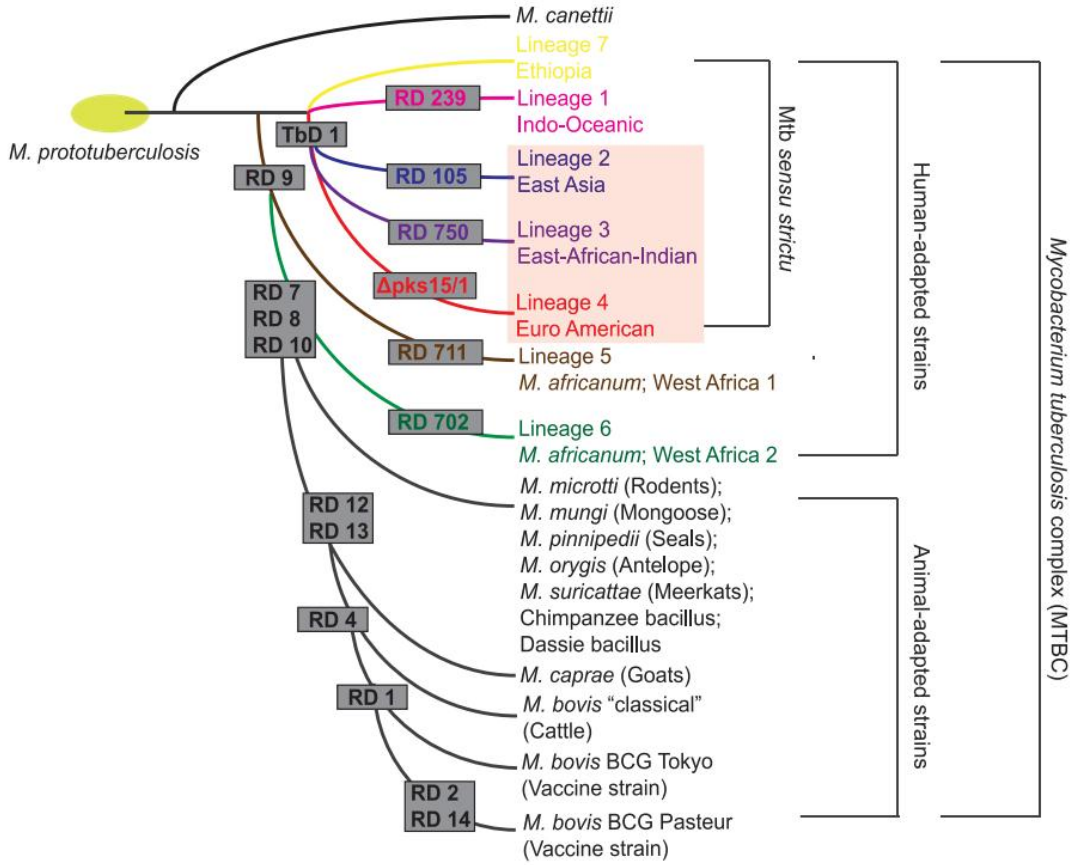


Figure 8. Evolutionary relationship between selected members of the Mtbc *(Tientcheu et al., 2017. Eur. J. Immunol, 47: 432–445).*

1.10. Tuberculosis burden: Globally and in India

Tuberculosis remains as a crucial infectious disease and a public health concern worldwide even today. Globally, in 2019, an estimated 10 million people were infected with *Mycobacterium tuberculosis*. Tb deaths among the HIV negative was estimated to be around 1.2 million and an additional ~2 lakhs death among the HIV positive individuals. Since 1998, India falls among the Tb "high burden" countries accounting for 87% of the world's cases and is among the three countries with the highest prevalence of global MDR-Tb burden. The estimated incident cases per year, showed that India followed by Indonesia are the two countries that contributes largely to the global increase in Tb. The Tb incidence in India was 28 lakhs which accounts for about a one-fourth of the world's cases (Global tuberculosis report, 2020).

1.11. Lifestyle of the Mizo tribe and the burden of Tuberculosis in Mizoram

Mizoram is a hilly tribal state in the north eastern part of India sharing borders with Myanmar and Bangladesh, contributing only about 0.09% of the Indian population (Census of India, 2011). Within the state, community welfare is given great importance and different forms of community services (*Hnatlang*) are held in various localities in which the people actively participate.

They also believe and practice a moral code (*Tlawmngaihna*), thus making them helpful and courteous to other people. Another common practice in both the gender is consumption of tobacco in various forms (smoking, tobacco-infused water aka '*Tuibur*', snuff tobacco mixed with lime) as well as alcohol among the men (Ghatak et al., 2016). The unique lifestyle highlights the Mizo tribe as compared to other Indian tribes (Pachuau et al., 2022). Small scale agriculture, Jhum Cultivation, dairy farming and livestock are the main occupation of the people apart from agro forest produce as the minor trade.

Mizoram with a population of 12.5 lakhs, has notified 2944 Tbpatients (both public and private sectors) in 2019. Mizoram also has 2575 (86%) Tb patients with known HIV status (India TB Report, 2020). Immunodeficiency caused by HIV is being regarded as the most significant risk factor for tuberculosis. As per the National AIDS Control Organization & ICMR-National Institute of Medical Statistics 2018, Mizoram had recorded 2.04% adult HIV prevalence which is the highest in the country.

Mizoram implemented RNTCP and Programmatic Management of Drug Resistant Tuberculosis (PMDT) in 2003, which is the former DOTS Plus. Sputum direct microscopy (Ziehl Neelsen stain) was the gold standard for Tb diagnosis. There are eleven districts in Mizoram of which 35 RNTCP direct microscopy centers are located in eight districts (https://health.mizoram.gov.in/page/tuberculosis#main-navbar). In spite of the tremendous efforts and progress achieved by National Tuberculosis Eradication Program (NTEP) (erstwhile Revised National Tuberculosis Control Program), tuberculosis is still a burden in certain peripheral areas of India such as Mizoram.

In Northeast India, there are only two places among the list of certified laboratories for performing Drug Susceptibility Testing (DST) and Line Probe Assay (LPA): IRL Guwahati, Assam for DST and LPA, Nazareth Shillong, Meghalaya for LPA (India TB Report, 2019). Currently, sputum smear microscopy using Ziehl Neelsen stain and Cartridge Based Nucleic Acid Amplification Test (CB-NAAT) otherwise known as GeneXpert are the only diagnostic means implemented in the state of Mizoram. The equipment is being utilized by Mizoram since the end of 2014 and from 1 September 2017, it is being used as a Universal Drug Susceptibility Test which is a nationwide programme to test for resistance to the first-line Tb drugs, especially Rifampicin. Culture and LPA are outsourced to neighbouring state such as Assam (Guwahati). Due to the unavailability of culture facility, this study was taken up to characterize local strains, which will help establish whether initial infection of individuals could result from a heterogeneous mixture of Mtb strains as well as to establish the genes and their SNPs responsible for drug resistance. The extent of heterogeneity in the initial acquired infections might prove great importance in the designing of informed treatment regimen for newly diagnosed tuberculosis patients.

Information on the underlying genetic pattern of drug resistance can provide insights into the pattern of mutation accountable for rifampicin resistance over the past years. A PubMed and Google Scholar search strategy using keywords such as Northeast India, Xpert Mtb/RIF, Genexpert, *rpo*B gene, Mizoram RR-Tb did not provide any information on *rpo*B gene and other gene mutations from North East India. Due to the unavailability of published data on the magnitude of RR-Tb from the state of Mizoram using molecular diagnostic tools, this study was conducted.

Chapter 2: Objectives

Objective 1: To determine the epidemiological risk factors of tuberculosis in Mizoram.

Objective 2: To determine the distribution and frequency of common mutations in *rpo*B gene of *Mycobacterium tuberculosis* detected by Xpert Mtb/RIF and identification of household location of Rifampicin Resistant-Tb cases in Mizoram.

Objective 3: Screening of antibiotic sensitivity of *M. tuberculosis* strains isolated from patients.

Objective 4: To determine gene specific mutations for drug resistance and the lineage of *M. tuberculosis* strains in Mizoram.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1. Ethical approval of the study

The ethical approval for this study was obtained from the Institutional Ethics Committee (IEC), Civil Hospital Aizawl (B.12018/1/13-CH (A)/IEC/63, dtd. 28-03-2017), Mizoram. Written consent was obtained from all the participants in this study.

3.1.2. Study design

The patients recruited for this study were from the District Tb Center (DTC), Falkawn and Synod Hospital, Durtlang, Aizawl, Mizoram. Different approaches were utilized to carry out the objectives:

Objective 1: Patients who were microbiologically confirmed or clinically diagnosed for Tb (both pulmonary and extrapulmonary) were consecutively enrolled from December 2017 to June 2020 for obtaining the risk factors of tuberculosis. The mode of diagnosis comprises any of the following tests: Chest X-ray, ZN stain (microscopy), Mantoux, ADA, CT scan, Biopsy, FNAC, Ultrasound, GeneXpert (CB-NAAT), Culture (MGIT) and/or Line Probe Assay (LPA) which is outsourced to neighboring states' Intermediate Reference Laboratory (IRL). Patients' written consent were obtained and were interviewed using a well-designed questionnaire for collection of data on Tb symptoms, socioeconomic, demographic, food and lifestyle factors.

Objective 2: Data were evaluated retrospectively from archived results for all the types of specimens received and tested using Xpert Mtb/RIF assay from December 2014 to May 2021. Samples detected positive for the presence of Mtb along with Rifampicin resistance were incorporated in the study.

Objective 3: AFB smear positive/ GeneXpert positive sputum samples were collected from Pulmonary Tb patients for liquid culture from June 2019 to May 2020. Antibiotic sensitivity against first line Tb drugs was performed.

Objective 4: GeneXpert Mtb/RIF was carried out on phenotypically rifampicin resistant and susceptible isolates. Conventional PCR using five antibiotic resistance genes, sanger sequencing and LSP-PCR was also carried out.

3.1.3. Inclusion and Exclusion criteria

Inclusion criteria for the recruitment of patients in the study:

1. **Objectives 1, 3 & 4:** Written informed consent and willingness to participate in the study, ≥15 years of age.
2. **Objectives 1, 2, 3 & 4:** Both genders, new and previously treated cases included.
3. **Objective 2:** All ages included (neonates, children, young adults and old age).
4. **Objectives 1&2:** Pulmonary and extrapulmonary samples/data included.

Exclusion criteria for the patients' recruitment:

1. Patients not willing to participate.
2. Extrapulmonary, HIV positive and diabetes cases (Objectives 3 & 4).
3. Pregnant ladies (Objectives 1, 3 & 4).

3.2 Methods

3.2.1. Methods for Objective 1

The case-control study was performed with patients visiting District Tb Center, Falkawn and Synod Hospital, Durtlang, Aizawl, Mizoram which are visited by TB patients from all districts of Mizoram. A total of 400 Tb patients and 840 healthy controls were enrolled and the healthy controls were with no previous history of Tb, no immunocompromised state (HIV) and non-diabetic. The estimated sample size for a robust statistical analysis (http://www.raosoft.com/samplesize.html) was 385 for a population size of 10,97,206 (Census of India, 2011).

The participants were categorized based on gender (female and male), age (15-45 years and >45 years), marital status (married- currently living with spouses and single- unmarried, widows, widowers, divorcees). Body Mass Index (BMI) score of 18.5 -24.9 was classified as normal weight, <18.5 as underweight, 25-29.9 as overweight, > 30 as obese (www.thecalculatorsite.com). For convenience, the BMI was classified as: Ideal (normal weight) and non-ideal (underweight, overweight and obese).

Education level was grouped into two classes ('Matriculation or class 10' and 'higher secondary & above'), Occupation ('employed' and 'unemployed') and Family income ('> INR 30,000 per month' and '< INR 30,000 per month'). The housing condition ('receiving proper sunlight during the day' and 'moist or damp without any proper sunlight'), Number of people living under one roof ('1 to 6 members' and '>6 family members'), physical activity in the form of exercise ('Regular' and 'Irregular'), Water supply source ('Govt. municipal' and 'Other sources'- includes river water and/or collection of spring water) were categorized into two classes each. The Govt. Municipal water is pumped from rivers and treated to make it potable.

The following factors/variables were classified into 'Yes' or 'No': dwelling in a crowded house; cross-ventilation system in house; participation in mass gathering; travel history; contact history with pulmonary Tb; having diabetes or HIV (checked at the Tb Clinic for random blood sugar by Accu-chek and HIV Tri-Dot); BCG vaccination (via. verbal response from participants); Consumption of tobacco products (smoke or smokeless forms - snuffed tobacco, gutkha, shikhar etc.); exposure to passive smoking; Tuibur habit (tobacco infused water); alcohol consumption history and current status; Susceptibility to respiratory infection; Previously diagnosed as Tb (laboratory test or clinically including a positive Tb skin test); Any family member with current or history of Tb; number of family members succumbed to Tb and Neighbor's suffering from Tb.

3.2.2 Statistical analysis for Objective 1

Analysis of the epidemiological data was performed using IBM Statistical Package for Social Sciences (SPSS v20, Madrid, Spain). The variables were

dichotomized for associated risk factors and bivariate analysis was performed. The p-value (≤ 0.05 was considered significant), odds ratio, 95% CI were calculated using logistic regression model after adjusting for confounding factors.

3.2.3. Methods for Objective 2

Study population

A total number of 13,927 samples suspected for tuberculosis were subjected to GeneXpert assay. 473 samples were removed due to invalid report, error, duplicates or no results and thus 13,454 samples were included. *M. tuberculosis* was detected in 2,894 (21.5%) cases, out of which 460 (15.9%) were Rifampicin Resistance detected and 2,434 (84.1%) were Rifampicin Resistance not detected cases (Figure 9). Socio-demographic and available clinical information were reviewed and analyzed for Rifampicin Resistant Tb cases only.

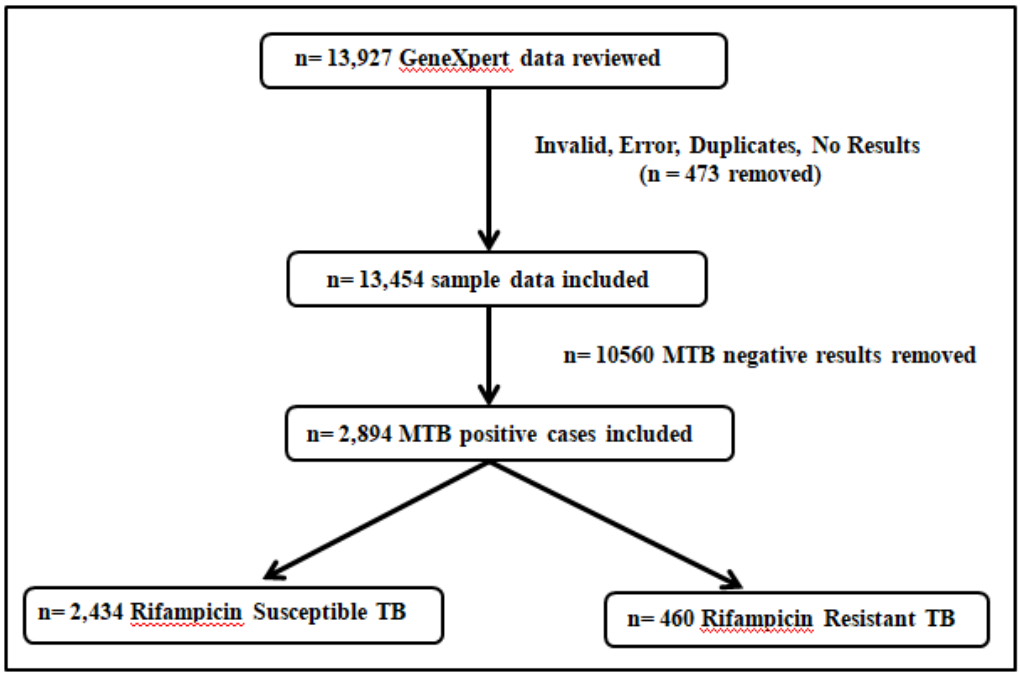


Figure 9. Flowchart for analysis of samples from RR-TB patients by Xpert Mtb/RIF

3.2.4 GeneXpert test

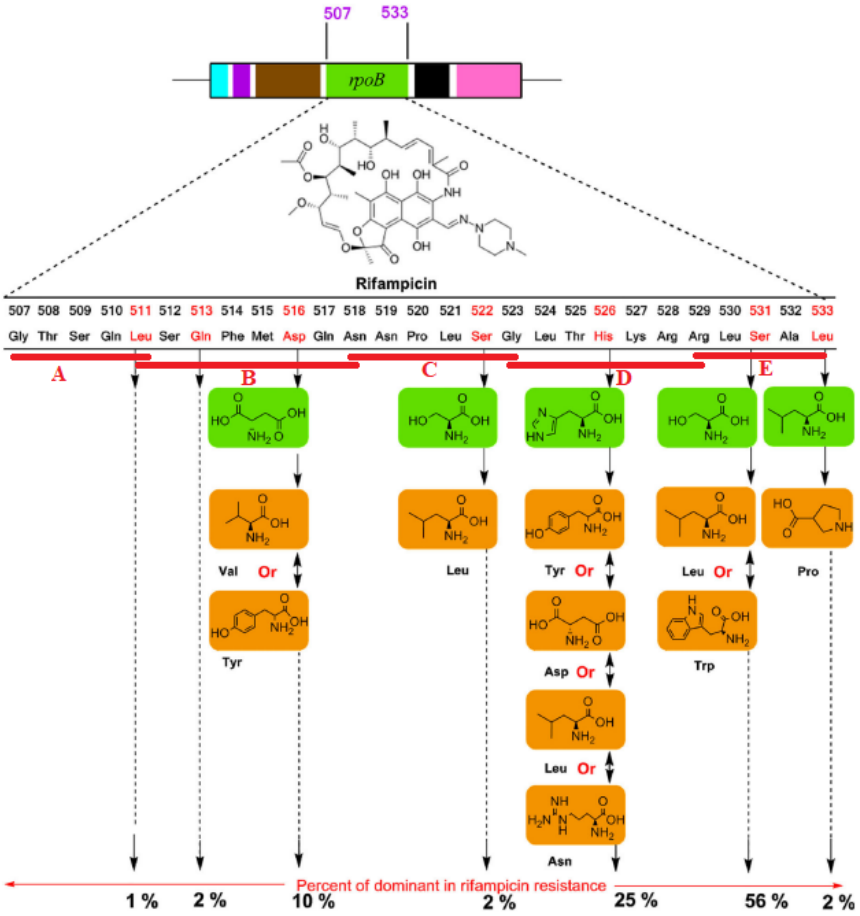


Figure 10. Schematic representation of *rpo*B gene mutations associated with the drug resistance *(Source: Swain et al., 2020)*

GeneXpert Mtb/RIF assay was recommended by WHO for the early diagnosis of Tb as well as for the detection of Rifampicin resistance in 2011. This fully automated cartridge based nucleic acid amplification test has revolutionized Tb control program as it requires only 130 Tb bacilli/ml of sputum for a positive result. It can be used for pulmonary and extrapulmonary samples (Takhar, 2016). Xpert Mtb/RIF assay G4 (Cepheid, CA, USA) is equipped with the potential of detecting mutation in *rpo*B gene that confers Rifampicin Resistance by utilizing five overlapping probes designated as probe A (codons 507-511), probe B (codons 511-518), probe C (codons 518-523), probe D (codons 523-529) and probe E (codons 529-533) (Alemu et al., 2020). It is being utilized worldwide with a sensitivity of

94.4% and specificity of 98.3% for diagnosis of rifampicin resistance (Boehme et al., 2011) and provides semi-quantitative results based on the probes' Cycle Threshold (Ct) i.e., number of PCR cycles required to amplify the DNA of Mtb to a detectable level. The results are reported as "High" (Ct<16), "Medium (Ct 16-22), "Low" (Ct 22-28) or "Very low" (Ct>28). Presence of *rpo*B mutations can change the hybridization pattern between the amplicon and the probes(s) which corresponds to the mutated site thereby causing a variation between the Ct values of the probes and when hybridization is inhibited, it results in missing probes. However, in samples without mutated *rpo*B, all the 5 probes match exactly to the amplified Mtb DNA. The G4 version of Xpert MTB/RIF software interprets sample results as resistant to Rifampicin if the difference between two probes (the first and last Ct) is >4 cycles (ΔCt>4) (Ocheretina et al., 2016). Some of the most common gene mutations associated with drug resistance in the *rpo*B gene are shown (Figure 10).

The tested samples were categorized into pulmonary (sputum, broncho alveolar lavage (BAL), gastric lavage, tracheal aspirate) and extrapulmonary (lymphnode aspirate, pleural fluid, ascitic fluid, cerebrospinal fluid, pus/abscess from various sites). Results of the Xpert Mtb/RIF assay were categorized into the following:1) Mtb not detected. 2) Mtb detected; Rifampicin Resistance not detected. 3) Mtb detected; Rifampicin Resistance Detected. 4) Invalid. 5) Error. 6) No result. For the tests having Mtb detected along with Rifampicin Resistance detected, the missed probe types as well as the DNA amounts were assessed.

3.2.5. Household identification

The localities of patients belonging to Aizawl District were entered in excel sheet, coded, segregated and mapped with the constituency they belong using the following link as a reference (https://ceo.mizoram.gov.in/state_profile). It was further arranged in descending order of RR-Tb prevalence.

3.2.6. Statistical Analysis for Objective 2

The data on the patient profile were coded and analyzed using Statistical Package for Social Sciences (SPSS v20.0, IBM Corp, USA). Bivariate analysis was

used to characterize clinical and demographic variables like frequencies of specimen type, DNA quantity, probe types, gender and multivariate analysis for age group. Logistic regression models were used to characterize the demographic and clinical variables with respect to probe types. The 460 Rifampicin Resistant Tb (Cases) were also analyzed against 920 Rifampicin Sensitive Tb (Control). P-value ≤ 0.05 was considered to be statistically significant.

3.2.7. Methods for Objective 3

Sample Collection

A total of 88 sputum samples from pulmonary tuberculosis patients (new and previously treated) were randomly collected in a sterile screw capped wide mouth container. The collected sputum specimens were transported to the laboratory for detection of AFB by Ziehl Neelsen (ZN) Stain. In addition, GeneXpert test was performed in accordance to the manufacturer's instructions using Xpert Mtb/RIF assay G4 (Cepheid, Sunnyvale, CA, USA) as well as liquid culture using MGIT (Mycobacterium Growth Indicator Tube) system.

3.2.8. Preparation of smear, staining using Ziehl Neelsen stain for AFB

Smear Preparation

Sputum smear microscopy allows a fast and dependable identification of patients with pulmonary tuberculosis (PTb) and has a low sensitivity requiring 5000–10,000 bacilli / mL of sputum to obtain positive results (Rasool et al., 2019). Smear examination allows presumptive diagnosis of Tb because the AFB in a smear may be acid-fast organism, other than Mtb. Unused slides, free from grease and scratches was used and sputum smear was prepared (Figure 11) and stained (Figure 12) as follows:

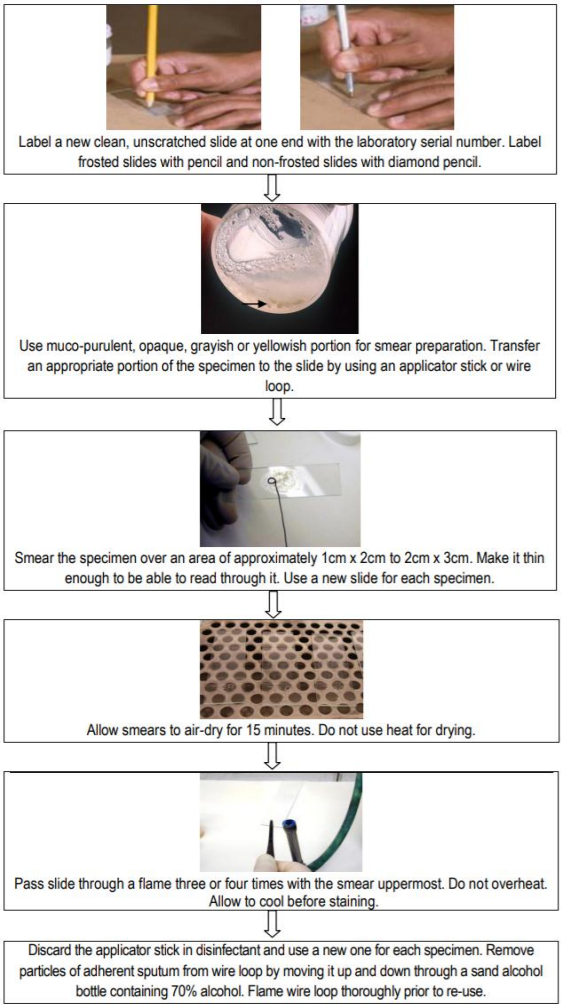


Figure 11. Technique of smear preparation for ZN staining

(Source: Standard Manual for Laboratory Technicians on Sputum Smear Microscopy, 2nd Edition)

Zielh Neelsen Staining for Acid Fast Bacilli

Stains used for Ziehl Neelsen staining

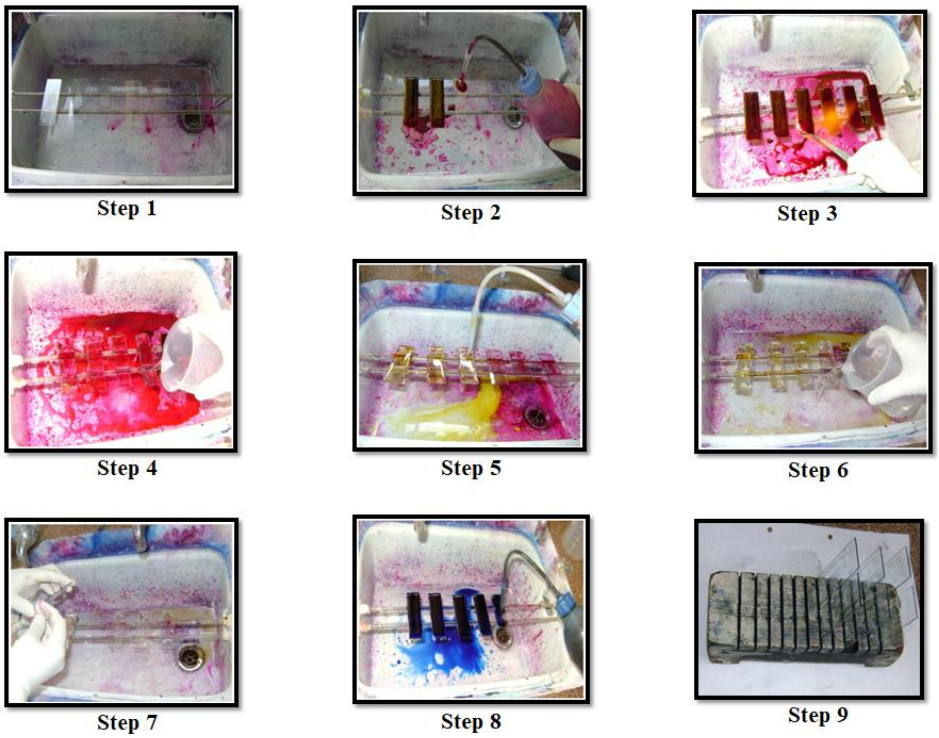


Figure 12. Steps employed in ZN staining

(Source: Standard Manual for Laboratory Technicians on Sputum Smear Microscopy, 2nd Edition)

Step 1: The slides were placed on a staining rack with the smeared slide facing up.

Step 2: The entire surface of the slide was flooded with 1% Carbol Fuchsin solution for 5 minutes. The slides were intermittently heated slowly till steam arises and care was taken not to boil the stain.

Step 3: Each slide was rinsed individually in running water until all the free stain was washed away. Excess water was drained off by tilting the slides.

Step 4: The slides were flooded with decolorizing solution i.e., 25% H_2SO_4 and left for 3 minutes.

Step 5: The slides were rinsed thoroughly with water and excess water was drained off. The back of the slides were wiped with cotton soaked in decolorizer to clean the dried stains.

Step 6: The slides were flooded with a counterstain i.e., 0.1% Methylene blue and left for 1 minute and rinsed thoroughly with running water. The rinsed slides were kept upright in a slide holding rack and allowed to airdry.

3.2.9 Microscopic Examination of stained slides

The stained slides were examined using 100 X oil immersion. Grading of AFB smears was done as per WHO and IUALTD recommendation (Table 1) (Technical Guide, 2000).

No. of Acid-fast bacilli (AFB)	Fields	Report
No AFB	In 100 immersion fields	Negative for AFB
1 to 9 AFB	In 100 immersion fields	Positive scanty Record exact figure
10 to 99 AFB	In 100 immersion fields	1+
1 to 10 AFB	Per field (examine 50 fields)	2+
>10 AFB	Per field (examine 20 fields)	3+

Table 1. Grading of AFB-stained smear

3.2.10. Principle for Mtb culture using liquid media (MGIT 960)

The culture of Mtb from pulmonary samples (sputum) was performed using automated liquid culture BACTEC MGIT 960 system (BD, USA). A fluorescent compound embedded at the bottom of the MGIT tubes is sensitive to the presence of oxygen dissolved in the broth. The level of fluorescence corresponds to the oxygen consumed by the organisms and in turn is proportional to the number of bacteria in the tube. The instrument flags the tube as positive after a certain level of fluorescence is attained (approx. 10^5 to 10^6 CFU/ml). Mycobacteria Growth Indicator Tube (MGIT) uses specialized tubes which are incubated at 37^{o}C and the instrument scans the tube every 60 minutes for increased fluorescence. Culture tubes which remain inactive for 42 days and showing no viable signs of positivity are declared as negative and removed from the instrument.

MGIT manual 2006 (Find Diagnostics) was followed. In brief, the BD BACTEC MGIT Growth Supplement [OADC - OADC-Oleic acid (0.1 g), Albumin (50 g), Dextrose (20 g), Catalase (0.03 g)] (Ref 211886 BD, USA) is added to each MGIT tube to provide essential substances for the growth of mycobacteria. Oleic acid is utilized by tubercle bacteria and involved in the metabolism. Albumin acts as a protective agent by binding toxic free fatty acids, thereby enhancing their recovery. Dextrose acts as an energy source and Catalase destroys toxic peroxides in the medium. The digestion and decontamination of the sputum samples was carried out using NaLC-NaOH. (Ref RM3142-25G, Ref TC460M-500G) to ensure that all other respiratory floras, apart from Mycobacteria, are removed. Contamination is reduced when supplementing the tubes with BD BBL MGIT PANTA (Polymixin- 6000 units, Amphotericin B- 600 µg, Nalidixic acid- 2400µg, Trimethoprim- 600 µg and Azolcillin- 600 µg) (Ref 245124) antibiotic mixture prior to inoculation with a clinical specimen. PANTA will inhibit the growth of any respiratory flora that escaped the killing via. the digestion-decontamination procedure.

3.2.11. Procedure for Digestion-Decontamination of specimens for AFB culture by NaLC-NaOH method

Reagent Preparation

Solution 1- 4% NaOH solution was prepared by weighing 4 grams NaOH in 100 ml distilled water. Solution 2- 2.9% Sodium Citrate Solution was prepared by weighing 2.9 grams of Sodium Citrate powder in 100 ml distilled water. Fresh NaOH-Sodium Citrate solution was prepared by adding equal volumes of solutions 1 and 2. All the prepared solutions and buffers were sterilized by moist heat using autoclave at 15 psi, 121ºC for 15 minutes.

Buffer Preparation

Solution 3- Disodium hydrogen phosphate (Na_2HPO_4) was prepared by weighing 9.47 grams in 1000 ml distilled water. Solution 4 - Potassium di-hydrogen phosphate (KH_2PO_4) was prepared by weighing 9.07 grams in 1000 ml distilled water. Phosphate buffer was prepared by mixing equal volumes of solution 3 and 4. The pH was adjusted to 6.8

3.2.12. Culture steps employed

Step 1: MGIT tubes were labelled including negative control tubes.

Step 2: PANTA was reconstituted with OADC by mixing 15 ml of growth supplement (OADC) to PANTA powder. It was mixed by inverting till it becomes a clear solution.

(**Note:** Reconstituted PANTA can be used for 5 days when stored at 2- 4ºC)

Step 3: 50 ml of the sterile NaOH-Sodium Citrate solution was transferred to Tarson conical tube. To this 0.25 grams of NaLC was added (0.5 grams of NaLC powder was added per 100 ml of NaOH-Sodium Citrate solution). The solution was mixed by inversion.

(Note: The NaLC-NaOH solution should ideally be prepared 2 hours prior to carrying out the digestion-decontamination process. The solution should ideally be used within 24 hrs).

Step 4: 800 µl of PANTA-OADC mixture was added into each MGIT tubes along the side wall including negative control tubes.

Step 5: Approx. 5 ml of AFB smear positive sputum sample was transferred into pre-labelled 50 ml Tarson tube. To this equal volume of NaLC-NaOH solution was added. To highly mucopurulent samples, a tiny pinch of extra NaLC powder is added to the sputum.

Step 6: The cap was tightened and the tube was vortexed for about 30 seconds. The tubes were periodically swirled by hand to ensure proper mixing. The specimen was allowed to stand at room temperature (RT) for 15 minutes with intermittent swirling.

Step 7: Sterile phosphate buffer (pH 6.8) was added up to 45 ml mark from the sides of the tube wall including negative control tubes. Mix well by inverting the tubes.

Step 8: The specimens were concentrated by centrifugation at 3000xg for 20 minutes using cold centrifuge (4ºC).

Step 9: After centrifugation, the supernatant was carefully decanted from the reverse side of the pellet.

Step 10: 1-2 ml of fresh phosphate buffer (pH 6.8) was added into each tubes including negative control tube.

Step 11: The tubes were then gently vortexed to resuspend the pellets and wait for 1-2 minutes for aerosols to settle down.

Step 12: 500 µl of the resuspended pellet was aseptically inoculated into pre-labelled MGIT tubes that was pre-mixed with growth supplement. The caps were tightened and the tubes were then inverted five times to ensure proper mixing. The cap was decontaminated using 70% isopropyl alcohol.

Step 13: The tubes were then loaded into MGIT 960 system.

In between adding the samples to different tubes, separate filter tips were used for each tube and the micropipettes were cleaned with 70% isopropyl alcohol. All the digestion-decontamination procedures along with first line drug susceptibility were carried out at Zoram Medical College, Falkawn and Synod Hospital, Durtlang using a Biosafety Cabinet Type II A2. For personal protection, N95 mask, double gloves, surgical cap, face shield and PPE were worn while carrying out the work. All the waste generated were segregated at the source of generation and discarded in color coded bins. Items that came in contact with the specimen as well as the culture were treated with 5% phenol and immersed overnight. After autoclaving, final disposal was done as per the Mizoram Pollution control board followed by the respective hospitals.

3.2.13. AFB positive sputum samples processed for culture

A total of the 88 sputum AFB positive samples were digested and decontaminated. 32 samples were not included in the study since bacilli could not be recovered upon culture due to scanty bacilli as well as contamination. The remaining 56 samples were included in the study.

3.2.14. Sterility check of MGIT positive tubes

The tubes that flagged positive underwent sterility check via. plating out on sheep blood agar. Approx. 20 µl of the culture broth was transferred to a sterile sheep blood agar, labelled and incubated at 37 ºC overnight. Sterility was checked the next day by examining the plate for the absence of growth. If colonies appear, the liquid culture is contaminated and needs further digestion-decontamination.

3.2.15. Checking for the presence of Cord formation

The mycobacterial cord factor, Trehalose-6,6-dimycolate (TDM) is the major virulence factor of Mtb and is the most abundant cell wall lipid which causes granuloma formation (Lang, 2013). The positive MGIT tubes were vortexed and allowed to stand unopened for 5 minutes for aerosols to settle down. In a new and clean glass slide, one drop of 1% Bovine Serum Albumin pH 7.0 (REF TC348, HiMedia) was added. To this 500 µl of the culture supernatant was added and mixed

by pipetting, allowed to air dry in Bio Safety Cabinet, heat fixed and stained using ZN stain. The slides were checked under low power (10X) and high power (100X) objectives for the presence of cord formation/ presence of AFB.

3.2.16. TBc Identification Test (TBc ID)

Principle

The BD MGIT^{TM} TBc Identification Test (Ref 245159) is a quick chromatographic immunoassay for the qualitative detection of Mtb complex (Mtbc) antigen from AFB smear-positive MGIT tubes. The device can detect the following species of the Mtbc: *M. tuberculosis, M. bovis, M. africanum* and *M. microti*. It detects the MPT64 Ag, a mycobacterial protein fraction, secreted from Mtbc cells during culture. When samples are added to the test device, MPT64 antigen binds to anti-MPT64 antibodies conjugated to visualizing particles on the test strip. The antigen-conjugate migrates and is captured by a second specific MPT64 antibody applied to the membrane which is visualized as pink to red line, if the antigen is present.

Test Procedure

Step 1: The TBc ID was placed on a flat surface and labelled with sample ID.

Step 2: The AFB smear positive tubes were thoroughly mixed by vortexing the tubes.

Step 3: The tube caps were removed and using a sterile pipette and filter tips, 100 µl of the sample was pipetted and transferred to the sample well on the device. The results were read within 15 minutes.

After passing the sterility check on blood agar and observing for the presence of cord formation or AFB along with a positive TBc ID, samples were further processed for first line drug susceptibility.

3.2.17. First Line Drug Susceptibility Test using MGIT 960 system

The positive tubes that had passed sterility, microscopy and identification check were subjected to first line drug susceptibility test using the four antibiotics, namely, Streptomycin (1.00 µg/ml), Isoniazid (0.10 µg/ml), Rifampicin (1.00 µg/ml) and Ethambutol (5.00 µg/ml) using BD BACTEC MGIT^{TM} 960 SIRE Kit (Ref 245123) as well as Pyrazinamide (100 µg/ml) provided the cultures are young (one to two days old). From the third day onwards to the fifth day, a 1:5 dilution was employed. Growth Control (GC) was included for each sample tested. The first line DST procedure are as follows (Figure 12):

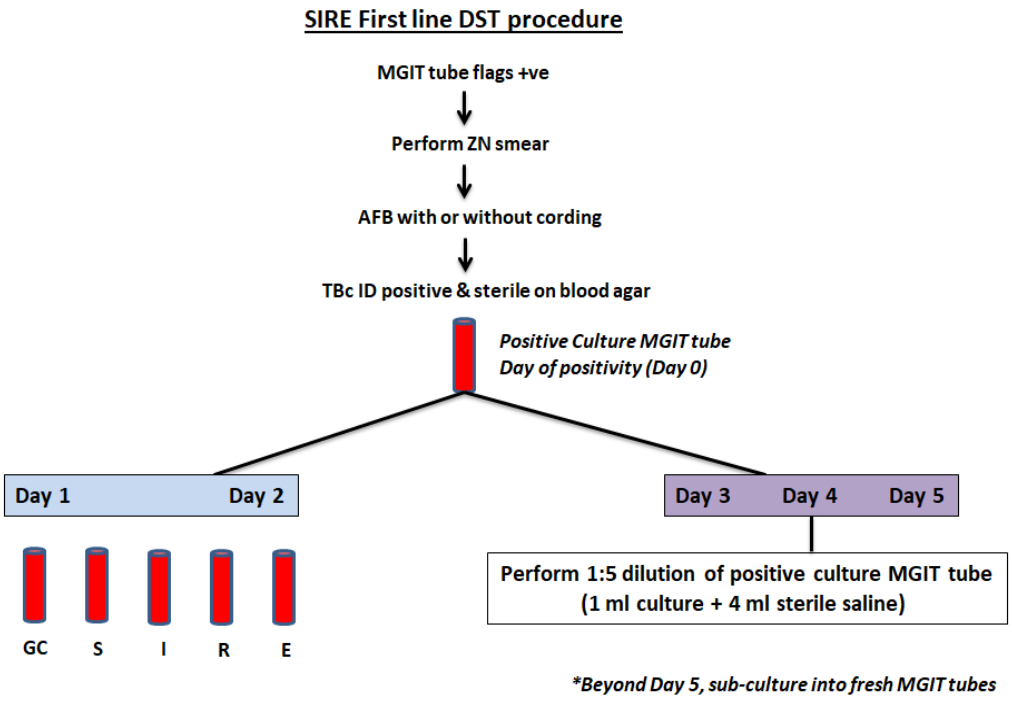


Figure 13. Flowchart of first line DST procedure.

Step 1: Five MGIT tubes were wiped with 70% isopropyl alcohol and labelled as Growth Control (GC), Streptomycin (S), Isoniazid (I), Rifampicin (R), Ethambutol (E).

Step 2: 800 µl of SIRE supplement was added in all the tubes including the GC tube.

Step 3: 100 µl of the drug was added in the respective tubes. No drug was added in the GC tube.

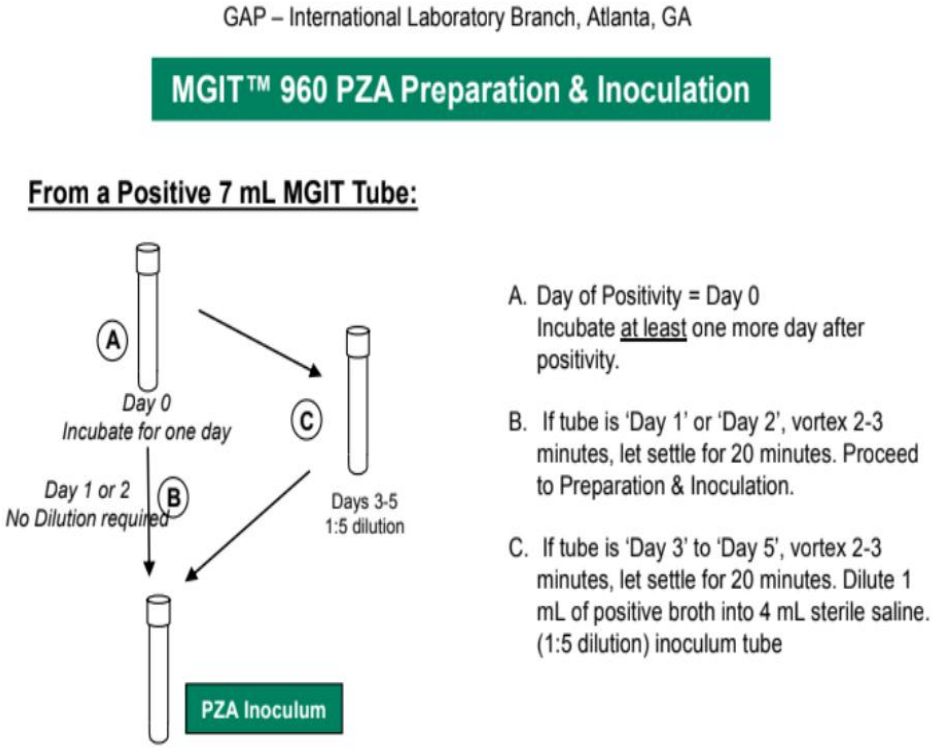
Step 4: 500 µl of positive culture from MGIT tubes was added into each drug tubes except GC tube.

Growth Control (GC) tube preparation and inoculation

A 1:100 dilution of positive culture was prepared by adding 100 µl of positive culture and 10 ml of sterile normal saline. From this suspension, 500 µl was inoculated into the tube labelled GC.

For pyrazinamide, a different protocol was followed as follows (Figure 14).

(Source: CDC, Global Aids Program, International Laboratory Branch)



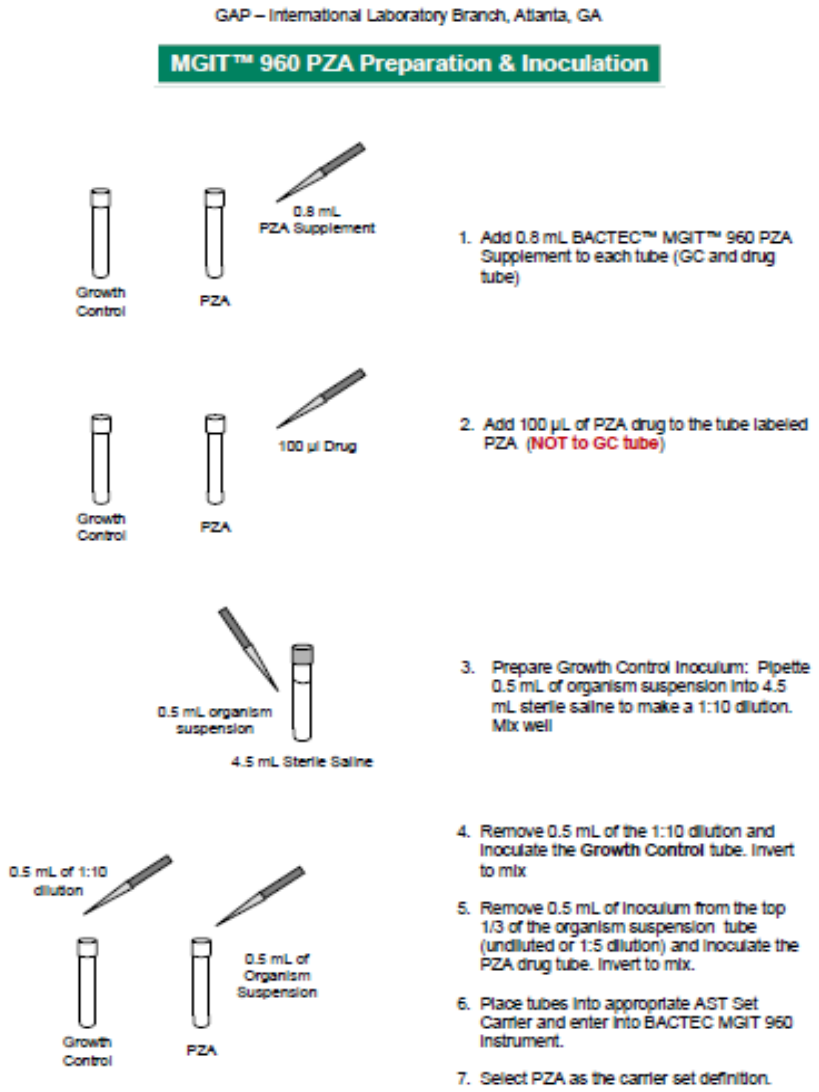


Figure 14. Flowchart of Pyrazinamide DST

All the tubes were incubated at 37ºC in the MGIT 960 system.

3.2.18. Methods for Objective 4

GeneXpert MTB/RIF was carried out on phenotypically rifampicin resistant and susceptible isolates. For determining the gene specific mutations for drug resistance and the lineage of *M. tuberculosis* strains in Mizoram, the DNA of Mtb was extracted from culture positive isolates that had passed the sterility check. Conventional PCR using five antibiotic resistance genes, sanger sequencing and LSP-PCR was also carried out.

3.2.19. DNA extraction

Growth from MGIT tubes were sub-cultured on LJ media using BBL™ Lowenstein-Jensen Medium Slants (Ref 220909) for DNA extraction and further molecular testing. DNA was extracted from 44 LJ culture growth as follows: A loop full of the growth was emulsified in 2 ml of 0.9% normal saline. This was gently vortexed and Mtb was heat killed at $80^{O}C$ for 20 minutes (Doig et al., 2002). Extraction was done as per manufacturer's instructions with few modifications. It was centrifuged in a cooling centrifuge at 14000 rpm for 5 minutes (eppendorf Centrifuge 5430 R). The supernatant was discarded and the pellet was emulsified in 1 ml of BD Difco™ Middlebrook 7H9 Broth (Ref 271310). This was treated using 2.5 ml of lysis buffer (Trueprep AUTO Mtb Sample Pre-treatment Pack, molbio diagnostics, Ref 60204AS20) containing glass beads and shaken intermittently, allowing it to stand at RT for 10 minutes. It was loaded into Universal Cartridge (Trueprep AUTO Universal Cartridge Based Sample Prep Kit, molbio diagnostics, Ref 60203AR50) and loaded into nucleic acid extractor machine (Trueprep AUTO v2, molbio diagnostics).

3.2.20. Quantitation of DNA and PCR amplification

Isolated DNA was run on a 0.8% agarose gel electrophoresis (BioRad) to check the quality and the DNA concentration was quantified using a µdrop MultiSkan SkyHigh Spectrophotometer (Thermoscientific).

Polymerase Chain Reaction (PCR) was run on five antibiotic resistance genes that has been related with resistance towards first line ATT (Anti-tuberculosis treatment) drugs. PCR was carried out for 35 cycles in a 10 µl reaction and the PCR product was observed in a 1.5% Agarose Gel Electrophoresis (Bio-Rad). The primers and conditions for each primer is given (Table 2).

Genes	Primer Sequences	Product Size	Annealing temp.	Reference
katG	F- GAAACAGCGGCGCTGATCGT R- GTTGTCCCATTTCGTCGGGG	210 bp	54^{o}C	Homolka et al., 2010
rpoB	F- TCGCCGCGATCAAGGAGT R- GTGCACGTCGCGGACCTCCA	158 bp		
embB	F- CCGACCACGCTGAAACTG R- GTAATACCAGCCGAAGGGATCCT	368 bp		Jain et al., 2008
rpsL	F- TTC TTGACACCCTGCGTATC R- GTCAAGACCGCGGCTCTGAA	272 bp		Chaoui et al., 2009
pncA	F- GGCGTCATGGACCCTATATC R- CAACAGTTCATCCCGGTTC	670 bp		Nguyen et al., 2003

Table 2. Primer sequence and conditions for Antibiotic Resistance Genes

3.2.21. Principle of Sanger Sequencing *(Source: Applied Biosystems)*

Sanger sequencing commonly referred to as "Chain termination technique" was developed by an English scientist Frederick Sanger and his colleagues in 1977. This approach is used to determine the nucleotide base sequence in a fragment of DNA. It is considered as the "Gold standard" for verifying next-generation sequencing data, with a base accuracy of 99.99 percent. For DNA to be extended using normal DNA polymerases, a -OH group must be present at the 3’ carbon on deoxyribose. The template DNA of interest to be sequenced is first produced as single-stranded DNA. Short oligonucleotide DNA primer, complementary to the template DNA is annealed as a starting point for DNA synthesis. A polymerase lack 3’ to 5’ exonuclease proof reading activity extends the primer by adding the corresponding dNTP to the template DNA strand in the presence of the four deoxynucleotide triphosphates (dNTPs). The reaction is carried out in one tube. Four ddNTPs (ddATP, ddCTP, ddGTP, and ddTTP) tagged with a unique fluorescent dye are mixed together and employed to terminate polymerization whenever they are incorporated into DNA to determine which nucleotide is integrated into the chain of nucleotides. ddNTPs lack an oxygen atom on the ribonucleotide, making it unable to create a bond with the following nucleotide. In this way DNA fragments are synthesized. A long, thin acrylic-fibre capillary separates DNA fragments by size (instead of an electrophoresis gel).

A beam of narrow laser light is then used to excite the fluorescent dyes bound to DNA. From both the bottom and top, laser light is directed into the plane of the capillaries. A little quantity of laser light is absorbed by the dyes and released in all directions as longer wavelength light which is then imaged and a detector analyzes the colour of the resulting emitted light, with 4 colours for the 4 different bases (Figure 15).

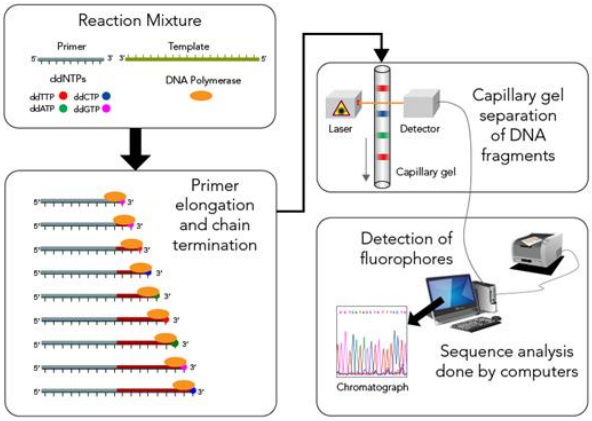


Figure 15. Diagrammatic representation of Sanger Sequencing principle *(Adopted from Applied Biosystems)*

3.2.22. Protocol for Sanger Sequencing

It was performed using the following steps:

PCR clean-up

1) 6µl of PCR products were taken in PCR tubes and labelled.

2) 2.5µl of ExoSAP-IT™ PCR Product Clean-up Reagent were added in each sample and spun down *(ExoSAP contains Exonuclease I and Shrimp Alkaline Phosphatase. Exonuclease I remove excess primers and short nucleotides; SAP removes dNTPs).*

3)The samples were incubated at 37^{o}C for 15 to 30 mins, 80^{o}C for 15 mins *(The enzymes are activated in 37^{o}C and denatures at 80^{o}C).*

Gel Electrophoresis

2 µl of the samples were loaded on a 2% agarose gel to check if the PCR clean-up was successful. Samples having clean bands of interest were taken up for the next step.

Cycle PCR

Cycle PCR cocktail was prepared for a total volume of 10µl using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as follows (Table 3):

Component	~ 300 bp product		~ 600 bp product	
	Concentration	**Volume**	**Concentration**	**Volume**
BigDye™ Terminator v3.1 5X Sequencing Buffer (ABI)	0.5x	2.25 µl	0.5x	0.5 µl
BigDye™ Terminator v3.1 Ready Reaction Mix (ABI)	0.5x	0.25 µl	0.5x	1.75 µl
Primer (Forward/Reverse)	0.5pmol	0.5 µl	0.5pmol	0.5 µl
Template (PCR product)	10 ng	0.8 to 1.5 µl	20 ng	0.8 to 1.5 µl
Millipore Water		Make up to 10 µl		Make up to 10 µl
Total		10 µl		10 µl

Table 3. Protocol for Cycle PCR

The samples were then spun down and cycle sequencing was performed in a thermal cycler according to the conditions below (Table 4):

Parameters		**25 cycles**			
	Initial Denaturation	Denaturation	Annealing	Extension	Hold
Temperature (^{o}C)	96	96	54^{o}C	60	4
Duration	1 min	10 secs	10 secs	4 min	

Table 4. Conditions for cycle sequencing

Cycle PCR Cleanup

Precipitation

Solution I and Solution II were prepared accordingly: -

Solution I – 125mM EDTA: Millipore water, 1:5 ratio

Solution II – 3M Sodium acetate:100% chilled alcohol, 1:25 ratio

1) The cycle PCR products were then transferred onto a 0.5 µl tube and labelled.

2) 12µl of Solution I were added in each tube. Vortexed for 5 mins and then spun down.

3) 52µl of Solution II was then added in each tube. Vortexed for 5 mins and spun down.

4) The samples were further incubated in the dark for 30 mins and centrifuged at 14,000 rpm for 50 mins.

Decanting – The tubes were inverted and spun at 0.2rcf for 1 min.

Alcohol washing

5) 200µl of 75% alcohol were then added in each sample. Voxtexed for 5 mins and spun down.

6) Samples were then incubated in the dark for 15 mins and centrifuged at 14,000 rpm for 50 mins.

Decanting – The tubes were inverted and spun at 0.2 rcf for 1 min.

7) The samples were then air dried in a Laminar Airflow for 30 to 45 mins.

Hi-Di™ Formamide

8) The samples were then resuspended in 10 µl Hi-Di™ Formamide. Vortexed for 5 mins, spun down and incubated in the dark for 15 mins.

Denaturation

9) Samples were then denatured on a thermal cycler at 96^{o}C for 2 mins and immediately transferred onto ice for 1 min and then loaded on the Genetic Analyzer 3500 (ABI).

3.2.23. Analysis of Sanger Sequencing

Sequencing was done using 3500 Genetic Analyzer automated sequencer (ABI) in the Department of Biotechnology, Mizoram University and analyzed for confirmation and presence of variants in the genes. The sequences were aligned using Clustal Omega with reference sequences (*Mycobacterium tuberculosis* H37Rv) obtained from Mycobrowser (Sievers et al., 2011; Kapopoulou et al., 2011).

3.2.24. Genotyping of *Mycobacterium tuberculosis* isolates from Mizoram

Genotyping of Mtb lineage was done using Long Sequence Polymorphism (LSP-PCR) otherwise known as Regions of Differences (RDs). Based on LSP (RD), the major Mtb lineages were classified using four primers for Indo-Oceanic (Lineage 1), Beijing (Lineage 2), East-African Indian (Lineage 3) and Euro-American (Lineage 4). The primers and PCR conditions used for classification of lineages are shown (Table 5).

LSP markers	Primer Sequence	Amplicon (bp)	Annealing Temp.	Interpretation	Reference
RD105	F: TGTGGTGCTTGGGCCTGAGAG R: AATCGTGGTGATCCCGGAGCG	435	68°C	(-) = Beijing (+) = Non-Beijing	(Hanchaina et al., 2014).
RD239	F: GTGAGCTGAAGAAGCTCGTCCC R: ATCACCGTCAAACCGTTCACGAC	1,053/210	68°C	(210 bp) = IO (1,053 bp) = Non-IO	
RD750	F: 5'CACAGACAATTCCAACCCATTTGG R: CGCATGCCGCACATGCAAGTAC	948/158	67°C	(158 bp) = EAI (948 bp) = non EAI	
7bp pks15/1	F: GGCCGCGGCCCGCGGCC R: GGCTCAATCAAGTCGAACATGGG	332	66°C	(-) = EuA (+) = non EuA	

Table 5. PCR conditions for the classification of Mtb lineages

(-) absent; (+) present; IO Indo-Oceanic; non-IO, not Indo-Oceanic; EuA, Euro-American; non-EuA, not Euro-American; EAI, East Africa Indian; non EAI, non-East African Indian.

Chapter 4: Results

4.1. Comparison between pulmonary and extrapulmonary Tb patients

4.1.1. Case Status

Four hundred (400) Tb patients were categorized as per the RNTCP classification into pulmonary (n=288) and extrapulmonary (n=112). The mean age of patients was found to be 33.1 years. The two groups are further broken down into new cases, relapse, treatment failure and defaulters (Figure 16).

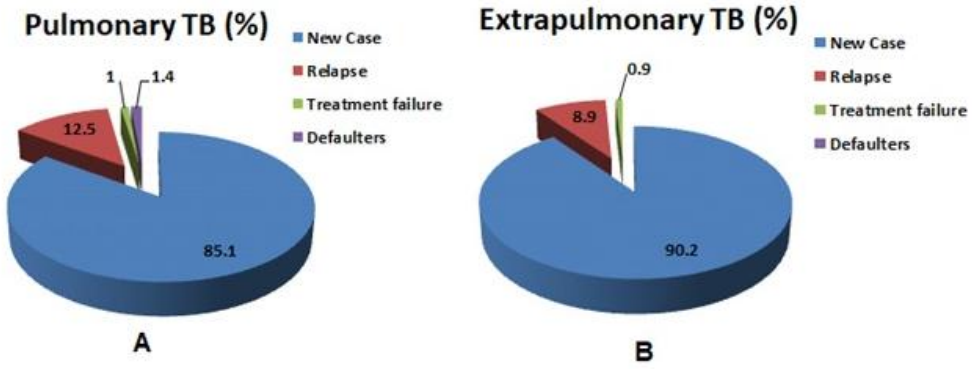


Figure 16. Comparison of cases between pulmonary and extra-pulmonary Tb.

4.1.2. Clinical Symptoms

Clinical presentation such as fever, cough, weight loss, chest pain and night sweats were more prominent among pulmonary TB patients (Table 6).

Clinical Presentation	Symptom	Pulmonary TB [n=288] (%)	Extra-pulmonary TB [n=112] (%)
Fever > 2 weeks	No	106 (36.8)	67 (59.8)
	Yes	182 (63.2)	45 (40.2)
Cough > 2 weeks	No	53 (18.4)	70 (62.5)
	Yes	235 (81.6)	42 (37.5)
Weight loss	No	50 (17.4)	46 (41.1)
	Yes	238 (82.6)	66 (58.9)
Chest Pain	No	112 (38.9)	65 (58.0)
	Yes	176 (61.1)	47 (42.0)
Night Sweats	No	98 (34.0)	68 (60.7)
	Yes	190 (66.0)	44 (39.3)

Type of Patient	New case	245 (85.1)	101 (90.2)
	Relapse	36 (12.5)	10 (8.9)
	Treatment failure	3 (1.0)	1 (0.9)
	Defaulters	4 (1.4)	0 (0)

Table 6. Clinical presentation of Tb patients from Mizoram, Northeast India.

4.1.3 Mode of diagnosis of Tb Infection

The common mode of diagnosis among pulmonary Tb are GeneXpert (40%), smear microscopy- Ziehl Neelsen Stain (32.6%) whereas for extrapulmonary Tb it is X-ray (40.1%) and FNAC (25.9%) (Figure 17).

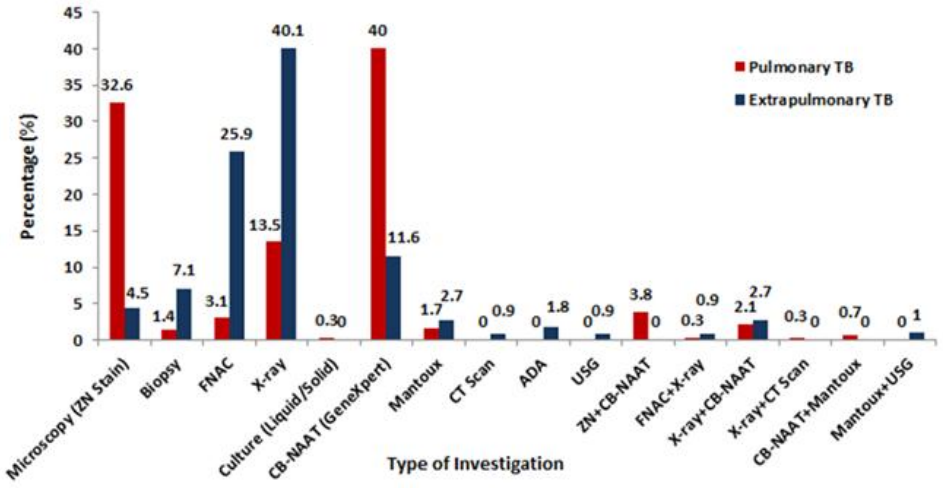


Figure17. Mode of diagnosis of Tb among pulmonary and extrapulmonary cases

4.1.4 Associated Risk Factors

Statistical analysis was performed to explore the risk factors of tuberculosis using Bivariate analysis (Table 7).

Variables		TB Patient (%)	Healthy Control (%)	p-value	OR (95% CI)
Sex	Male	207 (51.7)	496 (59.0)	**Reference**	
	Female	193 (48.3)	344 (41.0)	**0.015**	1.344 (1.058-1.708)
Age (in years)	15-45	327 (81.8)	721 (85.8)	**Reference**	
	>45	73 (18.2)	119 (14.2)	0.063	1.353 (0.983-1.861)
Marital Status	Married	191 (47.8)	342 (40.7)	**Reference**	
	Single	209 (52.3)	498 (59.3)	0.019	0.751 (0.591-0.955)

BMI Score	Ideal Weight	244 (61)	47 (5.6)	**Reference**	
	Non-ideal weight	156 (39)	793 (94.4)	0.000	0.038 (0.027-0.054)
Education	Higher Secondary & above	154 (38.5)	627 (74.6)	**Reference**	
	Upto Matriculation	246 (61.5)	213 (25.4)	0.000	4.702 (3.646-6.064)
Occupation	Employed	170 (42.5)	549 (65.4)	**Reference**	
	Unemployed	230 (57.5)	291 (34.6)	0.000	2.552 (2.000-3.258)
Family income (per month)	>30,000 per month	84 (21.0)	349 (41.5)	**Reference**	
	<30,000 per month	316 (79.0)	491 (58.5)	0.000	2.674 (2.027-3.527)
Reside in a crowded house	No	244 (61.0)	644 (76.7)	**Reference**	
	Yes	156 (39.0)	196 (23.3)	0.000	2.101 (1.625-2.716)
Cross Ventilation	Yes	232 (58.0)	304 (36.2)	**Reference**	
	No	168 (42.0)	536 (63.8)	0.000	0.411 (0.322-0.524)
Condition of housing	Sunlight	279 (69.8)	751 (89.4)	**Reference**	
	Damp or Moist	121 (30.3)	89 (10.6)	0.000	3.660 (2.694-4.971)
No. of people living under one roof	1-6 members	289 (72.3)	658 (78.3)	**Reference**	
	>6 members	111 (27.8)	182 (21.7)	0.018	1.389 (1.056-1.826)
Water supply source	Govt. municipal	339 (84.8)	783 (93.2)	**Reference**	
	Other sources (spring water, river etc.)	61 (15.2)	57 (6.8)	0.000	2.472 (1.686-3.625)
Exercise	Regularly	51 (12.8)	127 (15.1)	**Reference**	
	Irregularly	349 (87.2)	713 (84.9)	0.266	1.219 (0.860-1.728)
History of contact with Pulmonary TB	No	292 (73)	628(74.8)	**Reference**	
	Yes	108 (27)	212 (25.2)	0.508	1.096 (0.836-1.435)
Mass gathering events	No	263 (65.8)	306 (36.4)	**Reference**	
	Yes	137 (34.3)	534 (63.6)	0.000	0.299 (0.233-0.383)
Travel history	No	295 (73.8)	734 (87.4)	**Reference**	
	Yes	105 (26.3)	106 (12.6)	0.000	2.465 (1.823-3.333)
HIV Status	Negative	374 (93.5)	840 (100)	**Reference**	
	Positive	26 (6.5)	0 (0)	0.000	1.070 (1.042-1.098)
Diabetes	No	349 (87.3)	840 (100)	**Reference**	
	Yes	51 (12.8)	0 (0)	0.000	1.146 (1.104-1.190)
BCG Vaccine	Yes	256 (64)	703 (83.7)	**Reference**	
	No	144(36)	137 (16.3)	0.000	2.886 (2.194-3.797)
Alcohol	No	205 (51.2)	672 (80.0)	**Reference**	
	Yes	195 (48.8)	168 (20.0)	0.000	3.805 (2.937-4.929)
Smoking	No	204 (51.0)	511 (60.8)	**Reference**	
	Yes	196 (49.0)	329 (39.2)	0.001	1.492 (1.174-1.897)
Other tobacco products (Gutkha, Shikhar etc)	No	262 (65.5)	509 (60.6)	**Reference**	
	Yes	138 (34.5)	331 (39.4)	0.096	0.810 (0.632-1.038)
Tuibur	No	369 (92.3)	768 (91.4)	**Reference**	
	Yes	31 (7.8)	72 (8.6)	0.625	0.896 (0.578-1.390)
Family members/colleagues smoke tobacco	No	109 (27.3)	284 (33.8)	**Reference**	
	Yes	291 (72.8)	556 (66.2)	0.020	1.364 (1.049-1.773)
Exposure to passive smoking	Rarely or never	211 (52.8)	578 (68.8)	**Reference**	
	Regularly	189 (47.3)	262 (31.2)	0.000	1.976 (1.547-2.524)

Prone to respiratory infection	No	231 (57.8)	523 (62.3)	**Reference**	
	Yes	169 (42.2)	317 (37.7)	0.128	1.207 (0.947-1.538)
Previously diagnosed with TB	No	319 (79.8)	840 (100)	**Reference**	
	Yes	81 (20.3)	0 (0)	0.000	1.254 (1.194-1.317)
Positive TB skin test	No	374 (93.5)	840 (100)	**Reference**	
	Yes	26 (6.5)	0 (0)	0.000	1.070 (1.042-1.098)
Family members with ongoing TB	No	369 (92.3)	791 (94.2)	**Reference**	
	Yes	31 (7.8)	49 (5.8)	0.199	1.356 (0.851-2.162)
Family members with history of TB	No	254 (63.5)	728 (86.7)	**Reference**	
	Yes	146 (36.5)	112 (13.3)	0.000	3.736 (2.811-4.966)
Members in family with TB so far	None	230 (57.5)	687 (81.8)	**Reference**	
	1 – 5 members	170 (42.5)	153 (18.2)	0.000	3.319 (2.547-4.324)
Neighbors with TB	No	284 (71.0)	789 (93.9)	**Reference**	
	Yes	116 (29.0)	51 (6.1)	0.000	6.319 (4.426-9.022)
Frequency of contact with a neighbor suffering from TB	Never	307 (76.8)	799 (95.1)	**Reference**	
	Frequently	93 (23.2)	41 (4.9)	0.000	5.903 (3.996-8.722)

Table 7. Bivariate analysis of the risk factors of tuberculosis in Mizoram.
p-value less than 0.05 is significant. Odds Ratio is the strength of association between an exposure and an outcome (OR > 1 - greater odds of association; OR = 1 - no association; OR < 1 - lower odd of association).

After adjusting for confounding factors, patients who are single have a 2.13 times higher risk of contracting Tb as compared to married people. Ideal weight reduced the risk of Tb by 0.031 times. With lesser level of educational qualification, the higher was the chance of contracting Tb and being less educated (upto Class 10), increased the chance by 4.57 times. Unemployed individuals had a risk of 2.79 times for contracting the disease. In this study, out of the 230 unemployed Tb patients, 84.7% (n=195) consume alcohol, 85.2% (n=196) smoke, 60% (n=138) use other tobacco products and 13.4% (n=31) engage in tuibur (tobacco infused smoke) practice.

Tb was prevalent among patients from low socio-economic status (family income < Rs.30,000 per month). People with low income had 2.43 times the risk of contracting Tb. Housing that are moist or damp have 2.60 times risk of harboring Tb infection compared to that received proper sunlight. Cross ventilation also had a positive impact (p-0.000).

One very interesting and less-established risk factor that requires immediate attention is the source of domestic water supply, other than the Government Municipal agency. The river water or spring water collection had 2.313 times risk of harboring tubercle bacilli. Regular engagement in societal activities involving community level mass gatherings are also the risk factors for contracting Tb (p-0.000). Individuals with recent travel history were at five times higher risk and individuals not receiving BCG vaccine were at 3.0 times higher risk of contracting Tb. Alcohol consumption increased the risk of infection by 7.17 times and 48.8% (n=195) patients consumed alcohol (81.5% males; 18.5% females). The risk of Tb increased by 4.40 times with a neighbor infected with Tb (Table 8).

Variables		TB Patient (%)	Healthy Control (%)	Unadjusted p-value	Unadjusted OR (95% CI)	Adjusted p-value	Adjusted OR (95% CI)
Sex	Male	207 (51.7)	496 (59.0)	**Reference**			
	Female	193 (48.3)	344 (41.0)	0.015	1.344 (1.058-1.708)	0.077	1.619 (0.949-2.763)
Marital Status	Married	191 (47.8)	342 (40.7)	**Reference**			
	Single	209 (52.3)	498 (59.3)	0.019	0.751 (0.591-0.955)	**0.002**	**2.135 (1.320-3.453)**
BMI Score	Ideal Weight	244 (61)	47 (5.6)	**Reference**			
	Non-ideal weight	156 (39)	793 (94.4)	0.000	0.038 (0.027-0.054)	**0.000**	**0.031 (0.018-0.052)**
Education	Higher Secondary & above	154 (38.5)	627 (74.6)	**Reference**			
	Upto Matriculation	246 (61.5)	213 (25.4)	0.000	4.702 (3.646-6.064)	**0.000**	**4.572 (2.824-7.404)**
Occupation	Employed	170 (42.5)	549 (65.4)	**Reference**			
	Unemployed	230 (57.5)	291 (34.6)	0.000	2.552 (2.000-3.258)	**0.000**	**2.791 (1.816-4.289)**
Family income (per month)	>30,000 per month	84 (21.0)	349 (41.5)	**Reference**			
	<30,000 per month	316 (79.0)	491 (58.5)	0.000	2.674 (2.027-3.527)	**0.001**	**2.342 (1.422-3.856)**
Reside in a crowded house	No	244 (61.0)	644 (76.7)	**Reference**			
	Yes	156 (39.0)	196 (23.3)	0.000	2.101 (1.625-2.716)	0.160	1.383 (0.880-2.175)

Cross Ventilation	Yes	232 (58.0)	304 (36.2)	**Reference**			
	No	168 (42.0)	536 (63.8)	0.000	0.411 (0.322-0.524)	**0.000**	**0.289 (0.187-0.446)**
Condition of housing	Sunlight	279 (69.8)	751 (89.4)	**Reference**			
	Damp or Moist	121 (30.3)	89 (10.6)	0.000	3.660 (2.694-4.971)	**0.000**	**2.604 (1.535-4.418)**
No.of people living under one roof	1-6 members	289 (72.3)	658 (78.3)	**Reference**			
	>6 members	111 (27.8)	182 (21.7)	0.018	1.389 (1.056-1.826)	0.100	1.497 (0.926-2.419)
Water supply source	Govt.municipal	339 (84.8)	783 (93.2)	**Reference**			
	Other sources (spring water, river etc.)	61 (15.2)	57 (6.8)	0.000	2.472 (1.686-3.625)	**0.017**	**2.313 (1.160-4.613)**
Mass gathering events	No	263 (65.8)	306 (36.4)	**Reference**			
	Yes	137 (34.3)	534 (63.6)	0.000	0.299 (0.233-0.383)	**0.000**	**0.261 (0.168-0.405)**
Travel history	No	295 (73.8)	734 (87.4)	**Reference**			
	Yes	105 (26.3)	106 (12.6)	0.000	2.465 (1.823-3.333)	**0.000**	**5.006 (2.876-8.711)**
BCG Vaccine	Yes	256 (64.0)	703 (83.7)	**Reference**			
	No	144(36.0)	137 (16.3)	0.000	2.886 (2.194-3.797)	**0.000**	**3.033 (1.895-4.854)**
Alcohol	No	205 (51.2)	672 (80.0)	**Reference**			
	Yes	195 (48.8)	168 (20.0)	0.000	3.805 (2.937-4.929)	**0.000**	**7.172 (4.060-12.671)**

Smoking	No	204 (51.0)	511 (60.8)	**Reference**			
	Yes	196 (49.0)	329 (39.2)	0.001	1.492 (1.174-1.897)	0.088	0.626 (0.365-1.073)
Family members/colleagues smoke tobacco	No	109 (27.3)	284 (33.8)	**Reference**			
	Yes	291 (72.8)	556 (66.2)	0.020	1.364 (1.049-1.773)	0.274	1.338 (0.794-2.255)
Exposure to passive smoking	Rarely or never	211 (52.8)	578 (68.8)	**Reference**			
	Regularly	189 (47.3)	262 (31.2)	0.000	1.976 (1.547-2.524)	0.260	1.319 (0.814-2.138)
Family members with history of Tb	No	254 (63.5)	728 (86.7)	**Reference**			
	Yes	146 (36.5)	112 (13.3)	0.000	3.736 (2.811-4.966)	0.133	2.008 (0.809-4.986)
Members in family with Tb so far	None	230 (57.5)	687 (81.8)	**Reference**			
	1 – 5 members	170 (42.5)	153 (18.2)	0.000	3.319 (2.547-4.324)	0.381	1.475 (0.618-3.524)
Neighbors with Tb	No	284 (71.0)	789 (93.9)	**Reference**			
	Yes	116 (29.0)	51 (6.1)	0.000	6.319 (4.426-9.022)	**0.005**	**4.405 (1.548-12.534)**
Frequency of contact with a neighbor suffering from Tb	Never	307 (76.8)	799 (95.1)	**Reference**			
	Frequently	93 (23.2)	41 (4.9)	0.000	5.903 (3.996-8.722)	0.780	0.844 (0.257-2.772)

Table 8. Binary logistic regression of the demographic factors influencing Tb.
Confounding factors were adjusted. p-value < 0.05 is statistically significant.

4.2. Analysis of Rifampicin Resistant Tb cases in Mizoram

4.2.1. Socio-demographic and clinical information of RR-Tb patients

Among the 460 RR-Tb cases, 57.8% (n=266) were males and 42.2% (n=194) were females. A high number of cases were observed in the age group of 25-34 years (34.5%, female=74; male=85) followed by 35-44 years (20.4%,female=33; male=61), 15-24 years (19.3%, female = 44; male=45), 45-54 years (11%, female=14; male=37), 55-64 years (6%, female=8; male = 20), >65 years (4.7%, female=7; male=15), 5-14 years (2.3%, female=8; male = 3) and 0-4 years (1.3%, female=6; male=0) (Figure 18). The mean age was 34 years. The predominant age group among extrapulmonary Tb was also between 25-34 years (n=29, 44.6%).

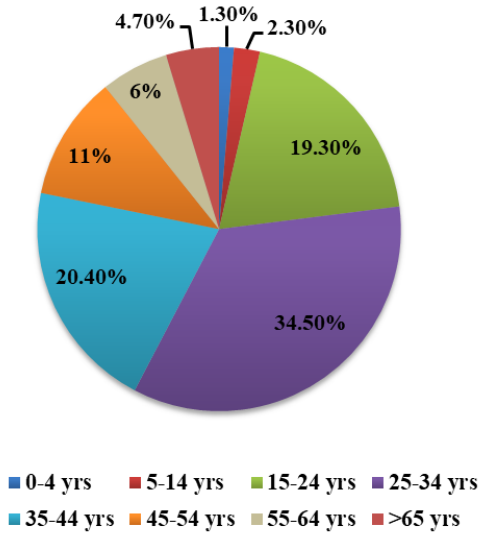


Figure 18. Age group of Rifampicin Resistant Tb patients in Mizoram

4.2.2. Sample type vs semi-quantitative Analysis

Majority of the specimens were pulmonary (n=395, 85.9%) such as sputum, gastric lavage, BAL/bronchial wash and tracheal aspirate while the remaining were extrapulmonary (n=65, 14.1%) such as lymph node aspirate, pleural fluid, cerebrospinal fluid, abscess and FNAC from various sites. As Xpert Mtb/RIF assay can semi-quantitatively quantify DNA present in the samples, the amount of DNA among the 460 RR-Tb cases were segregated as follows: High (n=107, 23.26% i.e., 104 from sputum samples, 1 each from gastric aspirate, liver abscess and pleural

fluid, respectively); Medium (n=129, 28% i.e., 125 from sputum samples, 3 from Bronchoalveolar Lavage (BAL) and 1 from FNAC of lymph node); Low (n=126, 27.39% i.e., 92 sputum samples, 11 lymphnode FNAC, 9 samples from abscess, 7 pleural fluid, 2 trachael aspirate, 2 gastric aspirate, 1 BAL, 1 ascitic fluid and 1 CSF); Very low (n=98, 21.3% i.e., 3 abscess, 61 sputum, 1 ascitic fluid, 2 Bronchoalveolar Lavage (BAL), 6 CSF, 12 lymphnode FNAC, 2 gastric aspirate, 11 pleural fluid).

4.2.3. Mutation in *rpo*B probes between pulmonary and extrapulmonary samples

In this study, probes A, B and D had higher mutation among extrapulmonary samples as compared to pulmonary samples. Whereas, probes C and E had higher mutation among pulmonary samples (Figure 19).

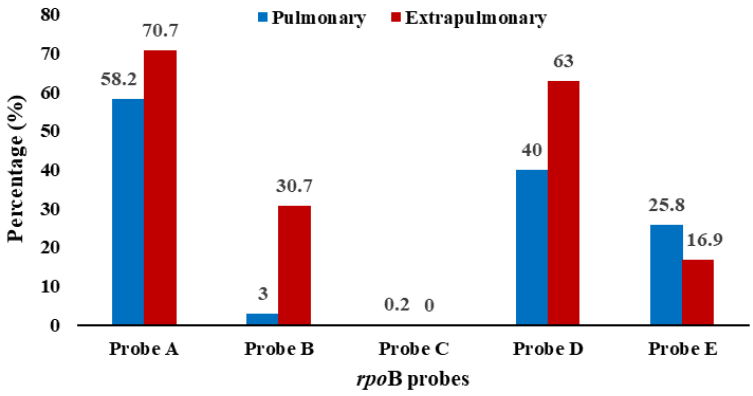


Figure 19. Comparison of *rpo*B probes mutation between pulmonary and extrapulmonary samples

4.2.4. Mutation in *rpo*B probes between pulmonary and extrapulmonary samples with respect to Gender

RR-Tb was predominant among pulmonary Tb as compared to extra-pulmonary forms among the males. On the contrary, RR-Tb was more in females suffering from extrapulmonary Tb (Figure 20).

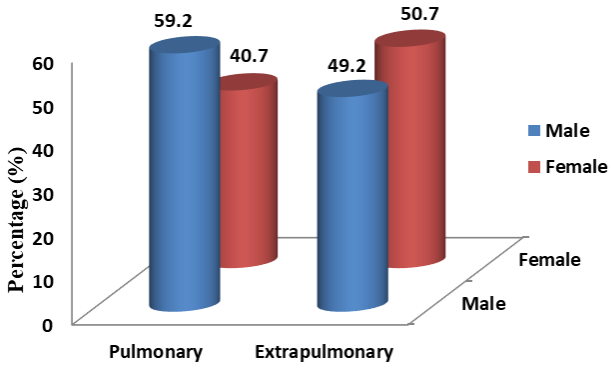


Figure 20. Prevalence of RR-Tb in comparison to gender and sample type

4.2.5. Characteristics of RR-Tb cases diagnosed via. GeneXpert in Mizoram

The number of rifampicin resistant Tb detected via. GeneXpert from 2014 till May 2021 was segregated year wise according to the different age groups as follows (Table 9):

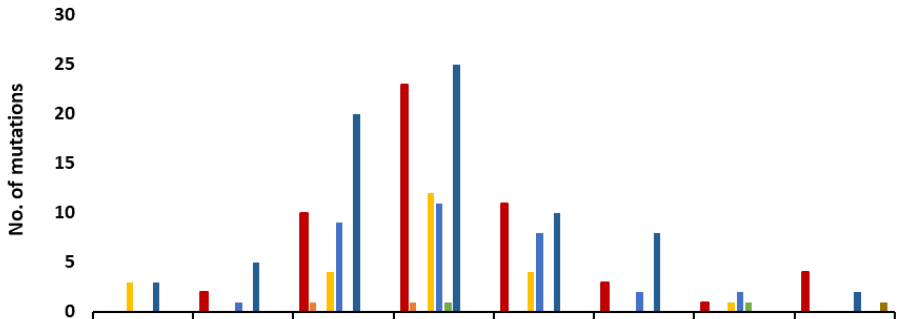
Age Group	Year wise number of Rifampicin Resistant Tb cases detected using GeneXpert Mtb/RIF (%)							
	2014 [n=52]	2015 [n=47]	2016 [n=43]	2017 [n=55]	2018 [n=64]	2019 [n=98]	2020 [n=76]	2021 [n=25]
0-4 years	0(0)	0(0)	0(0)	2(3.6)	1(1.6)	2(2.0)	1(1.3)	0(0)
5-14 years	2(3.8)	1(2.1)	0(0)	2(3.6)	2(3.1)	0(0)	3(4.0)	1(4.0)
15-24 years	9(17.3)	8(17.0)	12(27.9)	8(14.5)	15(23.4)	16(16.3)	16(21.1)	5(20.0)
25-34 years	16(30.8)	15(31.9)	11(25.6)	21(38.2)	26(40.6)	35(35.7)	27(35.5)	8(32.0)
35-44 years	10(19.2)	6(12.8)	12(27.9)	15(27.3)	11(17.2)	19(19.5)	14(18.4)	7(28.0)
45-54 years	7(13.5)	9(19.1)	4(9.3)	3(5.5)	5(7.8)	16(16.3)	6(7.9)	1(4.0)
55-64 years	5(9.6)	6(12.8)	3(7.0)	1(1.8)	3(4.7)	8(8.2)	1(1.3)	1(4.0)
≥ 65 years	3(5.8)	2(4.3)	1(2.3)	3(5.5)	1(1.6)	2(2.0)	8(10.5)	2(8.0)

Table 9. Year wise number of Rifampicin Resistant Tb detected via. GeneXpert segregated with respect to age group.

4.2.6. Mutation in *rpo*B probes between male and female Tb patients

The mutation frequency in males were: (i) Single probe mutation: probe A- 24.4% (65/266), probe B- 3.8% (10/266), probe C- 0.4% (1/266), probe D- 7.9% (21/266) and probe E- 28.2% (75/266); (ii) Multiple probe mutation: probes AD- 29.3% (78/266), probes AE- 0.8% (2/266), probes DE- 0.4% (1/266) and probes ADE – 0.4% (1/266). All probes positive RR-Tb were found in 5 samples. On the other

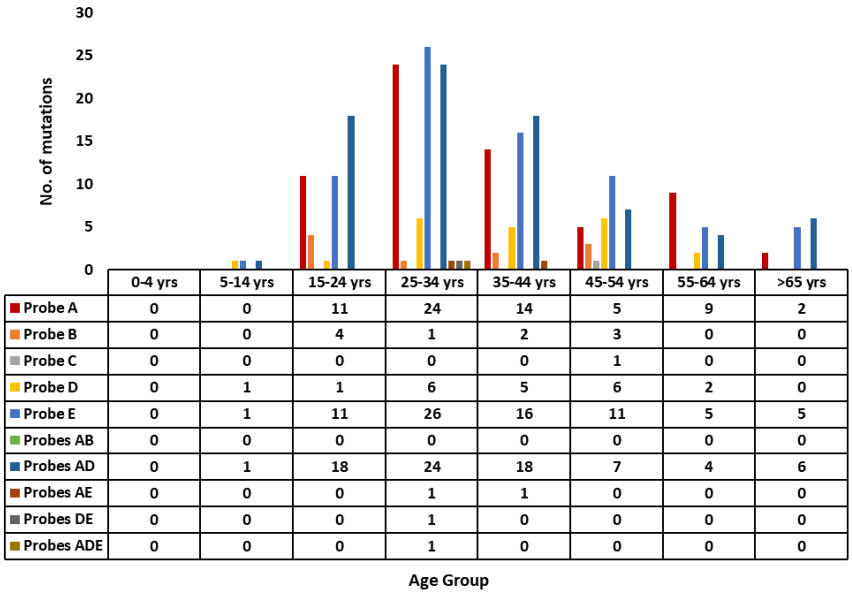
hand, the mutation frequency in females were: (i) Single probe mutation: probe A- 27.8% (54/194), probe B- 1% (2/194), probe C- 0%, probe D- 12.4% (24/194) and probe E -17% (33/194). (ii) Multiple probes mutation: probes AB – 1% (2/194), probes AD – 37.6% (73/194) and probes ADE – 0.5% (1/194). All probes positive RR-Tb were found in 12 samples. The results showed that mutations in probes A, B, C, E, AD, AE and DE were more frequent among males whereas probes D and AB mutations were more among females. In addition, different age groups among males and females displayed varied probe mutations (Figure 21 A and 21 B). The overall mutations in individual and combined probes are shown in Figure 21 C.



	0-4 yrs	5-14 yrs	15-24 yrs	25-34 yrs	35-44 yrs	45-54 yrs	55-64 yrs	>65 yrs
Probe A	0	2	10	23	11	3	1	4
Probe B	0	0	1	1	0	0	0	0
Probe C	0	0	0	0	0	0	0	0
Probe D	3	0	4	12	4	0	1	0
Probe E	0	1	9	11	8	2	2	0
Probes AB	0	0	0	1	0	0	1	0
Probes AD	3	5	20	25	10	8	0	2
Probes AE	0	0	0	0	0	0	0	0
Probes DE	0	0	0	0	0	0	0	0
Probes ADE	0	0	0	0	0	0	0	1

Age Group

Figure 21 A. Age group and probe mutations among female RR-Tb cases



	0-4 yrs	5-14 yrs	15-24 yrs	25-34 yrs	35-44 yrs	45-54 yrs	55-64 yrs	>65 yrs
Probe A	0	0	11	24	14	5	9	2
Probe B	0	0	4	1	2	3	0	0
Probe C	0	0	0	0	0	1	0	0
Probe D	0	1	1	6	5	6	2	0
Probe E	0	1	11	26	16	11	5	5
Probes AB	0	0	0	0	0	0	0	0
Probes AD	0	1	18	24	18	7	4	6
Probes AE	0	0	0	1	1	0	0	0
Probes DE	0	0	0	1	0	0	0	0
Probes ADE	0	0	0	1	0	0	0	0

Figure 21 B. Age group and probe mutations among male RR-Tb cases

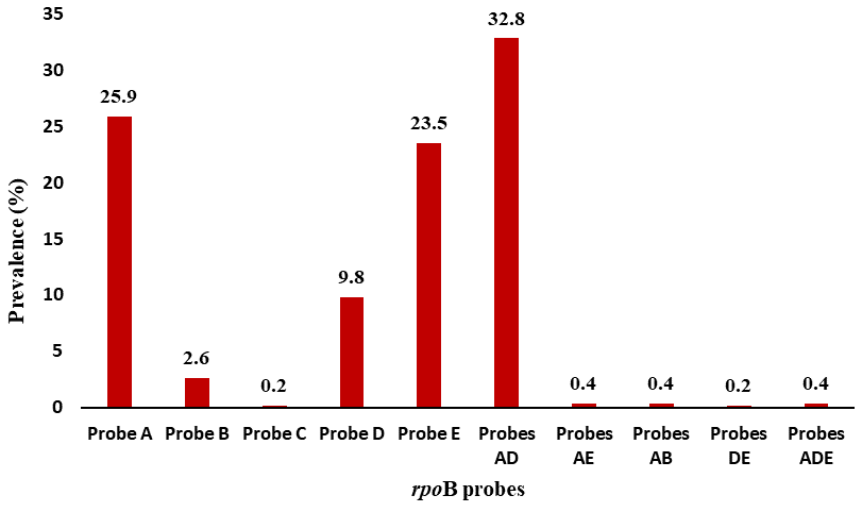
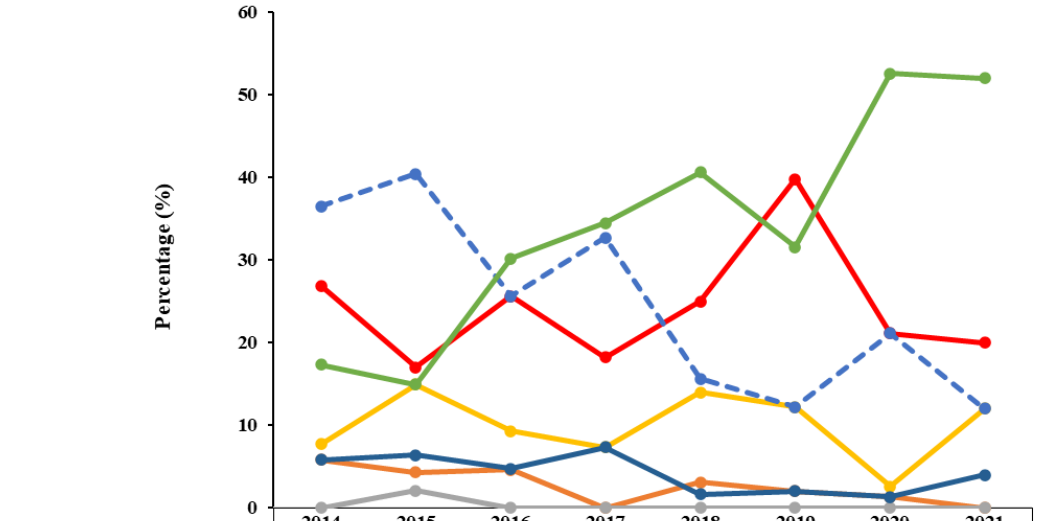


Figure 21 C. Overall mutations in individual and combined probes (2014 - 2021).

4.2.7. Pattern of Xpert Mtb/RIF probes mutation during 2014 – 2021.

In this retrospective study, it was observed that throughout the eight years (2014 to May 2021), single probe mutations were observed as follows: probe A (codons 507-511), n=119 (25.9%) had the most common mutation as compared to the other four probes. This was followed by probe E (codons 529-533), n=108 (23.5%), probe D (codons 523-529), n=45 (9.8%), probe B (codons 512-518), n=12

(2.6%) and probe C (codons 518-523), n=1 (0.2%). There was no probe B mutation in the year 2017 as well as 2021 till the study time period. Also, mutation in probe C (codons 518-523) was observed only once (in 2015) within these 8 years. Within these eight years, mutation combinations were also observed as follows: Probes AB (n=2, 0.4%), probes AD (n=151, 32.8%), probes AE (n=2, 0.4%), probes DE (n=1, 0.2%) and probes ADE (n=2, 0.4%). However, 17 (3.7%) RR-Tb cases had no missed probe types (all probes positive) and the ΔCt for these samples were all >4 cycles. Including all the mutations (single and multiple), the Genexpert probe mutations throughout eight years are shown in Figure 22. Among the 460 RR-Tb cases, 98 (21.3%) samples had "very low" DNA quantity which is correlated with the missing probes as (i) single probe mutation: Probe A (n=23, 23.5%), B (n=0), C (n=1, 1%), D (n=9, 9.2%), E (n=15, 15.3%), (ii) multiple probes mutation: Probes AD (n=44, 44.9%), AE (n=1, 1%) and ADE (n=2, 2%) and all probes positive (n=3, 3%).

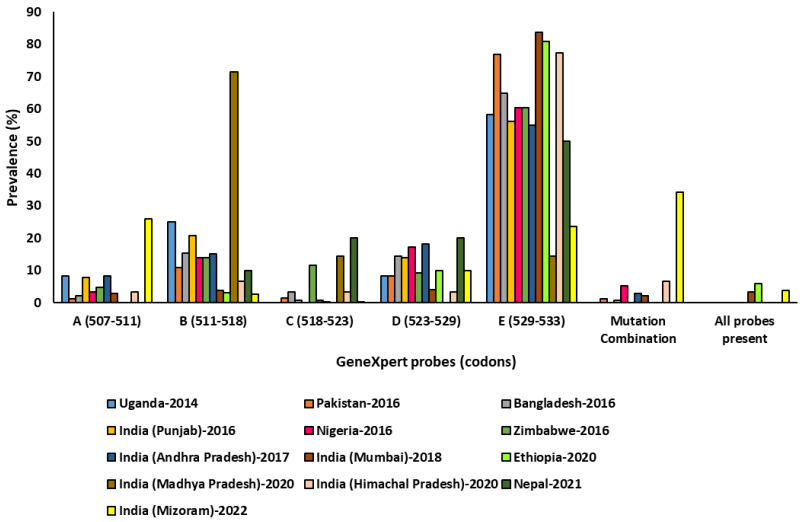


	2014	2015	2016	2017	2018	2019	2020	2021
Probe A	26.9	17	25.6	18.2	25	39.8	21.1	20
Probe B	5.8	4.3	4.6	0	3.1	2	1.3	0
Probe C	0	2.1	0	0	0	0	0	0
Probe D	7.7	14.9	9.3	7.3	14	12.2	2.6	12
Probe E	36.5	40.4	25.6	32.7	15.6	12.2	21.1	12
Mutation Combination	17.3	14.9	30.2	34.5	40.6	31.6	52.6	52
All probe positive	5.8	6.4	4.7	7.3	1.6	2	1.3	4

Figure 22. Pattern of *rpo*B probes mutation during eight years in Mizoram

4.2.8. Comparison of Rifampicin Resistant Tb probes mutation studies performed using GeneXpert system

Twelve studies employing Xpert Mtb/RIF were retrieved from 2014 to 2021. These studies reported probe mutations conferring rifampicin resistance and were compared with the current study (Figure 23).



Study	Country	Total Number of RR-TB	Probe mutations					Mutation combinations	All Probes Positive
			A	B	C	D	E		
Mboowa *et al.*, 2014	Uganda	12	1	3	0	1	7	0	0
Ullah *et al.*, 2016	Pakistan	408	5	44	6	34	314	5	0
Rahman *et al.*, 2016	Bangladesh	91	2	14	3	13	59	0	0
Kaur *et al.*, 2016	India (Punjab)	130	10	27	1	18	73	1	0
Ochang *et al.*, 2016	Nigeria	58	2	8	0	10	35	3	0
Metcalfe *et al.*, 2016	Zimbabwe	43	2	6	5	4	26	0	0
Reddy *et al.*, 2017	India (Andhra Pradesh)	171	14	26	1	31	94	5	0
Kanade *et al.*, 2018	India (Mumbai)	686	20	26	2	27	575	14	22
Alemu *et al.*, 2020	Ethiopia	100	0	3	0	10	81	0	6
Kumar *et al.*, 2020	India (Himachal Pradesh)	31	1	2	1	1	24	2	0
Sharma *et al.*, 2020	India (Madhya Pradesh)	7	0	5	1	0	1	0	0
Adhikari *et al.*, 2021	Nepal	10	0	1	2	2	5	0	0
Sailo *et al.*, 2022	India (Mizoram)	460	119	12	1	45	108	158	17

Figure 23. Comparison of the prevalence of probes mutation conferring rifampicin resistance in different countries

The above previous studies conducted within and outside India have reported the most frequent probe mutation occurring at E, except for one study by Sharma et al. (2020) where probe B was found to be the most mutated site. The present study reports the most frequent mutation occurring at probe A, followed by probe E. This study also reports very high number of mutation combinations as well as high number of RR-Tb detected using cycle threshold >4 cycles (all probe positive RR-Tb).

4.2.9. Association of socio-demographic data and mutations present at each probe among all RR-Tb cases

The study of the association of demographic characteristics of patients to rifampicin resistance showed the following: using bivariate analysis, the frequency of mutation at probes A (p=0.009; OR=1.670; CI=1.137-2.453) and D (p=0.007; OR=1.668; CI=1.146-2.426) were statistically significant for females. For sample type, the frequency of mutation at probes A (p=0.047; OR=1.782; CI=1.009-3.149) and D (p=0.001; OR=2.450; CI=1.432-4.189) were statistically significant for extrapulmonary Tb. The frequency of mutation at probe D site was also statistically significant for samples with low (p=0.000; OR=2.915; CI=1.685-5.042) and very low (p=0.000; OR=3.283; CI=1.837-5.867) DNA quantity (Table 10). Multivariate analysis showed that among the various age groups, the frequency of mutation at probe A site was statistically significant for younger age groups: 15-24 years (p=0.015; OR=2.394; CI=1.183-4.847) and 25-34 years (p=0.032; OR=2.009; CI=1.061-3.802) (Table 11). Single and mutation combination of the *rpo*B probes observed were statistically significant (p=0.000) for ≤ 40 years of age.

Categories		Probe A		Probe B		Probe C		Probe D		Probe E	
		p-value	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**
Gender	Males	**Reference**		**Reference**		**Reference**		**Reference**		**Reference**	
	Females	**0.009**	**1.670 (1.137 – 2.453)**	0.302	0.539 (0.167 – 1.745)	-	-	**0.007**	**1.668 (1.146 – 2.426)**	0.003	0.503 (0.319 – 0.792)
Sample type	Pulmonary Tb	**Reference**		**Reference**		**Reference**		**Reference**		**Reference**	
	Extrapulmonary Tb	**0.047**	**1.782 (1.009 – 3.149)**	0.995	0.995 (0.218 – 4.549)	-	-	**0.001**	**2.450 (1.432 – 4.189)**	0.111	0.573 (0.288 – 1.136)
DNA Quantity	High	**Reference**		**Reference**		**Reference**		**Reference**		**Reference**	
	Medium	0.912	0.972 (0.581 – 1.625)	0.140	0.396 (0.116 – 1.353)	-	-	0.172	**1.471** (0.846 – 2.559)	0.700	0.897 (0.514 – 1.563)
	Low	0.190	1.420 (0.841 – 2.399)	0.044	0.200 (0.41 – 0.961)	-	-	**0.000**	**2.915 (1.685 – 5.042)**	**0.018**	0.479 (0.261 – 0.881)
	Very low	0.12	2.112 (1.182 – 3.773)	0.996	-	-	-	**0.000**	**3.283 (1.837 – 5.867)**	**0.029**	0.483 (0.251 – 0.929)

Table 10. Bivariate analysis of the demographic and other factors influencing *rpo*B probe mutation.

Categories (Age group)	Probe A		Probe B		Probe D		Probe E	
	p-value	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**
0-4 years	0.820	1.217 (0.224 - 6.615)	-	-	-	-	-	-
5-14 years	0.108	3.246 (0.771 - 13.661)	-	-	0.183	2.500 (0.649 – 9.635)	0.610	0.650 (0.124 – 3.405)
15-24 years	**0.015**	**2.394 (1.183 - 4.847)**	0.948	0.952 (0.218 - 4.161)	0.415	1.335 (0.666 – 2.677)	0.686	0.847 (0.380 -1.891)
25-34 years	**0.032**	**2.009 (1.061 – 3.802)**	0.157	0.308 (0.060 – 1.575)	0.780	1.095 (0.578 – 2.077)	0.962	0.983 (0.476 – 2.028)
35-44 years	0.156	1.643 (0.827 – 3.265)	0.256	0.348 (0.056 – 2.153)	0.831	0.927 (0.463 – 1.857)	0.885	1.059 (0.486 – 2.307)
55-64 years	0.472	1.405 (0.557 - 3.543)	0.657	0.593 (0.059 – 5.981)	0.154	0.476 (0.172 – 1.322)	0.962	0.974 (0.337 – 2.819)
>65 years	0.074	2.609 (0.910 – 7.478)	-	-	0.983	0.989 (0.358 – 2.733)	0.873	1.096 (0.354 – 3.393)

Reference Category: 45-54 years; Positive (No mutation)

Table 11. Multivariate analysis of the demographic factor (age) influencing *rpo*B gene probes mutation.

Treating tuberculosis with rifampicin resistant (cases) and rifampicin sensitive (controls), variables such as gender, sample type and DNA quantity were compared between the two. However, no significant association was observed, i.e., p=>0.05 (Table 12).

Categories		**Cases n=460 [Rif- Resistant Tb] (%)**	**Controls n=920 [Rif- Sensitive Tb] (%)**	**p- value**	**OR (95% CI)**
Gender	Males	266 (57.8)	534 (58.0)	**Reference**	
	Females	194 (42.2)	386 (42.0)	0.939	1.009 (0.804 – 1.266)
Sample type	Pulmonary Tb	394 (85.6)	784 (85.2)	**Reference**	
	Extrapulmonary Tb	66 (14.3)	136 (14.9)	0.829	0.966 (0.703 – 1.327)
DNA Quantity	High	107 (23.2)	175 (19.0)	**Reference**	
	Medium	129 (28.0)	263 (28.6)	0.177	0.802 (0.583 – 1.104)
	Low	126 (27.4)	275 (29.8)	0.077	0.749 (0.544 – 1.032)
	Very low	98 (21.3)	207 (22.5)	0.140	0.774 (0.551 – 1.088)

Table 12. Bivariate analysis of Rifampicin Resistant and Sensitive tuberculosis.

To have a clearer insight of the probes' mutations occurring in all the RR-Tb cases in this study, this table shows the segregation of the different demographic factors with respect to the probes mutation which are also classified into single probe mutation as well as mutation combination/multiple probes mutation (Table 13).

Categories	Probe type										
	Single Probe Mutation (%)					Mutation Combination (%)					All probe positive
	A	B	C	D	E	AB	AD	AE	DE	ADE	
Gender											
Male (n=266)	65(24.4)	10(3.7)	1(0.4)	21(7.9)	75(28.1)	0(0)	78(29.3)	2(0.7)	1(0.4)	1(0.4)	12(4.5)
Female (n=194)	54(27.8)	2(1.0)	0(0)	24(12.4)	33(17.0)	2(1.0)	73(37.6)	0(0)	0(0)	1(0.5)	5(2.6)
Sample type											
Pulmonary (n=395)	107(27.0)	10(2.5)	1(0.2)	38(9.6)	98(24.8)	2(0.5)	118(29.9)	2(0.5)	1(0.2)	1(0.2)	17(4.3)
Extrapulmonary (n=65)	12(18.5)	2(3.1)	0(0)	7(10.8)	10(15.4)	0(0)	33(50.8)	0(0)	0(0)	1(1.5)	0(0)
Age in years											
0-4 (n=6)	0(0)	0(0)	0(0)	3(50)	0(0)	0(0)	3(50)	0(0)	0(0)	0(0)	0(0)
5-14 (n=11)	2(18.2)	0(0)	0(0)	1(9.1)	2(18.2)	0(0)	6(54.5)	0(0)	0(0)	0(0)	0(0)
15-24 (n=89)	21(23.6)	5(5.6)	0(0)	5(5.6)	20(24.5)	0(0)	38(42.7)	0(0)	0(0)	0(0)	0(0)
25-34 (n=159)	47(29.5)	2(1.2)	0(0)	18(11.3)	37(23.2)	1(0.6)	49(30.8)	1(0.6)	1(0.6)	1(0.6)	2(1.2)
35-44 (n=94)	25(26.5)	2(2.1)	0(0)	9(9.6)	24(25.5)	0(0)	28(29.8)	1(1.1)	0(0)	0(0)	5(5.3)
45-54 (n=51)	8(15.7)	3(5.9)	1(1.9)	6(11.8)	13(25.5)	0(0)	15(29.4)	0(0)	0(0)	0(0)	5(9.8)
55-64 (n=28)	10(35.7)	0(0)	0(0)	3(10.7)	7(25.0)	1(3.6)	4(14.3)	0(0)	0(0)	0(0)	3(10.7)
>=65 (n=22)	6(27.3)	0(0)	0(0)	0(0)	5(22.7)	0(0)	8(36.4)	0(0)	0(0)	1(4.5)	2(9.1)

Table 13. Association of demographic data and mutations at different probe sites among all RR-TB cases.

4.2.10. Geographic distribution of RR-Tb isolates in various residential areas of Aizawl (Mizoram)

There are 40 legislative assembly constituencies in Mizoram, out of which 14 are in Aizawl District (the capital). Patients had provided their residential areas (localities) at the time of checkup at the clinic as well as sample investigation. Therefore, as per the details provided by the patients, the localities of the RR-Tb patients within Aizawl District (the capital) were investigated (Figure 24).

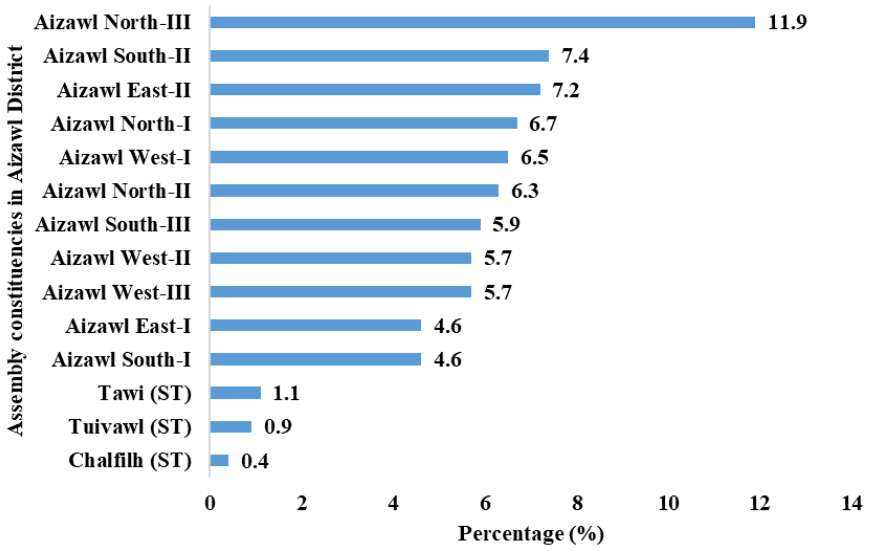


Figure 24. Geographical distribution (localities) of RR-Tb cases in Aizawl district

The areas included under each of these constituencies where RR-Tb patients reside as per the GeneXpert archive data are as follows:

Aizawl North -III (n=55, 11.9%) : Electric, Chanmari West, Hunthar, Edenthar, Zarkawt, Chanmari.

Aizawl South -II (n=34, 7.4%) : ITI, Kulikawn, Mission Veng, Model Veng, Dam Veng, Saikhamakawn, Salem Veng, Tlangnuam, Tuikhuahtlang.

Aizawl East-II (n=33, 7.2%) : Chhinga Veng, Saron Veng, Dawrpui, Armed Veng, Armed Veng South, Tuithiang.

Aizawl North-I (n=31, 6.7%) : Bawngkawn, Chaltlang, Durtlang.

Aizawl West-I (n=30, 6.5%) : Sairang, Rangvamual, Lungverh, Central Jail Veng, Tanhril, Chawnpui, Luangmual, Phunchawng, Sakawrtuichhun, Tuivamit, Zonuam, Zotlang, Govt.Complex.

Aizawl North-II (n=29, 6.3%)	**:** Ramthar, Ramhlun Venglai, Ramhlun North, Ramhlun, Ramhlun South, Ramhlun Vengthar, Aizawl Venglai, Laipuitlang, Ramhlun Sport Complex.
Aizawl South-III (n=27, 5.9%)	**:** Falkawn, Hualngohmun, Sailam, Kelsih, Hlimen, Sumsuih, Aibawk, Lamchhip, Muallungthu, Siaksuk.
Aizawl West-II (n=26, 5.7%)	**:** Tuikual North, Tuikual South, Dawrpui Vengthar, Dinthar, Vaivakawn, Kanan.
Aizawl West-III (n=26, 5.7%)	**:** Khatla, Bungkawn, Khatla East, Khatla South, Mission Vengthlang, Nursery, Maubawk, New Capital.
Aizawl East-I (n=21, 4.6%)	**:** Zemabawk, Zuangtui, Muanna Veng, Thuampui.
Aizawl South-I (n=21, 4.6%)	**:** Bethlehem, Bethlehem Vengthlang, Republic, College Veng, Upper Republic, Venghlui, Bethlehem Vengchhak, Mualpui.
Tawi (ST) (n=5, 1.1%)	**:** Tuikhurhlu, Mualpheng, Tlungvel, Thingsul.
Tuivawl (ST) (n=4, 0.9%)	**:** Darlawn, Sakawrdai.
Chalfilh (ST) (n=2, .04%)	**:** Phullen, Sesawng.

Mizoram has 11 districts and patients belonging to districts other than Aizawl were also enrolled and diagnosed as Rifampicin Resistant Tb in the District Tb Center-Falkawn, Aizawl and Synod hospital, Durtlang, Aizawl. Among these patients, the prevalence of RR-Tb as per the archived record of GeneXpert instrument are also shown in Figure 25.

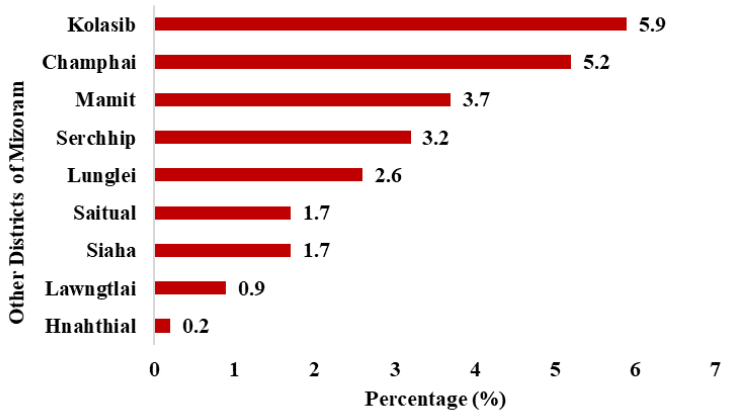


Figure 25. Patients from other districts of Mizoram diagnosed as RR-Tb at DTC, Aizawl district

The areas included under each of these districts are as follows:

Kolasib (n=27, 5.9%)	**:** Venglai, Bilkhawthlir, Diakkawn, Vairengte, North Chawnpui, Hortoki, Kawnpui, Bairabi, Hermon Veng, Salem Veng, Lungdai, Sethawn.
Champhai (n=24, 5.2%)	**:** Sialhawk, Chawngtlai, Ngopa, Bethel Veng, Hliappui, Kahrawt, New Chalrang, Venglai, Farkawn, Kawlkulh, Khawzawl, Ngaizawl, South Khawbung, Teikhang.
Mamit (n=17, 3.7%)	**:** Dampui, West Phaileng, Rengdil, Dampui South, Hmunpui, Lengpui, Saithah, Zawlnuam, Zawlpui.
Serchhip (n=15, 3.2%)	**:** Chhiahtlang, Hriangtlang, Thenzawl, Chhingchhip, Mualcheng.
Lunglei (n=12, 2.6%)	**:** Rahsi Veng, Venglai, Chawngte, Thingfal, Lunglawn, Bazar Veng.
Saitual (n=8, 1.7%)	**:** Saitual, Vanbawng, Suangpuilawn.
Siaha (n=8, 1.7%)	**:** Kawlchaw East, Meisatla, Council Veng, Meisavaih.
Lawngtlai (n=4, 0.9%)	**:** Parva, Salem, Kawlchaw West.
Hnahthial (n=1, 0.2%)	**:** Kanan Veng.

Within the study time period, Khawzawl District had no patients diagnosed as RR-Tb (using GeneXpert) at DTC, Falkawn and Synod Hospital, Durtlang.

4.3. MGIT Drug Susceptibility Report (DST)

After showing a positive result on TBc ID, sterility on blood agar and presence of cord formation, first line phenotypic drug susceptibility test (DST) was performed for 56 isolates and the results were obtained (Figure 26 A). The DST results were interpreted into three measures as resistant, sensitive and DST failed. Isoniazid antibiotic showed the highest drug resistance (32.1%). Streptomycin and ethambutol showed similar resistance pattern (26.8%), pyrazinamide (25%), while rifampicin showed the lowest resistance rate (19.6%). In addition, phenotypic drug susceptibility test also failed by a proportion of 16.1% for streptomycin, isoniazid, rifampicin and ethambutol while pyrazinamide alone failed by 35.7% (Figure 26 B).

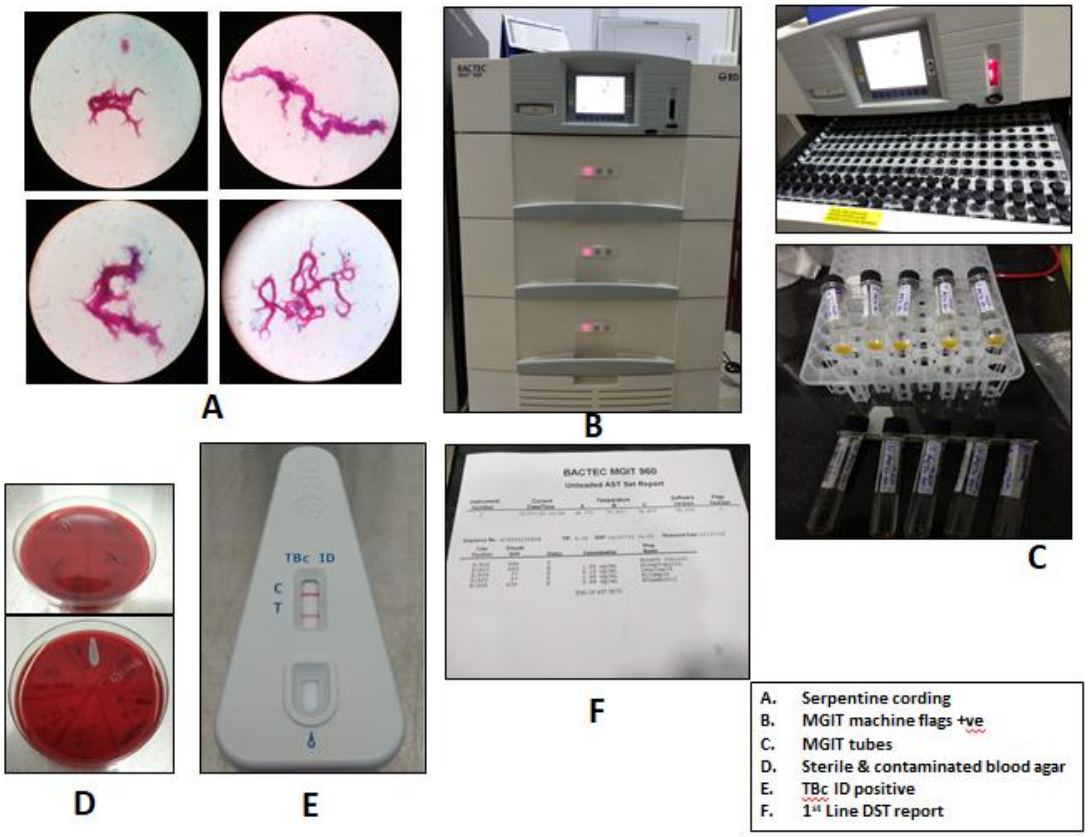


Figure 26 A. Result of liquid culture showing serpentine cording, positive TBc ID and DST.

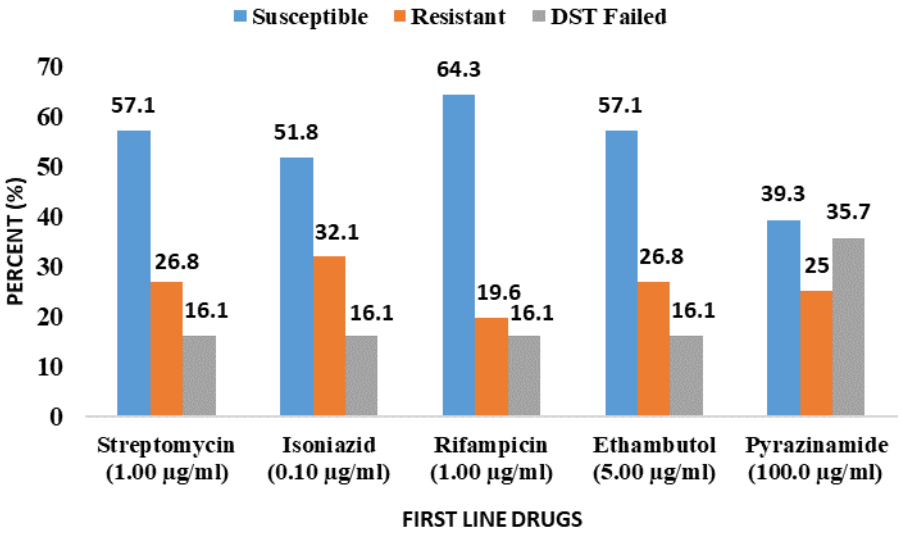


Figure 26 B. First line MGIT DST in pulmonary cases

4.3.1. PCR amplification and sequencing of *kat*G gene

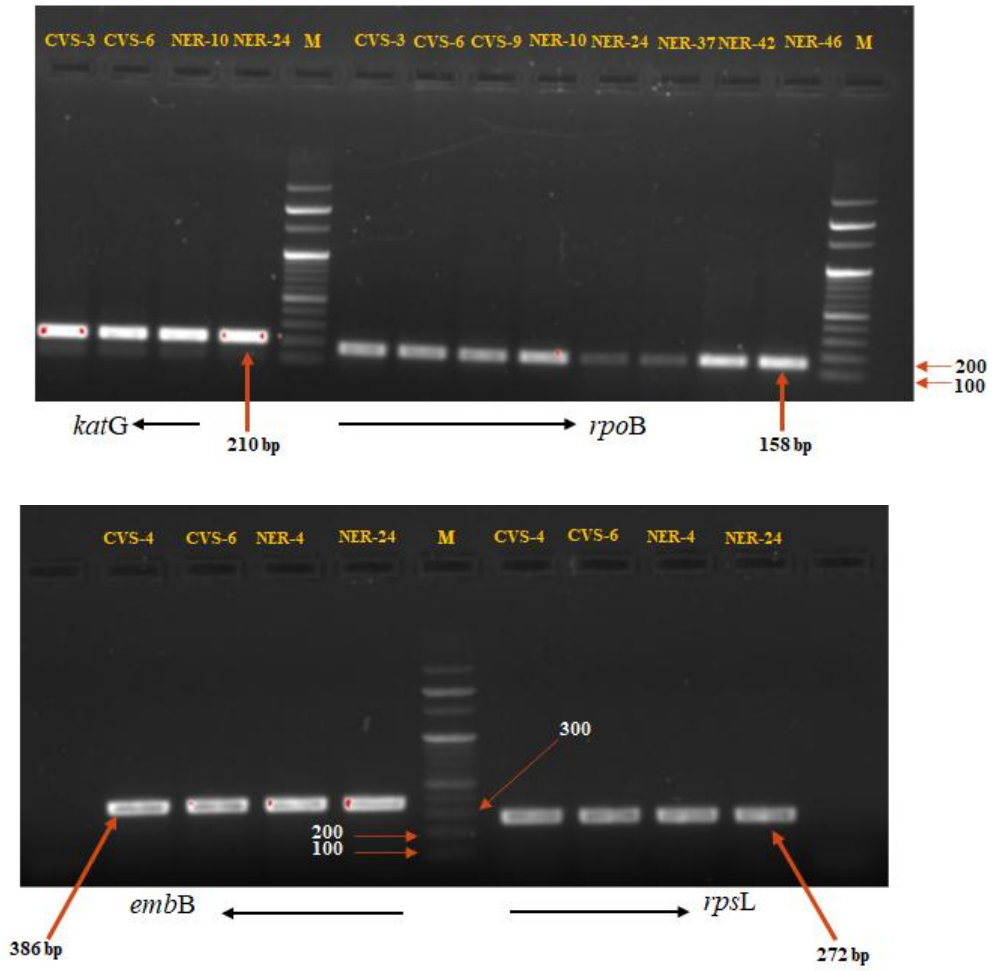
PCR amplification was performed for 26 samples against five antibiotic resistance genes. Of the 26 samples, 10 were INH resistant isolates and three from these samples (CVS-1, 13, 17) did not correlate with mutations in Sanger sequencing. Seven samples (CVS-3$^{\#}$, 6$^{\#}$, NER-10, 24, 37, 42, 46) had both INH (MGIT 960) and RIF resistance (Xpert Mtb/RIF) and defined as MDR. Among these, in 6 samples (CVS-6$^{\#}$, NER-10, 24, 37, 42, 46) the most common amino acid change was Serine to Threonine (85.7%) at codon 315 (AGC>ACC) of the *kat*G. The Ser315Asn substitution (AGC>AAC) was observed in one sample (CVS-3$^{\#}$). Mutations were not found in any of the INH susceptible isolates (Table 14).

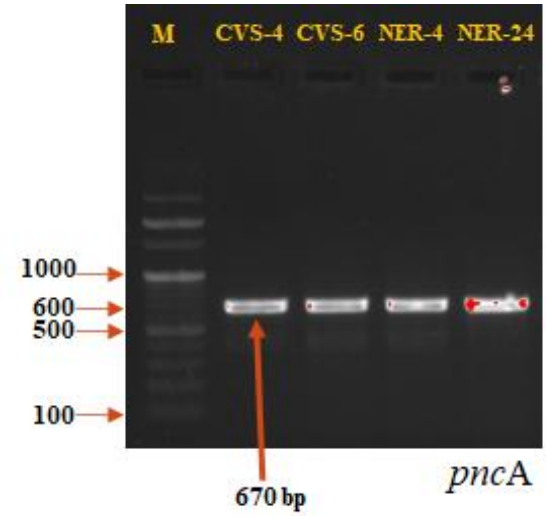
4.3.2. PCR amplification and sequencing *rpo*B gene

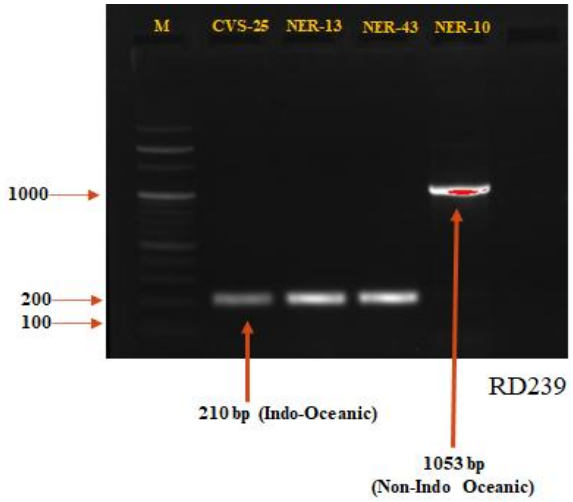
Apart from the concordant phenotypic (MGIT 960) and genotypic (Xpert Mtb/RIF) results, discordant DST results for Rifampicin were observed: 4 samples (NER-24, 42, CVS-3$^{\#}$, 9$^{\#}$) were MGIT 960 susceptible while resistant in Xpert Mtb/RIF and 3 samples (CVS-1, 13, 17) were rifampicin resistant in MGIT 960 and susceptible via. Xpert Mtb/RIF. The genexpert missing probes/ probe dropout results matched with the sanger sequence data of *rpo*B. Two samples (CVS-6$^{\#}$, NER-46) had probes dropout at both probes A (codons 507-511) and D (codons 523-529). While three samples (NER-10, 24, 42) had a probe dropout only at probe A and NER-37 had a single probe drop out at D.

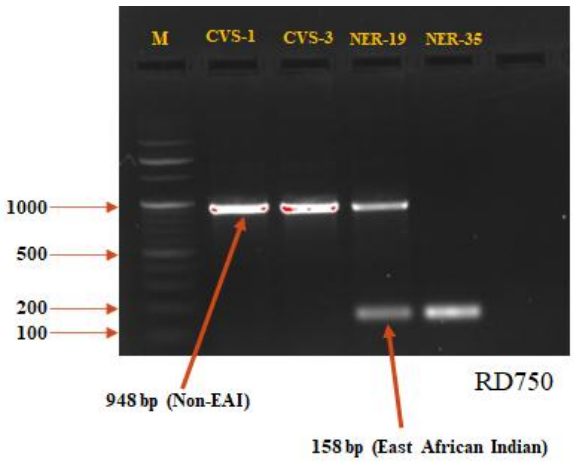
After DNA extraction, PCR amplification was done using antibiotic resistance genes and LSP markers, the band of which are visualized using gel electrophoresis (Figure 27). In this study, the mutations occurring at *rpo*B probe A (codons 507-511) upon sanger sequencing reveals an amino-acid substitution from Leucine to Proline (L511P, CTG →CCG) in five samples (CVS-6$^{\#}$, NER-10, 24, 42, 46). The most common mutation occurring at probe D (codons 523-529) revealed amino acid substitution from Histidine to Glutamine (H526Q, CAC → CAG) in two samples (CVS-6$^{\#}$, NER-46). Another less common mutation at probe D was amino-acid substitution from Histidine to Leucine (H526L, CAC →CTC) in one sample (NER-37). Both isoniazid (via. MGIT 960) and rifampicin (via. Xpert Mtb/RIF) were

resistant in one sample (CVS-3#), and hence, an MDR. In two samples (CVS-3#, 9#), the phenotypic DST of rifampicin (via. MGIT 960) was susceptible but resistant (via. Xpert Mtb/RIF). However, for these two samples, Genexpert result showed no probe drop out in all the five probes (all probes positive). The ΔCt was >4 resulting in instrument positivity (rifampicin resistance). The representative chromatogram images are shown (Figure 28).




M
CVS-4
CVS-6
NER-4
NER-24
1000
600
500
100
670 bp
pncA


M
CVS-25
NER-13
NER-43
NER-10
1000
200
100
RD239
210 bp (Indo-Oceanic)
1053 bp
(Non-Indo Oceanic)


M
CVS-1
CVS-3
NER-19
NER-35
1000
500
200
100
RD750
948 bp (Non-EAI)
158 bp (East African Indian)

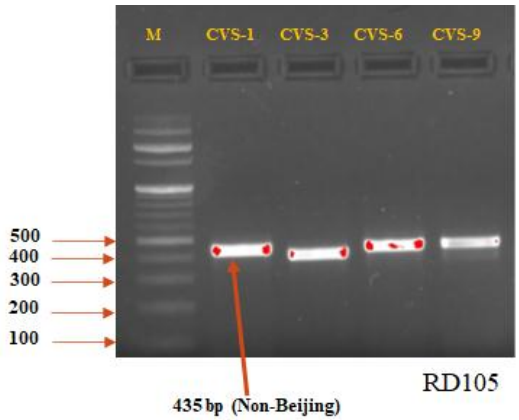
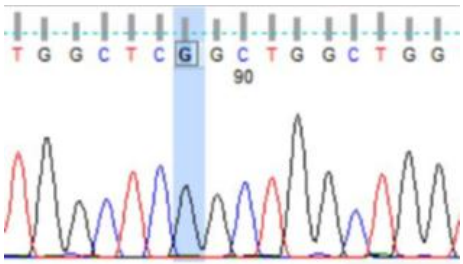
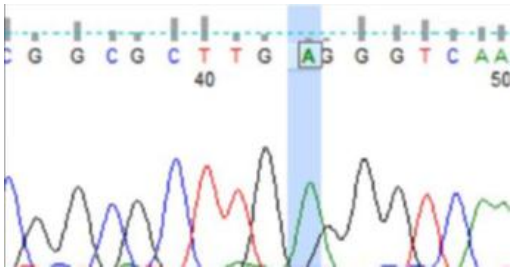


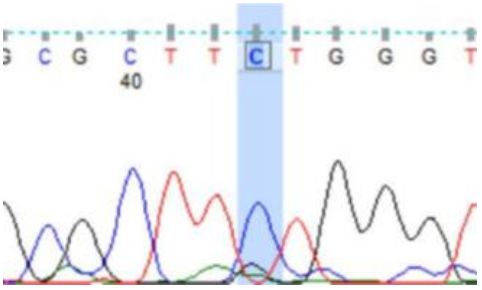
Figure 27. Representative gel images of antibiotic resistance genes and LSP markers.



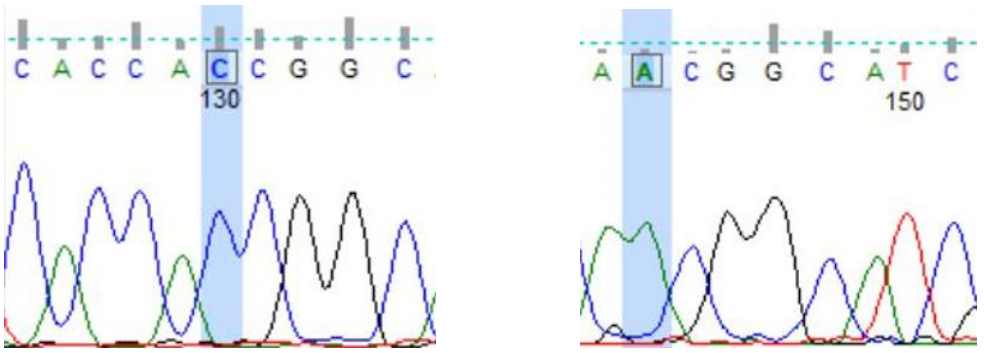
rpoB: CVS-6 (CTG to CCG → L511P)
Using Reverse Primer (CAG to CGG)



rpoB: NER-37 (CAC to CTC → H526L)
Using Reverse Primer (GTG to GAG)



rpoB: NER-46 (CAC to CAG → H526Q)
Using Reverse Primer (GTG to CTG)



katG: NER-24 (AGC to ACC → Ser315Thr) ***katG: CVS-3 (AGC to AAC → Ser315Asn)***

Figure 28. Representative Chromatogram of antibiotic resistance genes

4.3.3. PCR amplification and sequencing of *rps*L, *emb*B and *pnc*A gene for Streptomycin, Ethambutol and Pyrazinamide

For Streptomycin, Ethambutol and Pyrazinamide, the phenotypic DST resistant profile did not correlate with the sanger sequencing results. These samples were: 14 samples were Streptomycin resistant (NER-24, 31, 36, 37, 42, 46, CVS-6#, 8, 22, 23, 27, 13, 37, 38), 14 samples were Ethambutol resistant (NER-24, 37, 42, 43, 46, CVS-1, 6#, 8, 22, 23, 27, 13, 37, 38) and 14 samples were Pyrazinamide resistant (NER-10, 19, 35, 37, 38, 41, 42, 45, 46, CVS-6#, 8, 21, 36, 37).

4.3.4. Lineage of *M. tuberculosis*

Genotyping of the Mtb major lineages were attempted using Long Sequence Polymorphism (LSP-PCR), otherwise known as Regions of Differences (RDs). Out of the 26 samples, lineage was identified for 11 (42.3%) samples. Samples CVS- 25, NER-13, 43 (11.5%) were Indo-Oceanic (Lineage 1); NER-32 (3.8%) was Beijing (Lineage 2); NER-35, 46 (7.7%) were East-African Indian (Lineage 3). CVS-1, 3#, NER-453 (11.5%) were Euro-American (Lineage 4). CVS-24 and NER-19 (7.7%) samples had mixed infection of both Indo-Oceanic and East-African Indian (Lineage 1 & Lineage 3).

15 samples (57.7%) were unknown i.e., could not be identified by the LSP-PCR method employed for genotyping in this study (Table 14).

Sample Code	Isoniazid			Rifampicin								LSP markers
				Drug Susceptibility Test		GeneXpert Probes Result					Sanger Sequencing		
	Phenotypic DST (MGIT)	***kat*G codon change (G/C/A)**	**Amino acid change**	**Phenotypic DST (MGIT)**	**Genotypic Rif-Resistance (GeneXpert)**	**A (507-511)**	**B (512-518)**	**C (518-523)**	**D (523-529)**	**E (529-533)**	**SNP**	**Amino acid change**	**Lineage**
CVS-1	R	AGC	Ser315	R	Not detected	+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/***EuA**
CVS-3[#]	**R**	AA**C**	**Ser315Asn**	S	**Detected**	+	+	+	+	+	No SNP detected	No SNP detected	*Non-Beijing/non-IO/non-EAI/***EuA**
CVS-6[#]	**R**	A**C**C	**Ser315Thr**	R	**Detected**	**Neg**	+	+	**Neg**	+	C**T**G>C**C**G, CA**C**>CA**G**	**L511P, H526Q**	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-9[#]	S	AGC	Ser315	S	**Detected**	+	+	+	+	+	No SNP detected	No SNP detected	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-13	R			R	Not detected	+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-21	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-24	S			S		+	+	+	+	+	-	-	*Non-Beijing/***IO/EAI/***non-EuA*
CVS-25	S			S		+	+	+	+	+	-	-	*Non-Beijing/***IO/***non-EAI/non-EuA*
NER-4	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-10	**R**	A**C**C	**Ser315Thr**	R	**Detected**	**Neg**	+	+	+	+	C**T**G>C**C**G	**L511P**	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-13	S	AGC	Ser315	S	Not detected	+	+	+	+	+	-	-	*Non-Beijing/***IO/***non-EAI/non-EuA*
NER-19	S			S		+	+	+	+	+	-	-	*Non-Beijing/***IO/***non-IO/non-EAI/***EAI/***non-EuA*
NER-23	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-24	**R**	A**C**C	**Ser315Thr**	S	**Detected**	**Neg**	+	+	+	+	C**T**G>C**C**G	**L511P**	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-32	S	AGC	Ser315	S	Not detected	+	+	+	+	+	-	-	**Beijing/***non-IO/non-EAI/non-EuA*
NER-35	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/***EAI/***non-EuA*
NER-36	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non EAI/non-EAI/non-EuA*
NER-37	**R**	A**C**C	**Ser315Thr**	R	**Detected**	+	+	+	**Neg**	+	CA**C**>CT**C**	**H526L**	*Non-Beijing/non-IO/non-EAI*

NER-41	S	AGC	Ser315	S	Not detected	+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-42	**R**	A**C**C	**Ser315Thr**	S	**Detected**	**Neg**	+	+	+	+	C**T**G>C**C**G	**L511P**	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-43	S	AGC	Ser315	S	Not detected	+	+	+	+	+	-	-	*Non-Beijing/***IO***/non-IO/non-EAI/non-EuA*
NER-44	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-45	S			S		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/***EuA**
NER-46	**R**	A**C**C	**Ser315Thr**	R	**Detected**	**Neg**	+	+	**Neg**	+	C**T**G>C**C**G, CA**C**>CA**G**	**L511P, H526Q**	*Non-Beijing/non-IO/non EAI/***EAI***/non-EuA*
CVS-4	NA	AGC	Ser315	NA	Not detected	+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-17	R			R		+	+	+	+	+	-	-	*Non-Beijing/non-IO/non-EAI/non-EuA*

Table 14. Antibiotic resistance and genes associated with variants and Lineage of Mtb.

S- Susceptible, R- Resistant; NA- Not available; + positive for GeneXpert probe; Neg-GeneXpert probe dropout; # previously treated cases; **Non-Beijing** (435 bp); **Beijing** (absence of 435 bp); **IO** Indo-Oceanic (210 bp); **non-IO** non Indo-Oceanic (1,053 bp); **EAI** East Africa Indian (158 bp); **non-EAI** non-East African Indian (948 bp); **EuA** Euro-American (absence of 332 bp); **non-EuA** non Euro-American (332 bp).

Chapter 5: Discussion and Conclusion

5.1. Discussions

5.1.1. Risk factors of Tb

In the present study, the male counterparts were at high risk of Tb infection which could be due to greater exposure to the reported risk factors which is in concordance with the Global Tuberculosis Report (Global tuberculosis report, 2020). Single individuals were more prone because they tend to involve more in multiple activities of the community, thus becoming as a high-risk group for Tb and the rate of incidence in this study was twice as high when compared to married individuals, which is also in concordance with previous report (Horwitz, 1971). Non-ideal weights (underweight, obese, over weight) were more prone to develop Tb in their lifetime as they could indicate poor health. In contrast, overweight or obesity were found to be at low risk (Leung et al., 2007). Educational status is an important aspect of life and being educated can decrease the risk of infectious diseases wherein, the awareness and knowledge to seek an immediate medical help will be a priority among the educated. The thesis results also observed that less educated participants (upto matriculation) were at a higher risk of Tb infection (Senanayake et al., 2018). Tb was found to be high midst unemployed individuals (Przybylski et al., 2014).

Poverty and Tb go in parallel wherein being economically backward increases the risk of Tb infection because of overcrowding, poor living or working conditions, malnutrition, and limited access to treatments (Siroka et al., 2016). Over-crowding is a high-risk factor for Tb that can expose susceptible individuals to the infection (Clark et al., 2002). However, it was not a significant factor in this study. The cross-ventilation system in houses is a protective factor wherein adequate aeration effectively controls Tb outbreaks (Du et al., 2020). Moist or damp houses with poor lighting is also an important risk factor because of the risk of harboring tubercle bacilli, which helps them to evade the direct killing by sunlight (Aditama et al., 2019).

Tuberculosis spreads through air and there are other probable routes of transmission which are not fully understood. Water supply source for domestic consumption is another important route pertaining to tuberculosis transmission that is very often neglected. The state of Mizoram is a mountainous hilly region situated in the north eastern part of India. The hospitals in the state have no proper sewage treatment facility and with an improper drainage system in the state, the drains clog and overflows during the rainy seasons. Hence, the waste-waters from hospitals and Tb sanatorium flows down the drain and converges down to the rivers. The majority of the participants (cases and controls) receive the Government water supply (PHED water), while some living in the lower ridges/altitudes rely on the river water as well as spring water collections (tuikhur) for domestic use. In this study, it was observed that participants with water supply source other than the government water supply have two times higher chances of contracting Tb, which is of serious concern since virulent tubercle bacilli was isolated from river water even after five months of storage (Musehold, 1900). Virulent tubercle bacilli in settled sewage water was recovered even from adequately disinfected sputum (Greenberg & Kupka, 1957). As stated by previous studies, the source of water contamination could be either human or non-human (Ghodbane & Drancourt, 2013; Martinho et al., 2013). Since both human and animal excreta can harbor the tubercle bacilli, sewage contamination of water sources could potentially be an additional source (Monkongdee et al., 2009). Our previous 16S rRNA V3-V4 region based metagenomic study around Aizawl, Mizoram examined the discharge of solid and liquid wastes from domestic, municipal and hospital premises and found the presence of *Mycobacterium tuberculosis* in soil and water sediment samples from three river sites and solid waste dumping sites (De Mandal et al., 2019). The drawback of this study is that we did not collect water samples for culture-based study which can be taken up for future studies. The aforementioned risk factors suggest a holistic view of the possible routes of Tb transmission which should be reconsidered (Velayati et al., 2015).

Since the Mizo people believe in the ethics of "*Tlawngaihna*" - a moral code which makes them courteous, helpful to others during deaths and the spontaneous gathering to help the deceased family is a very common practice. There are many community services (*Hnatlang*) to be attended for their welfare or for church

welfare. The people share their drinking water (from the same bottle or glass), snuffed tobacco from the same pack along with a puff from the same cigarette. Being a closely knit society, these practices have been in existence within the Mizo community from times immemorial. These cultural activities lead to the gathering of huge crowds and such mass gathering events can be associated with public health risks. Also, the data on tuberculosis associated with mass gathering events are scarce. However, this study found that people participating in such events have a significant chance of contracting Tb, which is also in concordance with previous reports where Tb is transmitted during the Hajj pilgrimage (Zumla et al., 2016). Patients with a travel history developed the symptoms after completing their travel. Since, the Tb status of a traveler is unknown, there is a minimal chance of Tb transmission during travel (Rieder, 2001).

The protection efficiency of BCG vaccine remains debatable and can vary based on geographical regions or populations (Fine, 1995). In this study, significant difference was observed among the participants who did not receive BCG vaccine as compared to those who did. This was in contrast to a previous study where no statistical difference in Tb was observed (Masood et al., 2019). Alcohol consumption can be a major risk factor and burden for tuberculosis and a three-fold increase in the risk was reported (Imtiaz et al., 2017). This study observed a seven-fold increase in the risk of tuberculosis with alcohol consumption, which is much higher than the previous reports. The probable reasons could be impaired immunity and social mixing patterns among people who consume alcohol (Classen et al., 1999). Many studies have associated Tb with active or passive smoking (Alavi-Naini et al., 2012; den Boon et al., 2005; Leung et al., 2010), but in this study it was not significant. A large-scale epidemiological study or contact tracing is essential to identify tuberculosis exposure (Moonan et al., 2020) as transmission between neighbors had also been documented (Moravkova et al., 2011). Similarly, in this study a significant association between tuberculosis transmission with an infected neighbor had been found.

5.1.2 Discussion on GeneXpert mutation analysis

Tuberculosis was declared as a 'global public health emergency' by WHO (World Health Organization, 1993). Globally, a total of approx. 2 lakhs MDR/RR-Tb cases were detected and notified in 2019. Three countries were responsible for nearly half of the world MDR-Tb burden: India, China and Russian Federation. As per India Tb Report 2020, diagnosis through GeneXpert is done to all the patients with smear positivity upon follow up, including treatment failures of first line ATT. Xpert Mtb/RIF is also done for early diagnosis and initiation of appropriate treatment in patients living with HIV people, children's and extrapulmonary Tb cases, smear negative patients (who have an X-ray suspicious for Tb) as well as referred patients from private organizations (India Tb Report, 2020). MDR-Tb arises either due to inadequate therapy or poor compliance to adhere fully to an appropriate treatment regimen or counterfeit drugs. In spite of all these measures, Tb still remains a huge burden especially in remote hilly regions such as Mizoram and so far, reliable published information on the MDR/RR-Tb within the state is also still unavailable.

In the present study, from December 2014 to May 2021, there were 2,894 Tb cases detected via. Xpert Mtb/RIF in Mizoram and among these, the proportion of RR-Tb diagnosed was 15.9% (n=460). Among the RR-Tb cases in this study, males (57.8%) are predominant compared to females (42.2%). Reports from Central India (17%) (Desikan et al., 2014), New Delhi (17.9%) (Singhal et al., 2015) and Lucknow 27.8% (Jain et al.,2014) have also been documented. A review from Ethiopia reported that being male is an identified risk factor for MDR-Tb (Asgedom et al., 2018). In this study, productive age group between 25-34 years were the most affected which may be due to more exposure to open cases of Tb and similar findings have been reported from China and Ethiopia (Zhu et al.,2018; Alemu et al.,2020). Among the 460 RR-Tb cases, 85.9% were from pulmonary samples while 14.4% from extrapulmonary samples. High DNA quantity was observed in pulmonary samples (sputum) while low or very low amount was found in extrapulmonary samples. This is in concordance with a study from Ethiopia (Alemu et al.,2020) and may be due to the fact that the tubercle bacilli are obligate aerobe, requires oxygen for its metabolism. Apart from other organs, the lungs have higher oxygen pressure and thus induces a

higher multiplication rate resulting in higher bacillary load (Cardona & Ruiz-Manzano, 2004).

Overall, in this study, the most common RRDR *rpo*B gene mutation in the 81 bp were observed in codons 507-511 (25.9%), codons 529-533 (23.5%), codons 523-529 (9.8%), codons 511-518 (2.6%) and the least in codons 518-523 (0.2%) which corresponds to probes A, E, D, B and C, respectively. This strongly contradicts with findings of the studies from Ethiopia (Alemu et al., 2020), Madhya Pradesh (Sharma et al., 2020) and Nepal (Adhikari et al., 2021) where they reported no mutation in probe A region. Our results are also dis-concordant with the findings of the studies from Madhya Pradesh, India where the commonest mutation was found in probe B followed by probe C and E, while no mutations were found in probes A and D (Sharma & Singh, 2020). Similar studies from different parts of the world i.e., Uganda, Pakistan, Bangladesh, Nigeria, Zimbabwe, Ethiopia, Nepal and various cities from India like Punjab, Andhra Pradesh, Mumbai and Himachal Pradesh had reported probe E (codon 531-533) to be the commonest mutated site in the region of the RRDR *rpo*B gene. This might be due to higher mean relative fitness (Darwinian fitness) (Billington et al., 1999) and resistant mutants have a better ability to survive. However, next to probe E mutation, the order of the prevalence of probe mutation varies from region to region wherein E is followed by B, D, A and C (Kaur et al., 2016; Reddy et al., 2017; Uddin et al., 2020) and by D and B (Alemu et al., 2020; Kanade et al., 2019; Ochang et al., 2016; Yue et al., 2003). A small proportion of probe C mutation was reported from Pakistan, Bangladesh, Punjab, Zimbabwe, India (Andhra Pradesh, Mumbai, Himachal Pradesh & Madhya Pradesh) and Nepal (Ullah et al., 2016; Rahman et al., 2016; Kaur et al., 2016; Metcalfe et al., 2016; Reddy et al., 2017; Kanade et al., 2019; Kumar et al., 2020; Sharma et al., 2020; Adhikari et al., 2021) while Nigeria (Ochang et al., 2016), Ethiopia (Alemu et al., 2020) and Uganda (Mboowa et al., 2014) reported no probe C mutation. This study also reports probe C mutation occurring only once within a span of eight years. The least mutations detected by Xpert Mtb/RIF in probe C might be due to this specific site of RRDR being probably less susceptible to mutations conferring the resistance or because of less selection pressure in this region.

Similar to other studies on mutation combinations, this study also reported high mutation combinations (Reddy et al., 2017; Kanade et al., 2019; Ochang et al., 2016), which may be due to the intrinsic or acquired ability of the bacteria to adapt to drug exposure. A study from Bangladesh reported the occurrence of probe mutation combinations in retreatment Tb cases (Uddin et al., 2020). Unlike other studies, this study reports high prevalence of probe mutation combinations (n=158, 34.3%) which demands deeper insights to gain rational understanding. In addition, the mutation combinations observed in this study might also be retreatment cases as information on the initiation of ATT drugs prior to testing the samples is unknown. Among the 460 samples tested, this study also reports a "very low" bacillary load in 98 samples. Having a "very low" bacillary load on Xpert testing was significantly correlated with false rifampicin resistance (Ngabonziza et al., 2020). Being a retrospective study, samples were not collected and hence the results cannot be compared with culture (gold standard) or *rpo*B sanger sequencing to rule out rifampicin false positivity rate. Therefore, it is difficult to elucidate the implications of this finding.

In this study, the proportion of extrapulmonary Tb was more among females aged 25-34 years which is in concordance with a study from China where younger female patients are more likely to have extrapulmonary Tb (Pang et al., 2019). The predilection of extrapulmonary Tb in women may be linked to the limited facilities for access to healthcare apart from the less prevalence of other risk factors such as habit of smoking (Razanamparany et al., 2002; Sreeramareddy et al., 2008). Previous studies have reported the increased risk of mortality with smoking in men, but not in women (Lam, 2001). Smoking is not common in women and are relatively protected from the hazardous pulmonary effect of smoking which may be one of the factors for the differences in distribution (Musellim et al., 2005). This suggest that female gender and younger age could be an independent risk factor for Tb especially in high burden areas. Bangladesh had reported the geographical distribution of RR-Tb within their country (Uddin et al., 2020). Similarly, in the present study within Aizawl District, Aizawl North –III (11.9%) had the highest proportion of RR-Tb followed by Aizawl South –II (7.4%) and Aizawl East –II (7.2%) which demands further investigation of the affected areas. The identification of the residential areas will

prove useful for future epidemiological surveys, understanding the transmission pattern and severity of the disease. Our study has several limitations: being a retrospective study design, it lacks relevant information such as previous history of Tb treatment, HIV, diabetes and BCG vaccination status.

5.1.3. Genotyping of drug resistant isolates and lineage of Mtb

This is the first attempt to culture tubercle bacilli since there are no functional Mtb culture facilities implemented in Mizoram. INH resistance is often accompanied by loss and/or reduction of Catalase Peroxidase activity or *kat*G activity coded by *kat*G gene (Zhang et al., 1992). INH is a prodrug that requires cellular activation by *kat*G protein to its active form, before it can exert its toxic effect on the bacillus. Resistance often occurs due to a point mutation occurring in the *kat*G gene, thus preventing the activation of the pro-drug (Barry et al., 1998). More than 300 types of *katG* mutations have been identified, however, mutations at codon 315 of the gene are the most prevalent, with one particular amino acid substitution (serine to threonine) accounting for 95% of all *katG* 315 mutations (Vilchèze et al., 2014; Seifert et al., 2015). Hence, mutation in *kat*G is responsible for high level isoniazid resistance (Ando et al., 2010). Another study also reported a 93.6% prevalence of the mutation from both new and previously diagnosed cases of tuberculosis (Mokrousov et al., 2002). In this study, similar report was observed where the 7-INH resistant isolates which are also Rifampicin resistant had the most common substitution such as Ser315Thr (n=6/7, 85.7%) in *kat*G gene. It is also admissible that the Ser315Thr substitution is associated with MDR strains (Marttila et al., 1998). One isolate (14.3%) had a Serine substituted by Asparagine at codon 315 which is less common. Similar substitution was also reported by a study from China and Agra (India) (Liu et al., 2021; Jaiswal et al., 2017). Three samples (CVS-1, CVS-13, CVS-17) that had a phenotypic INH resistance, but is not reflected in the sanger report might possibly be due to the INH resistance mutation occurring in other genes such as *inh*A (Seifert et al., 2015).

In this study, for Rifampicin drug susceptibility test, discordant results between MGIT 960 and Xpert Mtb/RIF was observed in which 3 isolates (CVS-1,

CVS-13, CVS-17) lacked mutations in the RRDR region of the *rpo*B gene, though these isolates were phenotypically resistant to RIF. These differences might be attributed to different genotypes prevailing worldwide. In addition, since Xpert Mtb/RIF detects mutations only within the RRDR of the *rpo*B gene, and the mutations might be present outside the known target regions (Heep et al., 2001). In two samples (CVS-3$^{\#}$, CVS-9$^{\#}$), we observed a discrepancy between MGIT and Xpert Mtb/RIF where rifampicin was susceptible via MGIT, resistant via Xpert Mtb/RIF without any probe drop out and ΔCt was >4, however sanger sequencing revealed no mutation in *rpo*B region. This could be a false resistance indicated by Xpert Mtb/RIF as similar discrepancy results were reported from China (Qin et al., 2021). In two other samples (NER-24, 42), rifampicin was susceptible via. MGIT and resistant via. Xpert Mtb/RIF where probe A was drop out. In this discrepancy, the mutation observed was L511P (CTG → CCG). Similar disputed *rpo*B mutation was reported from South Korea (Jo et al., 2017). A study from Kuwait reported isolates as rifampin susceptible by the MGIT system while resistant by the Xpert assay. *rpo*B sequencing identified a silent (CTG521TTG) and a missense (GAC516TAC) mutation (Mokaddas et al., 2015). However, this was not the case in this study.

In Mizoram, the specific mutation in probe A (507-511) was L511P. However, this particular mutation was not observed in a study from Bangladesh (Rahman et al., 2016). Our study also reported the specific mutation occurring at probe D (523-529) as H526Q and H526L, which is not in concordance with another study from Bangladesh where they reported mutation as H526D and H526Y (Uddin et al., 2020). The mutations L511P and H526L have been reported to confer a low level of resistance (Hauck et al., 2009). A study from Treichville, Abidjan District reported H526Q mutation in a relapse case (Kouassi et al., 2016). In this study, phenotypic DST resistance was observed for Streptomycin, Ethambutol and Pyrazinamide, which do not correlate with sanger sequencing results. This may be probably because the primers used in this study does not cover the region where SNP's are present.

Though the number of samples studied maybe small, this study attempts to provide information on *M. tuberculosis* strain diversity in this remote region of northeast India where all the four major lineages (Lineage1, Lineage 2, Lineage 3 Lineage 4) were present. In a recent study, Beijing (Lineage 2) and East-African Indian (Lineage 3) were found in three of the northeastern states, Manipur, Nagaland and Tripura (Dusthackeer et al., 2021). In this study, among the 6 INH resistant isolates, five were unknown genotypes while one belonged to East-African Indian lineage (Lineage 3). Previous studies have revealed Indo-Oceanic (Lineage 1) and East-African Indian (Lineage 3) to be most prevalent in India and less common in other parts of the world (Gutierrez et al., 2006; Ahmed et al., 2009). This might explain the mixed strain infection observed in two samples in this study. A recent study from Nepal also reported mixed lineage infection of Indo-Oceanic and East-African Indian in a captive elephant (Paudel et al., 2019). Mixed strain infections particularly when strains with different drug resistance phenotypes are involved, it makes diagnosis, treatment and control more cumbersome and people with underlying morbidity may be prone to mixed strain infection (Asare-Baah et al., 2021; Kamakoli et al., 2017). A study from Botswana had reported that among new cases of tuberculosis, mixed strain infections but not heteroresistant infection were associated with poor treatment outcomes, using standard combination treatment regimens. (Shin et al., 2018). Previous studies have shown that in patients who harbored both MDR and susceptible strains, MDR strain was able to persist and grow during treatment with first-line regimens and, upon a switch to second-line regimens, the susceptible strain was able to re-emerge, compromising treatment outcome (van Rie et al., 2005). In addition, immune responses may be altered in patients with ongoing mixed strain Tb infections, rendering them more prone to reinfection (Warren et al., 2004).

Mizoram being located in a remote area, establishment of highly sophisticated diagnostic machines which requires high end technical expertise is still cumbersome. On most occasions, ATT is initiated based on the GeneXpert reports unless there is a need to outsource samples for LPA and culture. The global frontline molecular diagnostic tools such as GeneXpert and LPA have been developed based

on known genetic markers (Gagneux & Small, 2007; Thakur et al., 2015; Thirumurugan et al., 2015). Since, frontline molecular tests rely on a limited number of mutations, there have been several instances where phenotypic resistance could not be explained by known mutations associated with drug resistance (Rigouts et al., 2013; Banu et al., 2013, Ahmad et al., 2016). Thus, our findings also suggest that Tb diagnosis aided with sequencing technologies is mandatory to provide accurate diagnosis and treatment.

The molecular study relied on small number of samples and is thus difficult to elucidate the findings of this study. Being the first Mtb culture to be performed within the state, the phenotypic DST is yet to be certified and since it is not from a competent laboratory, it warrants further confirmation. The methodology used for lineage identification in this study is not sufficient enough to determine all the possible lineages. Hence, further studies using larger number of samples and employing better identification tools such as spoligotyping is needed to support our findings.

5.2 Conclusion

Tuberculosis is still a global public health concern claiming 1.5 million lives per year. It still associated with social stigma which is responsible for the strong barrier to health-seeking behavior, especially in women, and is a cause of significant suffering. Under most circumstances, an infected individual is frequently unable to seek timely help due to the fear of losing social status, future or current marital problems or adverse response and or reaction from the community.

Being a multifactorial disease, the infection is contributed by various risk factors. In the state of Mizoram, the unique social practices within the community might also serve to fuel Tb transmission and/or hinder the tuberculosis control program. Apart from the well-established risk factors (alcohol consumption, no BCG vaccine, lower level of education, damp or moist housing, neighbors with Tb, travel history) for Tb, this study highlights the need to investigate water sources used for domestic purposes as they can serve as a potential source of infection. From a public health standpoint, this highlights the needed of further research in this area.

Due to the existence of limited Tb diagnostic infrastructures within the state, this retrospective study being the first molecular data on *Mycobacterium tuberculosis* from the state provides genetic pattern of drug resistance accountable for rifampicin resistance over the past years. This will help local clinicians be acquainted with the possible mutations conferring drug resistance in tuberculosis and further aid in the initiation of correct treatment. Also, our findings from the retrospective study provides a baseline data on the magnitude of RR-Tb within the state and identification of the residential areas can help local health authorities in planning surveillance programs to control the spread of rifampicin resistant Tb.

Our study also provides information on the genotyping of mutations detected with GeneXpert as well as MGIT 960. It also provides information on the lineages of *M. tuberculosis* circulating in the state. Though the sample size may be small, it is the first of its kind to be reported from the state of Mizoram where culture facilities are presently unavailable. Due to the existence of genetic heterogeneity among *M. tuberculosis* isolates from various geographic zones, the utilization of sequencing technologies is essential in diagnostic laboratories to map the genetic variations, identify all the possible genotypes associated with drug resistance and trace the outbreaks. In addition, it can as well be used as a cautionary measure to prevent wrong diagnosis by sequencing discrepant results and guide in effective patient care. Our study forms a baseline data and gives preliminary information on tuberculosis within the state. Hence, more research with a greater number of samples along with better stratification is needed to further strengthen our findings.

Though the global target of Tb eradication is set to 2035, tuberculosis was, is and will remain a public health concern for at least another one or more decades to come. In view of the fact that peripheral areas of India inhabited by tribal people such as Mizoram and any other tribal inhabited state often lag behind in obtaining sophisticated diagnostic infrastructures for any disease. In view of tuberculosis, better diagnostic infrastructures utilizing molecular methods is a need of the hour in every corner of the country alongside with training of manpower for the same purpose. The responsibility of the health care personnel as well as clinical researchers is to strive for the correct identification of the infection along with its

associated mutations. This may be followed by initiation of timely interventions by the Clinicians comprising of administration of the "*right drug with the right dosage at the right time with the right patient without any interruption during treatment*". This in turn can serve as the initial step in aiding Tb eradication.

Chapter 6: Summary

The present study was carried out to explore the possible risk factors of tuberculosis in Mizoram, Northeast India using a structured questionnaire. Techniques such as GeneXpert Mtb/RIF was employed to determine the pattern of *rpo*B probes mutation among Rifampicin-Resistant tuberculosis patients within a specified time duration i.e., 2014 to May 2021 (retrospective study). The residential areas of Rifampicin Resistant tuberculosis patients within Aziawl District were also identified. In addition, Ziehl Neelsen Stain, liquid culture via. Mycobacterial Growth Indicator Tube (MGIT) was employed alongside with Sanger Sequencing (3500 Genetic Analyzer automated sequencer [ABI]) to locate the precise amino acid substitution (underlying molecular mechanisms) responsible for the mutation conferring drug resistance. In addition, the lineage of tuberculosis circulating in Mizoram was also identified using Long Sequence Polymorphism-PCR (LSP-PCR).

The significant risk factors were compared with the findings of other previous studies. The probe mutations observed in GeneXpert Mtb/RIF were also compared with similar studies from different countries. Similarly, the sequencing data were aligned using Clustal Omega with reference sequences (*Mycobacterium tuberculosis* H37Rv) obtained from Mycobrowser and analysed for confirmation and presence of variants in the genes. In addition, the lineages of *M. tuberculosis* identified were compared with the lineages found within and outside India.

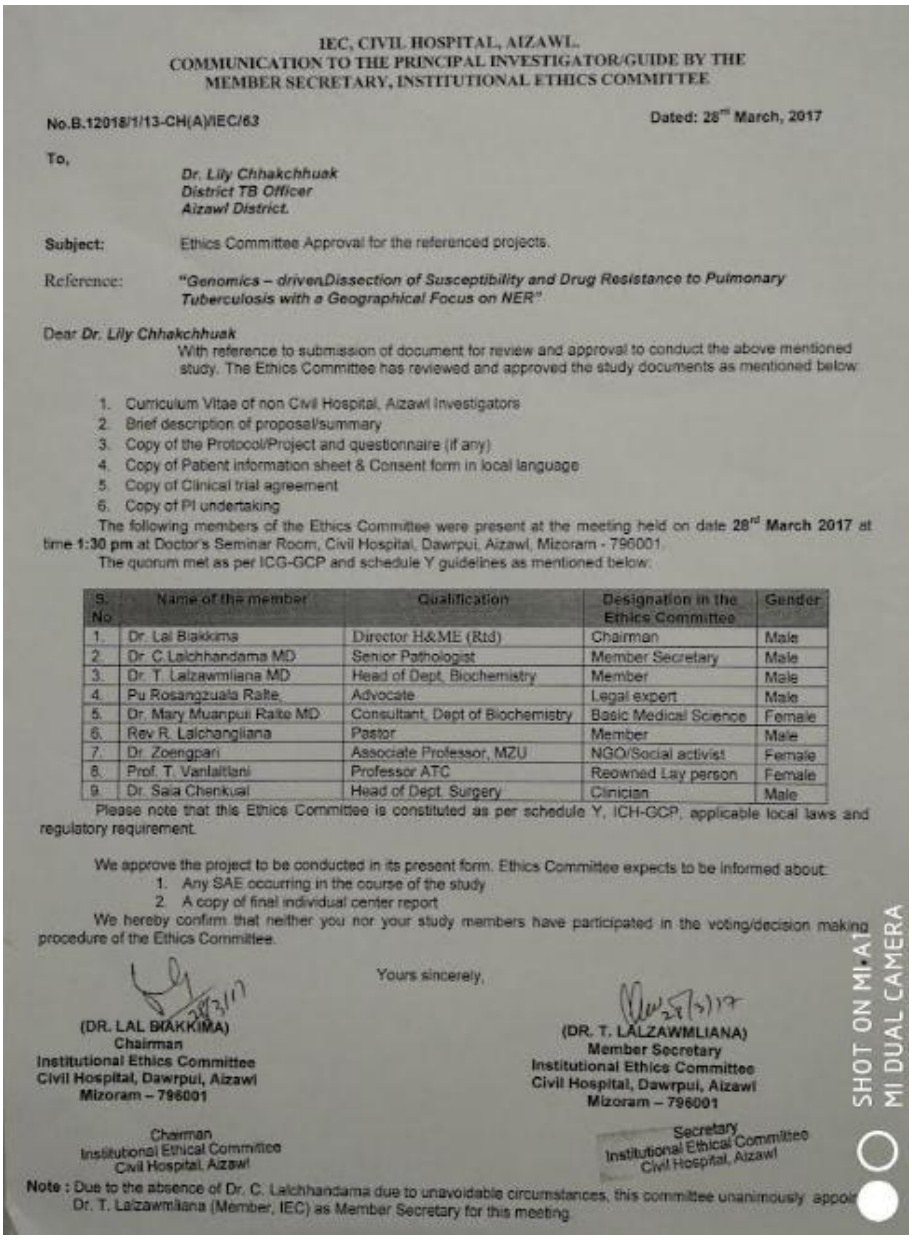
Significant findings of the study are highlighted below:

1. The most common modes of detection of *Mycobacterium tuberculosis* (Mtb) within Mizoram are GeneXpert (40%), smear microscopy- Ziehl Neelsen stain (32.6%) for pulmonary cases and for extrapulmonary cases, X-ray (40.1%) followed by FNAC- Fine Needle Aspiration Cytology (25.9%).
2. The symptoms of tuberculosis such as fever (>2 weeks), cough (>2 weeks), weight loss, chest pain and night sweats were more pronounced among pulmonary cases as compared to extrapulmonary cases. The mean age of tuberculosis patients (pulmonary and extrapulmonary) in Mizoram was 33.1 years.

3. Single marital status, non-ideal weight (underweight, obese, overweight), lower educational qualification (upto matriculation), unemployment, lower family income (<30,000 per month), lack of cross-ventilation system, damp or moist housing, participation in mass gathering events, history of travel, no BCG vaccination, alcohol consumption and neighbours with tuberculosis are all significant risk factors of tuberculosis in Mizoram.
4. Other sources of water supply (river water and spring water collection), though less-established was found to be an important risk factor of tuberculosis in Mizoram. This is the first world-wide report of the potential role of water source as a risk factor for Tb.
5. Rifampicin Resistant Tb in males was predominant in pulmonary samples, while in females it was predominant for extrapulmonary samples. Majority of the Rifampicin Resistant Tb cases in Mizoram falls between productive age groups of 25-34 years followed by 35-44 years.
6. Higher number of probes (A, B and D) mutations in Mtb were observed for extrapulmonary samples, while probes C and E mutations were more in pulmonary samples.
7. GeneXpert probe mutations in Mtb observed during eight years (2014-2021) are as follows: Single mutation: probe A (codons 507-511)- 25.9%; probe E (codons 529-533)- 23.5%; probe D (codons 523-529)- 9.8%; probe B (codons 512-518)- 2.6%; probe C (codons 518-523)- 0.2%.
8. Probes mutation combinations were also found in 158 Mtb samples: AB- 0.4%, AD- 32.8%, AE- 0.4%, DE- 0.2% and ADE- 0.4%.
9. Seventeen of the Rifampicin Resistant Tb cases had no missed probes (all probes positive) and the ΔCt for these samples were all >4 cycles.
10. This study reported the highest probe A mutation as well as mutation combinations in Mtb from Mizoram upon comparison with twelve other similar studies within and outside India.
11. Association studies using bivariate analysis showed the frequency of probe A ($p=0.009$) and probe D ($p=0.007$) mutations to be statistically significant for female gender.

12. Association studies using bivariate analysis showed the frequency of probe A (p=0.047) and probe D (p=0.001) mutations to be statistically significant for extrapulmonary samples.
13. The frequency of mutation at probe D site (p=0.000) was also statistically significant for samples with low and very low DNA quantity.
14. Association studies using multivariate analysis also showed that the frequency of mutation at probe A site was statistically significant for younger age groups, 15-24 years (p=0.015) and 25-34 years (p=0.032).
15. The residential areas of the majority of Rifampicin Resistant Tb patients within Aizawl District were found to be located in Aizawl North- III followed by Aizawl South-II and Aizawl East-II.
16. MGIT Mtb drug susceptibility report showed the highest drug resistance for Isoniazid (32.1%), followed by Streptomycin and Ethambutol (26.8% each), pyrazinamide (25%) and the least resistance by Rifampicin (19.6%).
17. Discrepancy in drug resistance using phenotypic and genotypic methods were observed for Isoniazid and Rifampicin.
18. Sequencing results of phenotypic Isoniazid resistant Mtb isolates showed Serine to Threonine substitution to be the most common at 315 amino-acid position. A lesser common substitution at this same position was Serine to Asparagine.
19. Sequencing of the *rpo*B gene of Rifampicin Resistant isolates with all probes' positive showed no single nucleotide polymorphism.
20. Sequencing of the *rpo*B gene of Rifampicin Resistant isolates with probes negative (mutation) showed an SNP of T>C (L511P) at probe A site and C>G (H526Q) at probe D. Another less common SNP at probe D was site was A>T (H526L).
21. Sequencing results showed no mutations for the other antibiotic resistance genes (*emb*B, *rps*L, *pnc*A).
22. Long Sequence Polymorphism -PCR showed the presence of Indo-Oceanic, Beijing, East African Indian and Euro American lineages of *Mycobacterium tuberculosis* in Mizoram along with mixed strain infection and other unidentified lineages.

Ethical Clearance Letter



IEC, CIVIL HOSPITAL, AIZAWL.
COMMUNICATION TO THE PRINCIPAL INVESTIGATOR/GUIDE BY THE MEMBER SECRETARY, INSTITUTIONAL ETHICS COMMITTEE

No.B.12018/1/13-CH(A)IEC/63 Dated: 28rd March, 2017

To,

Dr. Lily Chhakchhuak
District TB Officer
Aizawl District.

Subject: Ethics Committee Approval for the referenced projects.

Reference: ***"Genomics – drivenDissection of Susceptibility and Drug Resistance to Pulmonary Tuberculosis with a Geographical Focus on NER"***

Dear ***Dr. Lily Chhakchhuak***

With reference to submission of document for review and approval to conduct the above mentioned study. The Ethics Committee has reviewed and approved the study documents as mentioned below:

1. Curriculum Vitae of non Civil Hospital, Aizawl Investigators
2. Brief description of proposal/summary
3. Copy of the Protocol/Project and questionnaire (if any)
4. Copy of Patient information sheet & Consent form in local language
5. Copy of Clinical trial agreement
6. Copy of PI undertaking

The following members of the Ethics Committee were present at the meeting held on date **28rd March 2017** at time **1:30 pm** at Doctor's Seminar Room, Civil Hospital, Dawrpui, Aizawl, Mizoram - 796001.

The quorum met as per ICG-GCP and schedule Y guidelines as mentioned below:

S. No	Name of the member	Qualification	Designation in the Ethics Committee	Gender
1.	Dr. Lal Biakkima	Director H&ME (Rtd)	Chairman	Male
2.	Dr. C.Lalchhandama MD	Senior Pathologist	Member Secretary	Male
3.	Dr. T. Lalzawmliana MD	Head of Dept. Biochemistry	Member	Male
4.	Pu Rosangzuala Ralte,	Advocate	Legal expert	Male
5.	Dr. Mary Muanpui Ralte MD	Consultant, Dept of Biochemistry	Basic Medical Science	Female
6.	Rev R. Lalchangliana	Pastor	Member	Male
7.	Dr. Zoengpari	Associate Professor, MZU	NGO/Social activist	Female
8.	Prof. T. Vanlaltlani	Professor ATC	Reowned Lay person	Female
9	Dr. Saia Chenkual	Head of Dept. Surgery	Clinician	Male

Please note that this Ethics Committee is constituted as per schedule Y, ICH-GCP, applicable local laws and regulatory requirement.

We approve the project to be conducted in its present form. Ethics Committee expects to be informed about:

1. Any SAE occurring in the course of the study
2. A copy of final individual center report

We hereby confirm that neither you nor your study members have participated in the voting/decision making procedure of the Ethics Committee.

Yours sincerely,

(DR. LAL BIAKKIMA)
Chairman
Institutional Ethics Committee
Civil Hospital, Dawrpui, Aizawl
Mizoram – 796001

(DR. T. LALZAWMLIANA)
Member Secretary
Institutional Ethics Committee
Civil Hospital, Dawrpui, Aizawl
Mizoram – 796001

Chairman
Institutional Ethical Committee
Civil Hospital, Aizawl

Secretary
Institutional Ethical Committee
Civil Hospital, Aizawl

Note : Due to the absence of Dr. C. Lalchhandama due to unavoidable circumstances, this committee unanimously appoi Dr. T. Lalzawmliana (Member, IEC) as Member Secretary for this meeting.

SHOT ON MI A1
MI DUAL CAMERA

Structured Questionnaire used for collecting socio-demographic data of tuberculosis patients

PROFORMA FOR COLLECTION OF DATA FOR THE STUDY ON CLINICAL AND DEMOGRAPHIC FACTORS INFLUENCING THE RISK OF MULTI-DRUG RESISTANT TUBERCULOSIS INCIDENCE AMONG MIZO POPULATION

State TB Office, Falkawn/ Synod Hospital, Durtlang/ Department of Biotechnology, Mizoram University

Referring Dr: _____ **STOMZ/Synod Hospital No.:** _____ /_____

Referring Unit: _____ **MZU Reg. No./Date:** MZU/DBT/_____

PERSONAL HISTORY

Name *(Hming)*: **Sex: Male (1)/ Female (0)**

Age: _____ **Marital Status: Married (1) / Single (0)**

15-25 (1)
26-35 (2)
36-45 (3)
46-55 (4)
56-65 (5)
66-75 (6)
76-85 (7)

How long have you been married? _____ **{Unmarried (0) <5yrs (1) / 5 – 10yrs (2)/ >10yrs (3)}**

Height: _____ **Weight:** _____ **Education:** _____ **{Uneducated (3)/ Class 1-5 (2)/ Class 6-10 (1)/ Above 11 (0)}**

Occupation: _____ **{No occupation (1)/ Self-employed (2) [labor/driver/small business]/ other employees (0) [Govt. employee]}**

Family monthly income (approx.): <20000 (2) / 20000 – 30000 (1) / >30000 (0)

Permanent Address:

_____

PinCode_____ Tel/Mob.No. (atleast two numbers) _____

_____ E-mail: _____

Present Address: _____

PinCode _____Tel/Mob.No. _____

E-mail: _____

Lifestyle Factors

WORK-PLACE AND JOB INFORMATION:

Do you work in a crowded room? **Yes (1)/ No (0)**
(I thawhna hmun a chep em?)

Are there cross- ventilations? **Yes (0)/ No (1)**
(Tukverh in hawitawn a awm em?)

Condition of the working room: **Damp (2) / Moist (1)/ Sunlight (0)**
(Thawhna hmun awm dan) *Hmun Thim leh hmuar/Chiau/Ni eng hmu*

Is your job stressful? *(I hna a hahthlak viau em)*: **Yes (1) / No (0)**

RESIDENTIAL PLACE INFORMATION:

Do you reside in a crowded room? **Yes (1) / No (0)**
(I chenna hmun a chep em?)

Are there cross- ventilations? **Yes (0) / No (1)**
(Tukverh in hawitawn a awm em?)

Condition of the housing: **Damp (2)/ Moist (1)/ Sunlight (0)**
(Chenna hmun awm dan) *Hmun Thim leh hmuar/Chiau/Ra/Ni eng hmu*

Type of housing: house (0) / apartment (1) / shelter or nursing home (2)/ others (3) (specify) (Chenna hmun)

Number of people living under one roof? _____ **[1-3 (0) / 4-6 (1) / 7-9 (2) / >10 (3)]**
(In inchhungah mi engzat nge cheng?)

What is your water supply source? River (2) / Tube well (1) / Govt.municipal (0)
(In tui tlan eng ang chi nge?) *Lui / Tui khur / Sorkar tui sem (connection gangngai)*

How often do you exercise? *(Exercise I la ngai em?)*:

Never (5) / Less than once a week (4)/ Once a week (3)/ 2-3 times a week (2) / 4-6 times a week (1)/ Everyday (0)

Whether spend time with someone having pulmonary or extra-pulmonary TB:
(TB vei nen hun in hmang tam em?):
No (0) /Don't know (1)/ Yes (2)

Participate in mass gathering events: **No (0)/ Yes (1)**
(Piupunna ah I kal zing em?)

Recent travel destinations: **No travel (0)/ Travelled (1)**
(when last there and for how long)
(Tun hnaia zinchhuahna – a hun leh a hmun, cham rei zawng)

HEALTH STATUS

HIV status: **Positive (1) / Negative (0)**
(HIV I vei m?)

Diabetes: **Yes (1)/ No (0)**
(Zunthlum I nei em?)

BCG Vaccine uptake: **Yes (0)/ Not Sure (1) / No (2)**
(TB laka in venna vaccine I la tawm em?)

Malnourishment: **Yes (1) / No (0)**
(Chaw tha I hmu tawk em?)

Alcohol History:

Do you take alcohol? *(Zu I in em?)* **Yes (1) / No (0)**
At what age do you start taking alcohol? _____ **[<20yrs (2) / 20 - 30yrs (1) / >30yrs (0)]**
(Kum engzat I nih in nge zu I in)
Duration of alcohol consumption? *(Eng chen a rei nge zu I in)* **[<5yrs (0) / 5-10yrs (1) / >10yrs (2)]**

Tobacco history

Do you smoke? *(Mei I zu em?):* **Yes (1) / No (2)**
At what age did you start smoking? _____ **[<20yrs (2) / 20 - 30yrs (1) / >30yrs (0)]**
(Kum engzat I nih in nge zial I zuk)

Duration of Smoking? *(Eng chen a rei nge zu I in)* **[<5yrs (0) / 5-10yrs (1) / >10yrs (2)]**

Do you consume other tobacco products? *(Vaihlo a siam thil dang tihfl nei em?):* **Yes (1) / No (0)**

Duration of consumption of other tobacco products? *(Eng chen a rei nge)* **[<5yrs (0) / 5-10yrs (1) / >10yrs (2)]**

History of passive smoking:

Do any of your family member/colleagues smoke tobacco? **Yes (1) /No (0)**
(I chenpui/thawhpui ah mei zu an awm em?)
Frequency of exposure to passive smoking: **Rarely (0) / Continuously (1)**
(Engtianga zing in nge heng meizu ho bulah hian I awm)

PRESENT COMPLAINT *(Tuna a nat dan)*

DISEASE CLASSIFICATION: **Pulmonary (No.1) / Extra-pulmonary (No.2)**

SPECIFY SITE: _____

TYPE OF PATIENT:

- **(0) New (Newly diagnosed for the first time)**
- **(1) Relapse (Treatment completed and cured, smear turned negative but later turned positive again)**
- **(2) Treatment failure (Smear still positive after 5 months of treatment)**
- **(3) Defaulters (One month of treatment after which there is interruption)**

SYMPTOMS (Pulmonary)

Fever > 2 weeks? **Yes (1) /** **No (0)**
(Khawsik kar 2 aia rei)

Cough > 2 weeks? **Yes (1) /** **No (0)**
(Khawsik kar 2 aia rei)

Weight loss? ***Yes (1) /*** ***No (0)***
(Rihna tlahniam)

Chest pain? **Yes (1) /** **No (0)**
(Awmchhung na)

Night sweats? **Yes (1) /** **No (0)**
(Zan mut laia thlan)

HISTORY OF PAST ILLNESS *(A hma a lo nat tawh dan)*

Are you prone to any kind of respiratory infection? **Very prone (2) / sometimes (1) / never (0)**
(Awm lam natna I vei awl em?)

Were you previously diagnosed with TB? **Yes (1) /** **No (0)**
(A hma in TB I vei tawh thin em?)

If yes, in which year? _____
(Engtik kumah nge?)

Was it Pulmonary (No.1) or Extra-pulmonary (No.2)? _____
(Chuap TB nge Chuap Pawn TB?)

If Extra-pulmonary mention site: _____
(Chuap Pawn TB anih chuan i khai lai tak nge?)

Was it diagnosed with smear (ZN) (No.1)/ Biopsy (No.2)/ FNAC (No.3)/ X-ray (No.4)/ Culture- solid or liquid (No.5) / Others (CB-NAAT) (No.6) / Mantoux (No.7)? :
(I TB chu engtia hmuhchhuah nge a nih?

Did you receive ATT? **Yes (0) /** **No (1)**
(TB damdawi I ei em?)
If yes, duration of ATT taken? _____
(Eng chen a rei nge I ei?)

Completion of ATT course: **Completed (0) / Not completed (1)**
(TB damdawi course I ei za em?)

Did you receive your ATT at DOTS (under health care supervision)? **Yes (0) /** **No (1)**
(TB damdawi DOTS hnuaiah I ei em?)

Type of ATT taken: **RNTCP (free-supply, consecutive days) (0) / AKuRiT-4 (Private, daily dose) (1)**
(Eng ang damdawi nge I ei?)

Drug Category Taken? Cat I (1) / Cat II (2) / Cat III (3) / Cat IV (4)

During treatment, did you switch your drug at any point of time? **Yes (1) /** **No (0)**
(I treatment lak lain I damdawi I thlak em?)

If yes, give reason: _____
(Damdawi thlak chhan)

SUSPECTED CASE (X-ray, CT, Smear, Bipsoy and FNAC negative or not suggestive)

Were you ever Clinically diagnosed (suspected case) and treated for Tuberculosis at any point of time?
(Laboratory atang nilo in TB hmuhchhuah a enkawl I ni tawh em?): **Yes (1) /** **No (0)**
If yes, Pulmonary (No.1) or extrapulmonary (No.2)? _____ **(mention site)**
(Chuap TB nge Chuap Pawn TB?)

Did you receive ATT? **Yes (0) /** **No (1)**
(TB damdawi I dawng taw hem?)
If yes, duration of ATT taken? _____
(Eng chen a rei nge I dawn tawh?)

Completion of ATT course: **Completed (0) / Not completed (1)**
(I course I complete em?)

Did you receive your ATT at DOTS (under health care supervision)? **Yes (0) /** **No (1)**
(TB damdawi DOTS hnuai ah I la em?)

Type of ATT taken: **RNTCP (free-supply, consecutive days) (0) / AKuRiT-4 (Private, daily dose) (1)**
(Eng damdawi chi nge I ei?)

Drug Category Taken? Cat I (1) / Cat II (2) / Cat III (3) / Cat IV (4)

During treatment, did you switch your drug at any point of time? **Yes (1) /** **No (0)**
(TB damdawi a enkawl I nih laiin I damdawi thlak a ni em?)
If yes, give reason: _____
(A chhan?)

Have you ever had a *positive* reaction to a TB skin test? When? **No (0) / Yes (1)**
(Ban a thun TB test ah a vual sen lian ve tawh em? Engtikah?

Whether traditional medicines taken since last interview? **No (0)/ Yes (1)**
Specify if yes _____
(Zo damdawi tun hnaiah ei I nei em?)

FAMILY DETAILS

Any member in the family with on-going TB? **Yes (1) / No (0)**
(Chhungkua ah TB vei lai mek an awm em?)

If yes, Drug susceptible TB (0)/ Unknown (1) / MDR-TB (2)

Any member in the family with history of TB? **Yes (1) /No (0)**
(Chhungkua ah TB vei tawh an awm em?)

If yes, Drug susceptible TB (0)/ Unknown (1) / MDR-TB (2)

How many members in the family had TB so far? _____ **[None (0) / 1-3 (1) / >3 (2)]**
(In chhungkua ah mi engzat in nge TB vei tawh?)

Was it: Pulmonary (No.1) / Extra-Pulmonary (No.2)?

Did he/she receive ATT? **Yes (0) /** **No (1)**
(Tb damdawi a la em)
If yes, duration of ATT taken? _____
(TB damdawi ei chhung)

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Bio Data

Educational Qualification

Degree	School/College/Board/University	Division	Month and Year of Joining	Month and Year of Completing
M.Phil	Dept. of Biotechnology (Mizoram University)	Dist.	August 2015	May 2017
M.Sc. Med Microbiology	Christian Medical College, Vellore (The Tamil Nadu Dr.M.G.R Medical University)	I	August 2009	October 2012
B.Sc. MLT	RIPANS, Zemabawk (Mizoram University)	I	August 2005	October 2008
HSSLC (Class 12)	St.Paul's Higher Secondary School (Mizoram Board of School Education)	I	January 2003	July 2004
HSLC (Class 10)	St.Mary's High School (Meghalaya Board of School Education)	II	January 2001	June 2002

M.Phil Title of Dissertation: *"Studies on the prevalence of Human papillomavirus associated with cervical lesions among Mizo women".*

Awards

- Proficiency Award II (2nd rank) holder in BSc. MLT course, RIPANS, under Mizoram University.
- 1st rank holder in MSc. Medical Microbiology course, CMC, Vellore.

Skills developed

Knowledge of phlebotomy, microscopy, fungal and bacterial culture (both aerobes and anaerobes) including *Mycobacterium tuberculosis* liquid and solid culture. DNA Isolation, Gel electrophoresis, PCR and Sanger Sequencing.

Work Experience

YEAR/month-Start	Year/Month - Finish	Institution / Company Name	Institution / Company Address	Position
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January 2013	July 2015	Synod Hospital	Willow Mount Road, Durtlang, Mizoram	Microbiologist

Papers Published

1. **Christine Vanlalbiakdiki Sailo**, Mary Vanlalhruaii Tonsing, Zothan Sanga, Zothankhuma Chhakchhuak, Febiola Kharkongor, Vanlal Fela, Lily Chhakchhuak, Lalremruata Ralte, Lalnun Nemi, Nachimuthu Senthil Kumar. (2022). Risk factors of tuberculosis in Mizoram: First report of the possible role of water source. *Indian Journal of Tuberculosis*, https://doi.org/10.1016/j.ijTb.2022.03.003
2. **Christine Vanlalbiakdiki Sailo**, Zothanzami, Lalremruata Ralte, Zothan Sanga, Vanlal Fela, Febiola Kharkongor, Lily Chhakchhuak, Zothankhuma Chhakchhuak, Gracy Laldinmawii, Dhiraj Kumar, Nachimuthu Senthil Kumar. (2022). MGIT sensitivity testing and genotyping of drug resistant Mycobacterium tuberculosis isolates from Mizoram, Northeast India. Indian journal of medical microbiology, S0255-0857(22)00098-6. Advance online publication. https://doi.org/10.1016/j.ijmmb.2022.06.003
3. Mary Vanlalhruaii Tonsing, **Christine Vanlalbiakdiki Sailo**, Zothansanga, Lily Chhakchhuak, Zothankhuma Chhakchhuak, Bhaswati Pandit, Dhiraj Kumar, Partha Pratim Mazumder, Nachimuthu Senthil Kumar. (2020). Analysis of variants in mitochondrial genome and their putative pathogenicity in tuberculosis patients from Mizoram, North east India. *Mitochondrion*, 54:21-25. doi:10.1016/j.mito.2020.06.012.
4. **Christine Vanlalbiakdiki Sailo**, Zothan Zami, Souvik Ghatak, Lalnun Nemi, K Lalremmawia, Lalawmpuii Pachuau, Eric Zomawia, Zothan Siama, Nachimuthu Senthil Kumar. (2022). Prevalence of High-Risk HPV Types in Women with Negative Cervical Cytology in a State of Northeast India with a High Burden of Cervical Cancer. *Indian Journal of Gynaecologic Oncology*, 20, 8. https://doi.org/10.1007/s40944-022-00610-7
5. **Christine Vanlalbiakdiki Sailo**, Puja Pandey, Subhajit Mukherjee, Zothan Zami, Ralte Lalremruata, Lalnun Nemi, Nachimuthu Senthil Kumar. (2019).

Pathogenic microbes contaminating mobile phones in hospital environment in Northeast India: incidence and antibiotic resistance. *Trop Med Health*, 47:59. https://doi.org/10.1186/s41182-019-0190-5

6. Dipika Malakar, Probodh Borah, Leena Das, Vabeiryureilai Mathipi, **Christine Vanlalbiakdiki Sailo,** Rupam Dutta, Naba K. Deka and Nachimuthu Senthil Kumar. (2020). Prevalence and Virulence Gene Profiling of Listeria monocytogenes from Fish and Meat Samples from Aizawl, Mizoram. *J Pure Appl Microbiol*, 14(2):1359-1365 | https://doi.org/10.22207/JPAM.14.2.33
7. Vanlalruati S.C.Ralte, Archana Loganathan, Prasanth Manohar, **Christine Vanlalbiakdiki Sailo**, Zothan Sanga, Lalremruata Ralte, John Zothanzama, Sebastian Leptihn, Ramesh Nachimuthu and Nachimuthu Senthil Kumar. The Emergence of Carbapenem-Resistant Gram-Negative Bacteria in Mizoram, Northeast India. *Microbiol. Res*. 2022, 13(3), 342-349; https://doi.org/10.3390/microbiolres13030027 (registering DOI)
8. Vanramliana, Gabriel Rosangkima, Lalnunnemi, Ralte Lalremruata, **Christine Vanlalbiakdiki Sailo**, Hunropuia, Deborah Lalnghakmawii and Lalfakzuala Pautu. (2021). Detection and Molecular Characterization of Orientia tsutsugamushi from Suspected Scrub Typhus Patients in Mizoram, India. *Int.J.Curr.Microbiol.App.Sci*,10(10):514-523. https://doi.org/10.20546/ijcmas.2021.1010.061

Papers Communicated

1. **Christine Vanlalbiakdiki Sailo**, Ralte Lalremruata, Zothan Sanga, Vanlal Fela, Febiola Kharkongor, Zothankhuma Chhakchhuak, Lily Chhakchhuak, Lalnun Nemi and Nachimuthu Senthil Kumar (2022). Distribution and frequency of common mutations in rpoB gene of Mycobacterium tuberculosis detected by Xpert MTB/RIF and identification of residential areas of Rifampicin Resistant-TB cases: A first retrospective study in Mizoram, Northeast India (communicated in Journal of Clinical Tuberculosis and Other Mycobacterial Diseases).

Oral presentations

1. Oral presentation in the International Conference on Emerging Trends in Biotechnology (ICETB) organized by School of Bio Sciences and Technology, Vellore Institute of Technology along with Association of Biotechnology and Pharmacy, from December 14 - 16, 2020
2. Oral presentation in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on

Recent Advances in Science and Technology (IRSRAST), organized by NEAST, Mizoram University, during 16th–18th November 2020 (Virtual)

3. Oral presentation in the Workshop entitled "Research on Molecular Biology of Stress, Aging and Cancer Using Cell Culture Based Assays - Interventions by Natural Compounds" at DAICENTER, National Institute of Advanced Industrial Science & Technology (AIST) under Sakura Science Program (Government of Japan) during October 6-12, 2019.

Workshop / Conferences attended

1. North-East Autumn School on Human Genetics: Techniques and Statistical Analyses organized by Indian Statistical Institute, Kolkata and Mizoram University from 8th to 11th September 2015.
2. Workshop on "Exploring the Cancer Genomics" sponsored by State Biotech-Hub Facility, Department of Biotechnology (DBT), New Delhi held at Mizoram University from 22nd to 27th February 2016.
3. Training on "Gene Cloning, Protein Biochemistry, Structural Biology & Bioinformatics" organized by DBT Biotechnology/ Bioinformatic Training Centre, Advanced Centre for Treatment, Research & Education in Cancer, Navi Mumbai during 18th to 29th July 2016.
4. Workshop on "Understanding Basic Principles in Human Molecular Genetics" organized by Department of Biotechnology, Mizoram University sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi during 7th to 11th September 2016.
5. Workshop on "Statistical Methods in Biological Research" organized by Bioinformatics Infrastructure Facility (BIF), Department of Biotechnology, Mizoram University sponsored by Department of Biotechnology (DBT), New Delhi during 3rd to 5th November 2017.
6. Workshop on "Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches" organized by NIBMG, Kolkata held at Department of Biotechnology, Mizoram University during 19th to 24th November 2017.
7. Training on "Handling Infectious Pathogens in BSL-3 Facility" organized by BSL-3, School of Biotechnology, Jawaharlal Nehru University, New Delhi during 17th to 19th April 2018.
8. 3rd Advanced Research Training Workshop on "Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches" held at National Institute of Biomedical Genomics, Kalyani, Kolkata during 23rd to 31st July 2018.
9. Continuing Medical Education course on "The Concept and Application of Genomics in Clinical Medicine" jointly organized by Civil Hospital Aizawl and

Mizoram University, conducted by CSIR- Institute of Genomics and Integrative Biology (CSIR-IGIB), New Delhi held at Civil Hospital, Aizawl on 11th August 2018.

10. Participated in The 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) & International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends (BEHIET 2018) organized at the School of Life Sciences, Mizoram University, Aizawl, Mizoram during November 12t to 14, 2018. Title: Polymorphism in aldehyde dehydrogenase-2-gene associated with risk of tuberculosis in Mizo population.
11. Workshop on "A brief introduction to Bioinformatics and Systems Biology" organized by Department of Biotechnology, Mizoram University sponsored by Bioinformatics Infrastructure Facility, Department of Biotechnology (DBT), New Delhi during 13th to 14th December, 2018.
12. Workshop on Biosafety guidelines for working with a BSL3 pathogen, working with BSL3 pathogen inside a BSL3 lab, *Mycobacterium tuberculosis* culture, ex-vivo infection, MIC assay (by Alamar Blue), bacterial genomic DNA isolation, CFU plating, freezing and reviving of stocks and culturing of human macrophage like cell lines at Cellular Immunology group at ICGEB, New Delhi during 7th to 15th January, 2019.
13. Workshop on Clinical Microbiology and Biosafety in Tuberculosis research conducted by Foundation of Neglected Disease Research, Bengaluru during 21st to 25th January, 2019.
14. Workshop on Research on Molecular Biology of Stress, Aging and Cancer Using Cell Culture Based Assays - Interventions by Natural Compounds at DAICENTER, organized by National Institute of Advanced Industrial Science & Technology (AIST) under Sakura Science Program (Government of Japan), Tsukuba, Japan from Oct 6th to 12th, 2019.

Webinars Attended

1. The Importance of Symbiotic Microorganisms in Drug Discovery, Organized by Mizoram University on June 7, 2020
2. Study of biomarkers for diagnosis and prognosis of tuberculosis infection, Organized by Mizoram Univeristy on June 7, 2020
3. Contribution of B-1 Cells during Mycobacterium infection, Organized by Mizoram University June 7, 2020
4. Mizoram a Model State in India with Novel Strategies in Combating the Covid-19, Organized by Mizoram University, June 10, 2020
5. Mending the Broken Heart: The Evolution of Novel therapeutic Approach, Organized by Mizoram University, June 19, 2020
6. Role of Microenvironment in Cancer Progression, Organized by Mizoram University, August 18, 2020

7. Human Microbiome - An Invisible Organ, Organized by Mizoram University, September 5, 2020
8. Syndromic approach to diagnosis of infectious diseases. Organized by Anaesthesia & Critical Care Foundation, July 23, 2021
9. Bug Tales – Case presentation series, Organized by Society of Clinical Microbiologist, Mumbai on July 24, 2021
10. Managing Pediatric Sepsis, Organized by BD India on July 28, 2021
11. Role of Diagnostic Lab in Supporting Antimicrobial Stewardship, Organized by BD India in association with ISCCM Vizag Chapter on July 31, 2021
12. Approaches to Antimicrobial Stewardship. Where Are We?, Organized by BD India in association with ISCCM Vizag Chapter on July 31, 2021
13. Empirical Antimicrobial Therapy in MDR Era & Advantages of Early Microbiological Reports, Organized by BD India in association with ISCCM Vizag Chapter on July 31, 2021
14. BREAK THE BREAKPOINTS: An insight into Antibacterial and Antifungal interpretation, Organized by Society of Clinical Microbiologists with BioMerieux on August 27, 2021
15. Diagnosis & Management of Gram Negative MDROs: Interactive Pediatric Cases, Organized by BD India on August 18, 2021,
16. Designing Rational Combination Therapies to treat Chemoresistant Breast and Ovarian Cancers, Organized by Mizoram University on September 6, 2021
17. Crosstalk between Prostate Cancer and Microenvironment Reveals New Therapeutic Targets, Organized by Mizoram University on September 6, 2021
18. Prostate Cancer Progression: Novel Signaling Mechanisms and Mouse Models, Organized by Mizoram University on September 6, 2021
19. Automation of Microbiological ID & AST: Changing Concepts & Defeating Challenges, Organized by BD India on September 30, 2021.
20. Interpretation and Relevance of MIC values in Clinical Practice: Physician's perspective, Organized by BD India on September 30, 2021.

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Original Research Article

MGIT sensitivity testing and genotyping of drug resistant *Mycobacterium tuberculosis* isolates from Mizoram, Northeast India

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ABSTRACT

Purpose: Tuberculosis, a crucial infectious disease is still a health concern globally. India is among the countries with high MDR-TB burden. Currently, sputum smear microscopy using Ziehl Neelsen stain and GeneXpert are the only diagnostic means in Mizoram. This study was done to characterize local tuberculosis strains circulating in Mizoram.

Methods: Sputum was cultured using MGIT 960 and DST was performed for Streptomycin, Isoniazid, Rifampicin, Ethambutol and Pyrazinamide. GeneXpert test was done simultaneously. DNA was extracted using Trueprep AUTO v2, molbio diagnostics. Antibiotic Resistance Genes and LSP were amplified and sequenced.

Results: Ser315Thr was the most common mutation in *kat*G among MDR-TB isolates. GeneXpert probes A and D drop out upon sequencing showed L511P, H526Q and H526L mutation. The L511P and H526Q mutations were seen in new and treated cases. Discrepancy between MGIT 960 and GeneXpert were observed. LSP-PCR revealed that Indo-Oceanic, East-African Indian, Euro-American and Beijing lineages were found in Mizoram.

Conclusion: This study provides mutation information on the resistant genotypes detected with GeneXpert as well as MGIT 960. It also provides information on the lineages of *Mycobacterium tuberculosis* circulating in the state. Utilization of sequencing technologies is essential in diagnostic laboratories to rule out discrepant results and as a cautionary measure to prevent wrong diagnosis and treatment.

1. Introduction

Tuberculosis which is still a global health concern is caused by *Mycobacterium tuberculosis complex* (MTBc) comprising of human and animal adapted pathogens [1]. Resistance to different anti-tuberculosis drugs has been attributed to mutations in specific genes [2]. With the advent of GeneXpert, primary screening involves PCR-based detection of MTB DNA as well as a targeted SNP based PCR in the *rpo*B (RIF resistance) gene. The rationale behind using *rpoB* SNP is that it serves as a surrogate marker for Rifampicin, since >90% of Rifampicin resistant isolates also exhibit Isoniazid resistance [3]. Thus, a positive result indicates confirmation of tuberculosis as well as multi-drug resistant infection.

MTB phylogeny consists of six major lineages (L) named according to their geographic distribution: L1 to L6 [4]. Few other lineages were recently identified in the African continent [5]. Previous studies have shown varied disease progression and relapse risk in different lineages [6]. MTB strains harbour insertions or deletions in the genome called Large Sequence Polymorphism (LSPs), also known as Regions of Difference (RDs) which are unique polymorphisms with mutations occurring only once in a species. Thus, RDs are irreversible and do not show homoplasy, hence have been used to differentiate MTB into its respective lineages [7].

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India is among the 3 countries with high Multidrug Resistant (MDR) TB burden and contributes largely to the global increase in TB [8]. Mizoram having a population of 12.5 lakhs, the total TB patients notified from both public and private sector in 2019 was 2944 [9]. Currently, sputum smear microscopy using Ziehl Neelsen stain and Cartridge Based Nucleic Acid Amplification Test (CB-NAAT) or GeneXpert are the only diagnostic means implemented in the state of Mizoram.

This study aims to characterize the MTB strains which will help to establish: 1) whether initial infection of individuals could result from a heterogeneous mixture of MTB strains; 2) the status of MDR and the associated variants; and 3) the lineage of the MTB strains present in the region. This is the first study from Northeast India on the extent of heterogeneity in the initial acquired infections which might prove great importance in the designing of informed treatment regimen for tuberculosis patients as well as future monitoring of the MDR/virulent strains in the region.

2. Materials and methods

2.1. Sample collection

Participants were recruited from District TB Center, Falkawn and Synod Hospital, Durtlang, Mizoram during 2019–2020. This includes new and previously treated pulmonary TB cases, aged 15 years and above, both genders without HIV infection. A total of 88 sputum smear positive samples were collected in a screw capped container.

2.2. GeneXpert test

Xpert MTB/RIF assay G4 (Cepheid) was performed in accordance to the manufacturer's instructions with a reported sensitivity of 94.4% and specificity of 98.3% for diagnosis of rifampicin resistance [3]. Presence of *rpo*B mutations can change the dynamics of hybridization, resulting in differences between the Ct values of the probes. Inhibition of hybridization results in probe dropout. However, in samples without mutated *rpo*B, all the 5 probes match exactly to the amplified MTB DNA. The G4 version of Xpert MTB/RIF software interprets sample results as resistant to Rifampicin, if the difference between two probes (the first and last Ct) is >4 cycles ($\Delta Ct > 4$) [10].

2.3. Culture and drug susceptibility test using MGIT 960

Culture of MTB from sputum was performed using automated liquid culture BACTEC MGIT 960 system (BD, USA) in accordance with FIND Diagnostics. The workflow for selection of samples is shown in Fig. 1. Drug susceptibility testing (DST) was performed from growth positive tubes using first line drugs - Streptomycin (1.00 µg/ml), Isoniazid (0.10 µg/ml), Rifampicin (1.00 µg/ml) and Ethambutol (5.00 µg/ml) using BD BACTEC MGIT™ 960 SIRE Kit and Pyrazinamide (100 µg/ml), provided the cultures are young i.e., one to two days old. From the third day onwards to the fifth day, a 1:5 dilution was employed [11] (see Fig. 2). Subculture was also maintained on LJ media using Lowenstein-Jensen Medium Slants for DNA extraction and further molecular testing (Supplementary Table 1).

2.4. DNA extraction

DNA was extracted from 44 LJ cultures. A loop full of the growth was emulsified in 2 ml of 0.9% normal saline, vortexed and heat killed at 80 °C for 20 min [12]. It was centrifuged in a cooling centrifuge at 14,000 rpm for 5 min, the supernatant discarded and the pellet was emulsified in 1 ml of 7H9 Broth. Lysis buffer was added and finally transferred into Universal Cartridge (Trueprep) and loaded into nucleic acid extractor machine (Trueprep AUTO v2). Extraction was done as per manufacturer's instructions.

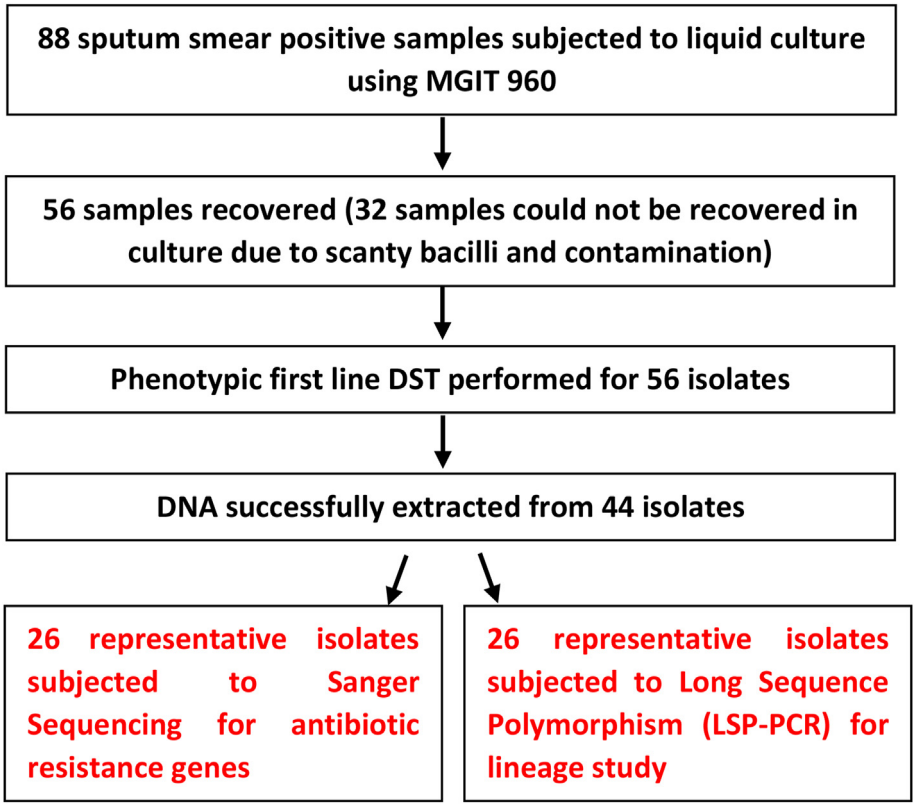

Fig. 1. Flowchart representing the workflow for selection of samples

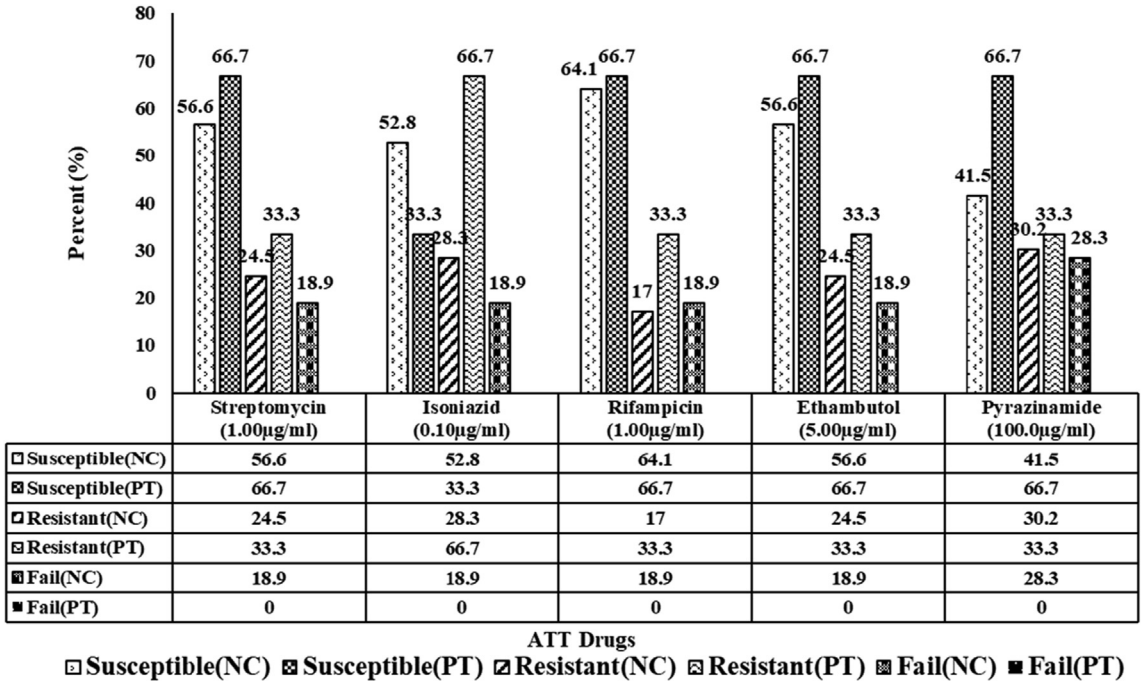

Fig. 2. Drug Susceptibility of new and previously treated cases for first line anti-tuberculosis drugs using MGIT 960. NC-New Case; PT- Previously Treated.

2.5. DNA quantitation, PCR amplification and sequencing

The isolated DNA was checked on a 0.8% agarose gel electrophoresis (BioRad), quantified using a µdrop MultiSkan SkyHigh Spectrophotometer (ThermoScientific). Polymerase Chain Reaction (PCR) was run on five antibiotic resistance genes towards first line ATT (Anti-tuberculosis treatment) drugs. The primer sequences and conditions for each primer is given in Supplementary Table 2. PCR was carried out in a 10 µl reaction and the PCR product was observed in a 1.5% Agarose Gel Electrophoresis (Bio Rad). Sequencing was done using 3500 Genetic Analyzer automated sequencer (ABI), analyzed for confirmation and presence of variants in the genes. The sequences were aligned using Clustal Omega with reference sequences (*Mycobacterium tuberculosis* H37Rv) obtained from Mycobrowser [13].

2.6. Genotyping of Mycobacterium tuberculosis isolates from Mizoram

Genotyping of MTB lineage was done using Long Sequence Polymorphism (LSP-PCR) otherwise known as Regions of Differences (RDs). Based on LSP (RD), the major MTB lineages were classified using four primers - Indo-Oceanic (L1), Beijing (L2), East-African Indian (L3) and Euro-American (L4). The primers and PCR conditions used for classification of lineages are shown in Supplementary Table 2.

3. Results

3.1. Drug susceptibility report

The phenotypic drug susceptibility for 56 samples (Fig. 1) was interpreted into three measures as: Resistant, Sensitive and DST failed. Isoniazid (30.4%) and pyrazinamide (30.4%) showed the highest drug resistance, Streptomycin and ethambutol showed similar resistance pattern (25.0%), while rifampicin showed the lowest resistance rate (17.9%). In addition, phenotypic drug susceptibility test also failed by a proportion of 17.9% for streptomycin, isoniazid, rifampicin and ethambutol while pyrazinamide failed by 26.8%.

3.2. Isoniazid (INH)

Out of the 26 representative samples, Phenotypic DST (MGIT) showed 34.6% resistance to INH and sequencing showed that 70% out of the resistant samples harboured SNP at codon 315 *katG* gene. Among these, the most common amino acid change observed was the substitution of Serine to Threonine (85.7%) occurring at (AGC > ACC). A single sample (14.3%) had a Ser315Asn substitution (AGC > AAC). Mutations were not found in any of the INH susceptible isolates and also in three INH resistant samples (Table 1).

3.3. Rifampicin (RIF)

The GeneXpert results showed that out of the 26 samples, two samples had probes dropout at both probes A (codons 507–511) and D (codons 523–529). A single probe A dropout was observed in 11.5% of samples and 3.8% of samples had a single probe drop out at D (Table 1).

Sequencing revealed an amino-acid substitution from Leucine to Proline (L511P, CTG → CCG) at probe A (codons 507–511) in the samples with Probe A drop out. The most common mutation occurring at probe D (codons 523–529) was an amino-acid substitution from Histidine to Glutamine (H526Q, CAC → CAG). A less common mutation observed for one sample at probe D was amino-acid substitution from Histidine to Leucine (H526L, CAC → CTC). For one sample (CVS-3$^{\#}$), both isoniazid (via. MGIT 960) and rifampicin (via Xpert MTB/RIF) were resistant, hence an MDR. However, in GeneXpert there was no probe drop out in all the five probes. The ΔCt was >4 resulting in the instrument calling it a rifampicin resistance. In addition, for this particular sample, the phenotypic DST for Rifampicin performed using MGIT had shown it as susceptible (Table 1).

Apart from the concordant phenotypic (MGIT 960) and genotypic (Xpert MTB/RIF) results, discordant DST results for Rifampicin were also observed for 7 samples: 6 samples showed as MGIT 960 susceptible while the Xpert MTB/RIF shows resistant. 3 samples were resistant to rifampicin via. MGIT 960 and susceptible via Xpert MTB/RIF. These results are shown in Supplementary Tables 3 and 4.

Table 1
Antibiotic resistance and genes associated with variants and Lineage of MTB.

Sample Code	Isoniazid			Rifampicin								LSP markers
				Drug Susceptibility Test		GeneXpert Probes Result					Sanger Sequencing		
	Pheno typic DST (MGIT)	*kat*G codon change (G/C/A)	Amino acid change	Pheno typic DST (MGIT)	Genotypic Rif-Resistance (GeneXpert)	A (507–511)	B (512–518)	C (518–523)	D (523–529)	E (529-533)	SNP	Amino acid change	Lineage
CVS-1	R	AGC	Ser315	R	Not detected	+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/*EuA
CVS-3[#]	R	AAC	Ser315Asn	S	Detected	+	+	+	+	+	No SNP detected	No SNP detected	*Non-Beijing/non-IO/non-EAI/*EuA
CVS-6[#]	R	ACC	Ser315Thr	R	Detected	Neg	+	+	Neg	+	CTG > CCG, CAC > CAG	L511P, H526Q	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-9[#]	S	AGC	Ser315	S	Detected	+	+	+	+	+	No SNP detected	No SNP detected	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-13	R			R	Not detected	+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-21	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-24	S			S		+	+	+	+	+	–	–	*Non-Beijing/*IO/EAI*/non-EuA*
CVS-25	S			S		+	+	+	+	+	–	–	*Non-Beijing/*IO/*non-EAI/non-EuA*
NER-4	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-10	R	ACC	Ser315Thr	R	Detected	Neg	+	+	+	+	CTG > CCG	L511P	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-13	S	AGC	Ser315	S	Not detected	+	+	+	+	+	–	–	*Non-Beijing/*IO/*non-EAI/non-EuA*
NER-19	S			S		+	+	+	+	+	–	–	*Non-Beijing/*IO/*non-IO/non-EAI/*EAI*/non-EuA*
NER-23	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-24	R	ACC	Ser315Thr	S	Detected	Neg	+	+	+	+	CTG > CCG	L511P	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-32	S	AGC	Ser315	S	Not detected	+	+	+	+	+	–	–	Beijing*/non-IO/non-EAI/non-EuA*
NER-35	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/*EAI*/non-EuA*
NER-36	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-37	R	ACC	Ser315Thr	R	Detected	+	+	+	Neg	+	CAC > CTC	H526L	*Non-Beijing/non-IO/non-EAI*
NER-41	S	AGC	Ser315	S	Not detected	+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*

(*continued on next page*)

Table 1 (*continued*)

Sample Code	Isoniazid			Rifampicin									LSP markers
				Drug Susceptibility Test		GeneXpert Probes Result					Sanger Sequencing		
	Pheno typic DST (MGIT)	*kat*G codon change (G/C/A)	Amino acid change	Pheno typic DST (MGIT)	Genotypic Rif-Resistance (GeneXpert)	A (507–511)	B (512–518)	C (518–523)	D (523–529)	E (529-533)	SNP	Amino acid change	Lineage
NER-42	R	ACC	Ser315Thr	S	Detected	Neg	+	+	+	+	CTG > CCG	L511P	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-43	S	AGC	Ser315	S	Not detected	+	+	+	+	+	–	–	*Non-Beijing/IO/non-IO/non-EAI/non-EuA*
NER-44	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
NER-45	S			S		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/EuA*
NER-46	R	ACC	Ser315Thr	R	Detected	Neg	+	+	Neg	+	CTG > CCG, CAC > CAG	L511P, H526Q	*Non-Beijing/non-IO/non EAI/EAI/non-EuA*
CVS-4	NA	AGC	Ser315	NA	Not detected	+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*
CVS-17	R			R		+	+	+	+	+	–	–	*Non-Beijing/non-IO/non-EAI/non-EuA*

S- Susceptible, R- Resistant; NA- Not available; + positive for GeneXpert probe; Neg-GeneXpert probe dropout; # previously treated cases; **Non-Beijing** (435 bp); **Beijing** (absence of 435 bp); **IO** Indo-Oceanic (210 bp); **non-IO** non Indo-Oceanic (1053 bp); **EAI** East Africa Indian (158 bp); **non-EAI** non-East African Indian (948 bp); **EuA** Euro-American (absence of 332 bp); **non-EuA** non Euro-American (332 bp).

3.4. Streptomycin, Ethambutol and Pyrazinamide

Phenotypic DST reported resistance for Streptomycin (14 samples), Ethambutol (14 samples) and Pyrazinamide (17 samples) (Supplementary Table 1). There were no mutations found in the genes corresponding to these antibiotics.

3.5. Lineage of M. tuberculosis

About 11.5% of the samples were Indo-Oceanic (L1), 7.7% samples were East-African Indian (L3) and 11.5% samples were Euro-American (L4). We also identified mixed infection of both Indo-Oceanic and East African Indian in two samples (7.7%). About 57.7% of samples which were not identified among the mentioned lineages were reported as non-Beijing, non- Indo-Oceanic, non-East-African India and non-Euro-American. A single sample out of the 26 samples belonged to Beijing (L2) (Table 1).

4. Discussion

This is the first attempt to culture tubercule bacilli in Mizoram, since there are no functional MTB culture facilities implemented. Limited scientific publications on MTB and drug resistance from this region necessitates this study. INH requires activation by *kat*G protein to exert its toxic effect on the bacillus. More than 300 *katG* mutations have been identified, however codon 315 (serine to threonine) accounts for 95% mutation [14]. In this study, similar report was observed where the 7-INH resistant isolates which are also Rifampicin resistant had the most common substitution as Ser315Thr (n = 6/7, 85.7%) in *kat*G gene. It is also admissible that the Ser315Thr substitution is associated with MDR strains [15]. A less common Ser315Asn substitution found in this study was also reported by a study from India [16]. The three samples that had a phenotypic INH resistance but is not reflected in the sanger report might possibly be due to the INH resistance mutation occurring in other genes such as *inh*A [14].

Rifampicin discordant results observed between MGIT 960 and Xpert MTB/RIF in 3 isolates (Supplementary Table 3) in which mutations were absent in the RRDR region of the *rpo*B gene though phenotypically resistant to RIF. This might be attributed to different genotypes prevailing worldwide and since Xpert MTB/RIF detects mutations only within the RRDR of the *rpo*B gene, the mutations in these strains might lie outside this region as described previously [17]. In two samples (CVS-3$^{\#}$, CVS-9$^{\#}$), we observed a discrepancy between MGIT and Xpert MTB/RIF where rifampicin was susceptible via. MGIT, resistant via. Xpert MTB/RIF without any probe drop out and ΔCt was >4, however sanger sequencing revealed no mutation in *rpo*B region. This could be a false resistance produced by Xpert MTB/RIF as similar discrepancy results were reported from China [18]. In two other samples (NER-24, NER-42), rifampicin was susceptible via. MGIT and resistant via. Xpert MTB/RIF where probe A was drop out. In this discrepancy, the mutation observed was L511P (CTG → CCG). Similar disputed *rpo*B mutation was reported from South Korea [19]. A study from Kuwait reported isolates as rifampin susceptible in MGIT, but resistant by Xpert assay. Sequencing of *rpo*B identified a silent (CTG521TTG) and a missense (GAC516TAC) mutation [20]. However, this was not the case in our study, suggesting diverse as yet unknown mechanisms, impacting rifampin susceptibility.

In this region, sanger sequencing of the specific mutation in probe A (507–511) was L511P. Our study also reported the specific mutation occurring at probe D (523–529) as H526Q and H526L which is not in concordance with another study from Bangladesh where they reported mutation as H526D and H526Y [21]. The mutations L511P and H526L have been reported to confer a low level of resistance [22]. A study from Treichville, Abidjan District reported H526Q mutation in a relapse case [23]. With respect to the other antibiotics tested, the phenotypic DST resistance observed for Streptomycin, Ethambutol and Pyrazinamide do not correlate with sanger sequencing results, probably because the primers used in this study does not cover the region where SNP's might be present.

Though the number of samples studied maybe small, this study attempts to provide information on *M. tuberculosis* strain diversity in this remote region of northeast India where all the four major lineages (L1, L2, L3 L4) are present. In a recent study which is under review, Beijing (L2) and East-African Indian (L3) were found in three of the northeastern states, Manipur, Nagaland and Tripura [24]. Studies have revealed Indo-Oceanic (L1) and East-African Indian (L3) to be most widespread in India compared to other parts of the world [25]. This might explain the mixed strain infection observed in two samples in this study.

Mixed strain infections particularly when strains with different drug resistance phenotypes are involved, makes diagnosis, treatment and control more cumbersome and people with underlying morbidity may be prone to mixed strain infection [26]. A study from Botswana had reported that among new cases of tuberculosis, mixed strain infections (but not hetero-resistant infection) were associated with poor treatment outcomes using standard combination treatment regimens [27]. Previous studies have shown that in patients who harboured both MDR and susceptible strains, MDR strain was able to persist and grow during treatment with first-line regimens and upon a switch to second-line regimen, the susceptible strain was able to re-emerge, and thereby compromising treatment outcome [28]. Immune responses may be altered in patients with ongoing mixed strain TB infections, rendering them more prone to re-infection [29].

In Mizoram, establishment of highly sophisticated diagnostic machines requiring technical expertise is still cumbersome. Due to programmatic challenges, ATT is usually initiated based on GeneXpert reports. Since the global frontline molecular diagnostic tools including GeneXpert and LPA are based on inadequate number of known genetic markers, seldom, phenotypic resistance could not be found associated with known drug resistant mutations [30]. Thus, our findings suggest that routine diagnosis aided with sequence-based information is essential in diagnostic laboratories to map the genetic variations and identify all the genotypes associated with drug resistance to provide newer, accurate diagnosis, better anti-tubercular therapies, vaccines and effective patient care.

5. Conclusion

This study provides information on the specific mutations detected with GeneXpert and MGIT 960 as well as the lineages of MTB circulating in the state. Being the first MTB culture to be performed within the state, the phenotypic DST is yet to be certified since it is not from a competent laboratory. However, from our small set of samples, we report fundamental data that represents the present status of the disease in the region that can be further explored by expanding the genetic regions covered for the antibiotic resistance genes as well as stronger lineage identification markers with larger number of samples.

Authors Contribution

VF, FK, ZC, LC recruited the patients. CVS, RL, ZS, GL collected the samples and performed the culture. CVS, ZS, ZC, LC, FK, ZZ performed the MGIT and Xpert assays, molecular work and data analysis. CVS, ZZ, VF, NSK interpreted the data and wrote the manuscript. NSK, RL, GL, ZS, DK contributed to the concept and design of the study. NSK, DK, VF acquired the funds for the study. All authors have proof read and approved the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

Ethics approval

Approval for the study was obtained from the Institutional Ethics Committee (IEC) of Civil Hospital, Aizawl, Mizoram (B.12,018/1/13-CH (A)/IEC/63). All the participants had provided a written consent at the time of sample collection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijmmb.2022.06.003.

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Original article

Risk factors of tuberculosis in Mizoram: First report of the possible role of water source

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ABSTRACT

Background: Various risk factors of tuberculosis have been studied across the globe, but these may be altered over time and can be specific to geographical regions and there is not much information available from Northeastern region of India. This study aims to investigate the various risk factors of tuberculosis and analyze the presence of any less-established risk factors.

Methods: A total of 400 TB cases and 840 healthy controls were interviewed from December 2017 - June 2020. Logistic regression model was used to analyze associated risk factors. Patients were categorized into pulmonary and extrapulmonary TB.

Results: Clinical presentation such as fever, cough, weight loss, chest pain and night sweats were more prominent among pulmonary TB patients. The most common mode of diagnosis among pulmonary and extrapulmonary TB were GeneXpert and X-ray, respectively. Tuberculosis was found to be strongly prevalent among patients from lower socio-economic status, less educated, unemployed and improper housing condition. Other risk factors associated were alcohol consumption, neighbours with TB, travel history, no BCG vaccine, mass gathering, and non-ideal weight. An interesting less-established risk factor that demands attention is the source of water supply (p-0.017, OR-2.313, CI: 1.160–4.613), which was significant in this study.

Conclusion: Our data suggests that apart from all the well-established risk factors for TB, water supply might play a crucial role towards the transmission of TB, since proper

Abbreviations: TB, Tuberculosis; BCG, Bacille Calmette Guerin; DOTS, Directly Observed Treatment, Short Course; DR-TB, Drug resistant Tuberculosis; RNTCP, Revised National Tuberculosis Control Programme; FNAC, Fine Needle Aspiration Cytology.

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hospital waste water treatment is yet to be adopted in Mizoram, Northeast India. From a public health standpoint, this highlights the need for further research in this area.

1. Background

Mycobacterium tuberculosis which is a contagious disease has been hypothesized to live and evolve with mankind for more than 150 million years. More than a century has passed from the day of its discovery by Robert Koch[1] and till date mankind is unable to eradicate this deadly disease plaguing human. In the modern world, we now know that tuberculosis is a multifactorial disease and many factors contribute to the outcome of the disease.[2] Globally, in 2019 an estimated 10 million fell ill with *Mycobacterium tuberculosis*. Since 1998, India has been considered to be among the TB "high burden" countries that accounted for 87% of the world's cases. India with an estimated population of 135 crores, reports approximately 28 lakhs incidence of TB accounting for about a quarter of the world's TB cases.[3] The Revised National Tuberculosis Control Program (RNTCP) was launched in India in 1997. It has now covered the whole of India making it the second largest such program in the world (https://dghs.gov.in/content/1358_3_RevisedNationalTuberculosisControlProgramme.aspx).

Mizoram is a small hilly tribal state, which is less densely populated and is situated in the north eastern region of India. The people of Mizoram actively participate in *Hnatlang*, or community service as an important activity for community welfare. Above all, most Mizo tribes believed in the ethics of "*Tlawmngaihna*" moral code which made them courteous and helpful to others. Different forms of tobacco consumption - smoking, tuibur (tobacco smoke–infused water), dried form mixed with lime and placed in the gum of the user and alcohol consumption is a common practice among the adults in Mizoram.[4] The distinguishing lifestyle of the people of Mizoram makes them different from the other tribes in India.[5] The main occupation of the people is agriculture on small land holdings and Jhum cultivation, livestock and dairy farming and agro forest produce are also a minor trade of the region.

In 2003, Mizoram implemented RNTCP and Programmatic Management of Drug Resistant Tuberculosis (PMDT) which is the former DOTS Plus in 2011.After the launching of RNTCP in the state, sputum microscopy (Ziehl Neelsen stain) became the gold standard of TB diagnosis with a total of 34 RNTCP direct microscopy centers till date, located in all the eight districts of Mizoram (https://health.mizoram.gov.in/page/tuberculosis#main-navbar). It is among the few states where the Universal Drug Susceptibility testing using Cartridge Based Nucleic Acid Amplification Test (GeneXpert) was implemented in 2017. This is a nationwide programme to test every TB patient for signs of resistance to the first-line drugs, especially Rifampicin. Mizoram contributes only about 0.09% of the population in India[6] In 2019, as many as 2,944 TB cases had been notified from both public and private sector and TB-HIV co-infected patients diagnosed were 321.[7] The state also reported the country's highest adult HIV prevalence of 2.04%.[8] This shows that Mizoram is in dire need of a massive effort to eliminate TB and spread awareness about the risk factors.

Individuals with diabetes have a higher risk of TB infection, and the relative risk is reported to range from 1.16 -7.83.[9] Malnutrition leads to being under-weight which can increase the risk of developing active tuberculosis by 6–10 times.[10] Lifestyle factors may also be a risk factor contributing to the development of the disease. The person with the habit of smoking are at a higher risk of developing TB as smoking alters immune responses to the bacterium due to impairment of alveolar macrophage as a result of which it contributes to susceptibility to TB infection.[11] TB infections are two times higher in tobacco smokers as compared to non-smokers.[12] It has been estimated that reduction in TB risk will be achieved by completely eliminating tobacco smoking.[13] The consumption of alcohol impairs the immune system and the functioning of alveolar macrophage in eliminating the pathogens get compromised, which increases the susceptibility to TB.[14] A systematic review concluded that people who drink alcohol everyday or have an alcohol use disorder, are at a higher risk of active tuberculosis.[15]

From the above, the risk factors that contribute to the outcome of tuberculosis infection are vivid. However, though the modes of transmission of tuberculosis have been well elucidated, we speculate a possible chance of indirect-infection through ingestion of contaminated water. Hardly few reports have focused their studies on water supply sources of the TB infected persons.[16] This study was conducted to understand other possible risk factors that may play a role towards the transmission of tuberculosis in the hilly mountainous regions of Northeast India.

2. Methods

2.1. Ethics approval and consent to participate

Approval for the study was obtained from the Institutional Ethics Committee (IEC) of Civil Hospital, Aizawl, Mizoram (B.12018/1/13-CH (A)/IEC/63, Dated: 28-03-2017). All the participants in this study provided a written consent.

2.2. Study design

This was a case–control study of patients visiting DR-TB centre, Falkawn, Mizoram. A larger number of TB patients from the eight districts of Mizoram visit this center on a daily basis. Participants in this study were Mizo TB patients aged 15 and above, who were consecutively enrolled from December 2017 to June 2020. Pregnant women were excluded in this study. The participants were categorized into their respective

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gender (male and female). Age was divided into two groups: 15–45 years and >45 years. Marital status was categorized into two –married (who are currently living with spouses) and single (this includes unmarried, all divorcees, widows and widowers).Body Mass Index (BMI) score was calculated using online BMI Calculator – Metric and Imperial (www.the.calculator.site.com).A BMI of 18.5–24.9 was classified as normal weight, <18.5 as underweight, 25–29.9 as overweight, > 30 as obese. For convenience, the BMI were categorized into two- Ideal weight (normal weight) and Non-ideal weight (underweight, overweight and obese).

Education level was grouped into two –upto Matriculation (class 10) and higher secondary and above. Occupation was categorized into two – employed (govt. employee or private sector) and unemployed (no job at all). Family income was also categorized into two. Those having a monthly income of >401.57 USD and <401.57 USD (this amounts to INR 30,000 per month).The condition of housing was categorized as those who receive proper sunlight during the day and as those whose houses are either moist or damp without any proper sunlight. Number of people living under one roof was grouped into two – 1 to 6 members and >6 family members residing under a single roof. Water supply source was categorized into two – 'Govt. municipal' which is the PHED (Public Health Engineering Department) water supply. This water is pumped up from various river sources and treated using the protocols for water treatment in order to make it potable and to suffice various domestic purposes. 'Other sources' includes river water and/or collection of spring water. The frequency of physical activity in the form of exercise was also classified into two – Regularly and Irregularly.

The following factors/variables were classified into two categories ("Yes" or "No"):

Residing in a crowded house; presence of cross-ventilation system in their homes; History of contact with pulmonary TB; Participation in mass gathering events; History of recent travel; whether suffering from diabetes and HIV (This was done after participants had undergone a random blood sugar test using Accu-chek and HIV Tri-Dot at the TB Clinic); BCG vaccine uptake (via verbal response from participant); alcohol consumption (those who have had or have been consuming and those who never consumed alcohol in their lifetime); Consumption of tobacco products in the form of smoking; Other tobacco products (snuffed tobacco, gutkha, shikhar etc); Tuibur (tobacco infused water); exposure to passive smoking (either by family members or colleagues); Susceptibility to respiratory infection; Previously diagnosed as TB (either by laboratory tests or clinically including a positive TB skin test); Any family members with ongoing TB; Family members with history of TB; Number of family members succumbed to TB so far and Neighbours suffering from TB.

2.3. *Sample size calculation*

The online sample size calculator Raosoft, Inc. (http://www.raosoft.com/sample.size.html) was used. For a population of 10,97,206,[6] with 5% margin of error, 95% confidence level and 50% response distribution, the required sample size was calculated to be 385. Therefore, a total of 400 TB patients and 840 healthy controls were included in this study. The criteria for healthy controls are individuals with no previous history of TB, no predisposing factors for TB such as immunocompromised state (HIV) and non-diabetic.

2.4. *Study procedures*

Patients' consents were obtained after which an interview was taken using a structured questionnaire for collection of data on demographic, socioeconomic, lifestyle factors and TB symptoms. Patients included are both pulmonary and extrapulmonary TB who are detected as TB positive in the District TB Center, Falkawn via clinical symptoms accompanied by either any of the following tests - ZN stain (microscopy), GeneXpert (CB-NAAT), Chest X-ray, Mantoux, ADA, Biopsy, FNAC, CT scan, Ultrasound, Culture and/or Line Probe Assay which is outsourced to neighboring states' Intermediate Reference Laboratory (IRL).

2.5. *Statistical analysis*

Data were analyzed using IBM Statistical Package for Social Sciences (SPSS) 20.0 program (SPSS Iberica, Madrid, Spain). For associated risk factors, variables were dichotomized. Cross-tabulation was performed for bivariate analysis and after adjusting for confounding factors the p-value, odds ratio and 95% CI were calculated using logistic regression model.

3. Results

A total of 400 TB patients were categorized as per the RNTCP classification into pulmonary (n = 288) and extrapulmonary (n = 112) TB. The two groups are further broken down into new cases, relapse, treatment failure and defaulters (Supplementary Fig. 1). Clinical presentation such as fever, cough, weight loss, chest pain and night sweats were more prominent among pulmonary TB patients (Supplementary Table 1). The most common modes of diagnosis among pulmonary TB were GeneXpert (40%) followed by smear microscopy – Ziehl Neelsen Stain (32.6%) whereas the most common modes of diagnosis for extrapulmonary TB were X-ray (40.1%) followed by FNAC (25.9%) among the study group (Supplementary Fig. 2). The mean age of patients was found to be 33.1 years.

3.1. *Associated risk factors*

Patients who are single have a 2.13 times higher risk of contracting TB as compared to married people using bivariate analysis after adjusting for confounding factors (Supplementary Table 2). Ideal body weight reduced the risk of TB by 0.031 times. The educational qualification was inversely proportional as the chance of contracting TB was increased by 4.57 times with less education (up to Class 10) and the risk of contracting the disease was 2.79 times in the case of unemployed individuals. In this study, out of the 230 unemployed participants with TB, 84.7% (n = 195) consume alcohol, 85.2% (n = 196) indulge in smoking, 60% (n = 138) use other tobacco products and 13.4% (n = 31) do take tuibur (tobacco infused smoke).

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Tuberculosis was found to be strongly prevalent among patients from lower socio-economic status where family income per month was below 401.57 USD. In other words, people with low income have 2.43 times the risk of contracting TB. Thus, income factor has a significant role towards contracting TB. Houses that are moist or damp have a 2.60 times risk of harbouring TB infection as compared to houses that received good sunlight. Cross ventilation do seem to have a positive impact in the present study as expected (P-0.000).

One interesting less-established risk factor that demands attention are the source of water supply other than the Government Municipal supply. Individuals whose water source for domestic purpose is either river water or a collection of spring water have a 2.313 times of getting infected with TB. People who are heavily engaged in societal activities that accompanies frequent community mass gathering events are also at risk of contracting TB infection (P-0.000). Individuals who have had a recent travel history are at five times higher risk of contracting TB. There is also a 3.0 times higher risk of contracting TB among individuals who did not receive BCG vaccine. It is noteworthy that alcohol consumption increases the risk of TB infection by 7.17 times as compared to non-drinkers. Among the TB patients, 48.8% (n = 195) consume alcohol of which 81.5% (n = 159) are males, while 18.5% (n = 36) are females. Furthermore, the risk of spreading TB increases by 4.40 times when there is a neighbour suffering from TB (Table 1).

4. Discussion

In this study, females were less prone to develop TB, while the male counterparts are at higher risk which could possibly be due to greater exposure to many of the proven risk factors of TB and this is in concordance with the Global Tuberculosis Report.[17] Single individuals tend to mingle more in various activities conducted by the community as compared to married people, thus serving as a high risk group for TB. In the present study, the rate of TB incidence was twice as high among single individuals, which is in concordance with previous report.[18] Non-ideal weights such as underweight, obese or overweight are more prone to develop tuberculosis in their lifetime as compared to normal weight because non-ideal weight could signify poor health. However, obesity and overweight individuals were also found to be at a lower risk of developing active tuberculosis.[19] Education is an important aspect of life. Being educated possibly decreases the risk of TB wherein, the knowledge to seek an urgent medical assistance is a priority among the educated. This study also showed that participants who are less educated (up to matriculation) are at a higher risk of developing TB which is in concordance with previous report.[20] The incidence of TB was observed to be higher among unemployed individuals wherein unemployment favours the occurrence of the disease.[21]

Poverty and tuberculosis go in tandem where being economically poor increases the risk of infection with *M. tuberculosis* as compared to the general population because of overcrowding, substandard living or working conditions, poor nutrition, and less access to medical interventions.[22] Overcrowding is an important risk factor for tuberculosis where overcrowding exposes susceptible individuals to infectious TB,[23] however, this factor is not significant in the present study. The knowledge of indoor transmission of tuberculosis remains poor. In this study, the presence of cross-ventilation system in the house seems to be a protective factor which was also reported by a recent study where maintaining adequate cross-ventilation effectively controlled TB outbreaks.[24] Condition of the housing is another important risk factor because houses that are moist or damp with poor lighting conditions are at risk of harboring tubercle bacilli whereas direct sunlight can kill the tubercle bacilli within several hours.[25]

Though tuberculosis is well known for its air borne transmission, other possible routes of transmission are in question. Water supply for domestic purpose is yet another important aspect that is often neglected in recent studies pertaining to tuberculosis transmission. The state where the present study is conducted is a mountainous hilly region in the north eastern part of India. It has poor drainage system where during the rainy seasons, drains would seldom clog and overflow. In addition, there is no sewage treatment facility in the hospitals so far. Hence, waste-waters from various hospitals and TB sanatorium would flow down the drain and eventually find its way into the soil and also converge down into rivers especially during the monsoon seasons. Also, majority of the people receive the government water supply (PHED water) while some who are living in the lower ridges/altitude still rely on river water as well as spring water collection (tuikhur) for domestic purposes. In this study, we observed that participants whose water supply source is other than the government water supply such as river or spring water collection have two times higher chances of contracting TB. This is of great concern since previous studies have recovered virulent tubercle bacilli from river water even after five months of storage either in the dark or diffused daylight.[26] Also, a previous review report had stated the recovery of virulent tubercle bacilli in settled sewage water from an institution where sputum was adequately disinfected.[27] As observed from previous reports, the sources of water contamination could be either human or non-human source.[28,29] In addition since both human and animal excreta such as urine and stool can harbor the tubercle bacilli, sewage contamination of water sources could potentially be another source.[30] Our previous metagenome study around Aizawl city, Mizoram had isolated and characterized bacterial microbiome from soil sediment of drinking water and hospital waste water sites and found many pathogenic bacteria, including *M. tuberculosis*.[31] The limitations of the present study is that we did not collect water samples for examination since it was not part of our objective. Moreover, culturing *M. tuberculosis* is cumbersome and requires a different set of protocol. However, it can be taken up for future studies. The aforementioned risk factors suggested that a broader view of the possible routes of TB transmission should be reconsidered.[32]

As previously mentioned, since the people of Mizoram believed in the ethics of "*Tlawngaihna*" a moral code which made them courteous and helpful to others, if any person within the community demise, spontaneous gathering to help the family of the demise is a very common practice. In addition,

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Table 1 – Binary logistic regression of the demographic factors influencing TB.

Variables		TB Patient (%)	Healthy Control (%)	Unadjusted p-value	Unadjusted OR (95% CI)	Adjusted p-value	Adjusted OR (95% CI)
Sex	Male	207 (51.7)	496 (59.0)	**Reference**			
	Female	193 (48.3)	344 (41.0)	0.015	1.344 (1.058–1.708)	0.077	1.619 (0.949–2.763)
Marital Status	Married	191 (47.8)	342 (40.7)	**Reference**			
	Single	209 (52.3)	498 (59.3)	0.019	0.751 (0.591–0.955)	**0.002**	**2.135 (1.320–3.453)**
BMI Score	Ideal Weight	244 (61)	47 (5.6)	**Reference**			
	Non-ideal weight	156 (39)	793 (94.4)	0.000	0.038 (0.027–0.054)	**0.000**	**0.031 (0.018–0.052)**
Education	Higher Secondary & above	154 (38.5)	627 (74.6)	**Reference**			
	Upto Matriculation	246 (61.5)	213 (25.4)	0.000	4.702 (3.646–6.064)	**0.000**	**4.572 (2.824–7.404)**
Occupation	Employed	170 (42.5)	549 (65.4)	**Reference**			
	Unemployed	230 (57.5)	291 (34.6)	0.000	2.552 (2.000–3.258)	**0.000**	**2.791 (1.816–4.289)**
Family income (per month)	>401.57 USD	84 (21.0)	349 (41.5)	**Reference**			
	<401.57 USD	316 (79.0)	491 (58.5)	0.000	2.674 (2.027–3.527)	**0.001**	**2.342 (1.422–3.856)**
Reside in a crowded house	No	244 (61.0)	644 (76.7)	**Reference**			
	Yes	156 (39.0)	196 (23.3)	0.000	2.101 (1.625–2.716)	0.160	1.383 (0.880–2.175)
Cross Ventilation	Yes	232 (58.0)	304 (36.2)	**Reference**			
	No	168 (42.0)	536 (63.8)	0.000	0.411 (0.322–0.524)	**0.000**	**0.289 (0.187–0.446)**
Condition of housing	Sunlight	279 (69.8)	751 (89.4)	**Reference**			
	Damp or Moist	121 (30.3)	89 (10.6)	0.000	3.660 (2.694–4.971)	**0.000**	**2.604 (1.535–4.418)**
No.of people living under one roof	1-6 members	289 (72.3)	658 (78.3)	**Reference**			
	>6 members	111 (27.8)	182 (21.7)	0.018	1.389 (1.056–1.826)	0.100	1.497 (0.926–2.419)
Water supply source	Govt.municipal	339 (84.8)	783 (93.2)	**Reference**			
	Other sources (spring water, river etc.)	61 (15.2)	57 (6.8)	0.000	2.472 (1.686–3.625)	**0.017**	**2.313 (1.160–4.613)**
Mass gathering events	No	263 (65.8)	306 (36.4)	**Reference**			
	Yes	137 (34.3)	534 (63.6)	0.000	0.299 (0.233–0.383)	**0.000**	**0.261 (0.168–0.405)**
Travel history	No	295 (73.8)	734 (87.4)	**Reference**			
	Yes	105 (26.3)	106 (12.6)	0.000	2.465 (1.823–3.333)	**0.000**	**5.006 (2.876–8.711)**
BCG Vaccine	Yes	256 (64.0)	703 (83.7)	**Reference**			
	No	144(36.0)	137 (16.3)	0.000	2.886 (2.194–3.797)	**0.000**	**3.033 (1.895–4.854)**
Alcohol	No	205 (51.2)	672 (80.0)	**Reference**			
	Yes	195 (48.8)	168 (20.0)	0.000	3.805 (2.937–4.929)	**0.000**	**7.172 (4.060–12.671)**
Smoking	No	204 (51.0)	511 (60.8)	**Reference**			
	Yes	196 (49.0)	329 (39.2)	0.001	1.492 (1.174–1.897)	0.088	0.626 (0.365–1.073)
Family members/colleagues smoke tobacco	No	109 (27.3)	284 (33.8)	**Reference**			
	Yes	291 (72.8)	556 (66.2)	0.020	1.364 (1.049–1.773)	0.274	1.338 (0.794–2.255)
Exposure to passive smoking	Rarely or never	211 (52.8)	578 (68.8)	**Reference**			
	Regularly	189 (47.3)	262 (31.2)	0.000	1.976 (1.547–2.524)	0.260	1.319 (0.814–2.138)
Family members with history of TB	No	254 (63.5)	728 (86.7)	**Reference**			
	Yes	146 (36.5)	112 (13.3)	0.000	3.736 (2.811–4.966)	0.133	2.008 (0.809–4.986)
Members in family with TB so far	None	230 (57.5)	687 (81.8)	**Reference**			
	1–5 members	170 (42.5)	153 (18.2)	0.000	3.319 (2.547–4.324)	0.381	1.475 (0.618–3.524)
Neighbors with TB	No	284 (71.0)	789 (93.9)	**Reference**			
	Yes	116 (29.0)	51 (6.1)	0.000	6.319 (4.426–9.022)	**0.005**	**4.405 (1.548–12.534)**
Frequency of contact with a neighbor suffering from TB	Never	307 (76.8)	799 (95.1)	**Reference**			
	Frequently	93 (23.2)	41 (4.9)	0.000	5.903 (3.996–8.722)	0.780	0.844 (0.257–2.772)

The confounding factors have been adjusted. A p-value less than 0.05 (typically ≤0.05) is statistically significant. Odds Ratio is a measure of the strength of association between an exposure and an outcome. OR > 1 means greater odds of association with the exposure and outcome. OR = 1 means there is no association between exposure and outcome. OR < 1 means there is a lower odds of association between the exposure and outcome.

within the community, there are many community services (*Hnatlang*) to be attended, be it for the community welfare or for the church welfare. During such activities, people are accustomed to sharing drinking water from the same bottle or the same glass. Snuffed tobacco sharing from the same pack is also a common practice along with taking a puff from the same cigarette. Since the society is closely knit, this practice has been in existence within the Mizo community from times immemorial. All these cultural activities lead to the gathering of a huge crowd. Mass gathering events can be associated with public health risks. Till date, there are many undiagnosed tuberculosis cases who serve to fuel TB transmission within the community. Also, data on tuberculosis associated with mass gathering events are scarce. However, this study showed that people who participated in such events have a significant chance of contracting communicable diseases such as tuberculosis. This is also in concordance with previous report where TB is transmitted during the Hajj pilgrimage.[33]

Patients who have had a travel history upon verbal enquiry develop the symptoms after their travel. Since, the TB status of a person one travels with is unknown, there could be a chance of TB transmission during travel though the risk may be small.[34]

The protective efficacy of BCG vaccine remains controversial and varies from different geographical regions and populations.[35] In this study, statistical significance was observed among the participants who did not receive BCG vaccine as compared to those who did. This was in contrast to a previous report where no statistical difference in tuberculosis was observed among vaccinated and unvaccinated group.[36] Several studies have attributed alcohol consumption as a major contributor to the increase in risk and burden of tuberculosis, where nearly a three-fold increase in the risk of TB was observed among those with alcohol use disorder.[14] Our study has observed a much higher increase i.e., a seven fold increase in the risk of tuberculosis with alcohol consumption. Apart from impairing the immunity, the risk of TB increases due to specific social mixing patterns among people who consume alcohol.[37] Though numerous studies have linked tuberculosis with active and passive smoking,[38–40] we did not find any association in this study. An extensive epidemiological study or contact tracing to identify tuberculosis exposure is essential.[41] The transmission of tuberculosis between neighbours had also been documented.[42] Similarly, in this study we have identified a significant association between tuberculosis transmissions when there is a neighbor suffering from TB.

5. Conclusion

Tuberculosis is a multifactorial disease and is contributed by various risk factors. In the state of Mizoram, the unique social practices within the community might also serve to fuel TB transmission and/or hinder the tuberculosis control program. Apart from the well-established risk factors for TB, this study highlights the need to investigate water sources as they can serve as a potential source of infection. From a public health standpoint, this highlights the needed of further research in this area.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors' contributions

FK, VF, LC, ZC: recruited patients at the TB clinic; CVS,MVT, ZS: interviewed recruited patients, collect data, carried out the analysis; LR, LN, NSK: conceived and planned the experiments. All authors provided critical feedback, discussed the results and contributed to the final manuscript.

Consent for publication

The manuscript has been read and approved by all the authors, the requirements for authorship as stated earlier in this document have been met, and each author believes that the manuscript represents honest work.

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Conflicts of interest

The authors have none to declare

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijtb.2022.03.003.

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Analysis of variants in mitochondrial genome and their putative pathogenicity in tuberculosis patients from Mizoram, North east India

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ABSTRACT

Tuberculosis caused by *Mycobacterium tuberculosis* is one of the main global health concerns. In this study, the entire mitochondrial genome from blood samples of tuberculosis patients was analyzed to understand the possible mtDNA variants. The potential impact of non-synonymous substitutions on protein functions were determined using prediction tools. 28 non- synonymous variants were found of which 2 variants (MT-ND2 g. A > G4824 p.T119A and MT-ND6 g. T > C14180 p.Y165C) were found to be deleterious among the cases only. Majority of the variants lie in the D-loop of the non-protein coding region of the mitochondrial DNA. We propose that mutations in the mitochondrial genome need to be validated further to understand their association with tuberculosis.

1. Introduction

Tuberculosis (TB) is caused by a single infectious agent, *Mycobacterium tuberculosis* (Mtb) [28] and is one of the leading causes of concern with a yearly occurrence of 10 million infections [9]. In 2018, in India, new cases diagnosed were 2.8 million with deaths approximately 0.4 million. In Mizoram, the total population is as low as 11 lakhs, of which at least 2499 people were diagnosed with TB in 2018 (TB India [11]). TB spreads from a diseased person to healthy individuals through the air droplets generated by cough, sneeze or saliva. An individual is believed to have a maximum of 15% chance of becoming sick with TB. This is because most of the people have latent TB and thus being unable to spread the disease. Therefore, early detection and proper treatment is needed to reduce TB burden [9].

The complete human mitochondrial DNA (mtDNA) was first sequenced in 1981 [2]. MtDNA is a circular genome of 16.5 kb in size and has a non-coding displacement loop (D-loop) constituting only 6.8% of the entire mt genome. This region controls replication and transcription of mtDNA. The coding region is larger than the non-coding region, constituting about 93.2% of the genome. The coding region houses thirty-seven (37) genes and has 22 tRNAs, 13 proteins and 2 rRNAs that encode proteins involved in the electron transport chain. During oxidative phosphorylation, the mtDNAs are in continuous exposure to reactive oxygen species which makes them highly susceptible to mutations [17]. It has been suggested that a higher fraction of mitochondrial DNA mutation is likely to be functionally damaging and have distinct phenotypes as compared to nuclear mutations [26]. Thus, serving as an outstanding 'model system' for identification of genetic changes that will help solve the medical problems that genomic medicine will encounter.

Studies have been carried out to see the relation of mtDNA variations with different diseases [18]. Study of Oral cancer showed that an increase in lymph node metastasis was contributed by non-synonymous mutations in mitochondrial respiratory genes [19]. Mutations in mtDNA found in gastric cancer suggest that the mitochondrial genome can be used as a biomarker in cancer detection at an early stage [26]. The study of mutations in ovarian cancer in mtDNA revealed somatic mutations suggesting that mtDNA mutation may be represented in 12 s and 16 s rRNA genes, the cytochrome *b* gene and the D-loop regions. Instability of mtDNA might lead to tumorigenesis [17].

Not much work has been carried out to identify the underlying genetic variants in mitochondrial genome for tuberculosis in any

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population across the world. There are no scientific reports or evidence for host mitochondrial genetic factors linked to tuberculosis and this is the first study to explore the possible genetic variants which could predispose the population. In the present study, we hypothesize that mutations in the key genes involved in energy metabolism might be found in TB patients.

2. Materials and methods

2.1. Participants

Present study included tuberculosis patients visiting the DR-TB Hospital, Falkawn, Mizoram, Northeast India. Blood samples were collected in 2 ml EDTA vials and immediately stored at −20 °C until further processing. The Institutional Ethics Committee (IEC) of Civil Hospital, Aizawl, Mizoram had given the approval to carry out the work (B.12018/1/13-CH (A)/IEC/63, dtd 28-03-2017). Blood was collected with written informed consent from each study participant.

2.2. DNA amplification and sequencing of mtDNA

A total of seven TB patient's and twelve healthy control's blood samples were recruited for the study. DNA isolation from blood was done using QIAamp (Qiagen) as per manufacturer's recommendations. SequalPrep™ Long PCR Kit with dNTPs (Thermo Scientific) was used for amplification of whole mtDNA (16.6 kb) from blood in ABI 9700 thermal cycler (Thermo Scientific). Visualization and purification of the PCR products was done in 0.8% agarose gel electrophoresis and QIAquick Gel Extraction Kit (Qiagen), respectively. The purified amplicons were processed for sequencing library preparation using Nextera XT DNA library kit (Illumina). After purification, the libraries were pooled and sequenced in Illumina HiSeq-2500 to generate 2 × 100 bp reads. Sequencing was performed at CoTERI, National Institute of Biomedical Genomics (NIBMG), Kalyani, West Bengal, India.

2.3. Sequence analysis

The preliminary quality check of the FASTQ files was done using FASTQC [3]. BWA-MEM tool was used for alignment of the sequence data to the mitochondrial reference sequence (rCRS- revised Cambridge Reference Sequence) [15]. SAM tool was used to convert SAM file to BAM files, and reads below mapping quality of 40 were discarded [16]. QualiMap was used to check the quality of BAM files to align with the mtDNA reference sequence [8]. VarScan2 was used for detection of variants in the mtDNA [14]. Integrative Genome Viewer [20]and ANNOVAR [29]was used for the visualization and annotation of variants, respectively.

The mitochondrial sequence data was filtered using different approaches to help prioritize variants. The first filter (Filter1) involved removal of all the common variants between patients and healthy controls, giving only case specific variants. After which the non-synonymous variants underwent filtering through function prediction tools such as PROVEAN (http://provean.jcvi.org/index.php) and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/).

3. Results

Upon sequencing the entire mitochondrial DNA, a total of 83 variants were observed in the non-coding region (Table 1). A total of sixty-three variants were present in the D-loop region, fourteen variants in 16 s, three in 12 s, two in tRNA-Gln and one in tRNA-Thr. In coding region, a total of ninety-four variants were present in different genes, out of which sixty-six were synonymous, twenty-eight were non- synonymous and one was a frameshift substitution of which 2 variants were shown to be deleterious in Polyphen-2 and Provean. The two deleterious mutations were MT-ND2 g. A > G4824 p.T119A found in

Table 1
List of variants in the non-coding region of the mtDNA of TB patients.

Patient ID	Position	Reference	Variant	Gene	VarFreq (%)
TB-1	152	T	C	D-loop	99.89
	194*	C	T		99.8
	247	G	-A		91.05
	279*	T	C		99.87
	489	T	C		99.94
	2706	A	G	16 s	99.96
	15926*	C	T	tRNA-Thr	99.89
	16,223	C	T	D-loop	99.59
	16,234	C	T		99.92
	16325*	T	C		99.86
	16390*	G	A		99.81
	16,519	T	C		99.88
TB-2	302	A	AC		8
	385*	A	G		99.81
	489	T	C		99.93
	2150*	T	TAA	16 s	91.36
	2706	A	G		99.91
	3105	A	-CC		99.02
	16,183	A	AC	D-loop	8.68
	16,189	T	C		99.54
	16215*	A	G		99.54
	16,223	C	T		99.52
	16,316	A	G		99.92
	16325*	T	C		99.9
TB-3	302	A	AC		8.03
	663	A	G	12 s	99.67
	2706	A	G	16 s	99.96
	16179*	C	-A	D-loop	16.06
	16172*	T	C		99.51
	16356*	T	C		99.84
	16,362	T	C		99.84
	16,519	T	C		99.83
TB- 4	152	T	C		99.81
	247	G	-A		95.11
	302	A	AC		9.31
	489	T	C		99.91
	2352	T	C	16 s	99.91
	2706	A	G		99.95
	2780	C	T		99.8
	4363	T	C	tRNA-Gln	91.99
	16,185	T	C	D-loop	97.55
	16,189	T	C		99.63
	16,223	C	T		99.7
	16,260	C	T		99.8
	16,298	T	C		99.82
TB- 5	152	T	C		99.91
	234	A	G		99.97
	247	G	-A		90.5
	302	A	AC		9.06
	513	G	CA	D-loop	84.76
	745*	A	AT	12 s	99.89
	1927*	G	A	16 s	99.91
	2706	A	G	16 s	99.98
	10454*	T	C	D-loop	99.92
	16,093	T	C		99.29
	16111*	C	T		97.98
	16,129	G	A		99.61
	16,304	T	C		99.68
	16,311	T	C		99.88
	16,519	T	C		99.82
TB- 6	152	T	C		99.7
	200	A	G		97.83
	235	A	G		100
	302	A	AC		6.27
	513	G	-CA		84.6
	663	A	G	12 s	99.82
	2706	A	G	16 s	99.94
	16,223	C	T	D-loop	99.65
	16,290	C	T		99.77
	16,319	G	A		99.71
	16,362	T	C		99.98
TB- 7	152	T	C		99.92
	247	G	-A		90.9
	302	A	AC		9.41

(continued on next page)

Table 1 (*continued*)

Patient ID	Position	Reference	Variant	Gene	VarFreq (%)
	489	T	C		99.91
	2352	T	C	16 s	99.93
	2706	A	G		99.95
	2780	C	T		99.84
	4363	T	C	tRNA-Gln	99.88
	16,223	C	T	D-loop	99.75
	16,260	C	T		99.89
	16,298	T	C		99.88
	16,519	T	C		99.84

*Represents the variants that are present only in patients and not in healthy controls.

two patients (TB-3 and TB-6), and MT-ND6 g. T > C14180 p.Y165C found in one patient (TB-2) (Table 2).

4. Discussion

This is the first study conducted for sequencing of the mitochondrial DNA in tuberculosis patients to understand whether the mtDNA alterations occur between cases and controls. The human mitochondrial proteome consists of an approximately more than 1000 distinct proteins, 13 of which are encoded by mtDNA [4] and such proteins can enter the nucleus and regulate the expression of nuclear DNA. The mitochondrial and nuclear genomes co-evolved to independently cross-regulate each other and to perform complex cellular functions [5]. Proteins in the mtDNA are structural components of the electron

Table 2
List of variants in the coding region of the mtDNA of the patients with their functional predictions.

Patient ID	Position	Reference	Variant	Gene	Amino acid change	VarFreq (%)	Polyphen-2	Provean
TB-1	3780*	C	T	ND1	Synonymous	99.87	–	–
	6179*	G	A	COX1		99.86		
	7853*	G	A	COX2	V90I	99.83	Benign	Neutral
	8701	A	G	ATP6	T59A	99.87		
	9469*	C	T	COX3	T88I	99.81		
	9540	T	C	COX3	Synonymous	99.92	–	–
	10,398	A	G	ND3	T114A	99.92	Benign	Neutral
	10,400	C	T	ND3	Synonymous	99.84	–	–
	12,705	C	T	ND5		99.91		
	13356*	T	C	ND5		99.94		
	14233*	A	G	ND6		99.93		
	14,783	T	C	CYTB		99.94		
	15,043	G	A			99.89		
	15,301	G	A			99.93		
	15,326	A	G		T194A	99.93	Benign	Neutral
TB-2	3999*	T	C	ND1	Synonymous	99.88	–	–
	4907*	T	C	ND2		99.92		
	5231*	G	A	ND2		99.79		
	8701	A	G	ATP6	T59A	99.95	Benign	Neutral
	10,398	A	G	ND3	T114A	99.89		
	10,400	C	T	ND3	Synonymous	99.77	–	–
	11176*	G	A	ND4		99.79		
	12,705	C	T	ND5		99.94		
	12876*	C	T			99.87		
	12,417	C	-A		Frameshift substitution	3.52		
	14,180	T	C	ND6	Y165C	99.93	Deleterious	
	14,783	T	C	CYTB	Synonymous	99.97	–	–
	15,043	G	A			99.87		
	15,301	G	A			99.8		
	15440*	T	C			99.92		
	15530*	T	C			99.88		
	15671*	A	G		M309V	99.95	Benign	Neutral
TB-3	4248	T	C	ND1	Synonymous	99.93	–	–
	4824	A	G	ND2	T119A	99.86	Deleterious	
	8794	C	T	ATP6	H90Y	99.9	Benign	Neutral
	10646*	G	A	ND4L	Synonymous	99.88	–	–
	11611*	G	A	ND4		99.83		
	12,417	C	-A	ND5		3.24		
	13,708	G	A	ND5	A458T	99.77	Benign	Neutral
	14569*	G	A	ND6	Synonymous	99.8	–	–
	15613*	A	G	CYTB		99.85		
TB-4	4715	A	G	ND2		99.82		
	4841	G	A	ND2		99.93		
	6752	A	G	COX1		99.94		
	7196	C	A	COX1		99.86		
	8584	G	A	ATP6	A20T	99.83	Benign	Neutral
	8598	T	C		Synonymous	99.92	–	–
	8701	A	G		T59A	99.93	Benign	Neutral
TB-4	8945*	T	C	ATP6	M140T	99.93		
	9090	T	C	ATP6	Synonymous	99.93	–	–
	9540	T	C	COX3		99.98		
	10,398	A	G	ND3	T114A	99.95	Benign	Neutral
	10,400	C	T	ND3	Synonymous	99.81	–	–
	10,490	T	C	ND4L		99.93		
	10,653	G	A	ND4L	A62T	99.91	Benign	Neutral
	12,417	C	-A	ND5	Synonymous	3.34	–	–

(*continued on next page*)

Table 2 (*continued*)

Patient ID	Position	Reference	Variant	Gene	Amino acid change	VarFreq (%)	Polyphen-2	Provean
	12,705	C	T	ND5		99.85		
	14,783	T	C	CYTB		99.95		
	15,043	G	A			99.94		
	15,301	G	A			99.9		
	15,487	A	T			99.56		
	15,784	T	C			99.95		
TB- 5	3970	C	T	ND1		99.81		
	6392	T	C	COX1		99.92		
	6599*	A	G			99.89		
	6962	G	A			99.89		
	9053*	G	A	ATP6	S176N	99.77	Benign	Neutral
	10,609	T	C	ND4L	M47T	99.94		
	12,406	G	A	ND5	V24I	99.97		
	12,882	C	T		Synonymous	99.89	–	–
	13759*	G	A		A475T	99.56	Benign	Neutral
	13,928	G	C		S531T	99.93		
TB- 6	4248	T	C	ND1	Synonymous	99.87	–	–
	4824	A	G	ND2	T119A	99.86	Deleterious	
	8794	C	T	ATP6	H90Y	99.84	Benign	Neural
	12,705	C	T	ND5	Synonymous	99.97	–	–
	15,478	A	G	CYTB		99.92		
TB- 7	4715	A	G	ND2		99.91		
	4841	G	A	ND2		99.89		
	7196	C	A	COX1		99.78		
	8584	G	A	ATP6	A20T	99.76	Benign	Neutral
	8701	A	G		T59A	99.98		
	8598	T	C		Synonymous	99.93	–	–
	9090	T	C			99.88		
	9540	T	C	COX3		99.91		
	10,398	A	G	ND3	T114A	99.95	Benign	Neutral
	10,400	C	T	ND3	Synonymous	99.84	–	–
	10,490	T	C	ND4L		99.93		
	10,653	G	A	ND4L	A62T	99.85	Benign	Neutral
	12,705	C	T	ND5	Synonymous	99.87	–	–
	14,783	T	C	CYTB		99.95		
	15,043	G	A			99.88		
	15,301	G	A			99.93		
	15,487	A	T			99.78		
	15,784	T	C			99.88		

Synonymous variants have been mentioned.
*Represents the variants that are present only in patients and not in healthy controls.

transport chain playing a critical role in oxidative phosphorylation (OXPHOS) [10]. MOTS-c is a peptide encoded by the mtDNA which localize to the nucleus and controls nuclear gene expression by binding to the chromatin in response to metabolic stress [13]. This communication between the mitochondria and the nucleus is of special interest, so in the present study, we analyzed the mtDNA sequence variants in the TB patients. Susceptibility to TB is also known to be both acquired and inherited [23].

In our study, the presence of variants in the non-coding region of the mtDNA genome in cases might suggest its participation in development of the disease. We could also identify gene variants in the coding region which were deleterious as predicted by the function prediction tools. The mutation in MT-ND2 gene (T119A) and MT-ND6 gene (Y165C) were predicted to be deleterious/ damaging in Polyphen-2 and Provean. These variants upon structural function prediction showed that it is close to a highly conserved position. The mutation in these residues will cause an empty space in the interior of the protein, breaking the hydrogen bonds and thus disturbing the correct folding of the protein. Mitochondria are known to be a target for *Mycobacterium tuberculosis*, which inhibits the functioning of the mitochondria in the cell promoting the survival of *Mtb* leading to a successful infection [7]. MT-ND2 and ND6 genes are the most important subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) which functions in the transfer of electrons from NADH to the respiratory chain (https://www.genecards.org).

Mitochondrial DNA mutation leads to defect in oxidative phosphorylation (OXPHOS) which has an important role in energy metabolism of the cell and electron transport. mtDNA mutation leads to metabolic, neurological and muscular disorders, thus acting as a cause for human diseases [22]. Mutation in MT-ND2 gene impairs mitochondrial Complex I assembly leading to Leigh syndrome [27] and MT-ND6 results in Leber's Hereditary Optic Neuropathy, Mitochondrial Encephalopathy and Leigh Syndrome. mtDNA mutations commonly occurs in different forms of cancers and may be functional in cancer detections [12]. Evidences suggest that mitochondrial reactive oxygen species (ROS) which are oxygen derived molecule capable of oxidising other molecules is important in some physiological systems including regulation of immunity. The oxidative burst of the ROS mediated by the NADPH oxidases in phagosomes leads to indirect killing of *Mtb* in case of TB infection, appearing to be important in antimicrobial responses [24,1]. Studies have also shown that various immune cells signal through the mitochondrial ROS like the Toll-like receptor (TLR) [30], cytokines like TNFα [21], IFNγ [25], interleukin 1β (IL-1β) and interleukin-18 (IL-18) [6]. These evidences suggests that mutation in MT-ND2 and ND6 gene, which is a subunit of complex 1, results in reduced formation of ROS resulting in higher chance of infection.

The other mitochondrial genes under study were not found to be directly associated with TB; yet, their analysis in other populations may still be fruitful given their functional importance in TB pathogenesis. To know the impact of these novel variants identified, we need to validate it using functional studies which would provide a basis for treatment of the disease and control TB burden.

5. Conclusion

Our study is the first to investigate the mtDNA variants in TB patients in the coding (synonymous or non-synonymous) and non-coding regions. Moreover, this study can serve as a baseline data to understand the genetic pre-disposition to TB infection. However, the study has several limitations since it relied on small number of sample size. Large population study is required to overcome the negative aspect of sample size and include all the variables associated with socio-demographic factors and the presence of the variants related to TB.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2020.06.012.

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ORIGINAL ARTICLE

Prevalence of High-Risk HPV Types in Women with Negative Cervical Cytology in a State of Northeast India with a High Burden of Cervical Cancer

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Abstract

Cervical cancer has emerged as a major threat among the tribal communities of Mizoram where literatures on the prevalence of HPV genotypes are scarce. This study aimed to determine the prevalence of HPV in different cytological grades of cervical samples in relation to lifestyle and socio-demographic factors. Sanger sequencing was performed to confirm the seven high-risk HPV genotypes and logistic regression analysis was performed. The most prevalent genotypes were HPV 16 (45%), HPV 31 (18%), HPV 18 (13%), HPV 33 (11%), HPV 35 (9%), HPV 52 and 58 (2%). Cytological reports showed three Cx-Ca, two HSIL, one LSIL and 29 NILM. Multiple infections of HPV 16, 18, 31 and 33 were present in the Cx-Ca samples, HPV 16, 31 and 33 in HSIL samples, HPV 16, 33 and 35 in the LSIL sample, HPV 16, 18, 31, 35 52 and 58 in 8 NILM and single infection of HPV 16 or 33 in 9 NILM samples. This is the first report of epidemiology and genotype correlation from the Mizo-Mongoloid population showing prevalence of HPV infections in different cytological grades. There is a need to screen HPV status in parallel to PAP smear for better management of timely interventions.

Keywords HPV genotypes · PAP smear · Mizoram · Lifestyle factors · Socio-demographic factors

Introduction

Cervical cancer (Cx-Ca) is the fourth leading cause of cancer-related deaths in women worldwide with an estimation of 604,000 new cases and 342,000 deaths in 2020 [1]. In India, Cx-Ca is also the fourth most common cancer with 96,922 new cases every year (http://cancerindia.org.in). As per the latest National Cancer Registry Programme, Cx-Ca is the leading cancer among women in Mizoram during 2012–2016 [2]. Mizoram is a hilly state located in the northeastern part of India, sharing borders with Bangladesh, Myanmar and three of the eight sister states of northeast India—Assam, Tripura and Manipur. It is a small state with a total population of around 10.97 lakhs as per 2011 census and the female population accounts for approx. 5.4 lakhs (https://www.census2011). Mizoram is believed to be a part of a wave of migration from China and settle in the northeast India in the course of history. The ethnicity, lifestyle and food habits are distinct from mainland India. In this state, fermented food and smoked food are consumed on a daily basis. More importantly, tobacco use has been observed among women populace in different forms such as smoking and chewing. Besides these, ignorance, lack of high-end equipment and research may contribute largely to increase in Cx-Ca rate for the past many years and Papanicolaou (PAP) smear remains the only means of detection of cervical dysplasia. All these factors along with other known associated factors to Cx-Ca

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with high-risk HPV status might play a role in the high incidence of Cx-Ca in the population [3].

Sexually transmitted human papillomavirus (HPV) infection is the essential risk factor for the development of cervical dysplasia and invasive Cx-Ca [4]. However, limited studies on HPV genotyping in parallel with PAP test have been reported so far from the state of Mizoram. Better insights of the genotypic divergence of HPV are essential for initiation of HPV vaccination program. In our study, we hypothesized that besides women with abnormal PAP report, women with normal PAP report might harbor high-risk HPV genotypes. Since there are neither documented data nor previous published literature from this region, we therefore attempted to find out the prevalence of high-risk HPV genotypes owing to the fact that the presence and persistence of high-risk HPV viral infection in the cervix can ultimately result in unfavorable outcomes in the long term. In the present study, our primary objective was to determine the HPV genotypes in normal as well as in different grades of abnormal cytology in the cervical samples and to find the correlation of HPV infection with lifestyle and socio-demographic factors in a less explored region.

Materials and Methods

Study Population, Specimen Collection and Storage

The study included unvaccinated married women aged between 25 and 62 years presenting with different cervical lesions ranging from normal to cervical cancer. During enrollment, all the women who participated in this study underwent pelvic examination. The cervix(s) that were suspected to be unhealthy upon visual inspection by the Gynecologist(s) were scraped for cytology (PAP test). Women with healthy looking cervix who presented at the Gynecology OPD for checkup were also scraped. Unmarried women as well as pregnant women were not included in this study. Samples were taken only from the patients who gave their consent to participate in the study and in total 35 cervical scrapping samples were obtained using Ayer's spatula in 5 ml 1X PBS. Samples were collected by Gynecologists from Synod Hospital, Durtlang and Ebenezer Hospital, Aizawl, Mizoram during January to June 2016. They were immediately stored at -20 degrees until further processing. PAP-stained specimens were screened by Pathologists and were classified according to the Bethesda system 2001.

Isolation of DNA, PCR and Sanger Sequencing

DNA was isolated from cervical scrapings using phenol:chloroform:isoamyl alcohol method and quantified using The Qubit^{TM} 2.0 Fluorometer (Invitrogen) with some modifications as previously described [5]. Conventional PCR was carried out in 25 µl total reaction volume. Samples were first subjected to β-globin amplification (268 bp), MY09/11 and GP5 + /6 + [6–8]. HPV positive samples were genotyped using selected high-risk types—HPV 16, 18, 31, 33, 52 and 58 which are constituent of the HPV 9-valent vaccine formulation (https://www.merckvaccines.com) and HPV 35. The primers used in this study were designed using Primer3 software and Oligo-analyzer (Suppl. Table 1). PCR amplification products were subjected to electrophoresis (Bio-Rad) on 1.5% agarose gel and sequencing using 3500 genetic analyzer automated sequencer (ABI) was performed at Department of Biotechnology, Mizoram University, Mizoram, India. Sequences were confirmed by BLAST for identification of HPV genotypes as well as aligned with CLUSTALW program with their respective prototype sequence from PaVE Database [9].

Statistical Analysis

The associations between lifestyle and socio-demographic factors with seven HR-HPV genotypes infection in different cervical cytology grades were analyzed. Odds ratios, 95% confidence interval and P values were computed by binary logistic regression using SPSS 20.0 (SPSS Iberica, Madrid and Spain). P value < 0.05 was considered statistically significant.

Results

Out of 35 cervical samples analyzed, three samples were cytologically confirmed as cervical carcinoma (Cx-Ca), two as high-grade squamous intraepithelial lesions (HSIL), one as low-grade squamous intraepithelial lesions (LSIL) and the remaining 29 samples were negative for intraepithelial lesions or malignancy (NILM). Our patients comprised of age between 25 and 62 years, with the median age of 41 years. The overall HPV genotype among the afore-mentioned 35 samples with different cervical lesions is shown in Fig. 1. Multiple infections were found in 14 samples, single infection in 9 samples and 12 samples had no infection. The most prevalent HPV genotypes in our study were HPV 16 found in 20 (45%) samples followed by HPV 31 detected in 8 samples (18%). HPV 18 was found in 6 samples (13%), HPV 33 in 5 samples (11%), HPV 35 in 4

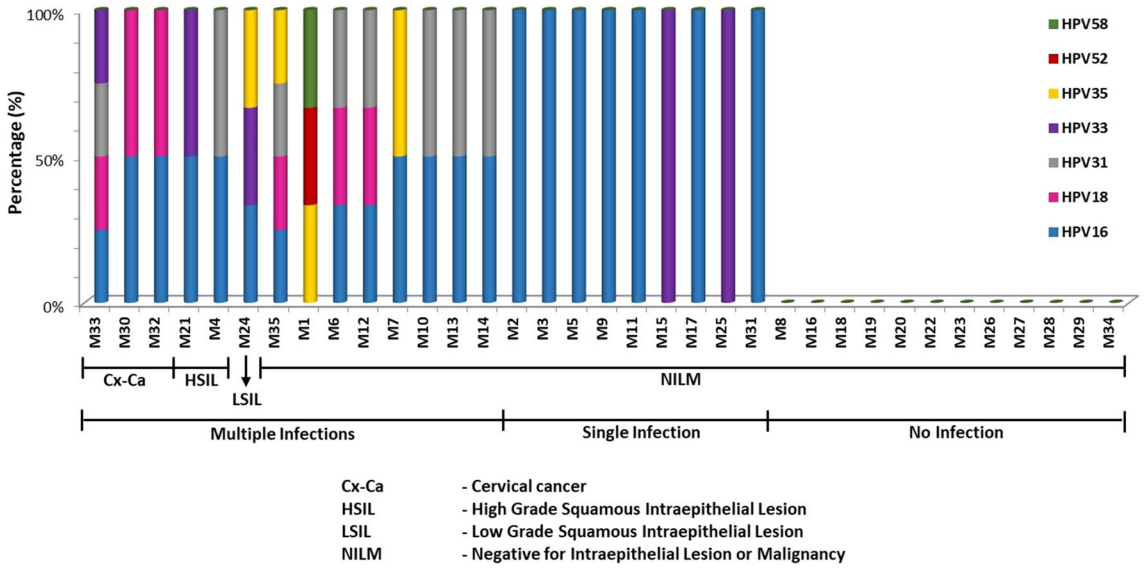

Fig. 1 Prevalence of HPV genotypes in the different cytological grades among the samples

samples (9%) and HPV 52 and 58 were found in single samples, respectively (Fig. 2).

HPV types 16 and 18 were detected in all the Cx-Ca samples (M33, M30 and M32). Multiple infections of HPV types 16, 18, 31 and 33 were detected in one of the Cx-Ca samples (M33). The distribution pattern of HPV types in the HSIL samples is HPV 16 and 33 (M21), HPV 16 and 31 (M4). In the LSIL sample (M24), three HPV types 16, 33 and 35 were found. We also observed multiple infections in 8 NILM samples: four HPV types 16, 18, 31, 35 (M35), three HPV types 35, 52 and 58 (M1), HPV genotypes 16, 18 and 31 (M6 and M12), two HPV types 16 and 31 (M10, M13 and M14) and HPV 16 and 35 (M7). In 9 NILM samples, a single infection of HPV 16 (M2, M3, M5, M9, M11, M17, M31) and HPV 33 (M15, M25) was detected (Fig. 1).

Regression analysis of lifestyle and socio-demographic factors with seven high-risk HPV tested showed that tobacco chewing had a high association with an OR of 2.857, however, nonsignificant ($p > 0.05$). Other factors with an OR of > 1 are age at marriage, > 2 times pregnancies, patients with miscarriage history, age at menarche at 14–16 years, patients having more than one sexual partner, use of contraceptive pills, patients who do not use contraceptive barriers, patients who have not done PAP test previously and tobacco smoking. However, these factors were not significant ($p > 0.05$) (Table 1).

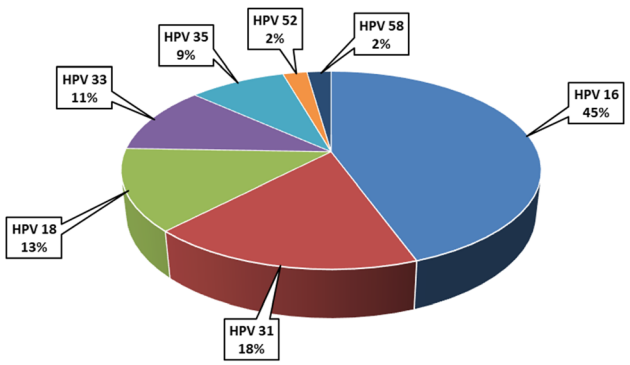

Fig. 2 Frequency of HPV genotypes in the patients' samples

Discussion

To our knowledge, this is the first study to assess the association between HPV genotypes and epidemiological factors among Mizo-Mongoloid population in northeast India. Within this study group, HPV 16 (45%), 31 (18%) and 18 (13%) were found to be predominant among the seven high-risk HPV genotypes screened (Fig. 2). Similar trend of genotype prevalence was also reported in East Asian countries (Japan, Mongolia and South Korea) [10]. In India, in a community-based multicentric cohort study comprising of 1484 women, HPV 16 and HPV 31 were the most common type observed [11]. Having a porous international border, the same pattern of genotype prevalence from Myanmar such as HPV16 (60.4%) followed by HPV31 (14.6%), HPV18 (12.5%) was observed in Mizoram [12]. Our report showed that HPV 16 was present in 14 out of the 29 the NILM samples. Others have also reported the prevalence of HPV 16 in NILM samples [13]. Moreover, consistent with our studies, multiple HPV infections have also been found in different cytological reports—LSIL, HSIL and NILM with high viral load [14]. The same trend was also observed in northeastern and northern India

Table 1 Regression analysis of seven high-risk HPV genotypes with lifestyle and socio-demographic factors

Factors	Variables	High-risk HPV genotypes		Total	Odds ratio	*p* Value	95% confidence interval
		Absent	Present				
Age at marriage	> 25	3	6	9	Reference		
	20–24 years	3	9	12	1.5	0.677	0.223–10.077
	< 20 years	5	9	14	0.9	0.907	0.154–5.258
Age at first pregnancy	> 25	2	8	10	Reference		
	20–24 years	5	10	15	0.5	0.471	0.076–3.293
	< 20 years	3	6	9	0.5	0.513	0.063–3.998
Total number of pregnancies	≤ 2	2	4	6	Reference		
	> 2	8	20	28	1.25	0.817	0.190–8.233
Total number of children	≤ 2 Children	2	8	10	Reference		
	3–4 Children	5	13	18	0.65	0.65	0.101–4.181
	> 4 Children	3	3	6	0.25	0.223	0.027–2.319
Place of delivery	Hospital	6	19	25	Reference		
	Hospital + Home	4	4	8	0.316	0.174	0.060–1.668
	Home	1	1	2	0.316	0.439	0.017–5.854
History of miscarriage	No	8	16	24	Reference		
	Yes	3	8	11	1.333	0.72	0.276–6.442
History of abortion	No	10	23	33	Reference		
	Yes	1	1	2	0.435	0.569	0.025–7.665
Regularity of menstrual cycle	Sometimes irregular	1	7	8	Reference		
	Regular	10	17	27	0.243	0.215	0.026–2.272
Age at menarche	11–13 years	3	7	10	Reference		
	14–16 years	6	16	22	1.143	0.874	0.220–5.928
	> 16 years	2	1	3	0.214	0.273	0.014–3.370
Sanitary pads	Self-made + Commercial	3	5	8	Reference		
	Commercial	8	19	27	1.425	0.674	0.273–7.439
Age at first intercourse	< 30 years	4	9	13	Reference		
	< 20 years	7	14	21	0.889	0.877	0.201–3.931
Total number of sexual partners	1 partner	9	18	27	Reference		
	> 1 partner	2	6	8	1.5	0.357	0.251–8.977
Contraceptive use of pills	No	8	15	23	Reference		
	Yes	3	9	12	1.6	0.556	0.335–7.639
Contraceptive barriers	Yes	8	15	23	Reference		
	No	3	9	12	1.6	0.556	0.335–7.639
History of UTI	No	1	4	5	Reference		
	Yes	10	20	30	0.5	0.558	0.049–5.083
Recurrence of UTI	No	7	21	28	Reference		
	Yes	4	3	7	0.25	0.115	0.045–1.402
Partners suffering from UTI	No	8	20	28	Reference		
	Yes	3	4	7	0.533	0.47	0.097–2.939
Knowledge of PAP test	Yes	6	14	20	Reference		
	No	5	10	15	0.857	0.834	0.204–3.610
PAP test done	Yes	7	15	22	Reference		
	No	4	9	13	1.05	0.949	0.239–4.615
Frequency of PAP test	≥ 3 times	2	4	6	Reference		
	2 times	1	4	5	2	0.624	0.125–31.975
	1 time	3	6	9	1	1	0.112–8.947

Table 1 (continued)

Factors	Variables	High-risk HPV genotypes		Total	Odds ratio	*p* Value	95% confidence interval
		Absent	Present				
	Never	5	10	15	1	1	0.134–7.451
Tobacco smoking	No	8	17	25	Reference		
	Yes	3	7	10	1.098	0.908	0.223–5.397
Tobacco chewing	No	4	4	8	Reference		
	Yes	7	20	27	2.857	0.207	0.559–14.603
Betel nut chewing	No	2	3	5	Reference		
	Yes	9	21	30	1.556	0.657	0.221–10.957

[15]. The presence of such multiple infections of high-risk genotypes even in NILM samples denotes a constantly associated factor for an increased risk of cervical disease severity [16].

Studies have shown that tobacco use (smoking or chewing) is associated with HPV prevalence [17, 18], incidence [19] and persistence [20]. Tobacco use has been a well-established risk factor for cervical cancer in the presence of HPV infection [21]. Tobacco use/smoking may increase viral load by weakening the cellular immune response which is supported by studies showing that smoking has adverse effects on both systemic and local immunity [22, 23]. Another possible mechanism for the association between cigarette smoking and HPV DNA load is that smoking may increase cell proliferation and turnover in the transformation zone of the cervix [24]. However, progression to cancer can take up to 10 to 15 years [25]. Given that 82% of our datasets are cytologically reported as NILM, we believe that use of tobacco might increase the risk of HPV multiple infections among the normal cytology. The Global Adult Tobacco Survey (2016–2017) reported that out of the 33.4% adult smokers in Mizoram, 14.3% were women [26]. This is an important factor to consider for future awareness in the population.

Early age at marriage or intercourse or first pregnancy has been reported to increase the risk of HPV infections in Indian women [27, 28]. Multiple sexually transmitted diseases (STDs) including HPV were found to be associated with early age at first intercourse [29]. HPV infections are likely to occur after the first sexual intercourse [30], on that note, early age at first intercourse/pregnancies may prolong the exposure to HPV infections. Notably, HPV prevalence increases as the pregnancy advances; however, it is still unclear whether HPV infections can cause miscarriage and vice versa [31]. With respect to contraceptive barriers, there is no strong evidence suggesting a valid association with the presence of HPV infection [32]. We also observed that more than half of the study participants did not undergo previous PAP test which is an associated risk factor in this population. Majority of the women in this study were not aware of HPV vaccine; thus, none of them have taken up which could be another important risk factor.

Globally, HPV infection has been a well-established risk factor or driver for cervical carcinoma as well as head and neck squamous cell carcinoma [33, 34]. HPV infection alone is cleared by the immune system; however, persistent infection in combination with lifestyle and socio-demographic risk factors enhances the infection to progress to cervical cancer [3, 35].

Conclusion

This is the first study showing the prevalence of high-risk HPV infections among Mizo-Mongoloid population. High-risk HPV infections were observed in all the cytological stages. High percentage (58.6%) of the NILM samples was positive for HPV infections (17/29), and of these, 27.5% had multiple HPV infections (8/29) which highlights the need for timely vaccination covering all the high-risk genotypes among the young populace. The synergistic effect of lifestyle risk factors, in the presence of high-risk HPV infection, might make the population prone to progression of cervical cancer in the long term which is not an unusual phenomenon. Hence, screening to identify early pre-cancers with HPV detection, cytology as well as colposcopy, initiation of awareness on the synergistic action of tobacco as well as early coitarche is highly imperative in the state. In view of the prevalence of infections other than HPV 16 and 18, there is a need for the newer vaccines covering the seven high-risk genotypes. Our findings might serve as baseline data which might contribute to better

prophylaxis, early diagnosis and provide timely interventions. Since our study relied on small number of samples, further studies with larger number of samples may be of significance to support these findings.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40944-022-00610-7.

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Author's Contributions CVS, LN, LP, KL, EZ and NSK contributed to formulating the questionnaire and study; LN, CVS, LP and KL contributed to the collection of samples and data; CVS, SG and ZZ performed the experiments; CVS, SG and ZS analyzed the data; CVS, SG, ZZ, ZS and NSK contributed to inferring the data and writing the manuscript.

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Data Availability The datasets generated and/or analyzed during the current study are not publicly available due to the fact that we do not wish to disclose our dataset since they are part of patients' medical history. However, they are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethics Statement Ethical approval was obtained from Institutional Review Board (IRB), Civil Hospital, Aizawl (B.12018/1/13-CH(A)/IEC/41) and Mizoram University (MZU/IHEC/2015/007), India. All the experiments were performed in accordance with relevant guidelines and regulations. Attested informed consent for study participation was obtained for this study. All authors read and approved the final manuscript.

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Pathogenic microbes contaminating mobile phones in hospital environment in Northeast India: incidence and antibiotic resistance

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Abstract

Background: The present study attempts to identify and determine the pattern of drug susceptibility of the microorganisms present in mobile phones of health care workers (HCWs) and non-HCWs in a hospital environment. Mobile phones of 100 participants including both genders were randomly swabbed from nine different wards/units and the bacterial cultures were characterized using VITEK 2 system.

Results: Forty-seven mobile phones were culture positive and a total of 57 isolates were obtained which consisted of 28 Gram-positive organisms and 29 Gram-negative organisms. The predominating organisms were *Acinetobacter baumannii* and *Staphylococcus hominis*. Among all the isolates from the mobile phones of HCW and non-HCWs, five isolates had ESBL and three isolates had colistin resistance. Incidentally, MRSA was not found on the mobile phones tested. The isolated organisms showed 100% susceptibility to linezolid, daptomycin, vancomycin, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin and tigecycline, while high resistance was shown against benzylpenicillin (75.0%), cefuroxime and cefuroxime axetil (56.5%). Non-HCWs' mobile phones were more contaminated as compared to HCWs ($P = 0.001$) and irrespective of individuals' gender or toilet habits, both Gram-positive and Gram-negative organisms were present on the mobile phones.

Conclusion: This study reports for the first time that the mobile phones of non-health care workers harbour more bacterial diversity and are more prone to cause transmission of pathogens. This study can serve to educate the public on personal hand hygiene practices and on maintaining clean mobile phones through antiseptic measures.

Keywords: Mobile phones, Microorganisms, Acinetobacter, Toilet, Healthcare workers, VITEK 2, Mizoram

Background

Mobile phones have become one of the most essential accessories of day-to-day life with recent advances in information sharing and social media applications [1]. The mobile phones contaminated with nosocomial pathogens is a potential mechanical vector for transferring pathogens with multidrug-resistant bacteria, especially extended-spectrum beta-lactamase (ESBL) across various wards in hospital settings [2]. During our daily activities, our hands come in contact with many surfaces containing germs and parallelly handle the phone. Over the course of time, these germs gradually accumulate on our phones and thus, phone and hand hygiene play a very crucial role in our health system. Most of the disease-causing bacteria are transferred from person to person through direct contact and fomites [3].

This study was conducted in Mizoram, which is a landlocked state situated in the north-east of India, with unique lifestyle and food habits. The people of this state are known for their regular consumption of boiled and fermented foods and there are strong evidences of the impact of fermented foods on the digestive health such

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as dysbiosis and well-being [4]. The effects of certain lifestyle and food habits influence the gut microbiota which in turn can inhabit the mobile phones of their owners [5]. This study aims to survey the diversity of culturable microorganisms present on mobile phones which can vary owing to differences in hand flora, diet, occupation, habits and health awareness.

The key questions addressed in this study are as follows: (1) What is the contamination rate of mobile phones in a hospital environment and what are the predominant pathogens present? (2) Which are the most resistant microorganisms and what are their antibiotic resistance profiles? (3) What are the most important factors responsible for mobile contamination? In this study, identification and pattern of drug susceptibility of microorganisms isolated from mobile phones of both health care workers (HCWs) and non-health care workers (non-HCWs) in a hospital environment was carried out.

Results

Of the total 100 mobile phones swabbed, 47 samples showed positive growth (47%)—from which 57 bacterial isolates were identified (28 Gram-positive organisms and 29 Gram-negative bacilli). Twenty-one mobile phones (44.7%) were culture positive for Gram-positive organisms alone (including single and multiple organisms), while 23 mobile phones (48.9%) were culture positive for Gram-negative bacilli (GNB) alone (including single and multiple organisms) and 3 mobile phones (6.4%) had mixed growth of both Gram-positive and negative organisms (Tables 3 and 4).

Antimicrobial susceptibility testing (AST)

The AST of Gram-positive cocci (GPC) and GNB from both HCWs and non-HCWs are shown in Tables 3 and 4. Among the GNBs, out of five ESBL isolates (sample code MP-25, MP-26, MP-71, MP-80, MP-92), four were *Enterobacter cloacae* (one patient attendant from the Gynaecology ward, two patient attendants from the Medical ward and one patient from the Orthopaedic ward) and one was *E. coli* (female ward attendant from the Surgical ward). It is noteworthy that in the total three *E. coli* strains isolated, two were non-ESBL (sample code MP-60, MP-82) from non-HCWs belonging to the Gynaecology ward and Surgical ward, while one ESBL strain (sample code MP-80) was from HCW of the Surgical ward. We also identified two strains of *Klebsiella pneumoniae* which were non-ESBL producers (Table 3).

Among the GNB, colistin resistance was shown by three isolates—*E. coli* (HCW, Surgical ward, sample code MP-80), *Moraxella* group and *S. paucimobilis* (non-HCWs, Gynaecology ward, sample codes MP-67 and MP-70). Intermediate drug resistance was shown by the *Moraxella* group against ceftriaxone and ampicillin as well as by *S. paucimobilis* against cefuroxime axitel and cefepime. These organisms were isolated from non-HCWs belonging to the Gynaecology ward. In GPC, intermediate resistance was shown against erythromycin by *S. cohnii* (non-HCW, Medical ward) and *S. xylosus* (HCW, k-ward) and against teicoplanin by *S. hominis* (HCW, ENT ward). Of the 29 GNB isolated, six isolates (*Pantoea* spp., *Oligella ureolytica*, *Klebsiella pneumoniae*, *Aeromonas salmonicida*, and two strains of *Acinetobacter lwoffii*) could not produce AST results when tested by VITEK 2 and therefore are not included in AST.

In this study, we observed that the most resistant GNB was *Enterobacter cloacae* (ESBL) isolated from non-HCW belonging to the Medical ward (sample code MP-26). This isolate showed resistance to 10 antibiotics (55.55%) out of the 18 antibiotics tested. On the contrary, the most susceptible GNB was *Sphingomonas paucimobilis* isolated from a HCW belonging to Microbiology laboratory (sample code MP-4). This isolate showed susceptibility to all the 18 antibiotics tested (100%) (Table 3). Conversely, the most resistant GPC was *S. haemolyticus* isolated from non-HCW belonging to the Medical ward (sample code MP-23). This isolate showed resistance to 10 antibiotics (62.5%) out of the 16 antibiotics tested. The most susceptible (100%) Gram-positive cocci were *S. sciuri* (isolated from HCW, ENT ward, sample code MP-39), *S. warneri* (isolated from non-HCW, ENT ward, sample code MP-31) and *S. hominis* (isolated from non-HCW, Gynae ward, sample code MP-69). These isolates showed susceptibility against all the antibiotics tested (Table 4).

Among the GPC, there were eight isolates of coagulase-negative *Staphylococcus* (CoNS) that had shown resistance to beta-lactams as well as modification of penicillin-binding protein (mecA). These included four strains of *S. haemolyticus* (sample codes MP-23, MP-24, MP-29, MP-35) which were from non-HCWs (Medical ward and ENT ward), three strains of *S. hominis* (sample codes MP-39, MP-47, MP-72) which were from HCWs (ENT ward) and non-HCWs (Gynaecology ward) and one *S. epidermidis* (sample code MP-85) from non-HCW (Surgical ward).

Single/multiple and mixed growth with respect to wards/units

Eight wards/units had either single/multiple and/or mixed growth of both Gram-positive and Gram-negative organisms. Interestingly, in the Orthopaedic ward, all the isolates obtained were GNBs. Mobile phones swabbed from ENT (72.7%) and Gynaecology (56.2%) wards were more contaminated as compared with other wards/units (Table 1).

Bacterial isolates from mobile phones in relation to different parameters

An informed verbal consent was obtained and a set of questionnaire was asked to all the participants pertaining

Table 1 Bacterial growth from mobile phones belonging to different wards/units

Wards/units	No. of samples	No. of growth (%)	Total no.			Organisms isolated
			Isolates	GNB's	GPC's/GPB	
Microbiology unit	05	02 (40.00)	02	01	01	1 *Sphingomonas paucimobilis*, 1 *Staphylococcus aureus* (MSSA)
Blood bank unit	04	01 (25.00)	01	00	01	1 *Staphylococcus cohnii*
Pathology unit	09	01 (11.11)	01	00	01	1 *Staphylococcus arlettae*
Medical ward	11	06 (54.54)	07	02	05	3 *Staphylococcus hemolyticus*, 2 *Enterobacter cloacae*, 1 *Staphylococcus cohnii* and 1 *Alloiococcus otitis*
ENT ward	11	08 (72.72)	13	03	10	2 *Staphylococcus warneri*, 2 *Staphylococcus hominis*, *1 Staphylococcus epidermidis*, 2 *Staphylococcus saprophyticus*, 1 *Acinetobacter lwoffii*, 1 *Pantoea* spp., 1 *Kocuria kristinae*, 1 *Acinetobacter hemolyticus*, 1 *Staphylococcus sciuri* and 1 *Staphylococcus hemolyticus*
K ward	04	01 (25.00)	01	00	01	1 *Staphylococcus xylosus*
Gynaecology ward	32	18 (56.25)	20	13	07	4 *Acinetobacter baumanii*, 1 *Enterobacter cloacae*, 4 *Staphylococcus hominis*, 2 *Acinetobacter lwoffii*, 2 *Moraxella group*, 1 *Sphingomonas paucimobilis*, 1 *Staphylococcus aureus*, 1 *Pantoea* spp., 1 *Staphylococcus epidermidis*, 1 *Klebsiella pneumoniae*, 1 *Erysipelothrix rhushiopathiae* and 1 *E.coli*
Surgical ward	15	05 (33.33)	07	05	02	2 *Escheriachia coli*, 1 *Acinetobacter baumanii*, 1 *Staphylococcus epidermidis*, 1 *Klebsiella pneumoniae*, 1 *Enterococcus* spp. and 1 *Oligella ureolytica*
Orthopaedic ward	09	05 (55.55)	05	05	00	2 *Acinetobacter lwoffii*, 1 *Acinetobacter baumanii*, 1 *Enterobacter cloacae* and 1 *Aeromonas salmonicida*

GNB Gram-negative bacilli, *GPC* Gram-positive cocci
K ward substance abuse ward, *ENT* ear, nose and throat ward

to gender, occupation [HCWs, non-HCWs (patients and patient attendants), their associated hospital wards/units, habit of cleaning their mobile phones and of carrying their phones to toilet (Table 2).

Out of 100 participants, 32 were males and 15 (46.9%) of their mobile phones were culture positive, whereas 68 were females and 30 (44.1%) of their mobile phones were culture positive (p value = 0.680; OR = 1.194; CI = 0.515–2.769). With respect to occupation, out of the 100 participants, 66 were non-HCWs and 34 were HCWs. The bacterial contamination rate of mobile phones among the non-HCWs was 59.1% and HCWs was 23.5%. We observed that non-HCWs mobile phones had more contamination (p = 0.001; OR = 4.694; CI = 1.848–11.922) as compared to HCWs (Table 2). With respect to occupation, the type of organisms isolated revealed that non-HCWs phones were more contaminated with *Acinetobacter baumannii* (n = 5) and *Acinetobacter lwoffii* (n = 5). On the contrary, there were no predominating organisms in the mobile phones of HCWs (Figs. 1 and 2).

With respect to the cleanliness of mobile phones, 23 participants had cleaned (without antiseptics) and 77 participants never cleaned their mobile phones (Table 2). Among these 23 participants, 10 samples were culture positive (43.4%) and maximum growth was contributed by *Enterobacter cloacae*, *Acinetobacter lwoffii* and *S. hominis*. The remaining were *Sphingomonas paucimobilis*, *S. cohnii*, *S. haemolyticus*, *S. warneri*, *Acinetobacter baumannii*, *Moraxella* group, *S. aureus* (MSSA) and *A. haemolyticus* (Fig. 3).

From the 77 uncleaned mobile phones, a total of 24 organisms were isolated (31.16%) (Table 2). *Acinetobacter baumannii* was abundant, followed by *S. hominis*, *S. haemolyticus*, *Acinetobacter lwoffii*, *S. epidermidis* and *E. coli*. Apart from the common organisms that grew both in the cleaned and uncleaned mobile phones, the uncleaned phones had additional growth of organisms such as *Klebsiella pneunoniae*, *S. epidermidis*, *S. saprophyticus*, *S. arlettae*, *S. sciuri*, *S. xylosus*, *Enterococcus* spp., *Alloiococcus otitis*, *Kocuria kristinae*, *Pantoea* spp., *E. coli*, *Erysipelothrix rhusiopathiae*, *Oligella ureolytica* and *Aeromonas salmonicida* (Fig. 3). The contamination rate was significantly higher in the uncleaned phones compared to the cleaned ones (statistical findings from Table 2).

Eighty-two (82) individuals used to carry their mobile phones inside the toilet, out of which 39 mobile phones (47.5%) were culture positive yielding 46 isolates (Table 2). In total, these isolates contained 25 types of organisms with a dominance of *S. hominis* (Fig. 4). Nine (50%) out of 18 mobile phones that were never carried inside the toilet were culture positive and from them, 11 isolates (containing 9 types of microbes) were identified with a dominance of *Acinetobacter baumannii* (Table 2, Fig. 4). There was no statistical significance in the number of microbial growth between the mobile phones that were and were not carried inside the toilet (p = 0.700) (Table 2).

Table 2 Relationship between bacterial isolate numbers and different parameters

No. of bacterial isolates						
Factors	No growth No. (%)	Growth per mobile phone[a] No. (%)	Total no. of isolates[b] No. (%)	*p* value	OR	CI
Gender						
Male	17 (53.12)	15 (46.88)	19 (33.33)	0.680	1.194	0.515–2.769
Female	38 (55.88)	30 (44.12)	38 (66.67)			
Occupation						
HCW	26 (76.47)	08 (23.53)	10 (17.54)	0.001	4.694	1.848–11.922
Non-HCW	27 (40.90)	39 (59.10)	47 (82.46)			
Mobile cleaning						
Yes	13 (56.52)	10 (43.48)	15 (26.32)	Reference	3.756	1.240–11.373
Never	39 (50.65)	38 (49.35)	42 (73.68)	0.019		
Use of mobile in the toilet						
Never	09 (50.00)	09 (50.00)	11 (19.30)	Reference	1.202	0.471–3.072
Yes	43 (52.44)	39 (47.56)	46 (80.70)	0.700		
			Wards/Units			
Surgical ward	10 (66.67)	05 (33.33)	07 (12.28)	Reference		
Pathology unit	08 (88.89)	01 (11.11)	01 (1.75)	0.246	0.250	0.024–2.594
Microbiology unit	03 (60.00)	02 (40.00)	02 (3.51)	0.787	1.333	0.165–10.743
Blood bank unit	03 (75.00)	01 (25.00)	01 (1.75)	0.751	0.667	0.054–8.161
Medical ward	05 (45.45)	06 (54.55)	07 (12.28)	0.284	2.400	0.484–11.891
ENT ward	03 (27.27)	08 (72.73)	13 (22.81)	0.055	5.333	0.968–29.393
K ward	03 (75.00)	01 (25.00)	01 (1.75)	0.751	0.667	0.054–8.161
Gynaecology ward	14 (43.75)	18 (56.25)	20 (35.10)	0.148	2.571	0.714–9.255
Orthopaedic ward	04 (44.44)	05 (55.56)	05 (8.77)	0.290	2.500	0.458–13.649

References represent the variable against which the tested factors were compared
OR odds ratio, is a measure of association between an exposure and an outcome; *CI* confidence interval, indicates a measurement precision. Narrow CI indicates high precision; Wide CI indicates low precision; *p* value, the probability of finding the observed results when the null hypothesis is true (indicates significance < 0.05); *ENT* ears, nose and throat; *K ward* substance abuse ward
[a]Growth observed per individual's mobile phone
[b]Total no. of bacterial isolates from each individual's mobile phone (includes > 1 organism per mobile phone)

Discussion

Nowadays, mobile phones are being owned and widely used everywhere by almost every individual irrespective of age, socio-economic status and educational level. It, therefore, has become an easy source of microorganism transmission. In the present study, 47% of the mobile phones were contaminated, whereas earlier studies had shown mobile phone contamination up to 94.5% and 30% [6, 7]. This variation could be attributed to knowledge of hand hygiene and sanitary practices and with rapid dissemination of awareness and precautions. This study proved that the mobile phones harbour both Gram-positive organisms (n = 28, i.e., 49.1%) and Gram-negative bacilli (n = 29, i.e., 50.9%) irrespective of belonging to HCWs or non-HCWs. Though this figure is not significantly high, more number of GNB was isolated. This is not in agreement with other studies where Gram-positive organisms (80%) were abundant rather than Gram-negative organisms (20%) [8, 9].

For GNB, the antibiotic resistance and susceptibility pattern among microbes isolated from mobile phones were almost similar to other previous studies [10], except for ampicillin (34.78%), piperacillin/tazobactam (4.35%) and cefuroxime axetil (56.52%) (Table 3). This low susceptibility to cefuroxime axetil can be attributed to the fact that it falls under the commercially available second generation of cephalosporins which were once widely used as therapy. Cefuroxime axetil was active against a wide range of organisms and its use decreased later due to the acquisition of resistance which led to the use of broader classes of cephalosporins like third, fourth and fifth generations [11].

In concordance with previous studies, GNB had 100% susceptibility to imipenem, meropenem, amikacin and

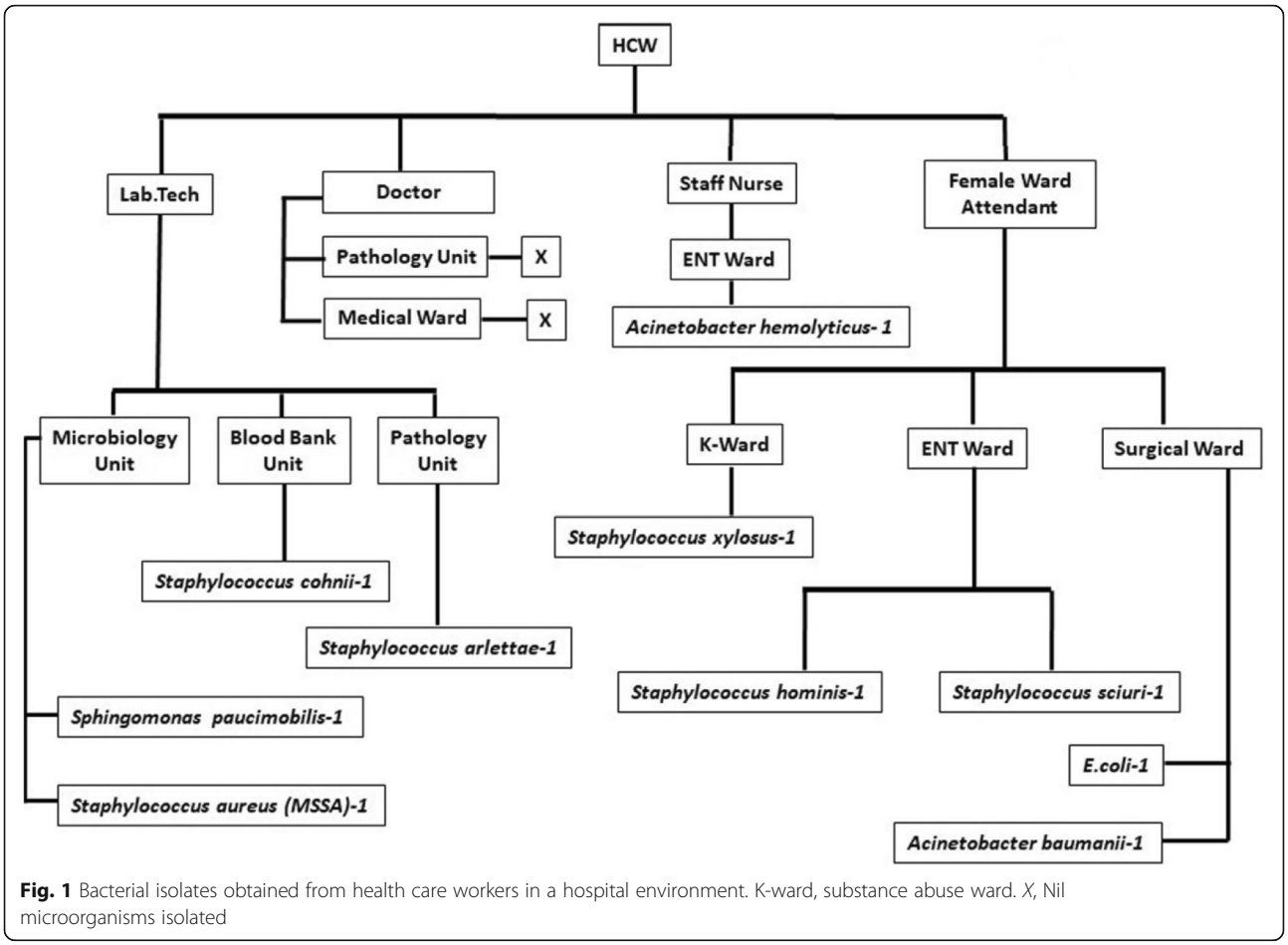

Fig. 1 Bacterial isolates obtained from health care workers in a hospital environment. K-ward, substance abuse ward. *X*, Nil microorganisms isolated

ciprofloxacin [12]. This can be attributed to the fact that the carbapenems such as imipenem and meropenem are reserved drugs which are seldom used, unless needed, and hence, GNB showed good susceptibility (100%) towards those drugs. This is in accordance with other previous studies [13]. It is noteworthy to mention that mobile phones of patient attendants (non-HCWs) and female ward attendants (HCWs) harboured extremely important nosocomial pathogens which were extended spectrum beta-lactamase (ESBL) microorganisms such as *Enterobacter cloacae* and *E. coli*. Such individuals can act as a source of ESBL transmission from patient-to-patient, patient-to-healthcare workers and patient/ward attendants to patients during hospitalization [14, 15]. In our study, of the three isolated *E. coli* strains, two were non-ESBL producers and one was ESBL producer. In addition, two *Klebsiella pneumoniae* strains isolated from patient attendants of surgery and Gynae wards were non-ESBL producer. That *E. coli* and *Klebsiella* can be both ESBL producer and non-ESBL producer is supported by a previous study [16].

Three colistin resistance strains were identified among all the GNB isolates obtained. These are *E.coli* (HCW, Surgical ward), *S. paucimobilis* and *Moraxella* group (non-HCWs, Gynaecology ward). However, only *E. coli* was flagged as polypeptides resistant by VITEK 2 system. Since colistin is a last resort drug and the associated resistance gene is transmissible to other drug naïve bacterial species as well as pathogens, carriage of the resistance gene to pathogens harbouring other resistance genes can give rise to super drug-resistant bacteria [17]. In this study, the presence of a colistin-resistant *E. coli* strain on a HCW signifies the alarming chances of it being transmitted across various wards which could eventually lead to a great hazard in clinical practice.

CoNS are well known opportunistic pathogens in hospital settings that result in high-end infections since they have the ability to adhere and invade epithelial cell lines, like HeLa [18] and form biofilms in prosthetic devices ultimately resulting in drug resistance [19, 20]. In this study, overall, 28 Gram-positive isolates were identified of which 22 were contributed by CoNS. It is not clear to us as to what extent these CoNS would have infected patients from this hospital.

As an antibiotic, benzylpenicillin is noted to possess efficacy against a wide variety of infections [10]. In the

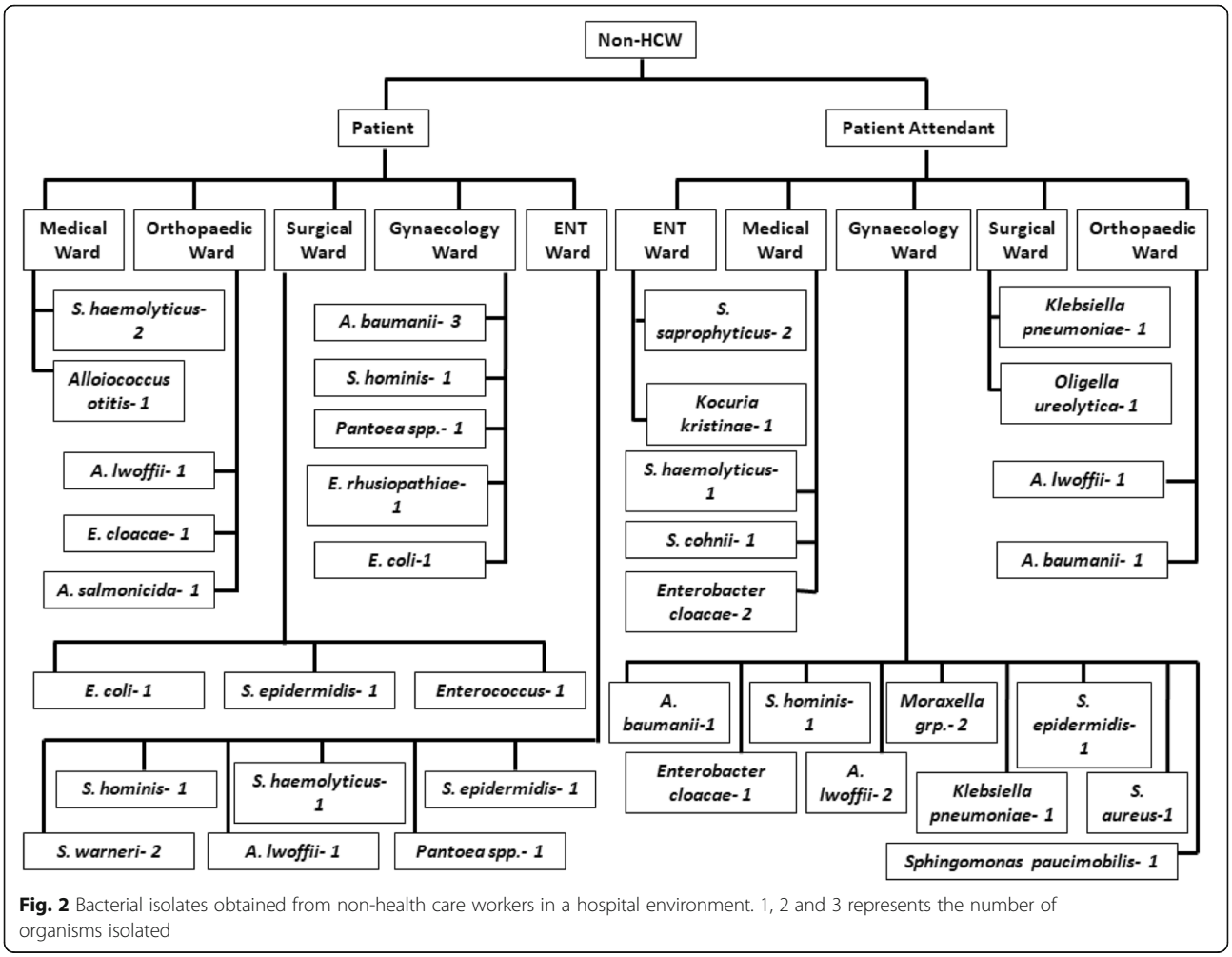

Fig. 2 Bacterial isolates obtained from non-health care workers in a hospital environment. 1, 2 and 3 represents the number of organisms isolated

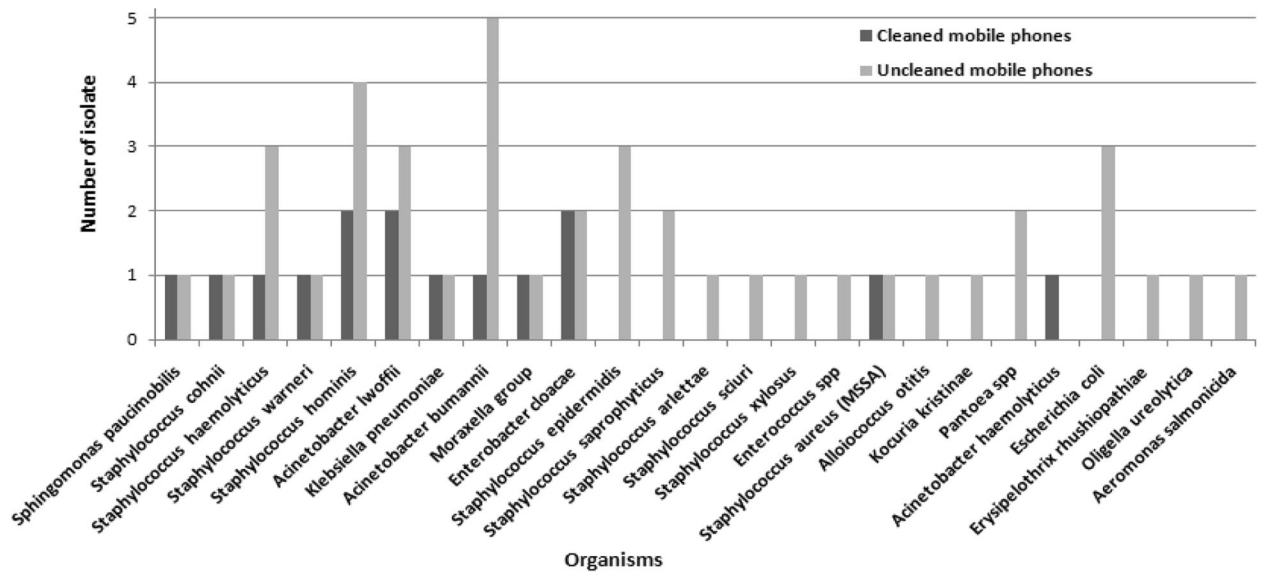

Fig. 3 Bacterial isolates isolated from mobile phones that were and were not cleaned. This figure shows that more number of microorganisms are present on uncleaned mobile phones

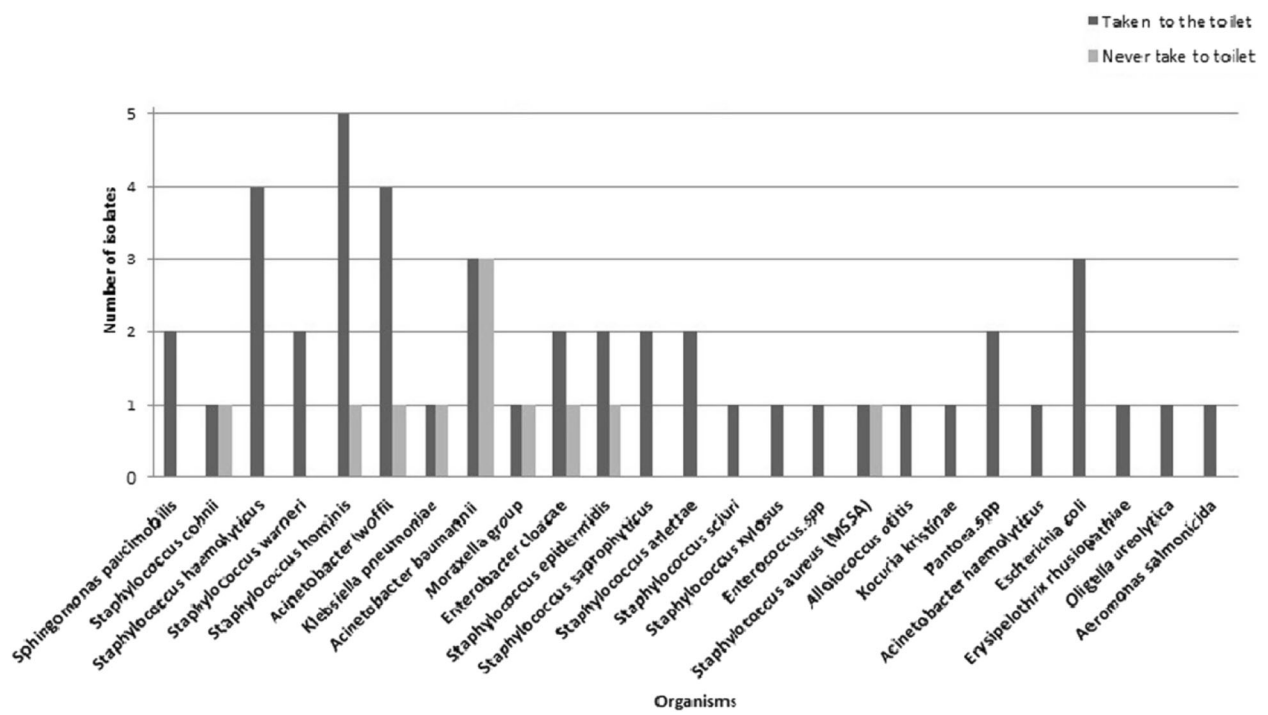

Fig. 4 Bacterial isolates from mobile phones that were and were not taken inside the toilet. This figure shows that more number of microorganisms are present on mobile phones that were taken inside the toilet

present study, the GPC had 75% resistance against benzylpenicillin, whereas it was found to be 100% in an earlier study [21]. This pattern of high resistance towards this antibiotic could be due to the rampant utilization of different groups of antibiotics including penicillin group of drugs, not only for empirical treatment but also for non-curative reasons like prophylaxis and metaphylaxis in animal feed stocks which thereby promotes drug resistance [22]. The GPC in our findings showed 100% susceptibility towards linezolid, vancomycin and tigecycline which is in total agreement with previous finding [23]. This could be due to the fact that they fall under second line drugs which are seldom used unless needed and hence showed good susceptibility [12].

In this study, no significant gender association was found between the organisms isolated from mobile phones of both male and female participants. With respect to occupation, mobile phones of non-HCWs (patient and patient attendants) were significantly contaminated (p = 0.001) as compared to those of HCWs and were predominantly contaminated with *A. baumannii* and *A. lwoffii*. This can be attributed to the proper hand hygiene awareness and practices among the HCWs resulting in the constant sterilization of hands either by hand-washing or using sanitizer upon touching patients and hospital devices. Hence, hand hygiene is the leading measure for preventing the transmission of antimicrobial resistance and reducing healthcare-associated infections [24].

As anticipated in this study, mobile phones which were never cleaned had more microorganisms (p = 0.019) and *A. baumannii*, *S. hominis*, *A. lwoffii*, *S. haemolyticus*, *S. epidermidis and E. coli* dominated other microorganisms (Fig. 3, Table 2). A similar finding was also reported by Koscova et al. [25] where the researchers also concluded that cleaning of mobile phones especially with antibacterial solutions can markedly reduce microbial contamination with an effective range of 36.8 to 100%.

Previous studies have regarded toilets as a source of microbial contamination and a hidden source of microbial transmission of pathogens across various individuals [26]. We found more number of microorganisms on mobile phones that were carried inside the toilet, and among them, *S. hominis* predominated (Fig. 4). Various sub-species of this species had been implicated for nosocomial outbreaks causing bloodstream infections in patients with underlying malignancies [27].

Our sampling was carried out during summer and the genus *Acinetobacter* as such are part of the human skin flora which shows seasonal variability of 53% during summer vs 32% during winter [28, 29]. In this study, GNBs such as *Acinetobacter lowffii*, *Acinetobacter baumannii* and *E. coli* predominated in the mobile phones taken inside the toilet, and interestingly, an equal number of *Acinetobacter baumannii* was also found among those mobile phones that were never taken inside the

Table 3 Antibiotic susceptibility and resistance pattern for GNB

Code No.	Site of Collection	Occupation	Name of Microorganisms	VITEK Card N280 Antibiotic Panels Tested Against Gram Negative Bacilli Isolated From Mobile Phones In a Hospital Environment																	AES Findings
				AMP	AMC	TZP	CXM	CXM Axetil	CTX	CRO	CFP/SUL	FEP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	CST	SXT	Phenotype Flagged
MP-4	Micro Lab	HCW	*S. paucimobilis*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None
MP-25	Med Ward	Non-HCW	*E. cloacae*	S	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	BETA-LACTAMS - ESBL(CTXM-LIKE)
MP-26	Med Ward		*E. cloacae*	S	R	R	R	R	R	R	R	R	S	S	S	S	R	S	S	S	R	
MP-31	ENT Ward		*A. lwoffii*	S	S	S	S	S	-	S	S	S	S	S	S	S	R	S	S	S	S	
MP-38	ENT Ward	HCW	*A. haemolyticus*	R	S	S	R	R	-	S	S	S	S	S	S	S	S	S	S	S	S	
MP-45	Gyn Ward	Non-HCW	*Pantoea spp.*	S	S	S	R	R	-	R	S	S	S	S	S	S	S	S	S	S	S	
MP-51	Gyn Ward		*A. baumannii*	R	R	S	R	R	-	S	S	S	S	S	S	S	S	S	S	S	S	
MP-57	Gyn Ward		*A. baumannii*	R	R	S	R	R	-	S	S	S	S	S	S	S	S	S	S	S	S	
MP-60	Gyn Ward		*E. coli*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
MP-63	Gyn Ward		*A. lwoffii*	S	S	S	S	S	-	S	S	S	S	S	S	S	S	S	S	S	S	
MP-67	Gyn Ward		*Moraxella group*	I	S	S	R	R	-	I	S	S	S	S	S	S	S	S	S	S	S	
MP-70	Gyn Ward		*S. paucimobilis*	S	S	S	S	I	-	S	S	I	S	S	S	S	S	S	S	R	S	
MP-71	Gyn Ward		*E. cloacae*	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	BETA-LACTAMS - ESBL
MP-76	Gyn Ward		*A. baumannii*	S	S	S	S	S	-	S	S	S	S	S	S	S	S	S	S	S	S	None
MP-80	Surg Ward	HCW	*E. coli*	R	S	S	R	R	R	R	S	R	S	S	S	S	S	S	S	R	S	BETA-LACTAMS - ESBL, POLYPEPTIDES-R
			A. baumannii	R	R	S	R	R	-	S	S	S	S	S	S	S	S	S	S	S	S	
MP-82	Surg Ward	Non-HCW	*E. coli*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None
MP-88	Surg Ward		*K. pneumoniae*	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
MP-92	Ortho Ward		*E. cloacae*	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	BETA-LACTAMS - ESBL
MP-97	Ortho Ward		*A. baumannii*	R	R	S	R	R	-	S	S	S	S	S	S	S	S	S	S	S	S	
MP-100	Ortho Ward		*A. lwoffii*	S	S	S	S	S	-	S	S	S	S	S	S	S	R	S	S	S	S	None
MP-59	Gyn Ward		*A. baumannii*	R	R	S	R	R	-	S	S	S	S	S	S	S	S	S	S	S	S	
			Moraxella group	S	S	S	S	S	-	S	S	S	S	S	S	S	R	S	S	R	S	
			K. pneumoniae	VITEK System could not produce AST results against these isolates																	
MP-35	ENT Ward		*Pantoea spp.*																		
MP-54	Gyn Ward		*A. lwoffii*																		
MP-87	Surg Ward		*O. ureolytica*																		
MP-95	Ortho Ward		*A. lwoffii*																		
MP-98	Ortho Ward		*A. salmonicida*																		

AMP ampicillin, *AMC* amoxicillin/clavulanic acid, *TZP* piperacillin/tazobactam, *CXM* cefuroxime, *CTX* cefotaxime, *CRO* ceftriaxone, *CFP/SUL* cefoperazone/sulbactam, *FEP* cefepime, *IPM* imipenem, *MEM* meropenem, *AMK* amikacin, *GEN* gentamicin, *NAL* nalidixic acid, *CIP* ciprofloxacin, *TGC* tigecycline, *CST* solistin, *SXT* trimethoprim/sulfamethoxazole, *S* susceptible, *R* resistant

Grey highlight—mobile phones having growth of > 1 organisms of the same Gram reaction

Pink highlight—mobile phones having mixed growth of both Gram-positive and Gram-negative organisms

toilet (Fig. 4). *Acinetobacter baumannii* has been reported to colonize the skin at very low levels and is an emerging multidrug-resistant pathogen in community and hospital environments which recognizes them as the most difficult GNB to control and treat [30–32]. *Acinetobacter* spp. have been reported to colonize up to 75% in hospitalized patients vs 43% of community dwellers and among these community dwellers, *Acinetobacter lowffii* contributed 58% [33]. Other microorganisms such as *S. cohnii*, *K. pneumoniae*, *Moraxella* group and *S. aureus* (MSSA) were also found in equal percentage in both the groups in the present study (Fig. 4).

We also observed that in our study, most of the microorganisms tested against the panel of antibiotics were of susceptible strains, which indicates that these microorganisms might have come from the community through the patients, patient attendants and/or hospital staff. Over time, such microorganisms can acquire resistance and infect patients when the opportunity arises and can even be fatal. Organisms like *S. aureus* (MRSA), CoNS and ESBL are well known for bloodstream infections leading to septicaemia and death [34]. The main drawback of this study apart from sample size is that an equal number of sampling from different sources could not be carried out due to the unavailability of an equal number of patients at the time of sample collection.

Conclusion

From this study, the most important factor associated with mobile contamination was being unaware of the facts that mobile phones can harbour microorganisms and mobile phones need to be cleaned with antiseptics. The novel finding of our work is that the mobile phones of non-health care workers are more contaminated and are more prone to cause transmission of pathogens. This study can create public awareness regarding the danger of transmission of germs through mobile phones and can educate the people about hand hygiene practices and regular cleaning of mobile phones with antiseptics.

Materials and methods

Sample collection

A total of 100 mobile phone swab samples were aseptically collected from a mission hospital using sterile cotton swab in screw-capped polypropylene tubes (HiMedia PW003-1x100NO) moistened with 9% sterile normal saline. The entire surface of the phone including the screen, buttons of keypads, sides and back, mouth and ear piece, volume and lock keys and the cover inside out were thoroughly swabbed [35]. Participants were both HCWs and non-HCWs and were randomly selected as per their convenience at the time of sample collection during the Summer period (March–May 2018). Mobile phone swab samples were collected from nine locations (six wards and three units) within the hospital as follows: 18 samples from three laboratory units (Microbiology—5, Blood bank—4 and Pathology unit—9) and 82 samples from six wards (Surgical ward—15, Medical ward—11, ENT ward—11, K-ward—4, Gynaecology ward—32, Orthopaedic ward—9) (Table 2). Further, the 82 samples were from patient attendants—37, patients—29, female ward attendants—4, staff nurse—11 and ward doctor—1 and the 18 samples were from Lab technicians—17 and laboratory doctor—1.

Sample processing and identification

All samples were processed at the Microbiology laboratory in the mission hospital, Mizoram, by plating on sheep blood agar plate (HiMedia MP1301), MacConkey agar (HiMedia MH081) and chromogenic media (HiCHROME UTI Agar M1353) using the three-phase streaking pattern and incubating aerobically at 37 °C for 24–36 h. Gram staining (HiMedia K001) was performed on isolated colonies and identified using VITEK 2 system (BioMérieux, Biotechnology Company; France). VITEK identification cards were selected as per the morphology and Gram reaction of the bacteria [36]. This automated system monitors the kinetics of bacterial growth, calculates it using a unique algorithm and follows a Clinical and Laboratory Standards Institute (CLSI) guidelines [37].

Table 4 Antibiotic susceptibility and resistance pattern for GPC/GPB

Code No.	Site of Collection	Occupation	Name of Microorganisms	VITEK Card P628 Antibiotic Panels Tested Against GPC/GPB Isolated From Mobile Phones in a Hospital Environment																AES Findings
				FOX Screen	Banzyl PEN	OXA	GEN	CIP	NVX	ERY	CLI	LZD	DAP	TEC	VAN	TET	TGC	RIF	SXT	Phenotype Flagged
MP-5	Micro Lab	HCW	*S. aureus*	NEG	R	S	S	R	R	R	S	S	S	S	S	S	S	S	S	None
MP-6	Blood Bank		*S. chonii*	NEG	R	S	S	S	S	R	S	S	S	S	S	S	S	R	S	
MP-10	Patho Lab		*S. arlettae*	AST not available																
MP-23	Med Ward	Non-HCW	*S. haemolyticus*	POS	R	R	R	R	R	R	R	S	S	S	S	R	S	R	R	BETA- LACTAMS MODIFICATION OF PBP(mecA)
			A. otitis	AST not available																None
MP-24	Med Ward		*S. haemolyticus*	POS	R	R	S	R	R	R	R	S	S	S	S	R	S	S	R	BETA- LACTAMS MODIFICATION OF PBP(mecA)
MP-28	Med Ward		*S. cohnii*	NEG	S	S	S	R	R	I	S	S	S	S	S	R	S	S	S	None
MP-29	Med Ward		*S. haemolyticus*	POS	R	R	R	R	R	R	R	S	S	S	S	S	S	R	S	BETA- LACTAMS MODIFICATION OF PBP(mecA)
MP-31	ENT Ward		*S. warneri*	NEG	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None
			S. hominis	NEG	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	
MP-33	ENT Ward		*S. saprophyticus*	NEG	R	S	S	S	S	R	R	S	S	S	S	S	S	S	S	
MP-34	ENT Ward		*S. warneri*	NEG	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
MP-35	ENT Ward		*S. haemolyticus*	POS	R	R	S	S	S	R	S	S	S	S	S	R	S	S	S	BETA- LACTAMS MODIFICATION OF PBP(mecA)
MP-36	ENT Ward		*S. saprophyticus*	NEG	R	S	S	S	S	R	R	S	S	S	S	R	S	S	S	None
			K. kristinae	AST not available																
MP-40	ENT Ward		*S. epidermidis*	NEG	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	
MP-39	ENT Ward	HCW	*S. hominis*	POS	R	R	S	S	S	R	S	S	S	I	S	S	S	S	S	BETA- LACTAMS MODIFICATION OF PBP(mecA)
			S. sciuri	NEG	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	None
MP-44	K Ward		*S. xylosus*	NEG	R	S	S	S	S	I	S	S	S	S	S	S	S	S	S	
MP-47	Gyn Ward	Non-HCW	*S. hominis*	POS	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	BETA- LACTAMS MODIFICATION OF PBP(mecA)
MP-53	Gyn Ward		*S. epidermidis*	NEG	R	S	S	S	S	R	S	S	S	S	S	S	S	S	R	None
MP-64	Gyn Ward		*E. rhushiopathiae*	AST not available																
MP-68	Gyn Ward		*S. aureus*	NEG	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	
MP-69	Gyn Ward		*S. hominis*	NEG	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
MP-72	Gyn Ward		*S. hominis*	NEG	R	R	S	S	S	R	S	S	S	S	S	R	S	S	S	BETA- LACTAMS MODIFICATION OF PBP(mecA)
MP-73	Gyn Ward		*S. hominis*	NEG	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	None
MP-82	Surg Ward		*Enterococcus spp.*	NIL	S	S	S(HL)	S	S	S	R	S	S	S	S	S	S	S	S	
MP-85	Surg Ward		*S. epidermidis*	POS	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	BETA- LACTAMS MODIFICATION OF PBP(mecA)

Fox Screen cefoxitin screen, *Benzyl Pen* benzylpenicillin, *OXA* oxacillin, *GEN* gentamicin, *CIP* ciprofloxacin, *NVX* levofloxacin, *ERY* erythromycin, *CLI* clindamycin, *LZD* linezolid, *DAP* daptomycin, *TEC* teicoplanin, *VAN* vancomycin, *TET* tetracycline, *TGC* tigecycline, *RIF* rifampicin, *SXT* trimethoprim/sulfamethoxazole. *HL* high level
Gray highlight—mobile phones having growth of > 1 organisms having the same gram reaction. Pink highlight—mobile phones having mixed growth of both Gram-positive and Gram-negative organisms
S susceptible, *R* resistant, *POS* positive, *NEG* negative

Antibiotic susceptibility testing

For antibiotic susceptibility test (AST), the VITEK 2 system was used as per manufacturer's instructions. For GNB, N280 antibiotic cards which included a panel of 18 antibiotics were used (Table 3). Etrapenem was not included since it did not produce any results upon testing by VITEK 2. For GPC, P628 antibiotic cards with a panel of 16 antibiotics were used (Table 4). Cefoxitin was used as a surrogate marker for methicillin-resistant *S. aureus*. Rifampicin did not produce any results upon testing and thus excluded.

Statistical analysis

Data were analysed using Statistical Package for Social Sciences (SPSS) version 20. Odds ratio (OR) and confidence interval (CI) were measured using logistic regression to analyse the factors associated with a possible source of contamination of mobile phones.

Abbreviations

AST: Antibiotic susceptibility testing; CI: Class interval; CoNS: Coagulase-negative *Staphylococci*; ENT: Ears, nose and throat; ESBL: Extended spectrum beta-lactamases; GNB: Gram-negative bacilli; GPC: Gram-positive cocci; HCW: Health care worker; K ward: Substance abuse ward; MRSA: Methicillin-resistant *Staphylococcus aureus*; Non-HCW: Non-health care worker; OR: Odds ratio; SPSS: Statistical Package for the Social Sciences

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Authors' contributions

CVS and PP were involved in sample collection and microbiological analysis. SM and ZZ were involved in the data analysis and interpretation of results. NSK, LN and RL have supervised and interpreted the reports of the research work. CVS, PP, ZZ and NSK drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

No datasets (culture deposits or sequence data) are generated and therefore are not applicable to this study.

Ethics approval and consent to participate

Ethical approval is not applicable in this study. However, verbal consent was obtained from all the study participants.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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The Emergence of Carbapenem-Resistant Gram-Negative Bacteria in Mizoram, Northeast India

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Abstract: (Background) Numerous reports on carbapenem resistance in different parts of India have been published, yet there are insufficient studies on the prevalence of antibiotic resistance in the northeast region of the country. This study evaluated the emergence of carbapenem resistance in the clinical isolates collected in Mizoram. (Results) A total of 141 Gram-negative clinical isolates were collected from the two hospitals, including the Civil Hospital in Lunglei and the Synod Hospital in Aizawl. The isolates include *Escherichia coli* (*n* = 62, 43.9%), *Klebsiella* spp. (*n* = 43, 30.4%), *Pseudomonas aeruginosa* (*n* = 9, 6.3%), *Serratia marcescens* (*n* = 3, 2.1%), *Proteus mirabilis* (*n* = 2, 1.4%), *Shigella* spp. (*n* = 4, 2.8%), *Enterobacter* spp. (*n* = 6, 4.2%) and *Acinetobacter* spp. (*n* = 12, 8.5%). The isolates were found to be resistant to meropenem (11%), colistin (48%), tigecycline (25%) and cefotaxime (50%). A total of four *E. coli* and one *Shigella sonnei* encoded the $bla_{\text{OXA-48-like}}$ gene. The $bla_{\text{CTX-M-1}}$ gene was detected in 13 isolates, of which eight were *E. coli*, two *Shigella flexneri*, and one isolates each of *K. pneumoniae*, *K. oxytoca* and *Shigella sonnei*, respectively. (Conclusion) Carbapenem-resistant *Enterobacteriaceae* are common among other parts of India, despite limited access to antibiotics, the emergence of resistance in the northeastern region is worrying.

Keywords: carbapenem-resistance; Gram-negative bacteria; *Enterobacteriaceae*; meropenem; antibiotic resistance

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1. Introduction

Antimicrobial resistance in bacterial pathogens is a widespread and critical challenge in clinical treatment resulting in high morbidity and mortality rates globally [1]. Gram-negative bacteria can result in severe complications or even treatment failure when caused by multi-drug resistant (MDR) strains that are increasingly common around the globe. Carbapenems, a highly effective class of broad-spectrum antibiotics usually reserved for MDR infections, are increasingly deployed, and as a result, resistance among Gram-negative bacteria has now become a worldwide concern [2]. Carbapenems are transpeptidase inhibitors that inhibit the peptide crosslinking during cell wall synthesis leading to autolytic activity (cell death) [3]. The basis of Carbapenem resistance mechanisms is either acquired via horizontal gene transfer or is based on intrinsic mechanisms, which include mutations

of the target site, enzymatic degradation of the antimicrobial compound, and changes in the efflux pump mechanisms. Among these, enzymatic degradation is the most prevailing mechanism [4]. Molecular mechanisms of carbapenemase enzymes have been proposed and are being classified as class A, class B and class D. Class A types are more often found in *Enterobacteriaceae* but rarely encountered in *P. aeruginosa*, which covers SME, NMC, KPC, IMI, GES-type of enzymes. Class B is found in *Acinetobacter* species, *P. aeruginosa*, and *Enterobacteriaceae* and includes VIM, SPM, GIM, and IMP-type enzymes. Class D includes the OXA-type, which is again more common among *Acinetobacter* [5].

The distribution of carbapenem-resistant *Enterobacteriaceae* is increasing, but the availability of data is limited in many countries. For lactose non-fermenters, the recorded rate of carbapenem resistance appears to be substantially higher than for lactose fermenters globally [6]. The virulence, as well as the pathogenicity of the carbapenem-resistant bacterial strains, were found to vary in the country in which a person is infected [7,8]. The epidemiological distribution of carbapenemase producers varies vastly. In Asian sub-continents, $bla_{\text{OXA-48-like}}$ and $bla_{\text{NDM-types}}$ are more common, while bla_{KPC} and other $bla_{\text{OXA-types}}$ are recorded quite often in the European countries. In northern America, half of the carbapenem-resistant *Enterobacteriaceae* (CRE) is found to be carbapenemase-producing, while bla_{NDM}, bla_{VIM} and $bla_{\text{OXA-48-like}}$ are less prevalent. In comparison to other Asian countries, the rate of carbapenem resistance was found to be significantly lower in India. Yet, in hotspots in India, an average of 13% of cases were registered as CRE [9].

In the northeast regions, in the states of Mizoram, Tripura, Nagaland and Meghalaya, the prevalence of carbapenem resistance were found to be below 5% due to less developed healthcare and the comparably reduced amounts of carbapenems used in these regions [10]. Based on this information, this study was conducted to assess the emergence of Gram-negative bacteria in Mizoram that are carbapenem-resistant, as well as to detect the presence of carbapenemase genes in the clinical isolates collected from two hospitals in the region.

2. Materials and Methods

2.1. Collection of Bacterial Isolates

A total of 141 Gram-negative bacterial isolates were obtained from Civil Hospital Lunglei (Lunglei District) as well as Synod Hospital Aizawl (Aizawl District), Mizoram, between 2018 and 2019. The isolates were transported as cryoprotectant stocks to the Antibiotic Resistance and Phage therapy research Laboratory at VIT, Vellore. All the isolates were sub-cultured and stored at −20 °C. The bacterial identification was performed using the VITEK identification system and the isolates were further confirmed by 16S rRNA identification using the universal primers 27F and 1492R. PCR products were Sanger sequenced (Eurofins Genomics Ltd., India) and analyzed using the NCBI-BLAST tool. The blast hit was analyzed against the available data from the NCBI database and the sequences were deposited in NCBI-GenBank [11].

2.2. Minimal Inhibitory Concentration (MIC)

A test to determine the MIC was performed for all the isolates using meropenem, cefotaxime, colistin and tigecycline (Sigma Aldrich, India). Briefly, Muller Hinton No. 2 cation-controlled broth (Hi-Media, India) was prepared and 100 µL of the broth was added to the 96 well-microtiter plates. The antibiotic to be tested was diluted by adding 100 µL of the respective antibiotic ranging from 0.25 µg/mL to 256 µg/mL. The bacterial culture at 0.5 McFarland standards (approx. 1×10^7–10^8 CFU/mL) was prepared and 5 µL was added to each well. The plates were incubated at 37 °C for 18 h. The results were recorded and interpreted according to CLSI guidelines (CLSI, 2019) [11].

2.3. DNA Isolation

Bacterial DNA was isolated from the clinical isolates following the boiling lysis method as described in our previous study [11]. Briefly, 10 colonies were inoculated into sterile distilled water and boiled for 10 min (temp: 95 °C) to break open the cells. The mixture

was allowed to cool down to room temperature and centrifuged at 8000× *g* for 5 min and the supernatant containing the DNA was used as a template for PCR analysis.

2.4. Screening of Beta-Lactamase Genes

The resistance genes screening was performed on both chromosomal and plasmid DNA. Chromosomal DNA was isolated by the boiling lysis method (above), while plasmid DNA was isolated using the Plasmid DNA isolation kit (Hi-Media, India). Carbapenem resistance genes bla_{NDM}, $bla_{\text{OXA-48-like}}$, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{DIM}, bla_{BIC}, bla_{GIM}, bla_{SIM} and bla_{AIM} and cefotaxime resistance genes, CTX-M group of genes: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-26}}$ and $bla_{\text{CTX-M-8}}$ were screened using PCR [12,13]. The integrase genes *intI1*, *intI2* and *intI3* were amplified (n = 16) by multiplex PCR using the primers described in a previous study [14]. The clonality of the bacterial species was determined (n = 141) by ERIC-PCR (enterobacterial repetitive intergenic consensus) using ERIC2 primers as described by Versalovic et al. [15]. ERIC-PCR results were analyzed by PyElph software (Python-based gel images analyzer) [16]. In brief, a DNA gel image was generated by photographing it with UV gel Doc. The DNA banding patterns (gel image) were inserted into PyElph software in JPEG format. The ERIC-PCR patterns obtained were interpreted and compared as described by Bilung et al. [17]. For cluster studies by ERIC-PCR, a dendrogram was generated using the unweighted pair group average (UPGMA) against a 1 kb DNA marker (HiMedia, Mumbai, India). The dendrogram was drawn for the entire genus separately and when the band similarity was greater than 80%, the clones were treated as related. The strains with carbapenem resistance genes are highlighted in red (Supplementary Figures S1–S7).

2.5. Identification of Carbapenemase Producers

The Modified Hodge test (MHT) was performed to detect the carbapenemase production in the resistant isolates. Briefly, an indicator organism, *E. coli* ATCC 25922, and the test bacteria were inoculated in MH broth and the turbidity was adjusted to 0.5 McFarland standard. Then, the indicator organism was plated onto Mueller–Hinton agar plates and the plates were allowed to dry for 10 min. Meropenem antibiotic disks (10 mcg) were placed at the centre of the agar plate. The test bacteria were streaked as a straight line from the edge of the disk towards the edge of the plate. The plates were incubated at 37 °C for 18 h. The results were interpreted according to the CLSI guideline [18].

2.6. Synergy Testing

The synergistic effect of the combination of two antibiotics was analyzed by the checkerboard method. The synergy was assessed using the two last-resort antibiotics, colistin and meropenem. Briefly, the test bacteria were diluted to 0.5 McFarland standard. Both the antibiotics were diluted in separate microtiter plates and combined into a single plate. In brief, meropenem (A) was diluted vertically from column A to H (1 µg/mL to 256 µg/mL) in 96 well microtiter plates marked as A and colistin (B) was diluted horizontally in rows from 1 to 12 in 96 well microtiter plate marked as B (1 µg/mL to 256 µg/mL). The diluted antibiotic from plate A was transferred to plate B onto their respective wells. The selected test bacteria were inoculated and the plates were incubated at 37 °C for 18 h. The synergistic effect was measured by calculating the Fractional Inhibitory Concentration (FIC) index of the antibiotic alone and in combination [19]. The FIC was calculated by a universal mathematical expression, FIC = (FIC A/=FIC B), where FIC A = (MIC of the antibiotic A in combination/MIC of the antibiotic A alone) and FIC B = (MIC of the antibiotic B in combination/MIC of the antibiotic B alone). Based on the value obtained at FIC, the results are interpreted as synergy (FIC $\leq$ 0.5), indifference (2 > FIC $\geq$ 0.5), and antagonistic (FIC $\geq$ 2).

3. Results

3.1. Bacterial Isolates and Carbapenem Resistance

The collected 141 non-repetitive Gram-negative clinical strains of bacteria included *E. coli* (*n* = 62, 43.9%), *Klebsiella* spp. (*n* = 43, 30.4%), *P. aeruginosa* (*n* = 9, 6.3%), *Serratia marcescens* (*n* = 3, 2.1%), *Proteus mirabilis* (*n* = 2, 1.4%), *Shigella* spp. (*n* = 4, 2.8%), *Enterobacter* spp. (*n* = 6, 4.2%) and *Acinetobacter* spp. (*n* = 12, 8.5%). The MIC results showed that 16/141 (11.3%) were resistant to meropenem, 68/141 (48.2%) were resistant to colistin, 35/141 (24.8%) to tigecycline and 71/141 (50.3%) to cefotaxime. MIC_{50} and MIC_{90} values for meropenem were 0.12 µg/mL, for tigecycline MIC_{50} and MIC_{90} value were 0.5 µg/mL and 0.25 µg/mL, for cefotaxime MIC_{50} and MIC_{90} value were 128 µg/mL and 0.12 µg/mL, for colistin MIC_{50} and MIC_{90} value were 16 µg/mL and 8 µg/mL. For none of these isolates we detected carbapenemase production by MHT. The isolates that showed co-resistance to colistin and meropenem was 4 (2.8%), tigecycline and meropenem was 9 (6.3%) and cefotaxime and meropenem was 12 (8.5%) (Figure 1).

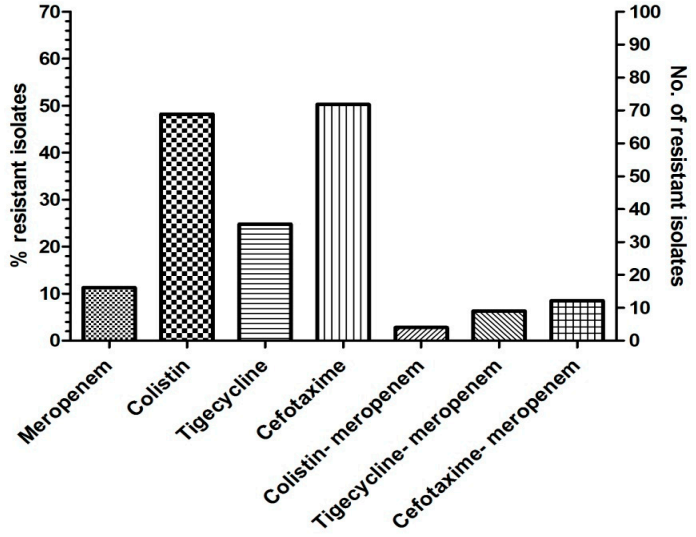

Figure 1. The distribution of antibiotic-resistant isolates among the Gram-negative bacterial isolates. A total of 141 Gram-negative bacteria were investigated in this study.

3.2. Molecular Studies

Of the 141 isolates, five carried $bla_{\text{OXA-48-like}}$ and 13 encoded the $bla_{\text{CTX-M-1}}$ gene. One isolate each of *E. coli* and *Shigella sonnei* was identified to carry both the $bla_{\text{OXA-48-like}}$ and the $bla_{\text{CTX-M-1}}$ gene. In total, 16/141 isolates carried resistance genes, $bla_{\text{OXA-48-like}}$ and $bla_{\text{CTX-M-1}}$ (Table 1). The $bla_{\text{OXA-48-like}}$ (5/141) carriers were *E. coli* (*n* = 4) and *Shigella sonnei* (*n* = 1). The isolates with $bla_{\text{CTX-M-1}}$ (13/141) were *E. coli* (*n* = 8), *Shigella flexneri* (*n* = 2), *K. pneumoniae* (*n* = 1), *K. oxytoca* (*n* = 1) and *Shigella sonnei* (*n* = 1). Other beta-lactamase genes such as bla_{KPC}, bla_{VIM}, bla_{BIC}, bla_{GIM}, bla_{DIM}, bla_{SIM} and bla_{AIM} were absent. None of the isolates had plasmid-borne resistance genes. A total of 16 isolates that carried resistance genes were identified at the species level and the sequences were deposited in GenBank.

Integron gene screening showed the presence of *intl1* in 13 isolates and interestingly, all the isolates were $bla_{\text{CTX-M-1}}$ positive (Table 1). The two isolates, *E. coli* and *S. sonnei*, that carried both $bla_{\text{CTX-M-1}}$ and $bla_{\text{OXA-48-like}}$ genes were positive for *intl1*. The ERIC-PCR dendrogram demonstrates that the $bla_{\text{CTX-M-1}}$ carrying carbapenem-resistant *E. coli* strains (EC46, EC48 and EC60) are closely related. Other strains of *E. coli* that carried $bla_{\text{OXA-48-like}}$ appear not related to each other. The ERIC-PCR results are shown as dendrograms in Supplementary Figures S1–S7.

Table 1. The distribution of beta-lactamase genes among clinical Gram-negative bacteria is investigated in this study.

S.no	Isolate ID	Bacteria	MIC				Resistance Gene	*Int* Gene
			Col	Tig	Mer	Cef		
1	EC 2	*E. coli*	1	**4**	**64**	**>128**	$bla_{\text{OXA-48-like}}$	-
2	EC 20	*E. coli*	2	<0.12	**32**	**>128**	$bla_{\text{OXA-48-like}}$	-
3	EC 15	*E. coli*	1	<0.12	**128**	**>128**	$bla_{\text{OXA-48-like}}$	-
4	SS 1	*Shigella sonnei*	2	<0.12	**128**	**>128**	$bla_{\text{OXA-48-like}}$ and CTX-M-1	*Intl1*
5	EC 17	*E. coli*	2	<0.12	**32**	**>128**	$bla_{\text{OXA-48-like}}$ and CTX-M-1	*Intl1*
6	SF 1	*Shigella flexneri*	2	**4**	**>128**	**>128**	CTX-M-1	*Intl1*
7	EC 4	*E. coli*	2	0.5	0.5	**>128**	CTX-M-1	*Intl1*
8	SF 2	*Shigella flexneri*	2	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
9	EC 8	*E. coli*	1	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
10	EC 12	*E. coli*	2	0.5	<0.12	4	CTX-M-1	*Intl1*
11	EC 13	*E. coli*	2	0.5	<0.12	**>128**	CTX-M-1	*Intl1*
12	KO 1	*K. oxytoca*	**16**	0.5	<0.12	**>128**	CTX-M-1	*Intl1*
13	KP 37	*K. pneumoniae*	**8**	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
14	EC 47	*E. coli*	**4**	<0.12	<0.12	**>128**	CTX-M-1	*Intl1*
15	EC 49	*E. coli*	**8**	0.5	<0.12	<0.12	CTX-M-1	*Intl1*
16	EC 61	*E. coli*	2	0.12	<0.12	**>128**	CTX-M-1	*Intl1*

Mer—Meropenem; Col—Colistin; Tig—Tigecycline; Cef—Cefotaxime; EC—*E. coli*; SF—*Shigella flexneri*; KO—*Klebsiella oxytoca*; SS—*Shigella sonnei*; KP—*Klebsiella pneumoniae*. The number in bold represents resistance by MIC. Bold numerals represent resistant isolates.

3.3. Combinational Activity of Colistin-Meropenem

The effect of antibiotic combinations on the bacterial cells was studied by the checkerboard method using colistin and meropenem. Isolates that showed resistance to both meropenem and colistin were chosen for this experiment. Four *K. pneumoniae* isolates (KP1, KP2, KP11 and KP12) were selected. The selected isolates had meropenem MIC of >128 μg/mL, while colistin MIC was 4 μg/mL (KP 1, KP2 and KP11) and 128 μg/mL (KP 12). All four isolates showed a reduced MIC during combinational studies. The FIC value for KP1, KP2, KP11 and KP12 were 1 (indifference), 0.5 (synergy), 1 (indifference) and 50 (antagonistic), respectively.

4. Discussion

Our findings illustrate the emergence of carbapenem resistance among Gram-negative bacteria in the northeastern region of India. The occurrence of 11.3% meropenem resistance, previously uncommon in the Mizoram region, is an indicator of the spread of resistance across India and possibly also globally. In addition, the higher prevalence of resistance to other last-resort antibiotics is highly concerning; we found resistance to colistin in more than 48% of the isolates, while almost a quarter of the strains are resistant to tigecycline.

In India, the prevalence of carbapenem-resistant bacterial infections is extensive, especially in the Southern and Northern regions, where the population density is high [20]. This is the first study to report the distribution of carbapenem resistance among Gram-negative bacteria from clinical samples collected from the two districts of Mizoram. The most common carbapenemase genes reported in the Indian isolates were bla_{NDM} and bla_{OXA} [20], though other beta-lactamase genes such as bla_{IMP}, bla_{SIM}, bla_{VIM} and bla_{AIM}, also contribute to carbapenem resistance. In this study, only the $bla_{\text{OXA-48-like}}$ carbapenemase gene was detected in the chromosomal DNA of *E. coli* and *Shigella sonnei*. To the best of our knowledge, this is the first report of a carbapenemase gene in *Shigella sonnei*. A recent report from

northeast India showed a low level of around 5% of carbapenem resistance, especially in the *Enterobacteriaceae* [21]. One of the previous studies in this region showed the presence of the $bla_{\text{NDM-1}}$ gene in *K. pneumoniae* and its association with insertion sequence (IS) elements [22]. In this study, integrase (*intl1*) genes were detected in *E. coli*, *Klebsiella* spp., and *Shigella* spp., which carried beta-lactamase genes. Their role in disseminating resistance remains to be investigated. Though plasmid-borne genes were reported to disseminate resistance among the bacterial community, none of the isolates had plasmid-borne resistance genes in this study. Possibly, the genetic information might be acquired by transduction/infection by temperate phages; recently, it was shown that around 5% of prophage sequences in the nosocomial pathogen *Acinetobacter baumannii* encode antimicrobial resistance genes, including bla_{OXA23} and $bla_{\text{NDM-1}}$ [23]. A recent study in the northeast region of Assam reported the presence of 18.9% of carbapenem-resistant *Enterobacteriaceae* [24]. The absence of carbapenemase production (detected by MHT) in the isolates carrying the $bla_{\text{OXA-48-like}}$ gene indicated that they were not efficient carbapenemase producers (or did not produce the enzyme at all). This implies that the strains are resistant to some other beta-lactamases.

Extended-spectrum cephalosporin-resistant isolates (n = 71) were also identified in this study. The common beta-lactamase genes found in the northeastern regions include $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$, SIM and VIM [22,25]. The presence of $bla_{\text{CTX-M-1}}$ in *E. coli* and *K. pneumoniae* in this study is one of the first reports in the Mizoram region. To add to this report, two *E. coli* isolates were found to carry both the $bla_{\text{CTX-M-1}}$ and $bla_{\text{OXA-48-like}}$ genes. This is a rather alarming finding for the northeastern region of India because it indicates faster dissemination of antibiotic resistance genes had occurred among the bacterial population. A study conducted by the state referral hospital in Falkawn, Mizoram, showed the emergence of a large number of MDR strains in this region [10]. Our study, which focused on two areas, Lunglei and Aizawl, found a high incidence of antibiotic resistance in Gram-negative bacteria to the last-resort antibiotics carbapenem, colistin and tigecycline. This indicates that resistance is disseminating to the northeastern parts of India, where the population is not dense. Possibly, the extensive use of colistin and other antibiotics in farming may contribute to the increasing numbers of resistant isolates as the resistance can spread from animal to human pathogens. In our study, carbapenem resistance was high among the *E. coli*, *K. pneumoniae* and *Shigella* spp. The discovery of Metallo-β-lactamases (MBL) and OXA-type carbapenemase in the clinical isolates of Gram-negatives are significant because the majority of the isolates are resistant to almost all -lactams and mostly involved in horizontal gene transfer [26,27]. A clonal similarity (ERIC-PCR) was not found between most of the resistant isolates, which is an indication that the resistance is currently just developing.

Clinical therapeutic options to treat carbapenem/colistin-resistant infections are mostly combinational therapies. Interestingly, the combination of two last-resort antibiotics, colistin and meropenem, showed a synergistic effect against four MDR *K. pneumoniae*. Although earlier studies showed synergy of the colistin-meropenem combination [28], this is one of the first against the clinical isolates collected from Mizoram. These results again support the combinational activity of two antibiotics against resistant bacterial pathogens. This study highlights the emergence of carbapenem-resistant bacteria in Mizoram, northeast India, and is a clear indication for immediate surveillance of AMR in this region. A high frequency of colistin, tigecycline, cefotaxime, and carbapenem resistance in association with beta-lactamase genes is a matter of concern to public health, not only in India but globally.

5. Conclusions

In this study, the emergence of carbapenem resistance and the high level of resistance developed towards cefotaxime, colistin and tigecycline are reported from the northeastern region of India, Mizoram, for the first time. A detailed investigation of the antibiotic susceptibility pattern and the exact cause of antibiotic resistance with the geographical location are some of the most crucial data to combat AMR. The present study showed the increased prevalence of carbapenem-resistant Gram-negative bacteria among clinical

isolates from Lunglei and Aizawl regions covering the disseminating resistance genes, $bla_{\text{OXA-48-like}}$ and $bla_{\text{CTX-M-1}}$ in northeast India. Similar to other studies from different parts of the world, this study also confirms the emergence of MDR bacteria against last-resort antibiotics with multiple resistance genes. This study contributes to the development of new strategies counteracting or limiting the spread of drug resistance in the northeastern region of India, the whole country and the world.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres13030027/s1, Figure S1: Dendrogram of *Enterobacter* spp. (EB) based on ERIC-PCR; Figure S2: Dendrogram of *Shigella* spp. based on ERIC-PCR. Ssp1—*Shigella* spp.; SS—*Shigella sonnei*; SF—*Shigella flexneri*; Figure S3: Dendrogram of *Klebsiella pneumoniae* (KP) based on ERIC-PCR; Figure S4: Dendrogram of *E. coli* (EC) based on ERIC-PCR; Figure S5: Dendrogram of *Acinetobacter* spp. based on ERIC-PCR; Figure S6: Dendrogram of *Pseudomonas aeruginosa* (PA) based on ERIC-PCR; Figure S7: Dendrogram of *Serratia marcescens* (SM) based on ERIC-PCR.

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Detection and Molecular Characterization of *Orientia tsutsugamushi* from Suspected Scrub Typhus Patients in Mizoram, India

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A B S T R A C T

Serologic and molecular tests were performed for the diagnosis and to detect *O. tsutsugamushi* genotypes that are circulating in the state of Mizoram, India. Blood samples from scrub typhus-suspected patients were collected from Synod Hospital, Durtlang, Mizoram. Weil-Felix and immunochromatographic test (ICT) were performed from the serum samples. Nested PCR (nPCR) amplification of 47kDa outer membrane protein antigen gene and 56kDa type-specific antigen gene were done from the whole blood. 141/177 (79.66%) and 134/177 (75.7%) cases showed the presence of antibody against scrub typhus by Weil-Felix and ICT assays respectively. 76/177 (42.93%) patients showed the presence of 47kDa OMP antigen gene by nPCR while 55/177 (31.07%) showed the presence of 56kDa TSA gene by nPCR. Phylogenetic analysis of 56kDa TSA gene sequence revealed that Karp-related genotype was the most common genotype in the study area followed by Kato-related genotype. In this study, a high degree of diversity of *O. tsutsugamushi* was observed similar to the observations reported from other parts of India. Nested PCR of 47kDa OMP antigen gene showed higher sensitivity as compared to nPCR amplification of 56kDa TSA gene suggesting it as the assay of choice for diagnosis of scrub typhus disease.

Introduction

Scrub typhus is an acute febrile illness. It is caused by the obligate intracellular bacterium *Orientia tsutsugamushi* and is transmitted to human by the bite of larval stages of thrombiculid mites (Seong *et al.*, 2001). Those, who inhabit regions infested with these vectors, are at high risk of acquiring scrub typhus (Tantibhedhyangkul *et al.*, 2011). It has been reported that scrub typhus disease causes illness in one million people each year,

with variable mortality rate (0–70%) and one billion people are at risk worldwide (Kelly *et al.,* 2009). Scrub typhus is endemic in geographical region known as "tsutsugamushi triangle", which extends from Northern Japan to Eastern Russia in the north, Northern Australia in the south and Pakistan and Afghanistan in the west (Xu *et al.,* 2017). An endemic focus of scrub typhus has also been described in southern Chile (Weitzel *et al.,* 2016). A number of genotypes such as Karp, Gilliam, Kuroki, Kato, Shimokoshi, Kawasaki etc. have been recognized based on the 56 kDa type-specific antigen (TSA), which is an immunodominant outer membrane protein unique to this bacterium. Karp is the most common genotype contributing about 50% of all scrub typhus cases (Nguyen *et al.,* 2017). Genotypic characterization and detailed understanding of genetic diversity of *O. tsutsugamushi* strains in endemic regions will be essential for the development of rapid diagnostics and vaccines. Outbreaks of scrub typhus have been reported from various parts of North, South and Eastern India (Gurung *et al.,* 2013; Varghese *et al.,* 2006; Bakshi *et al.,* 2007; Kumar *et al.,* 2014). In Mizoram state, India, scrub typhus cases has also been reported from both rural and urban areas (Lalrinkima *et al.,* 2017; Lalmalsawma *et al.,* 2017; Lalthazuali *et al.,* 2020).

However, there has been no report regarding the serotypes and genotypes of *O. tsutsugamushi* from this region. Mizoram is one of the eight states of North-east India, at the extreme end of the Himalayan ranges and lies between 92°15' and 93°26'E longitude and 21°58' and 24°35'N latitude (Rosangkima *et al.,* 2018). The state has two international borders, Bangladesh in the west and Myanmar in the east. The region is influenced by monsoons, with heavy rainfall during the month of May to September. The average annual rainfall is 254 centimetres. The objective of the present study was to perform serologic and molecular tests for the diagnosis of scrub typhus and to detect *O. tsutsugamushi* serotypes that are circulating in the state of Mizoram, India.

Materials and Methods

Patient recruitment and sample collection

A prospective study of acute febrile illness was conducted during July to December, 2020 in Synod Hospital, Durtlang, Mizoram, India, and the study was approved by the Institutional Ethical Committee of Synod Hospital, Durtlang, Mizoram. 177 febrile patients who visited the hospital and 48 non-diseased control samples were included in the study. Non-diseased control samples include healthy participants from outside the hospital without any symptoms of scrub typhus disease. Patients suspected to have rickettsial illness due to the fever persisting for at least 5 days or more were enrolled in the present study. Guardian of the patients between 14 and 18 years of age were given written informed consent to participate. Cases with fever of already known causes such as malaria, typhoid, etc. were excluded in the present study. A thorough clinical history was also investigated which include questions about recent exposure to tick habitats, recent travel, similar illness in close contacts and tick bites. However, absence of these features does not rule out tick-borne illness. 3ml of venous blood was collected from each patient, 2ml in ethylene diaminetetra acetic acid (EDTA) and 1 ml in a plain tube. Serum from plain tubes were collected and subjected to Weil-Felix and immunochromatographic test (ICT).

The EDTA blood samples were also subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen, GmBh, Germany) following the manufacturer's instructions. The extracted genomic DNA was stored at -20°C for use in nested PCR amplification.

Weil-Felix test

Serological testing of OXK, OX19 and OX2 antibodies were carried out following standard protocol using PROGEN proteus antigen suspensions obtained from Tulip Diagnostics (P) Ltd., Goa, India. The serum samples were diluted and the titre values of more than 1:80 were considered positive for scrub typhus.

Rapid Diagnostic Test

InBios rapid test (SD Bioline Tsutsugamushi test, SD Diagnostics, Korea), which is an immunochromatographic strip test (ICT) designed specifically for the qualitative detection of IgM to *O. tsutsugamushi* (Wahid *et al.,* 2014). It consists of ready-to-use antigen-coated strips and reagents. The test strip was precoated with lines, one for 'T' (*O. tsutsugamushi* antibody test line), and one for 'C' (control line). The colour intensity developed on the strip is directly related to the antibody concentration.

Nested PCR amplification of 47kDa OMP antigen gene

nPCR was performed for the detection of the 47kDa OMP antigen gene using OtsuFR263 and OtsuRP1133 primers. The reaction mixture (25µl) consisted of 12.5µl of 2X MasterMix (Takara), 0.75µl each of primers (10 pmol), 9µl of nucleus free water and 2µl of DNA template. PCR reaction cycle included an initial denaturation at 94 ºC for 7 min followed by denaturation at 94ºC for 30 sec, annealing at 60ºC for 1.5 min and extension at 72ºC for 1 min.

The final extension at 72ºC takes 7 min. The second cycle of nested PCR was prepared by using 1µl of first cycle nPCR product as template and OtsuFP555 and OtsuRP771 primers producing an amplicon size of 216 bp. Other reaction components and PCR cycle conditions were similar with those in the first PCR cycle except the annealing temperature of 56^{o}C (Srinivasan *et al.,* 2020). The PCR reaction was carried out using a ProFlex PCR System (Applied Biosystems, life technologies).

Nested PCR amplification of 56kDa TSA gene

A 56 kDa nested PCR was done in order to minimize the contamination and to get improved-sensitivity. The primers used in nested PCR were as described by Particia *et al.,* 2017 (Table 1) (Patricia *et al.,* 2017).

The first cycle of nested PCR was performed using P34 and P55 primers producing an amplicon size of 1003bp. The reaction mixture (25µl) consisted of 12.5µl of 2X MasterMix (Takara), 0.75µl each of P34 (10 pmol) and P55 (10 pmol), 9µl of Nucleus free water and 2µl of DNA template.

PCR reaction cycle included an initial denaturation at 94 ºC for 7 min followed by denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec and extension at 72ºC for 1 min. The final extension at 72ºC takes 7 min.

The second cycle of nested PCR was prepared by using 1µl of first cycle nested PCR product as template and P10 and P11 primers producing an amplicon size of 483 bp. Other reaction components and PCR cycle conditions were similar with those in the first PCR cycle (Teppawar *et al.,* 2019). The PCR reaction was carried out using a ProFlex PCR System (Applied Biosystems, life technologies). The diagnostic assays used in the present study (Weil-Felix, ICT, nPCR of 47kDa and 56kDa genes) were assessed for sensitivity and specificity using the MedCalc statistical software program (Belgium). A total of 48 healthy, non-diseased samples were used for specificity assessment.

DNA sequencing and phylogenetic analysis

The amplified PCR products of 56kDa TSA gene of *O. tsutsugamushi* were sequenced by Sanger's dideoxy method (Sanger *et al.,* 1997) on ABI 3730XL automated sequencer (AgriGenome Labs Pvt. Ltd., SmartCity Kochi, Kerala, India). Nucleotide sequences obtained from the present study were deposited in the GenBank data library under accession numbers MW620836 to MW620866. Sequences were edited using BioEdit Sequence alignment editor and subjected to phylogenetic tree construction. A phylogenetic dendrogram of the partial 56kDa protein genes in the present study as well as those obtained from the GenBank database was constructed by the maximum likelihood method using MEGA7 software (Kumar *et al.,* 2016). Sequence identity among 56kDa TSA genes was calculated by the Sequence Identity and Similarity (SIAS) programme (http://imed.med.ucm.es/Tools/sias.html).

Results and Discussion

A total of 177 scrub typhus-suspected patients were included in this study. The median age of the patients was 42.5 years (ranging from 15 to 86 years). 141/177 (79.66%) cases showed scrub typhus positive by Weil-Felix test, of which 53.1% were female and 26.5% were male. Immunochromatographic test showed 134/177 (75.7%) scrub typhus positive with 32.7% female and 42.9% male. Nested PCR of the 47kDa OMP antigen gene showed 76/177 (42.93%) positive with 20.3% female and 22.6% male. Nested PCR of 56kDa TSA gene also showed 55/177 (31.07%) positive with 16.4% female and 14.7% male (Table 2; Figure 1). The common symptoms noted among the patients included fever, vomiting, abdominal pain, myaglia, chills, headache and jaundice. Among the four diagnostic assays used for scrub typhus disease in the present study, Weil-Felix test showed the highest sensitivity of 79.66% (95% CI, 72.9 to 85.3) but lowest specificity of 87.41% (95% CI, 75.5 to 94.7). Nested PCR of 47kDa OMP antigen gene showed higher sensitivity of 42.94% (95% CI, 35.5 to 50.5) as compared to nPCR of 56kDa TSA gene showing sensitivity of 31.07% (95% CI, 24.3 to 38.4).

Neither 47kDa nor 56kDa nPCR showed positive for non-diseased samples, and also the primers used were species-specific primers designed for *O. tsutsugamushi*, thereby, revealing perfect specificity (100%) of both assays (Table 3).

The 56kDa TSA gene products (483 bp) were sequenced for randomly selected 37 positive samples. A phylogenetic tree was constructed for these 37 samples together with 40 reference strains (Figure 2). Phylogenetic and sequence analysis revealed that Karp-like strains predominated. Thirty one samples (83.7%) out of 37 samples analyzed in the present study clustered within the Karp-related genotype. Four samples (10.8%) clustered within the Kato-related genotype, while only one sample each clustered within the Gilliam and TA763-related genotypes. Karp-related genotypes were phylogenetically differentiated into four clades (clade 1, 2, 3 and 4). Seventeen samples were assigned into clade 1, three samples into clade 2, two samples into clade 3 and nine samples into clade 4. They were clustered with strains from Taiwan, Korea, Thailand, India, China, Japan and Vietnam. Within the partial 56 kDa TSA gene, Karp-related genotype from different clades showed sequence identity of 89.2% - 99.6%, while the sequence identity between Karp-related and Gilliam genotypes was 62% - 91% (Table 4). Scrub typhus is prevalent in Asia-Pacific area especially in rural areas of South-East Asia (Rapmund, 1984). However, it is difficult to diagnose due to its non-specific presentations, and lack of relevant laboratory tests (Sinha *et al.,* 2014).

In India, scrub typhus has been reported from many states accounting for up to 50% of undifferentiated febrile illnesses during cooler season (Mathai *et al.,* 2003; Abhilash *et al.,* 2015). Scrub typhus responds well to proper antibiotic treatment. However, delayed diagnosis may cause fatal complications (Silpapojakul *et al.,* 1991). Therefore, a rapid diagnosis of scrub typhus is important to get successful treatment.

Weil–Felix is the oldest test and is easy to perform but lacks sensitivity. Due to low antibody levels during the early stages of infection, serological tests may fail to diagnose scrub typhus (Huber *et al.,* 1012). This may reflects the low sensitivity of Weil-Felix test in some cases. However, during the later stages of infection, it showed higher sensitivity as observed in the present study. Therefore, it is recommended that the rapid card test should be interpreted in combination with other diagnostic assays and clinical findings (Wahid *et al.,* 2014). In the present study, Weil-Felix and ICT tests were performed as a screening test followed by nPCR targeting 47kDa OMP antigen gene and 56kDa TSA gene as confirmatory tests. The sensitivity (79%) and specificity (87%) of Weil-Felix test in the result of present study are slightly higher than the range of sensitivity and specificity given by the manufacturer (70% each). However, the sensitivity of ICT observed in the present study (75%) was quite lower than the range given by the manufacturer (99%) while, specificity (93%) was close to manufacturer range (96%). PCR-based molecular diagnosis targeting different genes have been used in scrub typhus cases. Although the sensitivity of these molecular methods for detection of the target gene is low, they have a very high specificity of up to 100%. The lower sensitivity of these PCR-based molecular methods reported from earlier studies (Kannan *et al.,* 2020) and in the present study could probably be due to the genetic variability associated with the target genes.

Table.1 Details of primer sets used in the present study.

Gene detected	Primer details		Product size
47kDa nPCR	(Cycle 1) OtsuFP263 (Forward) OtsuRP1133 (Reverse)	 5'-GTGCTAAGAAARGATGATACTTC-3' 5'-ACATTTAACATACCACGACGAAT-3'	216 bp
	(Cycle 2) OtsuFP555 (Forward) OtsuRP771 (Reverse)	 5'-TCCTTTCGGTTTAAGAGGAACA-3' 5'-GCATTCAACTGCTTCAAGTACA-3'	
56kDa nPCR	(Cycle 1) P34 (Forward) P55 (Reverse)	 5'-TCAAGCTTATTGCTAGTGCAATGTCTGC-3' 5'-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3'	483 bp
	(Cycle 2) P10 (Forward) P11 (Reverse)	 5'-GATCAAGCTTCCTCAGCCTACTATAATGCC-3' 5'-CTAGGGATCCCGACAGATGCACTATTAGGC-3'	

Table.2 Number of positive by different diagnostic assays per a total of 177 cases.

Weil Felix		ICT		nPCR (47kDa)		nPCR (56kDa)	
Female	Male	Female	Male	Female	Male	Female	Male
94	47	58	76	36	40	29	26
53.1%	26.5%	32.7%	42.9%	20.3%	22.6%	16.4%	14.7%

Table.3 Sensitivity and specificity of different diagnostic assays.

Diagnostic assays	Percent sensitivity		Percent specificity	
	Value	95% CI	Value	95% CI
Weil Felix	79.66%	72.97% to 85.33%	87.27%	75.52% to 94.73%
ICT	75.71%	68.70% to 81.83%	93.75%	82.80% to 98.69%
47kDa nPCR	42.94%	35.54% to 50.58%	100%	92.60% to 100%
56kDa nPCR	31.07%	24.34% to 38.45%	100%	92.60% to 100%

Table.4 DNA sequence identity (%) of partial 56kDa type-specific antigen gene among the present study samples with Karp and Gilliam genotypes.

Strain/sample	Karp	KP18	KP21	KP71	KP33	NT83	Gilliam
KP18	98.9						
KP21	94.4	87.5					
KP71	92.9	93.4	90.5				
KP33	94.7	89.2	93.4	95.8			
NT83	99.6	99.0	94.6	92.9	94.9		
Gilliam	90.9	66.9	76.7	72.3	77.2	76.7	
NT19	77.5	62.2	71.1	66.5	76.2	75.7	93.6

Fig.1 Bar diagram showing the percentage positivity of different diagnostic assays on scrub typhus suspected cases.

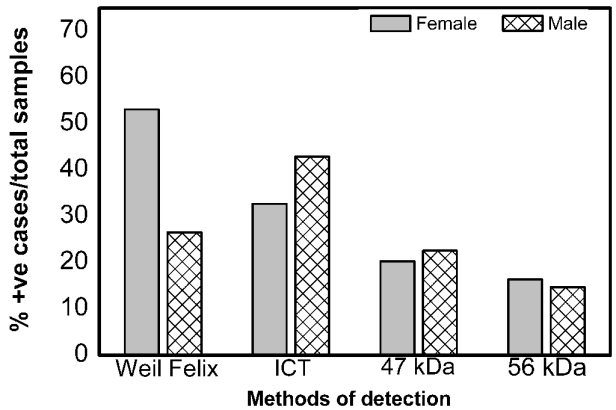
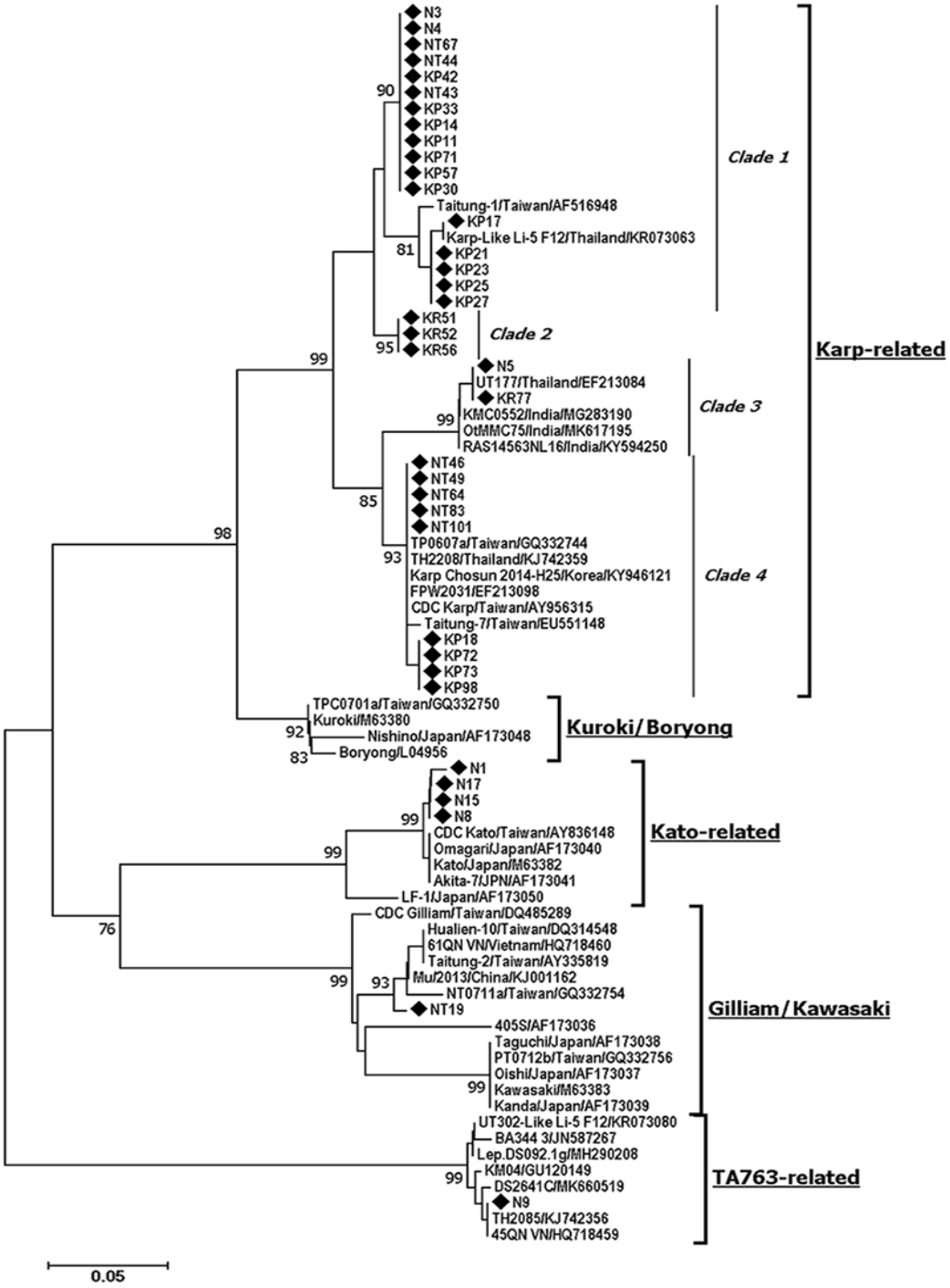


Fig.2 Phylogenetic dendrogram of partial 56kDa TSA gene sequences generated for 77 samples (37 from the present study; 40 from reference strains). The tree was constructed using the neighbour-joining method in MEGA7 software. Tree was statistically supported by bootstrapping with 1000 replicates. Percentage bootstrap support values greater than 75 were shown.



There are a large number of studies documenting the usefulness of 47kDa for diagnosing scrub typhus (Huber *et al.,* 2012; Kim *et al.,* 2011; Jiang *et al.,* 2004). It is as sensitive as nPCR of other genes. The sensitivity ranges from 40% to 80%, with specificity of up to 100% (Peter *et al.,* 2015; Watthanaworawit *et al.,* 2013; Janardhanan *et al.,* 2014). The result of present study presented the diagnostic efficiency of Weil-Felix, ICT and nPCR of 47kDa OMP and 56kDa TSA genes. Our study revealed highest sensitivity with Weil-Felix assay followed by ICT. However, these two assays exhibited comparatively lower specificity against molecular methods. Nested PCR of 47kDa OMP gene showed higher sensitivity (42.94%) as compared to nPCR of 56kDa TSA gene (31.07%). The lower sensitivity of nPCR of 56kDa TSA gene may be due to low-copy number of target DNA or excess number of host DNA.

More than 20 prototype strains of *Orientia tsutsugamushi* have been documented so far (Kelly *et al.,* 2009). However, in India, only a few studies have been conducted from the north, south and north-east India regarding the genetic diversity of circulating strains (Varghese *et al.,* 2013; Bora *et al.,* 2018). In the present study we have documented the genetic diversity of *O. tsutsugamushi* in Mizoram state, India. Phylogenetic analysis of 56kDa TSA gene in the present study revealed the presence of Karp-related, Kato-related, Gilliam-related and TA763-related strains in the study area. Karp-like strains predominated followed by Kato-related strains, while Gilliam and TA763-related strains were detected only on one sample each. In India, the presence of Gilliam and Karp prototypes along with other genotypes closely related to Kuroki, Boryong, Chuto and Kato have been reported (Patricia *et al.,* 2017; Kumar and Beena, 2017). Kato-like strain has also been reported to be very common across the country. In the present study area also Karp and Kato-related strains were comparatively more common than the other strains. The limitation of this study is that the present study was done over a short period of time and included only participants from a single Hospital in the state.

The most prevalent genotype identified in the present study was those in the Karp-related strain. However, considering the higher strain variation across the country, further prospective studies in this area will be required to identify the prevalent strains as well as antigenic variations. As compared to nPCR of 56kDa TSA gene, nPCR of 47kDa OMP antigen is more sensitive suggesting it as the assay of choice for diagnosis of scrub typhus disease.

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RESEARCH ARTICLE OPEN ACCESS

Prevalence and Virulence Gene Profiling of *Listeria monocytogenes* from Fish and Meat Samples from Aizawl, Mizoram

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Abstract

This survey aimed to study the occurrence of *Listeria* species in fish and meat samples and characterization of their virulence genes. Over all, *Listeria* spp. was found in 25.22% samples out of which 9.0% and 16.21% were *L. monocytogenes and L. innocua,* respectively. *L. monocytogenes* (n=10) belonged to 4b, 4d and 4e serovars. All the isolates revealed presence of virulence genes- *plcA* and *iap*, while *plcB* gene was also present in 90% of the isolates. The occurrence of *L. monocytogenes* in samples shows cogent evidence for their zoonotic potential and has public health significance.

Keywords: *Listeria* spp., Prevalence, Serotypes, Virulence gene, Antimicrobials, Susceptibility

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INTRODUCTION

The genus *Listeria* is facultatively anaerobic, rod-shaped bacteria and dispersed in food, environmental and clinical samples[1]. *L. monocytogenes* is a known pathogen, whereas the pathogenicity of *L. seeligeri*, *L. ivanovii* and *L. innocua* have been less documented[2]. *Listeria monocytogenes* is disseminated from animals to humans through fecal-oral route. The fatality rate is higher (30 to 75%) especially in pregnant women, neonates, aged people and people with grievous underlying disease conditions like immune-suppression[3]. Ninety percent of cases of *Listeria* contamination due to use of contaminated food products are reported[4].

Listeria monocytogenes was differentiated into 13 serotypes[5]. Only serotypes 1/2a, 1/2b, 1/2c and 4b are responsible for 95% of human illnesses[6]. In 2012 in the US, serotypes 4b (54%) and 1/2a (28%) were most commonly identified[7]. Many virulence determinants act as essential factors for the pathogenesis of *L. monocytogenes,* including *hlyA, inlA*, *inlB*, *inlC, inlJ*, *actA*, *iap* and *plcA* gene[8]. The *hlyA* gene is the main virulence determinant in *L. monocytogenes*; a hemolysin gene. The pore-forming cytolysin listeriolysin O is encoded by this *hlyA* gene. The product of *hlyA* is the first virulence factor which play a vital role in the *L. monocytogenes* pathogenesis, helps in intracellular parasitism[9].

Little information is available on *Listeria* contamination in animal originated foods from North-East India. This survey aims to estimate the *Listeria* spp*.,* identify the main serotypes and to screen the *L. monocytogenes* isolates for presence of different virulent genes.

MATERIALS AND METHODS

Collection of Samples

One hundred and eleven (111) samples, including fish gill, fish intestine and beef (15 each), chicken and pork (24 each) and mutton (18) were collected from various markets in Aizawl, Mizoram, a hilly state in the North-Eastern region India. The samples were collected aseptically by maintaining cold chain and were processed for microbiological analysis within 24 h. This study was conducted for a period of one year from July 2018 to June 2019.

Isolation of *Listeria*

For isolation of *Listeria*, ISO 11290 method was used. Each sample was inoculated aseptically in Half Fraser Broth followed by incubation at 30°C. It was subcultured in Fraser broth, further streaked onto PALCAM agar and incubated at 37°C. The grey-green colonies on PALCAM agar with diffuse black-zone was plated on Tryptone Soya Yeast Extract agar. Gram staining and Biochemical tests comprising catalase test, fermentation and acid production from various sugars, followed by MR/VP tests were performed for identification of the isolates. All the *Listeria* isolates and the control strain were preserved in nutrient agar stab (with 1.5% agar agar) and 16% glycerol for subsequent analysis. *L. monocytogenes* ATCC 13832 was utilized as the standard strain.

Confirmation of *Listeria* by PCR

Listeria were cultured in Brain Heart Infusion Broth for overnight for extraction of genomic DNA using Bacterial DNA isolation kit (Genaid, Taiwan). To confirm the suspected isolates, PCR was performed by amplifying *Listeria* genus-specific *prs* gene using specific primer[10] (1st BASE, Malaysia) (Table 1). To confirm *L. monocytogenes* isolates, primers specific for

Table 1. Primers used for identification of *Listeria* species

Species	Target Gene	Primers	Sequences	Product Size (bp)	References
Genus *Listeria*	*prs*	prs-F	5'-GCTGAAGAGATTGCGAA-3'		
		prs-R	5'-AGAAGCAAAGAAACCTTGG ATTTGCGG-3'	370	Michel *et al*.[10]
L. monocytogenes	*hlyA*	hlyA-F	5'-GCTTTTGACGCTGCCGTAAG-3'		
		hlyA-R	5'-GCAACGTATCCTCCAGAGTGATCG-3'	335	Designed.
L. innocua	*in9*	in9-F	5'-GGCTTCAGCGATTCTTCCG-3'		
		in9-R	5'-GCCCGATTTCCTCACTGTCTAA-3'	421	Tingting *et al*.[24]

Table 2. Primers used for identification of *L. monocytogenes* serogroups

Target Gene	Primer	Sequences	Product Size (bp)	Serotype Specificity	References
ORF2819	ORF2819-F	5'-AGCAAAATGCCAAAACTCGT-3'	470	1/2b,3b,4b, 4d,4e	Michel *et al*.[10]
	ORF2819-R	5'-CATCACTAAAGCCTCCCATTG-3'			
ORF2110	ORF2110-F	5'-AGTGGACAATTGATTGGTGAA-3'	597	4b,4d,4e	Michel *et al*.[10]
	ORF2110-R	5'-CATCCATCCCTTACTTTGGAC-3'			

Table 3. Primers used for detection of virulence-associated genes in *L. monocytogenes* isolates

Target Gene	Primer	Sequences	Product Size (bp)	References
plcA	plcA-F	5'-TCCGCTCTACCTGACACAAC-3'	317	The primers were designed in this study.
	plcA-R	5'-TCGTTGCTGTTTTGCTCGTC-3'		
plcB	plcB-F	5'-TTGCCGGGTTTTGCTAATGC-3'	323	
	plcB-R	5'-TAGTCCGCTTTCGCCCTTTT-3'		
iap	iap-F	5'-AGTGGCGCTGGTGTTGATAA-3'	605	
	iap-R	5'-GCTGTTTGTTGTTGCGTTGC-3'		

the virulence associated *hly*A gene[11] were self-designed using primer BLAST online tool. For detection of *L. innocua*, PCR amplification of *in9* gene was targeted[24]. A total volume of 25 µl mixture comprising 12.5 µl of 2X Mastermix, 0.5 µl (10 pmol) each of bi-directional primers, DNA (1 µl) and nuclease-free water (10.5 µl) was prepared for PCR. The amplification of *prs*, *hlyA* and *in9* genes was done by 5 min. denaturation at 95°C, accompanied by thirty cycles of 95°C (30 sec.), 53°C (30 sec.), 72°C (30 sec.) and final elongation at 72°C (5 min.).

Identification of serogroup

A simplex PCR was used to determine the serogroup using group-specific primers (Table 2). Amplification of ORF2819 was used to identify 1/2b and 3b serovars and amplification of both ORF2819 and OFR2110 to identify 4b, 4d and 4e serovars. This PCR does not differentiate within serovars.

A 25 µl volume of reaction mixture was prepared for PCR same as prepared for *Listeria* identification. *Listeria monocytogenes* (ATCC 13832) serotype 4b was used as the positive control. PCR conditions for amplification were as previously described by Michel *et al*.[10]

Virulence associated gene detection

After confirming the isolates by *hlyA* gene, a simplex PCR was done to determine the three virulence genes, *plcA, plcB* and *iap* (Table 3). The composition of reaction mixture was the same as for the other genes used in this study. PCR conditions for amplification of all the three virulence genes were standardized at denaturation at 95°C (5 min.), followed by thirty cycles of 95°C (30 sec.), 52°C (30 sec.), 72°C (30 sec.) and a final elongation at 72°C (5 min.).

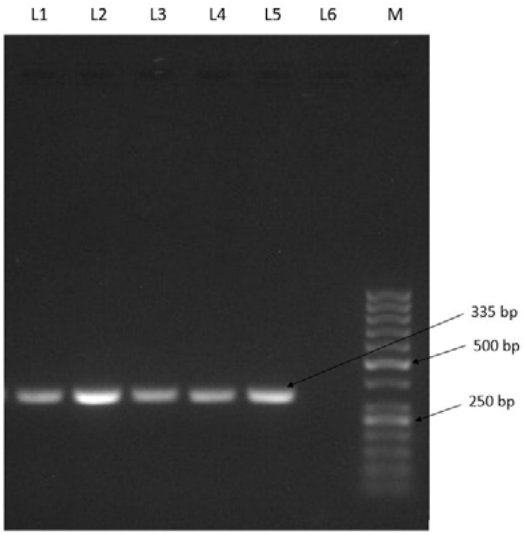
RESULTS AND DISCUSSION

Prevalence of *Listeria*

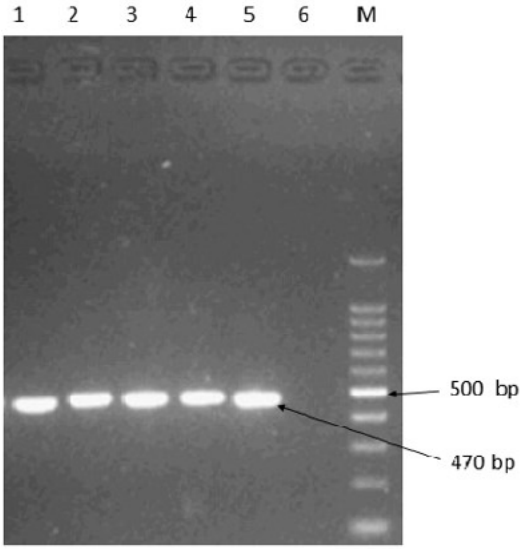
Occurrence of *Listeria* spp. collected from various markets in Aizawl is shown in Table 4. Out of 111 samples, 28 (25.22%) were positive for *Listeria*., 10 (9.0%) for *L. monocytogenes* and 18 (16.21%) for *L. innocua.* The *hlyA* gene was found in *L. monocytogenes* using PCR (Fig. 1). Incidence of *L. monocytogenes* rated higher in mutton and pork (16.6% each) followed by chicken (12.5%). The incidence of *L. innocua* was found in mutton (50%) followed by fish gill and pork (33.33% each), chicken (16.66%), fish intestine and beef (6.66%).

Nayak *et al*.[12] recorded the overall occurrence of *Listeria* in 9% of foods and the maximum incidence was found in milk samples (16%) followed by meat and fish (8% each), and milk (4%). Barbuddhe[13] and Nayak[14] with their co-workers reported occurrence of *Listeria* from 10.17% and 6.7% meat samples, respectively.

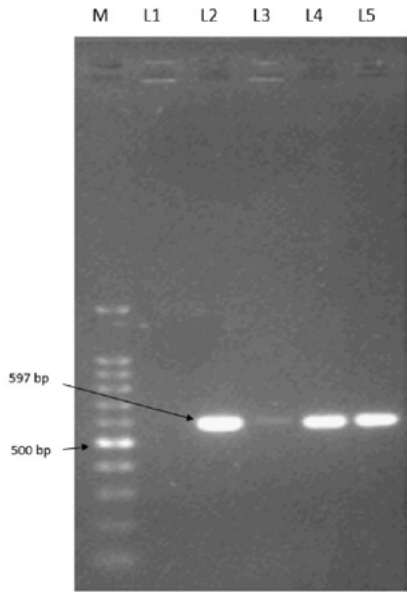
Murtiningsih and Sunarya[15] reported *L. innocua* as the superior species isolated from fish and seafood samples (11.3%). Variation in occurrence of *Listeria* spp. reported by different workers might be owing to the difference in specimen, source and variation in hygienic condition maintained in different locations.



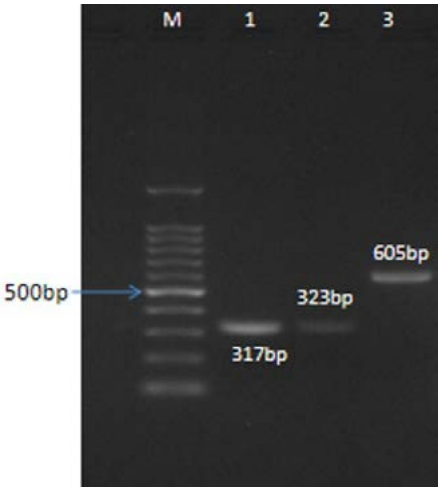
L1: Positive control (ATCC 13832), L2-L5: *Listeria* isolates, L6:Negative control, M:50bp Ladder
Fig.1. Identification of *L. monocytogenes* by PCR



L1: Positive control (ATCC 13832), L2-L5: *Listeria* isolates, L6: Negative control, M: 100 bp Ladder
Fig.2. Identification of *L.monocytogenes* serotypes (1/2b,3b,4b,4d,4e) isolates by PCR



M: 100 bp Ladder, L1: Negative control, L2: Positive control (ATCC 13832), L3-L5: *Listeria* isolates
Fig.3. Identification of *L.monocytogenes* serotypes (4b, 4d, 4e) isolates by PCR



M: 100 bp Ladder, L1: PCR Product (*plcA* gene), L2: PCR Product (*plcB* gene), L3: PCR Product (*iap* gene)
Fig.4. Virulent gene detection in *L.monocytogenes* by PCR

Table 4. Prevalence of *Listeria* spp. in various food samples

Type of Sample	No. of samples tested	No. of samples positive for Listeria spp. (%)		Total No. of Positive Samples (%)
		L. monocytogenes	*L. innocua*	
Fish gill	15	0	5 (33.3%)	5 (33.33%)
Fish intestine	15	0	1 (6.6%)	1 (6.66%)
Pork	24	4 (16.6%)	4 (16.6%)	8 (33.33%)
Mutton	18	3 (16.6%)	6 (33.3%)	9 (50%)
Chicken	24	3 (12.5%)	1 (4.16%)	4 (16.66%)
Beef	15	0	1 (6.6%)	1 (6.66%)
Total	111	10 (9%)	18 (16.21%)	28 (25.22%)

Serotyping

All *L. monocytogenes* showed PCR amplification of both ORF2819 and ORF2110, which identified them as serovars 4b, 4d or 4e (Fig.'s 2 and 3). Serovars 1/2a, 1/2b and 4b are linked with listeriosis[16] and 4b was linked with major outbreaks of listeriosis[17]. Thus, the presence of serovar 4b in foods inflicts greater warnings on human health[18]. Serotype 4b was proclaimed from 37.2% of foods in Turkey[19] as well in India and the United States of America with 60.4%[20] and 16.4%[21], respectively. The difference in rate of the prevalence might be due to contradiction in the types of foods selected for each investigation. According to Michel *et al*.[10], the serotypes 3a, 4d, and 4e are infrequent in foods, therefore the serovars 4b, 4d and 4e were marked as 4b serogroup. In the present study, all the isolated *L. monocytogenes* were confirmed as 4b serogroup which is a major public health concern, since isolates of *L. monocytogenes* belonging to this serogroup are potential human pathogens.

Virulence-associated genes

Virulence-associated gene profiling in *L. monocytogenes* was done by PCR amplification of three genes (Fig. 4). The *plcA and iap* genes showed presence in all the isolates, while *plcB* gene was present in 90% of the isolates.

In a study in North-East India, Pegu *et al*.[22] found virulence-associated genes, *hly*A (40.7%), *iap* (29.6%), *plc*A (40.7%) and *plc*B (22.2%) of 27 isolates from fish. The mechanism of pathogenicity of *L. monocytogenes* is stated to be usually associated with production of haemolysin encoded by *hly*A gene[23]. In our study, all the suspected isolates exhibited existence of *hlyA, plcA* and *iap* genes. This indicated that the isolates are potentially pathogenic. Occurrence of such pathogenic bacteria in meat has significant public health implications.

CONCLUSION

This seems to be the first study on isolation and serogrouping of *L. monocytogenes* in foods from Mizoram. The prevalence of serogroup 4b reported to be associated frequently with human listeriosis seems to be of serious public health concern. These virulent strains can contaminate other foods during processing, packaging or storage. Hence, large-scale screening of various food and food products sold in the markets of northeast India at regular intervals for the incidence of virulent strains is of utmost importance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication**.**

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors .

DATA AVAILABILITY

All datasets generated or analyzed in this research are included in the manuscript.

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Characterization of *Mycobacterium tuberculosis* associated with Pulmonary Tuberculosis in Mizoram using Genomic Approaches

BY

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Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Biotechnology of Mizoram University, Aizawl

Abstract

Introduction

Tuberculosis is an infectious, curable bacterial disease caused by *Mycobacterium tuberculosis* (Mtb). Mtb are irregular rods (0.3–0.5 μm diameter) of variable length (Wayne & Kubica, 1986). It is an obligate or facultative intracellular bacterium (Vandal et al., 2009) having a genome size of 4.4 million base pairs encoding 4,000 genes (Cole et al., 1998). They are acid-fast bacterium detectable by Ziehl–Neelsen stain and are neither Gram-positive or Gram-negative (Koch & Mizrahi, 2018). The illness was known to the world by different names across the 17^{th} and 18^{th} century. It is a remarkably ancient disease and is believed to survive over 70,000 years (MacDonald & Izzo, 2015). The first documented written information describing Tb in India was as early as 3300 years ago and 2300 years ago in China (Morse, 1967; Brown, 1941). Even though more than a century of Tb discovery by Robert Koch has passed, mankind is unable to eliminate this deadly disease till date.

The WHO declared Tb a Global Health Emergency in 1993 (World Health Organization, 1993). In the past, various attempts have been undertaken to control tuberculosis including the RNTCP (Revised National Tuberculosis Programme) which was launched in 1997. This programme strives to control Tb by interrupting the transmission chain using quick detection, identification and cure via. direct supervision of therapy thus preventing the development of drug resistant forms in the community. The standard anti-Tb drugs used are isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin for a period of 6-8 months. (WHO. Communicable Diseases Cluster, 1999; Davies, 2003). In 2007, Programmatic Management of Drug Resistant tuberculosis (PMDT) was launched under RNTCP. The World Health Organisation's (WHO) End Tb Strategy has set the target for Tb elimination globally at 2035. However, India has set its own target at 2025 (Kumar, 2017; Uplekar et al., 2015). Mizoram implemented RNTCP and (PMDT) in 2003, which is the former DOTS Plus. Sputum direct microscopy (Ziehl Neelsen stain) was the gold standard for Tb diagnosis. There are eleven districts in Mizoram of which 35 RNTCP direct microscopy centers are located in eight districts (https://health.mizoram.gov.in/page/tuberculosis#main-navbar).

On a global scenario, Tb is among the top 10 causes of death and the principal cause of death by a single pathogen. Globally, in 2019, an estimated 10 million people were infected with *Mycobacterium tuberculosis*. Tb deaths among the HIV negative was estimated to be around 1.2 million and an additional ~2 lakhs death among the HIV positive individuals. Since 1998, India falls among the Tb "high burden" countries accounting for 87% of the world's cases and is among the three countries with the highest prevalence of global MDR-Tb burden. The Tb incidence in India was 28 lakhs which accounts for about a one-fourth of the world's cases (Global tuberculosis report, 2020).

Tuberculosis is a multifactorial disease. Well established risk factors of tuberculosis can be summarised as: (a) bacillary load and proximity to an infectious case (b) immunosuppressive state, diabetes, malnourishment, health-care workers, extremes of age, socioeconomic and behavioural factors, tobacco, alcohol and demographic (ethnic) factors (Narasimhan et al., 2013).

In Northeast India, there are only two places among the list of certified laboratories for performing Drug Susceptibility Testing (DST) and Line Probe Assay (LPA) (India TB Report, 2019). Currently, sputum smear microscopy using Ziehl Neelsen stain and Cartridge Based Nucleic Acid Amplification Test (CB-NAAT) otherwise known as GeneXpert are the only diagnostic means implemented in the state of Mizoram. Culture and LPA are outsourced to neighboring state, Assam (Guwahati). Due to the unavailability of culture facility, this study was taken up to characterize local strains, which will help establish whether initial infection of individuals could result from a heterogeneous mixture of Mtb strains as well as to establish the genes and their SNPs responsible for drug resistance. The extent of heterogeneity in the initial acquired infections might prove great importance in the designing of informed treatment regimen for newly diagnosed tuberculosis patients.

Information on the underlying genetic pattern of drug resistance can provide insights into the pattern of mutation accountable for rifampicin resistance over the past years. A PubMed and Google Scholar search strategy using keywords such as Northeast India, Xpert Mtb/RIF, Genexpert, *rpo*B gene, Mizoram RR-Tb did not provide any information on *rpo*B gene and other gene mutations from North East India. Due to the unavailability of published data on the magnitude of RR-Tb from the state of Mizoram using molecular diagnostic tools, this study was conducted.

Objectives

The objectives of this study are as follows:

Objective 1: To determine the epidemiological risk factors of tuberculosis in Mizoram.

Objective 2: To determine the distribution and frequency of common mutations in *rpo*B gene of *Mycobacterium tuberculosis* detected by Xpert Mtb/RIF and identification of household location of Rifampicin Resistant-Tb cases in Mizoram.

Objective 3: Screening of antibiotic sensitivity of *M. tuberculosis* strains isolated from patients.

Objective 4: To determine gene specific mutations for drug resistance and the lineage of *M. tuberculosis* strains in Mizoram.

Inclusion criteria for the recruitment of patients in the study:

1. **Objectives 1, 3 & 4:**	Written informed consent and willingness to participate in the study, ≥15 years of age.
2. **Objectives 1, 2, 3 & 4:**	Both genders, new and previously treated cases included.
3. **Objective 2:**	All ages included (neonates, children, young adults and old age).
4. **Objectives 1&2:**	Pulmonary and extrapulmonary samples/data included.

Exclusion criteria for the patients' recruitment:

1. Patients not willing to participate.
2. Extrapulmonary, HIV positive and diabetes cases (Objectives 3 & 4).
3. Pregnant ladies (Objectives 1, 3 & 4).

Materials and Methods

Ethical Approval

The ethical approval for this study was obtained from the Institutional Ethics Committee (IEC), Civil Hospital Aizawl (B.12018/1/13-CH (A)/IEC/63, Dated. 28-03-2017), Mizoram. Written consent was obtained from all the participants in this study.

Study Design

The patients recruited for this study were from the District TB Center (DTC), Falkawn and Synod Hospital, Durtlang, Aizawl, Mizoram. Different approaches were utilized to carry out the objectives:

Objective 1: Patients who were microbiologically confirmed or clinically diagnosed for Tb (both pulmonary and extrapulmonary) were consecutively enrolled from December 2017 to June 2020 for obtaining the risk factors of tuberculosis. The mode of diagnosis comprises any of the following tests: Chest X-ray, ZN stain (microscopy), Mantoux, ADA, CT scan, Biopsy, FNAC, Ultrasound, GeneXpert (CB-NAAT), Culture (MGIT) and/or Line Probe Assay (LPA) which is outsourced to neighboring states' Intermediate Reference Laboratory (IRL). Patients' written consent were obtained and were interviewed using a well-designed questionnaire for collection of data on Tb symptoms, socioeconomic, demographic, food and lifestyle factors.

Methods for Objective 1

The case-control study was performed with patients visiting District Tb Center, Falkawn and Synod Hospital, Durtlang, Aizawl, Mizoram which are visited by TB patients from all districts of Mizoram. A total of 400 Tb patients and 840 healthy controls (no previous history of Tb, no immunocompromised state (HIV), non-diabetic) were enrolled. The estimated sample size for a robust statistical analysis (http://www.raosoft.com/samplesize.html) was 385 for a population size of 10,97,206 (Census of India, 2011). The participants were categorized based on gender (female and male), age (15-45 years and >45 years), marital status (married-currently living with spouses and single- unmarried, widows, widowers, divorcees). Body Mass Index (BMI) score of 18.5 -24.9 was classified as normal weight, <18.5 as underweight, 25-29.9 as overweight, > 30 as obese (www.thecalculatorsite.com). For convenience, the BMI was classified as: Ideal (normal weight) and non-ideal (underweight, overweight and obese).

Education level was grouped into two classes ('Matriculation or class 10' and 'higher secondary & above'), Occupation ('employed' and 'unemployed') and Family income ('> INR 30,000 per month' and '< INR 30,000 per month'). The housing condition ('receiving proper sunlight during the day' and 'moist or damp

without any proper sunlight'), Number of people living under one roof ('1 to 6 members' and '>6 family members'), physical activity in the form of exercise ('Regular' and 'Irregular'), Water supply source ('Govt. municipal' and 'Other sources'- includes river water and/or collection of spring water) were categorized into two classes each. The Govt. Municipal water is pumped from rivers and treated to make it potable.

The following factors/variables were classified into 'Yes' or 'No': dwelling in a crowded house; cross-ventilation system in house; participation in mass gathering; travel history; contact history with pulmonary Tb; having diabetes or HIV (checked at the Tb Clinic for random blood sugar by Accu-chek and HIV Tri-Dot); BCG vaccination (via. verbal response from participants); Consumption of tobacco products (smoke or smokeless forms - snuffed tobacco, gutkha, shikhar etc.); exposure to passive smoking; Tuibur habit (tobacco infused water); alcohol consumption history and current status; Susceptibility to respiratory infection; Previously diagnosed as Tb (laboratory test or clinically including a positive Tb skin test); Any family member with current or history of Tb; number of family members succumbed to Tb and Neighbor's suffering from Tb.

Statistical analysis for Objective 1

Analysis of the epidemiological data was performed using IBM Statistical Package for Social Sciences (SPSS v20, Madrid, Spain). The variables were dichotomized for associated risk factors and bivariate analysis was performed. The p-value (≤ 0.05 was considered significant), odds ratio, 95% CI were calculated using logistic regression model after adjusting for confounding factors.

Objective 2: Data were evaluated retrospectively from archived results for all the types of specimens received and tested using Xpert Mtb/RIF assay from December 2014 to May 2021. Samples detected positive for the presence of Mtb along with Rifampicin resistance were incorporated in the study.

Methods for Objective 2

Study population

A total number of 13,927 samples suspected for tuberculosis were subjected to GeneXpert assay. 473 samples were removed due to invalid report, error,

duplicates or no results and thus 13,454 samples were included. *M. tuberculosis* was detected in 2,894 (21.5%) cases, out of which 460 (15.9%) were Rifampicin Resistance detected and 2,434 (84.1%) were Rifampicin Resistance not detected cases. Socio-demographic and available clinical information were reviewed and analyzed for Rifampicin Resistant Tb cases only.

The tested samples were categorized into pulmonary (sputum, broncho alveolar lavage (BAL), gastric lavage, tracheal aspirate) and extrapulmonary (lymphnode aspirate, pleural fluid, ascitic fluid, cerebrospinal fluid, pus/abscess from various sites). Results of the Xpert Mtb/RIF assay were categorized into the following:1) Mtb not detected. 2) Mtb detected; Rifampicin Resistance not detected. 3) Mtb detected; Rifampicin Resistance Detected. 4) Invalid. 5) Error. 6) No result. For the tests having Mtb detected along with Rifampicin Resistance detected, the missed probe types as well as the DNA amounts were assessed.

Household identification

The localities of Rifampicin Resistant Tb patients belonging to Aizawl District were entered in excel sheet, coded, segregated and mapped with the constituency they belong using the following link as a reference (https://ceo.mizoram.gov.in/state_profile).

Statistical Analysis for Objective 2

The data on the patient profile were coded and analyzed using Statistical Package for Social Sciences (SPSS v20.0, IBM Corp, USA). Bivariate analysis was used to characterize clinical and demographic variables like frequencies of specimen type, DNA quantity, probe types, gender and multivariate analysis for age group. Logistic regression models were used to characterize the demographic and clinical variables with respect to probe types. The 460 Rifampicin Resistant Tb (Cases) were also analyzed against 920 Rifampicin Sensitive Tb (Control). P-value ≤ 0.05 was considered to be statistically significant.

Objective 3: AFB smear positive/ Genexpert positive sputum samples were collected from Pulmonary Tb patients for liquid culture from June 2019 to May 2020. Antibiotic sensitivity against first line Tb drugs was performed.

Methods for Objective 3

Sample Collection and processing

A total of 88 sputum samples from pulmonary tuberculosis patients (new and previously treated) were randomly collected in a sterile screw capped wide mouth container. The collected sputum specimens were transported to the laboratory for detection of AFB by Ziehl Neelsen (ZN) Stain. In addition, GeneXpert test was performed in accordance to the manufacturer's instructions using Xpert Mtb/RIF assay G4 (Cepheid, Sunnyvale, CA, USA) as well as liquid culture using MGIT (Mycobacterium Growth Indicator Tube) system. Culture positive samples showing cord formation, TBc ID positive and passing the sterility check (without any contamination on blood agar) were subjected to first line Drug Susceptibility Test (Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide).

Objective 4: To determine gene specific mutations for drug resistance and the lineage of *M. tuberculosis* strains in Mizoram.

Methods for Objective 4

Out of the 88 samples subjected to liquid culture, 56 samples had shown susceptibility report in at least one of the first line ATT drugs tested. GeneXpert MTB/RIF results were also available for these 56 isolates. For determining the gene specific mutations for drug resistance and the lineage of *M. tuberculosis* strains in Mizoram, the DNA of Mtb was extracted from culture positive isolates that had passed the sterility check. Isolated DNA was run on a 0.8% agarose gel electrophoresis (BioRad) to check the quality and the DNA concentration was quantified using a µdrop MultiSkan SkyHigh Spectrophotometer (Thermoscientific). Conventional PCR using five antibiotic resistance genes (*kat*G, *rpo*B, *emb*B, *rps*L, *pnc*A), sanger sequencing and Mtb lineage identification using LSP markers (RD105, RD239, RD750, 7bp pks15/1) was also carried out.

Results

Objective 1

- The most common modes of detection of *Mycobacterium tuberculosis* (Mtb) within Mizoram are GeneXpert (40%), smear microscopy- Ziehl Neelsen stain (32.6%) for pulmonary cases and for extrapulmonary cases, X-ray (40.1%) followed by FNAC- Fine Needle Aspiration Cytology (25.9%).

- The symptoms of tuberculosis such as fever (>2 weeks), cough (>2 weeks), weight loss, chest pain and night sweats were more pronounced among pulmonary cases as compared to extrapulmonary cases. The mean age of tuberculosis patients (pulmonary and extrapulmonary) in Mizoram was 33.1 years.
- Single marital status, non-ideal weight (underweight, obese, overweight), lower educational qualification (upto matriculation), unemployment, lower family income (<30,000 per month), lack of cross-ventilation system, damp or moist housing, participation in mass gathering events, history of travel, no BCG vaccination, alcohol consumption and neighbours with tuberculosis are all significant risk factors of tuberculosis in Mizoram.
- Other sources of water supply (river water and spring water collection), though less-established was found to be an important risk factor of tuberculosis in Mizoram. This is the first world-wide report of the potential role of water source as a risk factor for Tb.

Objective 2

- Rifampicin Resistant Tb in males was predominant in pulmonary samples, while in females it was predominant for extrapulmonary samples. Majority of the Rifampicin Resistant Tb cases in Mizoram falls between productive age groups of 25-34 years followed by 35-44 years.
- Higher number of probes (A, B and D) mutations in Mtb were observed for extrapulmonary samples, while probes C and E mutations were more in pulmonary samples.
- GeneXpert probe mutations in Mtb observed during eight years (2014-2021) are as follows: Single mutation: probe A (codons 507-511)- 25.9%; probe E (codons 529-533)- 23.5%; probe D (codons 523-529)- 9.8%; probe B (codons 512-518)- 2.6%; probe C (codons 518-523)- 0.2%.
- Probes mutation combinations were also found in 158 (34.3%) Mtb samples: AB- 0.4%, AD- 32.8%, AE- 0.4%, DE- 0.2% and ADE- 0.4%.
- Seventeen of the Rifampicin Resistant Tb cases had no missed probes (all probes positive) and the ΔCt for these samples were all >4 cycles.

- This study reported the highest probe A mutation as well as mutation combinations in Mtb from Mizoram upon comparison with twelve other similar studies within and outside India.
- Association studies using bivariate analysis showed the frequency of probe A (p=0.009) and probe D (p=0.007) mutations to be statistically significant for female gender.
- Association studies using bivariate analysis showed the frequency of probe A (p=0.047) and probe D (p=0.001) mutations to be statistically significant for extrapulmonary samples.
- The frequency of mutation at probe D site (p=0.000) was also statistically significant for samples with low and very low DNA quantity.
- Association studies using multivariate analysis also showed that the frequency of mutation at probe A site was statistically significant for younger age groups, 15-24 years (p=0.015) and 25-34 years (p=0.032).
- The residential areas of the majority of Rifampicin Resistant Tb patients within Aizawl District were found to be located in Aizawl North- III followed by Aizawl South-II and Aizawl East-II.

Objectives 3 & 4

- MGIT Mtb drug susceptibility report showed the highest drug resistance for Isoniazid (32.1%), followed by Streptomycin and Ethambutol (26.8% each), pyrazinamide (25%) and the least resistance by Rifampicin (19.6%).
- Discrepancy in drug resistance using phenotypic and genotypic methods were observed for Isoniazid and Rifampicin.
- Sequencing results of phenotypic Isoniazid resistant Mtb isolates showed Serine to Threonine substitution to be the most common at 315 amino-acid position. A lesser common substitution at this same position was Serine to Asparagine.
- Sequencing of the *rpo*B gene of Rifampicin Resistant isolates with all probes' positive showed no single nucleotide polymorphism.
- Sequencing of the *rpo*B gene of Rifampicin Resistant isolates with probes negative (mutation) showed an SNP of T>C (L511P) at probe A site and C>G

(H526Q) at probe D. Another less common SNP at probe D was site was A>T (H526L).

- Sequencing results showed no mutations for the other antibiotic resistance genes (*emb*B, *rps*L, *pnc*A).
- Long Sequence Polymorphism -PCR showed the presence of Indo-Oceanic, Beijing, East African Indian and Euro American lineages of *Mycobacterium tuberculosis* in Mizoram along with mixed strain infection and other unidentified lineages.

Discussion

Risk factors of Tb

In the present study, the male counterparts were at high risk of Tb infection which could be due to greater exposure to the reported risk factors which is in concordance with the Global Tuberculosis Report (Global tuberculosis report, 2020). Single individuals were more prone because they tend to involve more in multiple activities of the community, thus becoming as a high-risk group for Tb and the rate of incidence in this study was twice as high when compared to married individuals, which is also in concordance with previous report (Horwitz, 1971). Non-ideal weights (underweight, obese, over weight) were more prone to develop Tb in their lifetime as they could indicate poor health. In contrast, overweight or obesity were found to be at low risk (Leung et al., 2007). Educational status is an important aspect of life and being educated can decrease the risk of infectious diseases wherein, the awareness and knowledge to seek an immediate medical help will be a priority among the educated. It was also observed that less educated participants (upto matriculation) were at a higher risk of Tb infection (Senanayake et al., 2018). Tb was found to be high midst unemployed individuals (Przybylski et al., 2014).

Poverty and Tb go in parallel wherein being economically backward increases the risk of Tb infection because of overcrowding, poor living or working conditions, malnutrition, and limited access to treatments (Siroka et al., 2016). Over-crowding is a high-risk factor for Tb that can expose susceptible individuals to the infection (Clark et al., 2002). However, it was not a significant factor in this study. The cross-ventilation system in houses is a protective factor wherein adequate aeration effectively controls Tb outbreaks (Du et al., 2020). Moist or damp houses with poor lighting is also an important risk factor because of the risk of harboring

tubercle bacilli, which helps them to evade the direct killing by sunlight (Aditama et al., 2019).

Tuberculosis spreads through air and there are other probable routes of transmission which are not fully understood. Water supply source for domestic consumption is another important route pertaining to tuberculosis transmission that is very often neglected. The state of Mizoram is a mountainous hilly region situated in the north eastern part of India. The hospitals in the state have no proper sewage treatment facility and with an improper drainage system in the state, the drains clog and overflows during the rainy seasons. Hence, the waste-waters from hospitals and Tb sanatorium flows down the drain and converges down to the rivers. The majority of the participants (cases and controls) receive the Government water supply (PHED water), while some living in the lower ridges/altitudes rely on the river water as well as spring water collections (tuikhur) for domestic use. In this study, it was observed that participants with water supply source other than the government water supply have two times higher chances of contracting Tb, which is of serious concern since virulent tubercle bacilli was isolated from river water even after five months of storage (Musehold, 1900). Virulent tubercle bacilli in settled sewage water was recovered even from adequately disinfected sputum (Greenberg & Kupka, 1957). As stated by previous studies, the source of water contamination could be either human or non-human (Ghodbane & Drancourt, 2013; Martinho et al., 2013). Since both human and animal excreta can harbor the tubercle bacilli, sewage contamination of water sources could potentially be an additional source (Monkongdee et al., 2009). Our previous 16S rRNA V3-V4 region based metagenomic study around Aizawl, Mizoram examined the discharge of solid and liquid wastes from domestic, municipal and hospital premises and found the presence of *Mycobacterium tuberculosis* in soil and water sediment samples from three river sites and solid waste dumping sites (De Mandal et al., 2019). The drawback of this study is that we did not collect water samples for culture-based study which can be taken up for future studies. The aforementioned risk factors suggest a holistic view of the possible routes of Tb transmission which should be reconsidered (Velayati et al., 2015).

Since the Mizo people believe in the ethics of "*Tlawngaihna*" - a moral code which makes them courteous, helpful to others during deaths and the spontaneous gathering to help the deceased family is a very common practice. There are many community services (*Hnatlang*) to be attended for their welfare or for church

welfare. The people share their drinking water (from the same bottle or glass), snuffed tobacco from the same pack along with a puff from the same cigarette. Being a closely knit society, these practices have been in existence within the Mizo community from times immemorial. These cultural activities lead to the gathering of huge crowds and such mass gathering events can be associated with public health risks. Also, the data on tuberculosis associated with mass gathering events are scarce. However, this study found that people participating in such events have a significant chance of contracting Tb, which is also in concordance with previous reports where Tb is transmitted during the Hajj pilgrimage (Zumla et al., 2016). Patients with a travel history developed the symptoms after completing their travel. Since, the Tb status of a traveler is unknown, there is a minimal chance of Tb transmission during travel (Rieder, 2001).

The protection efficiency of BCG vaccine remains debatable and can vary based on geographical regions or populations (Fine, 1995). In this study, significant difference was observed among the participants who did not receive BCG vaccine as compared to those who did. This was in contrast to a previous study where no statistical difference in Tb was observed (Masood et al., 2019). Alcohol consumption can be a major risk factor and burden for tuberculosis and a three-fold increase in the risk was reported (Imtiaz et al., 2017). This study observed a seven-fold increase in the risk of tuberculosis with alcohol consumption, which is much higher than the previous reports. The probable reasons could be impaired immunity and social mixing patterns among people who consume alcohol (Classen et al., 1999). Many studies have associated Tb with active or passive smoking (Alavi-Naini et al., 2012; den Boon et al., 2005; Leung et al., 2010), but in this study it was not significant. A large-scale epidemiological study or contact tracing is essential to identify tuberculosis exposure (Moonan et al., 2020) as transmission between neighbors had also been documented (Moravkova et al., 2011). Similarly, in this study a significant association between tuberculosis transmission with an infected neighbor had been found.

Discussion on GeneXpert mutation analysis

In the present study, from December 2014 to May 2021, there were 2,894 TB cases detected via. Xpert Mtb/RIF in Mizoram and among these, the proportion of

RR-Tb diagnosed was 15.9% (n=460). Among the RR-Tb cases in this study, males (57.8%) are predominant compared to females (42.2%). Reports from Central India (17%) (Desikan et al., 2014), New Delhi (17.9%) (Singhal et al., 2015) and Lucknow 27.8% (Jain et al.,2014) have also been documented. A review from Ethiopia reported that being male is an identified risk factor for MDR-Tb (Asgedom et al., 2018). In this study, productive age group between 25-34 years were the most affected which may be due to more exposure to open cases of Tb and similar findings have been reported from China and Ethiopia (Zhu et al.,2018; Alemu et al.,2020). Among the 460 RR-Tb cases, 85.9% were from pulmonary samples while 14.4% from extrapulmonary samples. High DNA quantity was observed in pulmonary samples (sputum) while low or very low amount was found in extrapulmonary samples. This is in concordance with a study from Ethiopia (Alemu et al.,2020) and may be due to the fact that the tubercle bacilli are obligate aerobe, requires oxygen for its metabolism. Apart from other organs, the lungs have higher oxygen pressure and thus induces a higher multiplication rate resulting in higher bacillary load (Cardona & Ruiz-Manzano, 2004).

Overall, in this study, the most common RRDR *rpo*B gene mutation in the 81 bp were observed in codons 507-511 (25.9%), codons 529-533 (23.5%), codons 523-529 (9.8%), codons 511-518 (2.6%) and the least in codons 518-523 (0.2%) which corresponds to probes A, E, D, B and C, respectively. This strongly contradicts with findings of the studies from Ethiopia (Alemu et al., 2020), Madhya Pradesh (Sharma et al., 2020) and Nepal (Adhikari et al., 2021) where they reported no mutation in probe A region. Our results are also dis-concordant with the findings of the studies from Madhya Pradesh, India where the commonest mutation was found in probe B followed by probe C and E, while no mutations were found in probes A and D (Sharma & Singh, 2020). Similar studies from different parts of the world i.e., Uganda, Pakistan, Bangladesh, Nigeria, Zimbabwe, Ethiopia, Nepal and various cities from India like Punjab, Andhra Pradesh, Mumbai and Himachal Pradesh had reported probe E (codon 531-533) to be the commonest mutated site in the region of the RRDR *rpo*B gene. This might be due to higher mean relative fitness (Darwinian fitness) (Billington et al., 1999) and resistant mutants have a better ability to survive. However, next to probe E mutation, the order of the prevalence of probe mutation varies from region to region wherein E is followed by B, D, A and C (Kaur et al.,

2016; Reddy et al., 2017; Uddin et al., 2020) and by D and B (Alemu et al., 2020; Kanade et al., 2019; Ochang et al., 2016; Yue et al., 2003). A small proportion of probe C mutation was reported from Pakistan, Bangladesh, Punjab, Zimbabwe, India (Andhra Pradesh, Mumbai, Himachal Pradesh & Madhya Pradesh) and Nepal (Ullah et al., 2016; Rahman et al., 2016; Kaur et al., 2016; Metcalfe et al., 2016; Reddy et al., 2017; Kanade et al., 2019; Kumar et al., 2020; Sharma et al., 2020; Adhikari et al., 2021) while Nigeria (Ochang et al., 2016), Ethiopia (Alemu et al., 2020) and Uganda (Mboowa et al., 2014) reported no probe C mutation. This study also reports probe C mutation occurring only once within a span of eight years. The least mutations detected by Xpert Mtb/RIF in probe C might be due to this specific site of RRDR being probably less susceptible to mutations conferring the resistance or because of less selection pressure in this region.

Similar to other studies on mutation combinations, this study also reported high mutation combinations (Reddy et al., 2017; Kanade et al., 2019; Ochang et al., 2016), which may be due to the intrinsic or acquired ability of the bacteria to adapt to drug exposure. A study from Bangladesh reported the occurrence of probe mutation combinations in retreatment Tb cases (Uddin et al., 2020). Unlike other studies, this study reports high prevalence of probe mutation combinations (n=158, 34.3%) which demands deeper insights to gain rational understanding. In addition, the mutation combinations observed in this study might also be retreatment cases as information on the initiation of ATT drugs prior to testing the samples is unknown. Among the 460 samples tested, this study also reports a “very low” bacillary load in 98 samples. Having a “very low” bacillary load on Xpert testing was significantly correlated with false rifampicin resistance (Ngabonziza et al., 2020). Being a retrospective study, samples were not collected and hence the results cannot be compared with culture (gold standard) or *rpo*B sanger sequencing to rule out rifampicin false positivity rate. Therefore, it is difficult to elucidate the implications of this finding.

In this study, the proportion of extrapulmonary Tb was more among females aged 25-34 years which is in concordance with a study from China where younger female patients are more likely to have extrapulmonary Tb (Pang et al., 2019). The predilection of extrapulmonary Tb in women may be linked to the limited facilities for access to healthcare apart from the less prevalence of other risk factors such as habit of smoking (Razanamparany et al., 2002; Sreeramareddy et al., 2008). Previous

studies have reported the increased risk of mortality with smoking in men, but not in women (Lam, 2001). Smoking is not common in women and are relatively protected from the hazardous pulmonary effect of smoking which may be one of the factors for the differences in distribution (Musellim et al., 2005). This suggest that female gender and younger age could be an independent risk factor for Tb especially in high burden areas. Bangladesh had reported the geographical distribution of RR-Tb within their country (Uddin et al., 2020). Similarly, in the present study within Aizawl District, Aizawl North –III (11.9%) had the highest proportion of RR-Tb followed by Aizawl South –II (7.4%) and Aizawl East –II (7.2%) which demands further investigation of the affected areas. The identification of the residential areas will prove useful for future epidemiological surveys, understanding the transmission pattern and severity of the disease. Our study has several limitations: being a retrospective study design, it lacks relevant information such as previous history of Tb treatment, HIV, diabetes and BCG vaccination status.

Discussion on Genotyping of drug resistant isolates and lineage of Mtb

This is the first attempt to culture tubercle bacilli since there are no functional Mtb culture facilities implemented in Mizoram. INH resistance is often accompanied by loss and/or reduction of Catalase Peroxidase activity or *kat*G activity coded by *kat*G gene (Zhang et al., 1992). It is a prodrug that requires cellular activation by *kat*G protein to its active form, before it can exert its toxic effect on the bacillus. Resistance often occurs due to a point mutation occurring in the *kat*G gene, thus preventing the activation of the pro-drug (Barry et al., 1998). More than 300 types of *katG* mutations have been identified, however, mutations at codon 315 of the gene are the most prevalent, with one particular amino acid substitution (serine to threonine) accounting for 95% of all *katG* 315 mutations (Vilchèze et al., 2014; Seifert et al., 2015). Hence, mutation in *kat*G is responsible for high level isoniazid resistance (Ando et al., 2010). Another study also reported a 93.6% prevalence of the mutation from both new and previously diagnosed cases of tuberculosis (Mokrousov et al., 2002). In this study, similar report was observed where the 7-INH resistant isolates which are also Rifampicin resistant had the most common substitution such as Ser315Thr (n=6/7, 85.7%) in *kat*G gene. It is also admissible that the Ser315Thr substitution is associated with MDR strains (Marttila et al., 1998). One isolate (14.3%) had a Serine substituted by Asparagine at codon 315 which is less common.

Similar substitution was also reported by a study from China and Agra (India) (Liu et al., 2021; Jaiswal et al., 2017). Three samples (CVS-1, CVS-13, CVS-17) that had a phenotypic INH resistance, but is not reflected in the sanger report might possibly be due to the INH resistance mutation occurring in other genes such as *inh*A (Seifert et al., 2015).

In this study, for Rifampicin drug susceptibility test, discordant results between MGIT 960 and Xpert Mtb/RIF was observed in which 3 isolates (CVS-1, CVS-13, CVS-17) lacked mutations in the RRDR region of the *rpo*B gene, though these isolates were phenotypically resistant to RIF. These differences might be attributed to different genotypes prevailing worldwide. In addition, since Xpert Mtb/RIF detects mutations only within the RRDR of the *rpo*B gene, and the mutations might be present outside the known target regions (Heep et al., 2001). In two samples (CVS-3$^{\#}$, CVS-9$^{\#}$), we observed a discrepancy between MGIT and Xpert Mtb/RIF where rifampicin was susceptible via MGIT, resistant via Xpert Mtb/RIF without any probe drop out and ΔCt was >4, however sanger sequencing revealed no mutation in *rpo*B region. This could be a false resistance indicated by Xpert Mtb/RIF as similar discrepancy results were reported from China (Qin et al., 2021). In two other samples (NER-24, 42), rifampicin was susceptible via. MGIT and resistant via. Xpert Mtb/RIF where probe A was drop out. In this discrepancy, the mutation observed was L511P (CTG → CCG). Similar disputed *rpo*B mutation was reported from South Korea (Jo et al., 2017). A study from Kuwait reported isolates as rifampin susceptible by the MGIT system while resistant by the Xpert assay. *rpo*B sequencing identified a silent (CTG521TTG) and a missense (GAC516TAC) mutation (Mokaddas et al., 2015). However, this was not the case in this study.

In Mizoram, the specific mutation in probe A (507-511) was L511P. However, this particular mutation was not observed in a study from Bangladesh (Rahman et al., 2016). Our study also reported the specific mutation occurring at probe D (523-529) as H526Q and H526L, which is not in concordance with another study from Bangladesh where they reported mutation as H526D and H526Y (Uddin et al., 2020). The mutations L511P and H526L have been reported to confer a low level of resistance (Hauck et al., 2009). A study from Treichville, Abidjan District reported H526Q mutation in a relapse case (Kouassi et al., 2016). In this study,

phenotypic DST resistance was observed for Streptomycin, Ethambutol and Pyrazinamide, which do not correlate with sanger sequencing results. This may be probably because the primers used in this study does not cover the region where SNP's are present.

Though the number of samples studied maybe small, this study attempts to provide information on *M. tuberculosis* strain diversity in this remote region of northeast India where all the four major lineages (Lineage1, Lineage 2, Lineage 3 Lineage 4) were present. In a recent study, Beijing (Lineage 2) and East-African Indian (Lineage 3) were found in three of the northeastern states, Manipur, Nagaland and Tripura (Dusthackeer et al., 2021). In this study, among the 6 INH resistant isolates, five were unknown genotypes while one belonged to East-African Indian lineage (Lineage 3). Previous studies have revealed Indo-Oceanic (Lineage 1) and East-African Indian (Lineage 3) to be most prevalent in India and less common in other parts of the world (Gutierrez et al., 2006; Ahmed et al., 2009). This might explain the mixed strain infection observed in two samples in this study. A recent study from Nepal also reported mixed lineage infection of Indo-Oceanic and East-African Indian in a captive elephant (Paudel et al., 2019). Mixed strain infections particularly when strains with different drug resistance phenotypes are involved, it makes diagnosis, treatment and control more cumbersome and people with underlying morbidity may be prone to mixed strain infection (Asare-Baah et al., 2021; Kamakoli et al., 2017). A study from Botswana had reported that among new cases of tuberculosis, mixed strain infections but not heteroresistant infection were associated with poor treatment outcomes, using standard combination treatment regimens. (Shin et al., 2018). Previous studies have shown that in patients who harbored both MDR and susceptible strains, MDR strain was able to persist and grow during treatment with first-line regimens and, upon a switch to second-line regimens, the susceptible strain was able to re-emerge, compromising treatment outcome (van Rie et al., 2005). In addition, immune responses may be altered in patients with ongoing mixed strain Tb infections, rendering them more prone to reinfection (Warren et al., 2004).

Mizoram being located in a remote area, establishment of highly sophisticated diagnostic machines which requires high end technical expertise is still cumbersome. On most occasions, ATT is initiated based on the GeneXpert reports

unless there is a need to outsource samples for LPA and culture. The global frontline molecular diagnostic tools such as GeneXpert and LPA have been developed based on known genetic markers (Gagneux & Small, 2007; Thakur et al., 2015; Thirumurugan et al., 2015). Since, frontline molecular tests rely on a limited number of mutations, there have been several instances where phenotypic resistance could not be explained by known mutations associated with drug resistance (Rigouts et al., 2013; Banu et al., 2013, Ahmad et al., 2016). Thus, our findings also suggest that Tb diagnosis aided with sequencing technologies is mandatory to provide accurate diagnosis and treatment.

The molecular study relied on small number of samples and is thus difficult to elucidate the findings of this study. Being the first Mtb culture to be performed within the state, the phenotypic DST is yet to be certified and since it is not from a competent laboratory, it warrants further confirmation. The methodology used for lineage identification in this study is not sufficient enough to determine all the possible lineages. Hence, further studies using larger number of samples and employing better identification tools such as spoligotyping is needed to support our findings.

Conclusion

Tuberculosis is still a global public health concern claiming 1.5 million lives per year. It still associated with social stigma which is responsible for the strong barrier to health-seeking behavior, especially in women, and is a cause of significant suffering. Under most circumstances, an infected individual is frequently unable to seek timely help due to the fear of losing social status, future or current marital problems or adverse response and or reaction from the community.

Being a multifactorial disease, the infection is contributed by various risk factors. In the state of Mizoram, the unique social practices within the community might also serve to fuel Tb transmission and/or hinder the tuberculosis control program. Apart from the well-established risk factors (alcohol consumption, no BCG vaccine, lower level of education, damp or moist housing, neighbors with Tb, travel history) for Tb, this study highlights the need to investigate water sources used for domestic purposes as they can serve as a potential source of infection. From a public health standpoint, this highlights the needed of further research in this area.

Due to the existence of limited Tb diagnostic infrastructures within the state, this retrospective study being the first molecular data on *Mycobacterium tuberculosis* from the state provides genetic pattern of drug resistance accountable for rifampicin resistance over the past years. This will help local clinicians be acquainted with the possible mutations conferring drug resistance in tuberculosis and further aid in the initiation of correct treatment. Also, our findings from the retrospective study provides a baseline data on the magnitude of RR-Tb within the state and identification of the residential areas can help local health authorities in planning surveillance programs to control the spread of rifampicin resistant Tb.

Our study also provides information on the genotyping of mutations detected with GeneXpert as well as MGIT 960. It also provides information on the lineages of *M. tuberculosis* circulating in the state. Though the sample size may be small, it is the first of its kind to be reported from the state of Mizoram where culture facilities are presently unavailable. Due to the existence of genetic heterogeneity among *M. tuberculosis* isolates from various geographic zones, the utilization of sequencing technologies is essential in diagnostic laboratories to map the genetic variations, identify all the possible genotypes associated with drug resistance and trace the outbreaks. In addition, it can as well be used as a cautionary measure to prevent wrong diagnosis by sequencing discrepant results and guide in effective patient care. Our study forms a baseline data and gives preliminary information on tuberculosis within the state. Hence, more research with a greater number of samples along with better stratification is needed to further strengthen our findings.

Though the global target of Tb eradication is set to 2035, tuberculosis was, is and will remain a public health concern for at least another one or more decades to come. In view of the fact that peripheral areas of India inhabited by tribal people such as Mizoram and any other tribal inhabited state often lag behind in obtaining sophisticated diagnostic infrastructures for any disease. In view of tuberculosis, better diagnostic infrastructures utilizing molecular methods is a need of the hour in every corner of the country alongside with training of manpower for the same purpose. The responsibility of the health care personnel as well as clinical researchers is to strive for the correct identification of the infection along with its associated mutations. This may be followed by initiation of timely interventions by the Clinicians comprising of administration of the "*right drug with the right dosage*

at the right time with the right patient without any interruption during treatment". This in turn can serve as the initial step in aiding Tb eradication.

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